Universidade Estadual de Londrina

LARISSA STAURENGO FERRARI

CONTROLE DA ARTRITE SÉPTICA PELO TRATAMENTO COM UM DOADOR DE NITROXIL, O SAL DE ANGELI:

AVALIAÇÃO DO EFEITO E MECANISMO DE AÇÃO NA REDUÇÃO DA DOR, INFLAMAÇÃO E INFECÇÃO

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Orientador: Prof. Dr. Waldiceu Aparecido Verri Junior.

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BANCA EXAMINADORA

_____

Orientador: Prof. Dr. Waldiceu A. Verri Junior
Universidade Estadual de Londrina - UEL

_____

Prof. Dra. Lucy Megumi Yamauchi Lioni
Universidade Estadual de Londrina - UEL

_____

Prof. Dra. Jacinta Sanchez Pelayo
Universidade Estadual de Londrina - UEL

_____

Prof. Dra. Idessânia Nazareth da Costa
Universidade Estadual de Londrina - UEL

_____

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

Londrina, 11 de setembro de 2015.

Dedico este trabalho à Deus e à minha família.

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“Jamais considere seus estudos como uma obrigação, mas como uma oportunidade invejável para aprender a conhecer a influência libertadora da beleza do reino do espírito, para seu próprio prazer pessoal e para proveito da comunidade à qual seu futuro trabalho pertencer.” *Albert Einstein*

FERRARI, Larissa Staurengo. **Controle da artrite séptica pelo tratamento com um doador de nitroxil, o Sal de Angeli:** avaliação do efeito e mecanismo de ação na redução da dor, inflamação e infecção. 2015. 103 f. Tese (Doutorado em Patologia Experimental) – Universidade Estadual de Londrina, Londrina, 2015.

RESUMO

É cada vez mais evidente a necessidade da busca de novas terapias para o paciente com artrite séptica devido ao sofrimento prolongado, dor, destruição articular e perda de função. Neste trabalho estamos propondo a avaliação de uma nova terapia, o tratamento com o sal de Angeli (SA, $Na_2N_2O_3$), uma droga doadora da forma reduzida do óxido nítrico ($NO^{\bullet}$), o nitroxil (HNO/NO). Estudos recentes que recriam condições biológicas demonstram que o HNO é uma molécula endógena em nosso organismo por atuar como fator relaxante derivado do endotélio assim como o $NO^{\bullet}$ e, ainda, diferentemente do $NO^{\bullet}$ atua como papel como fator hiperpolarizante derivado do endotélio. Assim, as atividades endógenas do HNO e $NO^{\bullet}$ são iguais em alguns pontos e diferentes em outros, demonstrando que são moléculas dimstintas e com efeitos independentes. Quanto ao $NO^{\bullet}$ em geral, existem evidências do seu efeito microbicida, importante para a eliminação bacteriana, para redução do influxo de leucócitos para o foco inflamatório e para analgesia. O HNO tem a capacidade de apresentar os efeitos do $NO^{\bullet}$, como a analgesia. Nesse sentido, avaliamos primeiramente o efeito analgésico do doador SA, em modelos de dor manifesta [*p*fenil-bezoquinona (PBQ); ácido acético e formalina] para melhor compreensão dos mecanismos analgésicos do HNO, nos quais a multiplicação bacteriana não é um fator interferente. Os modelos de dor manifesta são caraterizados por um imediato comportamento, podendo ser contorções abdominais (PBQ e ácido acético) ou lambida/sacudidas (formalina) do membro estimulado, e são avaliados por 20-30 min. Tais comportamentos dolorosos foram reduzidos de maneira dose-dependente pelo tratamento subcutâneo com o SA, sendo o efeito máximo observado com 3 mg/kg. O efeito inibitório do SA foi prevenido pelo pré-tratamento com ODQ, KT5823 e glibenclamida que são inibidores farmacológicos da via cGMP/PKG/canais de K_+ATP-sensíveis. Além disso, o pré-tratamento com L-cisteina (“sequestrador” de HNO), confirmou que o efeito analgésico do SA depende do HNO. Em seguida, a partir da dose analgésica efetiva de SA de 3 mg/kg, verificamos o efeito dessa molécula no modelo crônico de artrite séptica induzida por *S. aureus*. O tratamento diário com SA inibiu significativamente a dor e as respostas inflamatórias (edema, recrutamento leucocitário, produção de citocinas, ativação de NF-κB e estresse oxidativo) resultando na prevenção da severidade da doença avaliados por parâmetros clínicos, conteúdo de proteoglicanos e osteoclastogênese. As características observadas parecem ser dependentes do efeito microbicida do HNO que diretamente inibiu o crescimento de *S. aureus* e o número de UFC na articulação. Como consequência desse efeito, o tratamento com SA também inibiu uma resposta inflamatória sistêmica em resposta à infecção (contagem de leucócitos no pulmão e concentração sérica de citocinas pró-inflamatórias). Assim, os resultados obtidos sugerem o potencial terapêutico do SA ou doadores de HNO na artrite séptica, e sustenta que o SA apresenta efeitos analgésicos intrínsecos independentemente da inibição da infecção.

Palavras-chave: Nitroxil. Artrite Séptica. *Staphylococcus aureus.* Dor. Inflamação.

FERRARI, Larissa Staurengo. **Angeli's Salt, a nitroxyl donor, inhibits septic arthritis in mice:** effect and mechanisms of action in reducing pain, inflammation and infection. 2015. 103 p. Thesis (Doctoral degree in Experimental Pathology) – Universidade Estadual de Londrina, Londrina, 2015.

ABSTRACT

It is increasingly evident the need for novel therapies for patients with septic arthritis due to prolonged suffering, pain, joint destruction and loss of function. Here, we present the evaluation of a novel therapy, the treatment with Angeli's salt (AS, $Na_2N_2O_3$), and a molecule that donates the reduced form of the nitric oxide ($NO^{\bullet}$), the nitroxil (HNO / NO^-). Recent studies that mimic biological conditions have demonstrated that the HNO is an endogenous molecule with a role as endotheliumderived relaxing factor similarly to $NO^{\bullet}$ and as endothelium-derived hyperpolarizing factor, a role that has not been assigned to $NO^{\bullet}$. Thus, endogenous activities of HNO and $NO^{\bullet}$ are similar in specific points and distinct in others. This shows that they are different and independent molecules. Regarding to $NO^{\bullet}$, in general, there is evidence of its microbicide effect, pivotal to clearance bacteria, to reduce the influx of leukocytes to the inflammatory foci and analgesia. The HNO is capable of presenting the $NO^{\bullet}$ effects, such as analgesia. In this sense, we firstly evaluated the analgesic effect of AS in overt pain-like behavior models [*p*-phenyl-bezoquinone (PBQ); acetic acid and formalin] for better understanding the analgesic mechanisms of HNO, in which the bacterial growth is not an influencing factor. These models induce a characteristic and quantifiable overt pain-like behavior described as abdominal contortions or paw flinches. The nociceptive response in these models were significantly reduced in a dose-dependent manner by subcutaneous pre-treatment with AS and the maximum effect observed was with 3 mg/kg dose. The inhibitory effect of AS was prevented by the pretreatment with ODQ, KT5823 and glibenclamide that are pharmacological inhibitors of the cGMP / PKG / ATP-sensitive K+ channels signaling pathway. Furthermore, the pretreatment with L-cysteine (a HNO scavenger) confirmed that the analgesic effect of AS depends on the HNO. Based on the effective analgesic dose of AS of 3 mg/kg, we observed the effect of HNO in a chronic model of septic arthritis induced by *S. aureus* intra-articular injection. The daily treatment with AS significantly inhibited the pain and the inflammatory response (edema, leukocyte recruitment, cytokine production, NF-kB activation and oxidative stress) resulting in the prevention of disease severity assessed by clinical score, joint proteoglycans content and osteoclastogenesis. The observed characteristics appear to be dependent on microbicide effect of HNO by directly inhibiting the growth of *S. aureus* and the CFU number in the knee joint. Because of the microbicide effect of HNO, the treatment with AS also inhibited a systemic inflammatory response due to local infection (leukocyte counts in the lungs and serum concentration of pro-inflammatory cytokines). Thereby, the present results suggest the therapeutic potential of AS or HNO donors in septic arthritis, and confirm that AS presents analgesic intrinsic effects independently of inhibiting infection.

Keywords: Nitroxyl. Septic Arthritis. *Staphylococcus aureus*. Pain. Inflammation.

LISTA DE ABREVIATURAS E SIGLAS

ATP	Adenosina Trifosfato
AP-1	Proteína Ativadora-1
Clf	Fator de Aglutinação
CP	Sorotipo Capsular
EDRF	Fator Relaxante Derivado do Endotélio
EROs	Espécies Reativas de Oxigênio
GCs	Guanilato Ciclase solúvel
GMPc	Guanosina Monofosfato cíclica
GSH	Glutationa Reduzida
GSNO	Nitrosoglutationa
HNO	Nitroxil
H_2O_2	Peróxido de Hidrogênio
H2S	Sulfeto de Hidrogênio
IAP	Proteína Associada à Integrina
IFN- γ	Interferon do tipo-γ
IL	Interleucina
DAMPs	Padrões Moleculares Associados ao Dano
MAPquinase	Proteíno-quinase Ativada por Mitógeno
MBC	Mínima Concentração Bacteriostática
MMP	Metaloproteinase
$Na_2N_2O_3$	Trioxidinitrato
NETs	Armadilhas Extracelulares de Neutrófilos
NF-κB	Fator de Transcrição Nuclear κB
NK	Células Natural Killer
NMDA	N- metil D- aspartato
NO	Óxido Nítrico
NO_2	Dióxido de Nitrogênio
NO_2^-	Nitrito
NO_3^-	Nitrato
N_2O_3	Trióxido de Nitrogênio
NOS	Óxido Nítrico Sintase
O_2^-	Ânion superóxido

OH	Radical hidroxila
$ONOO^-$	Peroxinitrito
OPG	Osteoprotegerina
PAMPs	Padrões Moleculares Associados ao Patógeno
PKG	Proteína Quinase G
PRs	Receptores de Reconhecimento Padrão
RANK	Receptor ativador de NF-κB
RANKL	Ligante do receptor ativador de NF-κB
S. aureus	*Staphylococcus aureus*
SOD	Superóxido Dismutase
TLR	Receptor do Tipo Toll
TNF-α	Fator de Necrose Tumoral-α
TSST-1	Toxina 1 da Síndrome do Choque Tóxico
VGEF	Fator do Crescimento Endotelial Vascular

SUMÁRIO

1 INTRODUÇÃO

1.1 ASPECTOS GERAIS DA INFLAMAÇÃO

A inflamação envolve vários fenômenos no nosso organismo que leva à uma sequência de eventos que podem ser desencadeados por diferentes estímulos (por ex. microrganismos, células necróticas, interações antígeno-anticorpo, agentes químicos, físicos ou térmicos, processos isquêmicos) (MEDZHITOV, 2008). O tipo e o grau da resposta inflamatória é dependente da natureza do agente agressor e de sua persistência (FREIRE; DYKE, 2000; MEDZHITOV, 2008). A representação macroscópica dos eventos coordenados que ocorrem durante a inflamação são os sinais clínicos conhecidos: eritema, calor, edema, dor e em algumas condições perda de função (revisado por FREIRE; DYKE, 2000).

A capacidade em desenvolver uma resposta inflamatória é essencialmente um mecanismo de defesa do organismo, cujo objetivo final é a remoção da causa da lesão e reparar, quando possível, suas consequências. Esse processo é acompanhado de vasodilatação das vênulas, acentuada permeabilidade dos capilares, aumento do fluxo sanguíneo e recrutamento leucocitário. No caso de invasão por microrganismos, a inflamação é fundamental para a eliminação e prevenção de uma infecção descontrolada (FREIRE; DYKE, 2000; MEDZHITOV, 2008). Sob condições normais, o processo inflamatório é terminado quando o agente lesivo é removido e todos os mediadores são dissipados ou inibidos fazendo com que o organismo retorne ao seu estado de homeostasia. Além disso, existem mecanismos anti-inflamatórios ativos que controlam a resposta e evitam que ela cause dano excessivo ao hospedeiro (FREIRE; DYKE, 2000; MEDZHITOV, 2008). Em contrapartida, em algumas situações e doenças, a resposta inflamatória pode ser exacerbada e persistente perdendo o caráter protetor (INSEL, 1996; MEDZHITOV, 2008).

Muitos tecidos e células estão envolvidos na resposta inflamatória, incluindo as células circulantes, células do tecido conjuntivo, da matriz extracelular e da membrana basal, fluídos e proteínas plasmáticas, mediadores químicos, (MEDZHITOV, 2008). No entanto, uma função crítica da inflamação é o encaminhamento de leucócitos para o local de inflamação, para que eles

desempenhem suas funções normais em defesa do hospedeiro. A sequência de eventos na jornada dos leucócitos do lúmen vascular para o interstício, conhecida por extravasamento, pode ser dividida em 3 etapas: 1. No lúmen: marginação, rolamento seguido de adesão firme do leucócito ao endotélio vascular. Eventos mediados por moléculas de adesão e seus respectivos ligantes; 2. A transmigração através do endotélio (também conhecida como diapedese); 3. Migração nos tecidos intersticiais em direção ao estímulo quimiotático onde se localiza o foco da lesão (revisado por KUMAR; ABBAS; FAUSTO, 2005; LEY et al., 2007).

A liberação de mediadores quimiotáticos como interleucina-1β (IL-1β), fator de necrose tumoral-α (TNF-α), C5a (componente do sistema complemento), prostanoides, fator de agregação plaquetário, histamina, quimiocinas (IL-8), leucotrienos (LTB_4) por células sentinelas , como por exemplo, macrófagos residentes e mastócitos, formam um gradiente de concentração dessas substâncias que é fundamental para o correto direcionamento dos leucócitos para o foco inflamatório (FREIRE; DYKE, 2000; revisado por KUMAR; ABBAS; FAUSTO, 2004). Cada leucócito responde a um estímulo quimiotático com diferentes taxas de velocidade. Como defensores primários, os neutrófilos aderidos respondem rapidamente à quimiotaxia e se dirigem para o foco da lesão (revisado por KUMAR; ABBAS; FAUSTO, 2004; LEY et al., 2007).

Uma vez nos tecidos, os neutrófilos em grande número tornam-se ativos por meio de cascatas de sinalização intracelulares desencadeadas pelo reconhecimento de moléculas específicas, conhecidas como padrões moleculares associados ao patógeno (PAMPs), expressas na superfície dos microorganismos (por ex. ácido lipoteicóico), ou ainda, os padrões moleculares associados ao dano (DAMPs), que podem ser componentes estruturais de células lesadas, como adenosina trifosfato (ATP). O reconhecimento dos PAMPs ou DAMPS ocorre por meio de receptores-sensores expressos pelos neutrófilos, assim como por outros leucócitos, conhecidos como receptores de reconhecimento padrão (PRRs) (FREIRE; DYKE, 2000; revisado por KUMAR; ABBAS; FAUSTO, 2005). Após a interação dos neutrófilos com seus estímulos agonistas, essas células rapidamente passam a liberar seus mecanismos microbicidas e pró-inflamatórios, dentre eles: fagocitose; produção de mediadores lipídicos; produção de óxido nítrico (NO), formação de espécies reativas de oxigênio (EROs) por uma “explosão respiratória” em que grande quantidade de oxigênio molecular consumido pela enzima NADPH

oxidase gera ânion superóxido (O_2^-), peróxido de hidrogênio (H_2O_2), radicais hidroxila (OH^-) e compostos halogenados; armadilhas extracelulares de neutrófilos (NETs); liberação de enzimas proteolíticas e transcrição de genes que resultam na produção e secreção de citocinas pró-inflamatórias [por ex. fator de transcrição- κB (NF- κB)] (NAUSEEF; BORREGAARD, 2014). À medida que a lesão amadurece, os neutrófilos se acumulam no tecido e iniciam um processo de morte programada (apoptose), sendo em seguida fagocitados por macrófagos (eferocitose), (PHILLIPSON; KUBES, 2011; FREITAS et al., 2012). Assim, o acúmulo inicial de neutrófilos é seguido por uma segunda onda de infiltração celular, surgindo progressivamente os eosinófilos, macrófagos e linfócitos que permanecem cerca de uma semana no local, isto é, se o agente lesivo for removido. (FREIRE; DYKE, 2000).

A regulação temporal da inflamação requer que os fenótipos celulares conservados e específicos para cada tipo de resposta sejam fundamentais para neutralizar o agente lesivo. No entanto, a falha na resolução da resposta inflamatória, ou a contínua ativação dos mecanismos inflamatórios, causam danos aos tecidos tornando-se lesões crônicas, conhecidas como doenças inflamatórias (FREIRE; DYKE, 2000). Essas condições merecem atenção especial, uma vez que além de lesões, podem gerar grande prejuízo para o paciente portador. Dessa maneira, o entendimento de mecanismos e moléculas envolvidos por meio de ensaios experimentais que recriem alguns elementos das condições inflamatórias crônicas, como a dor, contribuem como ferramentas de estudo para melhor compreender os mecanismos envolvidos e, o mais importante, propor novas abordagens terapêuticas.

1.2 Modelos de Dor Inflamatória

Diversos modelos animais de inflamação podem mimetizar condições inflamatórias crônicas em que a dor está envolvida. Esses modelos são importantes para o estudo de novos fármacos ou de novas terapias para o tratamento dos danos que envolvam a dor inflamatória, uma vez que, em muitos casos, as terapias existentes não são efetivas. Além disso, em algumas situações, as terapias analgésicas e anti-inflamatórias disponíveis na clínica são contraindicadas devido aos efeitos colaterais que provocam, como por exemplo, a

predisposição às infecções (PAVÃO-DE-SOUZA et al. 2012; STAURENGO-FERRARI et al., 2013).

De maneira geral, os modelos animais avaliam dois principais sintomas de dor: a) comportamentos do tipo dor manifesta ou b) hiperalgesia e a alodinia. Ambos resultam da complexa interação entre mediadores inflamatórios e neurônios nociceptivos, gerando a sensibilização dos nociceptores ou de maneira mais coloquial “receptores da dor”. Os mediadores inflamatórios, como as citocinas e prostaglandinas, liberados em hierarquia ativam os segundos mensageiros como a adenosina monofosfoato cíclica e as proteína quinases A e C que levam à a fosforilação dos canais iônicos da membrana neuronal. Como consequência dessa fosforilação, ocorre um aumento da corrente de influxo de íons cálcio e sódio e redução da corrente de efluxo de íons potássio. Consequentemente, há a diminuição do limiar do neurônio nociceptor (momento em que conseguimos detectar a “dor”) e aumento da excitabilidade da sua membrana neuronal, facilitando a ativação do nociceptor e a transmissão do impulso, resultando em hiperalgesia (VERRI et al., 2006).

Os modelos de dor manifesta são caracterizados por um estímulo inflamatório que induz um comportamento espontâneo como a sacudida do membro estimulado ou contorções abdominais em resposta ao agente flogístico injetado sem que haja estímulo mecânico ou térmico adicional. Esse comportamento declarado ocorre por meio da rápida produção de mediadores inflamatórios, como o TNF-α e IL-1β, que ativam os nociceptores em resposta ao estímulo nocivo. Os estímulos que geralmente são químicos (como a *ρ*-fenil-benzoquinona, ácido acético, formalina) ou biológicos (zimosan, CFA) (COLLIER et al., 1968; DUBUISSON; DENNIS, 1977; VERRI et al., 2006) são amplamente utilizados pela facilidade, replicabilidade, servindo como base de “*screening*” e determinação do mecanismo de ação de novas drogas (VERRI et al., 2006).

Em situações de hiperalgesia existe a necessidade de dispor de técnicas e equipamento específicos que atuam como estímulos adicionais, como a placa quente (hiperalgesia térmica) ou o analgesímetro, como a versão digital dos filamentos de Von Frey (hiperalgesia mecânica), uma vez que os estímulos *per se* não induzem comportamento doloroso espontâneo (VERRI et al., 2006; STAURENGO-FERRARI et al., 2013). A hiperalgesia é o denominador comum de algumas condições inflamatórias crônicas em que as alterações nos limiares

nociceptivos são detectadas, como no caso de artrites inflamatórias de origem infecciosa, como por *Staphylococcus aureus* (*S. aureus*), ou induzida por adjuvantes.

1.3 INFECÇÕES E ARTRITE

A colonização do corpo humano por extensa carga microbiana tem sido foco de pesquisas nas últimas décadas com fluxo constante para sua associação com o desenvolvimento da autoimunidade ou predisposição à infecções (MATHEW; RAVINDRAN, 2014). O papel dos microrganismos como vírus, bactérias, fungos e parasitas tanto na causa como na iniciação do processo inflamatório articular é complexo, mas tem sido extensamente apreciado (MATHEW; RAVINDRAN, 2014). Neste contexto, a organização mundial da saúde (OMS) em conjunto com o Conselho de Pesquisa de Artrite & Reumatismo, em 1974, classificaram em 4 grupos, os tipos de artrite relacionados às infecções (DUMOND, 1976):

1. **Grupo 1**: Neste grupo estão incluídas as artrites do tipo séptica ou infecciosa que são caracterizadas pela presença de microrganismos viáveis na articulação.
2. **Grupo 2:** Este grupo compreende os tipos de artrites pós-infecciosas com antígenos bacterianos detectados nas articulações, como em alguns casos de artrite reumatóide ou alguns tipos de artrites virais.
3. **Grupo 3:** Este grupo inclui as artrites reativas (ReA) com infecções originadas no sistema urogenital ou gastrointestinal, causando uma doença inflamatória articular, porém, o microrganismo não é detectado na articulação.
4. **Grupo 4:** Neste grupo estão as artrites inflamatórias desencadeadas por microrganismos, mas nem o microrganismo nem seu produto ou antígeno estão presentes nas articulações, como na doença de Lyme.

As variações dos tipos de artrite são geralmente determinadas tanto pelo microrganismo quanto pelos fatores atípicos do hospedeiro. Idade, genética, susceptibilidade, gênero, presença de co-morbidades e as condições articulares são

alguns dos fatores críticos. Quanto aos microrganismos é importante considerar os fatores virulência, habilidade desse microrganismo de produzir substâncias tóxicas, liberação dos produtos microbianos e tropismo favorável nos tecidos (CHANDRASEKARAN, 2006).

Dando enfoque na artrite do tipo infecciosa ou séptica, esse tipo de artrite acomete tipicamente articulações maiores como joelhos ou quadris, porém outras articulações podem ser afetadas (COLAVITE; SARTORI, 2014). A incidência dessa infecção geralmente é monoarticular, como em 75% dos pacientes. Não obstante, casos poliarticulares não são incomuns e a prevalência tem aumentado nos últimos anos, especialmente em pacientes que são mais predispostos ao desenvolvimento dessa (ESPINOZA; GARCIA, 2013). A ocorrência estimada da artrite séptica em países industrializados é cerca de 6 casos em 100.000 pessoas por ano, com maiores taxas sendo encontradas naqueles com idade inferior a 15 ou superior a 55 anos de idade (NADE, 2000). O risco mais importante a ser considerado na artrite séptica é a pré-existência de patologias articulares, especialmente nos pacientes com artrite reumatoide ou cirurgias prostéticas articulares. Nesses casos, além da lesão prévia da região articular que predispõe à colonização dos microrganismos, o tratamento com imunomoduladores, como Terapias anti-TNF (infliximab); ou imunossupressores, (corticoides), afeta a responsividade imunológica à infecção e limita o processo de eliminação do patógeno (TARKOWSKI, 2006). Nessas condições, a incidência de artrite séptica aumenta para 70 casos a cada 100.000 pessoas (KAANDORP, 1998).

A artrite séptica é geralmente considerada uma infecção secundária por escapes dos microrganismos da corrente sanguínea para os tecidos articulares. Estratégias do hospedeiro, como os anexos endoteliais, processos de transcitose e paracitose, e o transporte dos patógenos por fagócitos, estão envolvidos na tentativa de erradicar a infecção. No entanto, a ocorrência desses mecanismos proporciona a disseminação do agente infeccioso do sangue para as articulações ou outros tecidos (EDWARDS, 2011). Ocasionalmente, traumas como injeções intra-articulares, artroscopias e cirurgias para substituição das articulações também podem diretamente introduzir microrganismos nas cavidades articulares (ESPINOZA; GARCIA-VALLADARES, 2013).

Considerando que a sinóvia é uma estrutura altamente vascularizada e livre de barreiras de membrana, essa região é um ambiente

favorável para penetração e estabelecimento de diferentes microrganismos como bactérias, vírus, parasitas e fungos (MATHEW; RAVINDRAN, 2014). Todavia, *S. aureus* e *Neisseria gonorrhoea* são bactérias com forte predileção pelas cavidades articulares por aderirem facilmente ao tecido sinovial e por produzirem toxinas que proporcionam a colonização durante a bacteremia (SWITALSKI et al., 1993; HERRMANN et al., 1988).

1.3 ARTRITE SÉPTICA INDUZIDA POR *S. AUREUS*

1.3.1 Fatores de Virulência Estafilocócicos Relacionados à Artritogenicidade

S. aureus é uma bactéria gram-positiva com características oportunistas e de resiliência. Responsável por sérias infecções agudas e crônicas, *S. aureus* é o agente causal mais comum da artrite séptica tanto em crianças como em adultos, sendo responsável por 40% dos casos na Inglaterra e países de Gales, 56% dos casos na França, 37% dos casos na Austrália tropical (COLAVITE; SARTORI, 2014). Esse número aumenta para 80% em pacientes, que concomitantemente, apresentam artrite reumatóide, ou ainda, outras alterações metabólicas como o diabetes mellitus (GOLDENBERG, 1998).

A predominância de *S. aureus* como principal microrganismo causador da artrite séptica é atribuída a uma variedade de fatores de virulência que estão associados com a habilidade dessa bactéria em colonizar as articulações independentemente do seu efeito sobre a imunidade do hospedeiro (EDWARDS; MASSEY, 2011). Alguns desses elementos de virulência, entre eles as adesinas, permitem que a bactéria se fixe em certos tipos de tecidos iniciando a infecção. Dois principais tipos de adesinas foram descritos como responsáveis pela ancoragem de *S. aureus* nas articulações: os fatores de aglutinação A e B (ClfA e B) e as proteínas de ligação a fibronectina (FnBPA e B) (JOSEFSSON et al., 2001; COLAVITE; SARTORI, 2014). As adesinas FnBP A e B expressas por *S. aureus* reconhecem fibronectina, fibrinogênio e elastina (SINHA et al., 1999). Essas proteínas capacitam a aderência estafilocócica e permitem a invasão de diferentes tipos celulares presentes no tecido sinovial, como as células epiteliais, endoteliais, fibrolastos e osteoclastos. A adesão ocorre através da formação de uma ponte entre as proteínas de ligação à fibronectina da bactéria e os receptores da células do hospedeiro

(SINHA et al., 1999; MASSEY et al., 2001), providenciando um mecanismo de evasão estafilocócico das defesas do hospedeiro (COLAVITE; SARTORI, 2014). Ademais, adesinas de ligação ao colágeno também parecem estar envolvidas na patogenicidade de *S. aureus* durante a artrite séptica por meio da conexão de alta afinidade das células bacterianas à cartilagem (GILLASPY; PATTI; SMELTZER, 1997).

Mais recentemente, a capacidade de formação de biofilme foi considerada determinante nas infecções induzidas por *S. aureus* (FOSTER et al., 2013). Biofilmes são comunidades de células bacterianas presentes sobre uma superfície e presas às substâncias da matriz extracelular do hospedeiro. Essas comunidades parecem ter afinidade pelas estruturas articulares que servem como suporte e manutenção para a bactéria (BROOKS, 2012; COLAVITE; SARTORI, 2014).

A elevada virulência de *S. aureus* comparada a outros agentes infecciosos é, em parte, pelas estratégias de evasão apresentadas por este patógeno. Alguns destes mecanismos de evasão ocorrem pela expressão de uma cápsula extracelular, pela liberação de peptídeos formilados, ou ainda, pela produção de moléculas dotadas de propriedades superantigênicas. Todos esses mecanismos foram correlacionados com maior artritogenicidade (COLAVITE; SARTORI, 2014). Cápsulas extracelulares contém polissacarídeos, entre 11 serotipos capsulares reportados, os tipos 5 (CP5) e 8 (CP8) parecem ser os mais frequentes durante infecções. Usando mutantes para CP5 que não expressam a cápsula em comparação à cepa de CP5 normal, Nilsson e colaboradores (1997) demonstraram menor frequência dos sinais de artrite séptica induzida por *S. aureus* e também um desenvolvimento mais atenuado da doença (NILSSON et al., 1997). Diferentemente de células eucarióticas, bactérias iniciam a síntese proteica com resíduos dos aminoácidos fomil-metionina na porção final *N*-terminal, dando origem aos peptídeos formilados (KOZAK, 1999). É conhecido que peptídeos formilados são potentes quimioatraentes para neutrófilos como passo inicial na eliminação bacteriana. Com relação à artrite séptica, foi demonstrado que essas estruturas funcionam como importante fator de virulência na artrite estafilocócica, parcialmente por mediar o recrutamento de neutrófilos, os quais contribuem substancialmente para o dano articular (GJERSTSSON et al., 2012).

Em adição à esses mecanismos, *S. aureus* produzem e secretam

amplo número de enzimas e toxinas que estão implicadas na artrite séptica. Por exemplo, *S. aureus* produzem toxinas de dano à membrana: hemolisinas (α, β, γ, δ) e leucocidina. Todas essas toxinas danificam eritrócitos, mas diferem no seu mecanismo de ação. A combinação entre as hemolisinas do tipo α e γ promovem a virulência estafilócica na artrite (TARKOWSKI, 2006). Também é importante destacar que algumas toxinas estafilocócicas com propriedades superantigênicas são determinantes na artrite séptica experimental, tanto que o uso de anticorpos neutralizadores toxina 1 da síndrome do choque tóxico (TSST-1) afeta a capacidade de *S. aureus* em desencadear a artrite séptica (ABDELNOUR; BREMELL; TARKOWSKI, 1994). Mais especificamente, a neutralização por anticorpos monoclonais dos receptores vβ de células T que reconhecem a TSST-1 leva a uma regulação negativa da doença e prevenção da morte do hospedeiro induzida por *S. aureus* (ABDELNOUR et al., 1994).

Apesar do reconhecimento da multiplicidade de fatores de virulência, ainda estamos longe de identificar exatamente quais fatores são essenciais para infecção articular e quais podem ser alvos de novas terapias. Sendo assim, é necessário reconhecer a participação da resposta do hospedeiro na modulação da infecção.

1.3.2 Imunopatogênese da Artrite Séptica

A artrite séptica permanece como uma das mais graves doenças articulares devido a sua característica de rápida progressão. A mortalidade dos pacientes com artrite séptica é alta (10-25%). A maioria dos pacientes desenvolvem um declínio funcional articular e cerca de 50% desenvolvem disfunção permanente da articulação (GOLDENBERG, 1998; KAANDORP et al., 1997). Já é bem estabelecido que, em adição aos fatores de virulência, a resposta imunológica do hospedeiro contribui significativamente para o dano articular. Ao longo dos anos, os mecanismos imunológicos que causam a morbidade têm sido explorados e acredita-se que a intensa resposta do hospedeiro, ao invés da bactéria, possa assumir o papel de “vilão” (HENNINGSON et al., 2012). Assim, entender os mecanismos envolvidos na artrite séptica, bem como sugerir novas terapias para modulação destes mecanismos é de grande importância.

Articulações dolorosas, edemaciadas, eritematosas e com aumento

da temperatura local são sinais clínicos apresentados por pacientes com artrite séptica ou também observadas em modelos experimentais. Tais sinais são resultados da interação do microrganismo com o hospedeiro (ROWTON, 2013). Morfologicamente, as articulações afetadas mostram-se com uma hipertrofia e proliferação do tecido sinovial, proliferação vascular, infiltração de células inflamatórias, que precedem os eventos críticos que levam a morbidade do paciente com artrite séptica. Dentre eles: a erosão óssea, a destruição da cartilagem e a formação de *pannus* (tecido de granulação composto que passa a preencher a cavidade articular) (TARKOWSKI, 2006; COLAVITE; SARTORI, 2014).

O recrutamento leucocitário para o local de injúria celular é uma das etapas essenciais da defesa do organismo contra um agente agressor. Nos estágios iniciais dos processos inflamatórios, como na resposta contra bactérias, o leucócito predominante é o neutrófilo (PHILLIPSON; KUBES, 2011). De fato, os fluídos sinoviais na artrite séptica são quase exclusivamente neutrofílicos, mesmo nos períodos crônicos da doença (CHAKRAVARTI et al., 2009). A química dos mecanismos envolvidos na resposta pelos neutrófilos resulta na morte dos microrganismos, mas também na morte da própria célula. Entretanto, a perda dessa célula é compensada pela habilidade do organismo em produzir novos neutrófilos a uma taxa prodigiosa. Por outro lado, a capacidade de tê-los faz com que ocorra um acúmulo nos tecidos causando danos colaterais ao tecido circundante (SEGEL et al., 2011).

O papel crucial dos neutrófilos na artrite séptica foi demonstrado experimentalmente pela primeira vez por Verdrengh e Tarkowski (1997). Por meio da utilização do anticorpo monoclonal, RB6-8C5, observou-se que os camundongos tratados com o anticorpo apresentaram maior carga bacteriana no sangue e nos rins 24 e 48 horas após a inoculação de *S. aureus,* bem como maior frequência de artrite quando comparados aos controles. Refletindo a severidade da doença, os níveis de citocinas pró-inflamatórias como o TNF-α, IL-6 e o interferon do tipo-γ (IFN- γ) estavam maiores naqueles animais que tiveram seus neutrófilos depletados. Esse conjunto de dados foi coletado no período inicial da doença, demonstrando que os neutrófilos desempenham papel protetor crucial na fase inicial da infecção por *S. aureus* (VERDRENGH; TARKOWSKI, 1997). Em contrapartida, nos períodos crônicos da artrite séptica, uma proeminente característica da doença é a destruição da cartilagem causada pelo aglomerado de neutrófilos, que ainda, quimioatraem

outros leucócitos como os macrófagos ativados e linfócitos T (COLAVITE; SARTORI, 2014). Nesse sentido, o influxo de neutrófilos para as articulações representa um atrativo para o desenvolvimento de novas estratégias terapêuticas para o tratamento da artrite séptica.

Acredita-se que uma parte apical da resposta dos neutrófilos é iniciada por meio da interação entre PAMPs e PRRs, em especial os receptores do tipo Toll (TLR). Por meio dessa interação, os neutrófilos passam a decodificar a ameaça e adaptar o aparato antimicrobiano necessário. Um dos mais bem caracterizados TLR em infecções estafilocócica, assim como na artrite séptica, é o TLR2. Esse subtipo de receptor tem capacidade de reconhecer diferentes agonistas presentes em *S. aureus*, como por exemplo, o ácido lipoteicóico e o peptidoglicano (FOURNIER, 2013). O amplo repertório de reconhecimento do TLR2 é parcialmente devido à capacidade de formar heterodímeros com TLR1 ou TLR6, expandindo o espectro sem alterar as vias de sinalização tradicionais que levam à ativação de NF-κB, proteína ativadora-1 (AP-1) ou proteíno-quinases ativadas por mitógenos (MAP quinases) que promovem a transcrição de produtos pró-inflamatórios, como citocinas e metaloproteases (FARHAT et al., 2008). De fato, além de TLR2, os TLR1 e 6 são expressos em amostras de condrócitos de pacientes com artrite séptica e em conjunto promovem papel catabólico sobre a cartilagem por meio da ativação de NF-κB e expressão de IL-1β e IL-6 e MMP-13 (PAPATHANASIOU et al., 2010). Ademais, o TLR-2 também é responsável por iniciar a artropatia destrutiva envolvida na artrite séptica por meio da expressão do fator do crescimento endotelial vascular (VGEF), responsável pelo remodelamento tecidual na doença. A expressão do VGEF ocorre em resposta à ativação de AP-1/quinase regulada por sinal extracelular (ERK) (VAROGA et al., 2006).

Um dos mecanismos lesivos dos neutrófilos que são compartilhados por outros fagócitos pode ser via produção de EROs. Essas moléculas são carreadas pelos fagócitos durante um processo inflamatório. Em inflamações agudas e crônicas, o ânion superóxido, peróxido de hidrogênio e peroxinitrito são produzidos a uma taxa que supera a capacidade defensiva endógena dos sistemas enzimáticos ou não enzimáticos, como a superóxido dismutase (SOD) e glutationa reduzida (GSH) em removê-los, resultando em injúria mediada por radicais e num processo conhecido como estresse oxidativo (SALVEMINE; DOYLE; CUZZOCREA, 2006). Essas moléculas liberadas no citosol ou mesmo no ambiente extracelular alteram o

estado “redox” da célula e oxidam componentes celulares, alterando sua função (MACDONALD; GALLEY; WEBSTER, 2003). O aumento de EROs tem sido bem documentado nas articulações sinoviais de pacientes com artrite inflamatória. O superóxido, em particular, degradada os tecidos cartilaginoso e ósseo (CUZZOCREA, 2006). Em adição, o peroxinitrito, gerado pela reação entre óxido nítrico (NO) e ânion superóxido, diminui a funcionalidade dos subtipos de SOD na cartilagem osteoarticular podendo comprometer as defesas antioxidantes (SALVEMINI; DOYLE; CUZZOCREA, 2006). Na artrite infecciosa experimental índices elevados de peroxidação lipídica dos tecidos articulares foram acompanhados de alteração do estado antioxidante e lesão tecidual. Essa condição foi mensurada nos termos de depleção dos níveis de GSH e da atividade das enzimas SOD e catalase (MAL et al., 2012). Assim, baseados no conceito de remoção do superóxido para a modulação do curso de inflamação e na restauração do balanço redox na artrite séptica, o tratamento combinado com antibióticos e antioxidantes parece eficaz na eliminação do microrganismo, restabelecimento do balanço oxidativo e melhora de sequelas das infecções (MAL et al., 2012; MAL et al., 2013).

Entre as moléculas que agem de maneira sinérgica ou aditiva aos radicais livres destacam-se as citocinas. Esses mediadores, como TNF-α, IL-1β e IL-6, primariamente produzidos por macrófagos residentes e após o reconhecimento da bactéria, por neutrófilos, são os maiores responsáveis pela inflamação grave na artrite séptica (COLAVITE; SARTORI, 2014). De maneira importante, essas citocinas trabalham em colaboração induzindo e mantendo o recrutamento ativo de neutrófilos e suas funções. Como consequência, ocorre a perda do conteúdo de proteoglicanos/cartilagem e destruição óssea, que são os eventos responsáveis pela morbidade do paciente com artrite séptica (CHAKRAVARTI et al., 2009). Além de trabalhar em conjunto com a NAPDH oxidase, o TNF-α induz a atividade excessiva de células de reabsorção óssea, osteoclastos, num processo que está intimamente ligado com a destruição e perda dos ossos da articulação. O TNF-α, considerada a maior citocina osteoclastogênica, ativa o NF-kB e promove a sobrevivência dos osteoclastos. Além disso, em conjunto com outras citocinas como IL-6, IL-15, IL-17, IL-18, promove a ativação do sistema: ligante do receptor ativador de NF-κB (RANKL)/ receptor ativador de NF-κB (RANK)/ osteoprotegerina (OPG) (KWAN et al., 2004). Esse sistema é a via final comum através dos quais muito dos fatores

osteotrópicos modulam seus efeitos sobre os ossos (KWAN et al., 2004; HENNINGSSON et al., 2012).

Além de neutrófilos, células natural-killer (NK) e linfócitos parecem estar envolvidos na patogênese da artrite séptica, porém pouco se conhece sobre a participação dessas células. Com relação aos linfócitos, os linfócitos B parecem não contribuir para o curso da infecção da artrite séptica (GJERTSSON et al., 2000). Em contraste, as células T, principalmente do fenótipo $CD4^+$, são detectadas nas articulações de camundongos infectados com *S. aureus* (ABDELNOUR et al., 1994). Nesse sentido, pesquisadores demonstraram as subpopulações de linfócitos T envolvidas na artrite séptica e o duplo papel dessas células. Por exemplo, a administração de IFN-γ antes ou depois da inoculação de *S. aureus* reduz a mortalidade mas eleva o desenvolvimento dos sinais de artrite por acentuar o padrão de resposta Th1 (ZHAO; NILSSON; TARKOWSKI, 1998). Por outro lado, camundongos deficientes para IL-12, que é uma citocina responsável por manter o padrão Th1, apesar de não apresentarem sinais de piora da artrite séptica, sucumbem à infecção pela ausência da produção de IFN-γ e da resposta Th1 (HULTGREN; STENSON; TARKOWSKI, 2000), ou mesmo, pela ausência da produção de NO, um fator importante para erradicar *S. aureus* na artrite séptica (McINNES et al., 1998).

No caso do padrão Th17, o papel diferencial da IL-17 também é notado nos diferentes modelos utilizados. Oposto ao modelo de artrite por infecção sistêmica por *S. aureus*, os camundongos deficientes para IL-17 têm maior sinovite, erosão e diminuída depuração de bactérias, sugerindo que a IL-17 é mais relevante na defesa local da infecção do que na resposta sistêmica para prevenir a piora da infecção e consequentemente da lesão articular (HENNINGSSON et al., 2010).

Apesar do número crescente de mediadores envolvidos na artrite séptica, as ações destes ocorrem de modo coordenado. Como resultado, terapias que vem sendo sugeridas e direcionadas contra alguns mediadores devem ser mais eficazes do que outras para que melhore a inflamação sem comprometer a resposta do hospedeiro frente à infecção (HENNINGSSON et al., 2010).

1.3.3 Abordagens Terapêuticas na Artrite Séptica

Até o momento, as terapias disponíveis são limitadas em tratar a

infecção por promover a eliminação bacteriana e ao mesmo tempo limitar a resposta do hospedeiro para prevenir a destruição articular (HENNINGSSON et al., 2010). Sob essa perspectiva, a velocidade e precisão da terapia para artrite séptica são fundamentais para controlar o agravamento da doença.

Na ocorrência de suspeita de artrite séptica, uma amostra sanguínea e aspiração do líquido sinovial articular devem ser coletados para dar início à administração de antibióticos (NADE, 2003). No entanto, antes mesmo do resultado dos procedimentos de rotina estarem disponíveis, é imperativo iniciar o tratamento com antibióticos de amplo espectro. Adicionalmente, existe um consenso de que o tratamento deve incluir a concomitante remoção de qualquer material purulento para alívio de sintomas, como a dor (COAKLEY et al., 2006).

Mesmo sendo bem estabelecido que a eliminação da bactéria é essencial para controlar a artrite séptica, somente a antibioticoterapia não é suficiente para impedir o processo de destruição articular. Nesse sentido foi descrito que mesmo após o completo tratamento com antimicrobianos, pacientes portadores de artrite séptica recuperam apenas por volta de 46-50% da sua original função articular. Isso ocorre como resultado da local destruição gerada pela resposta imune contra a bactéria e pela presença de fragmentos bacterianos que permanecem na articulação e são capazes de manter a resposta inflamatória articular (ALI et al., 2015). Essa percepção levou a associação de antibióticos com substâncias capazes de neutralizar essa resposta imunológica exagerada como os glicocorticóides ou terapias anti-TNF-α (ODIO et al., 2003; ALI et al., 2015). No entanto, o caráter imunossupressor dessas drogas, a longo prazo, pode perpetuar um ciclo destrutivo entre infecção e resposta inflamatória culminando com a permanente disfunção articular. Assim, identificar moléculas com papel duplo, ou seja, com caráter bactericida e ao mesmo tempo com propriedades anti-inflamatórias é essencial para o tratamento da artrite séptica.

1.4. NITROXIL

Nas últimas décadas, os efeitos biológicos do NO e seus congêneres têm sido intensamente pesquisados. Muito desse interesse provém da descoberta da geração de $NO^{\bullet}$ (sem carga), e do seu importante papel em diferentes condições fisiológicas, incluindo a homeostase vascular, agregação plaquetária,

angiogênese, inflamação, modulação de infecções e estados de dor (FURCHGOTT; ZAWADSKI, 1980; FURLONG et al., 1987; BENJAMIM et al., 2000; CURY et al., 2011). Considerando que os sistemas biológicos são oxidantes, a maior parte da literatura tem focado nos efeitos patofisiológicos de seus produtos oxidados, como o peroxinitrito ($ONOO^-$), nitrito (NO_2^-), nitrato (NO_3^-), dióxido de nitrogênio (NO_2) e trióxido de dinitrogênio (N_2O_3). Por outro lado, os estados reduzidos, como o nitroxil (HNO/NO^-), até recentemente eram objetos de pouca atenção (DONZELLI et al., 2008). Sob condições biológicas, as espécies reduzidas do $NO^{\bullet}$ são protonadas (HNO; nitroxil) (IRVINE et al., 2008; STAURENGO-FERRARI et al., 2014).

O interesse por essa molécula quimicamente distinta do $NO^{\bullet}$ foi renovado com evidências de que o HNO pode ser produzido de forma endógena, segue uma via de sinalização inteiramente separada e apresenta efeitos únicos que não são induzidos pelo $NO^{\bullet}$ (FUKUTO et al., 2005, IRVINE et al., 2008). Assim, além da diferença estrutural, o $NO^{\bullet}$ e HNO também tem atividades biológicas endógenas diferentes confirmando que são entidades diferentes (ANDREWS et al., 2009)

Com relação à produção endógena do HNO, acredita-se que possa ocorrer por meio de vias distintas. Estudos bioquímicos têm demonstrado que o ânion nitroxil pode ser formado pela ação da enzima óxido nítrico sintase (NOS) (HOBBS et al., 1994; SCHMIDT et al., 1996; RUSCHE et al., 1998), particularmente, na ausência do cofator requerido pela NOS, a tetraidrobiopterina, ou após a oxidação de intermediários produzidos pela NOS, como a Nω-hidroxi-L-arginina (FUKUTO et al., 1992; PUFAHL et al., 1995) e hidroxilamina (DONZELLI et al., 2008). Além disso, o ânion nitroxil pode ser formado por fontes independentes da NOS, por meio da redução do $NO^{\bullet}$ pelo citocromo c mitocondrial (SHARPE; COOPER, 1998), pela xantina oxidase (SALEEM; OHSHIMA, 2004), hemoglobina (GOW; STAMLER, 1998), por meio da superóxido dismutase (SOD) (FUKUTO et al., 2005), ou ainda, em pH fisiológico, pelas reações de S-nitrosotióis com outras espécies de tióis [como o sulfeto de hidrogênio (H_2S) ou nitrosoglutationa (GSNO)] (ARNELLE; STAMLER, 1995; WONG et al., 1998). Essas evidências de biossíntese do HNO são sustentadas pelo desenvolvimento de métodos que permitem a detecção em alcances de variações sensíveis (JOHNSON et al., 2014).

Uma importante reação do nitroxil é sua dimerização, uma propriedade que exclui a conveniente e direta acessibilidade do HNO para estudos das propriedades bioquímicas e farmacológicas do nitroxil (JOHNSON et al., 2014;

JOROLAN, et al., 2015). Portanto, muito dos estudos que demonstram a biologia do HNO requer o uso de moléculas doadoras (JOHNSON et al., 2014). O mais bem estudado, estabelecido e caracterizado doador de HNO é o trioxidinitrato de sódio ou sal de Angeli ($Na_2N_2O_3$ ou Angeli's salt) (BONNER; RAVID, 1975). Este sal inorgânico é solúvel em base e libera espontaneamente HNO com uma taxa constante de 4,6 x 10^{-4}/segundo entre pH 4-8 (BONNER; RAVID, 1975). Em pH ácido, o Sal de Angeli pode ser tonar doador de $NO^{\bullet}$, possivelmente pela protonação da região não básica, resultando em um mecanismo de decomposição diferente (DUTTON; FUKUTO; HOUK, 2004). No entanto, em condições fisiológicas cerca de 98% do produto de decomposição do Sal de Angeli é o HNO (ZELLER et al., 2009).

Similarmente ao $NO^{\bullet}$, o HNO exerce seus efeitos biológicos por interagir principalmente com a enzima guanilato ciclase solúvel (GCs) e assim gerar o segundo mensageiro guanilato monofosfato cíclico cíclico (GMPc) (IRVINE et al., 2008). Algumas vertentes vêm sendo propostas para explicar a interação HNO-GCs-HNO. Por exemplo, em situações de estresse oxidativo, pela ativação da SOD, o HNO pode ser oxidado a $NO^{\bullet}$ e interagir com a GCs para gerar GMPc (ZELLER et al., 2009). Porém, esse processo oxidativo não deve ocorrer em microambientes inflamatórios em que a atividade da SOD é limitada devido à sobrecarga de ânion superóxido, como em modelos de inflamação induzido pela carragenina (ZARPELON et al., 2013). Ademais, apenas 1-2% das moléculas doadas pelo sal de Angeli se decompõe à $NO^{\bullet}$ e para que essa liberação ocorra é necessária a presença da glutationa reduzida (GSH), cujos níveis caem durante a inflamação. Nesse sentido, a possível relevância do primeiro mecanismo é limitada. Uma outra explicação (mecanismo independente do $NO^{\bullet}$) sugere que o nitroxil pode ser diretamente ativado pela forma oxidada GCs, a qual é insensível ao $NO^{\bullet}$ (IRVINE et al., 2008;), Essa explicação considera que os efeitos do HNO não são explicados pela simples conversão a $NO^{\bullet}$, pois sabe-se que o HNO em artérias do mesentério de ratos estimula canais de K^+ dependentes de voltagem (Kv) enquanto o $NO^{\bullet}$, no mesmo leito vascular, promove ativação de canais de K+ ativados por cálcio (KCa) (MISTRY; GARLAND, 1998; SAMPSON et al., 2001; IRVINE et al., 2008;), ou seja, são moléculas diferentes com ações, e em algumas situações, independentes.

Outros efeitos farmacológicos do HNO também têm sido observados em diferentes modelos e doenças como crescimento tumoral e angiogênese (NORRIS et al., 2008; BASUDHAR et al., 2013), superação da insuficiência cardíaca

(SABBAH et al., 2013), alcoolismo (DEMASTER et al., 1998) e modelos de isquemia-reperfusão (PAGLIARO et al., 2003; JOROLAN, et al., 2015). Além disso, há dois outros estudos que evidenciam a participação do nitroxil no SNC por modular atividade do receptor NMDA. Kim et al. (1999) demonstraram, em cultura de células corticais de ratos, que o HNO modifica o resíduo de cisteína na subunidade do receptor NMDA, resultando na redução do influxo de íons cálcio e, consequentemente, protegendo os neurônios contra a excitotoxicidade induzida pelo NMDA (agonista de receptores glutamatérgicos tipo NMDA). Todavia, Colton (2001) verificou que o HNO potencializa a corrente elétrica induzida pelo glutamato em células HEK293 transfectadas com receptores NMDA recombinante de rato. Esses resultados conflitantes podem, em parte, ser explicados pela diferença no tipo de célula e abordagem experimental utilizada.

Mesmo compartilhando algumas propriedades com o $NO^{\bullet}$, o HNO possui vantagens terapêuticas sobre o $NO^{\bullet}$ principalmente por não desenvolver tolerância pelo uso contínuo (IRVINE et al., 2008). Outro fator importante é que aparentemente doadores de HNO como o sal de Angeli atuam independente da formação de peroxinitrito diferentemente de doadores de $NO^{\bullet}$ e desempenham atividade antioxidante por meio da doação do seu átomo de hidrogênio ou por meio da ativação da enzima heme-oxigenase (NAUGHTON et al., 2002).

Sob o contexto acima e considerando que as propriedades versáteis do nitroxil facilitam seu uso como agente farmacológico, recentes estudos conduzidos em nosso laboratório demonstraram que o doador de HNO, sal de Angeli, apresenta efeito analgésico em modelos de dor inflamatória induzida por diferentes estímulos (carragenina, lipopolissacarídeo, formalina, prostaglandina E_2, citocinas. Esse efeito analgésico do HNO é independente do $NO^{\bullet}$, pois o tratamento com L-cisteína, "*um sequestrador*" de HNO, sobre os animais que receberam estímulo inflamatório reverte o efeito analgésico do sal de Angeli, confirmando a especificidade do doador (ZARPELON et al., 2013). Um outro aspecto importante do efeito analgésico do HNO é que seu efeito é da inibição da produção produção de TNF-α e IL-1β e ativação da via GMPc/ proteína quinase G (PKG)/canais de potássio ATP sensíveis (ZARPELON et al., 2013). Esse é um importante mecanismo analgésico periférico, ativado, por exemplo, pela morfina e anti-inflamatórios não esteroidais [ex. inibidores da ciclooxigenase (COX) diclofenaco], que são os fármacos de escolha na maioria das condições dolorosas (CUNHA et al., 2010;

CURY et al., 2011).

A utilização da morfina ou mesmo do diclofenaco é limitado pelo desenvolvimento de tolerância dependente da ativação dos receptores opióides ou pelo desenvolvimento de efeitos gastrintestinais (STAURENGO-FERRARI et al., 2013). Dessa maneira, a ativação das mesmas vias de sinalização envolvidas no mecanismo de ação dessas drogas pelo HNO mas sem a inibição da COX ou evitando a ativação de receptores opióides na indução da analgesia, fazem do HNO uma abordagem analgésica promissora por não induzir efeitos colaterais. Contudo, ainda não se sabe o efeito do nitroxil em modelos de dor e infecção crônica como na artrite séptica.

Considerando que os quadros de artrite séptica merecem atenção especial, uma vez que além da lesão articular podem ocorrer alterações de comportamento, sociais e econômicas, que resultam em grande prejuízo para o paciente e que o HNO, assim como outros óxidos de nitrogênio, possa ter características microbicidas é cabível sugerir que o tratamento com o Sal de Angeli seja eficaz na artrite séptica, atuando em duas vertentes: a) efeito microbicida direto sobre a bactéria e consequentemente redução da inflamação por uma menor ativação das células fagocíticas devido a menor carga bacteriana e b) pelas propriedades analgésica e anti-inflamatória isoladamente independente da proliferação bacteriana.

2 OBJETIVOS

Avaliar se o tratamento com o sal de Angeli (um doador de nitroxil) reduz o processo infeccioso inflamatório articular no modelo de artrite séptica induzido por *S. aureus.*

2.1 OBJETIVOS ESPECÍFICOS

Avaliar se o doador de nitroxil, Sal de Angeli: 1) reduz a dor por ativação da via GMPc/PKG/canais de K+ ATP-sensíveis (p.ex. utilizando co-tratamentos com inibidores dessa via) em modelos de dor manifesta em que a multiplicação bacteriana não é um interferente; 2) reduz o processo inflamatório por mecanismos relacionados à diminuição da produção de citocinas quimioatraentes e expressão de fatores oxidantes; 3) Induz a expressão das enzimas antioxidantes, heme-oxigenase-1 e glutationa redutase; 4) reduz a lesão articular 5) apresenta efeito bactericida direto sobre *S. aureus* determinado pela mínima concentração bacteriostática (MBC) e consequentemente uma diminuição da detecção de bactérias na articulação; 5) previne a disseminação da infecção sistêmica nos camundongos com artrite séptica.

3 CONCLUSÕES GERAIS

No presente trabalho estamos propondo avanços quanto à abordagem terapêutica em modelo de artrite séptica induzida por *S. aureus* em camundongos. Com os resultados alcançados, podemos concluir:

- O tratamento com o doador de HNO, sal de Angeli, é eficaz na redução dos comportamentos de dor manifesta induzida por diferentes estímulos algésicos. Esse efeito foi confirmado pelo tratamento com o sequestrador de HNO, L-cisteína que preveniu o efeito analgésico do doador. Ademais, o mecanismo ativado para induzir a analgesia é dependente da via cGMPC/PKG/ canais de K^+_{ATP}-sensíveis. Importantemente, os efeitos do HNO são intrínsecos independentemente da inibição da infecção.
- O tratamento diário com o sal de Angeli, iniciado após a inoculação bacteriana, reduz a inflamação articular no modelo de artrite séptica. Especificamente, houve uma redução hiperalgesia mecânica, edema, recrutamento leucocitário local e melhora dos sinais clínicos da artrite séptica. Os efeitos inibitórios foram mais pronuciados a partir do 7º dia após a infecção e mantidos até a fase crônica da doença (28º dia).
- Como consequência da redução da inflamação, principalmente do recrutamento de neutrófilos, os camundongos tratados com sal de Angeli apresentaram uma redução da perda do conteúdo de proteoglicanos e uma menor expressão da via osteoclastogênica sistema RANKL/RANK/OPG, que são os marcadores de lesão de cartilagem e óssea, respectivamente. Esses parâmetros avaliados são responsáveis pela morbidade na artrite séptica.
- O tratamento com sal de Angeli reduz a produção de TNF-α, IL-1β, IL-33, IL-17 e IFN-γ que são mediadores envolvidos na resposta contra *S. aureus*, e ao passo que reduz a produção dessas citocinas reduz a ativação de NF-kB no tecido articular dos camundongos com artrite séptica.
- Em adição à redução da resposta inflamatória, os animais tratados com sal de Angeli apresentaram uma redução nos parâmetros de estresse oxidativo que parecem estar associados com a inibição da expressão das enzimas NADPH oxidase e ciclooxigenase. Simultaneamente, o HNO também induziu a expressão de enzimas antioxidantes, como a heme-oxigenase e glutationa redutase, na tentativa do

estabelecimento do balanço “redox” do ambiente inflamatório.

- O HNO tem propriedade bactericida por diretamente inibir o crescimento de *S. aureus “in vitro”* ao passo que reduz o número de UFC nas articulações. Nesse sentido, os efeitos anti-inflamatórios obtidos pelo tratramento com o sal de angeli é também pelo efeito microbicida do HNO.
- O tratamento com o sal de Angeli, previne o desenvolvimento de uma resposta inflamatória sistêmica (contagem de leucócitos pulmões e concetração séria de citocinas pró-inflamatórias) justamente pelo efeito bactericida sobre *S. aureus.*
- Os dados obtidos acrescentam uma nova perspectiva sobre o tratamento para artrite séptica com o HNO por mecanismos que envolvem efeitos microbicidas, ações anti-inflamatórias, ações antioxidantes e redução da gravidade da doença.

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ANEXOS

ANEXO A

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Nitroxyl inhibits overt pain-like behavior in mice: Role of cGMP/PKG/ATP-sensitive potassium channel signaling pathway

Larissa Staurengo-Ferrari [a], Ana C. Zarpelon [a], Daniela T. Longhi-Balbinot [a], Mario Marchesi [b], Thiago M. Cunha [c], José C. Alves-Filho [c], Fernando Q. Cunha [c], Sergio H. Ferreira [c], Rubia Casagrande [d], Katrina M. Miranda [b], Waldiceu A. Verri Jr [a,*]

[a] Departamento de Patologia, Centro de Ciências Biológicas, Universidade Estadual de Londrina, Londrina, Brazil
[b] Department of Chemistry and Biochemistry, University of Arizona, Tucson, USA
[c] Department of Pharmacology, Ribeirão Preto Medical School, University of São Paulo, São Paulo, Brazil
[d] Department of Pharmaceutical Sciences, University Hospital (Health Science Centre), Londrina State University, Parana, Brazil

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ABSTRACT

Background: Several lines of evidence have indicated that nitric oxide (NO) plays complex and diverse roles in modulation of pain/analgesia. However, the roles of charged and uncharged congeners of NO are less well understood. In the present study, the antinociceptive effect of the nitroxyl (HNO) donor, Angeli's salt ($Na_2N_2O_3$; AS) was investigated in models of overt pain-like behavior. Moreover, whether the antinociceptive effect of nitroxyl was dependent on the activation of cGMP (cyclic guanosine monophosphate)/PKG (protein kinase G)/ATP-sensitive potassium channels was addressed.
Methods: The antinociceptive effect of AS was evaluated on phenyl-p-benzoquinone (PBQ)- and acetic acid-induced writhings and *via* the formalin test. In addition, pharmacological treatments targeting guanylate cyclase (ODQ), PKG (KT5923) and ATP-sensitive potassium channel (glybenclamide) were used.
Results: PBQ and acetic acid induced significant writhing responses over 20 min. The nociceptive response in these models were significantly reduced in a dose-dependent manner by subcutaneous pre-treatment with AS. Furthermore, AS also inhibited both phases of the formalin test. Subsequently, the inhibitory effect of AS in writhing and flinching responses were prevented by ODQ, KT5823 and glybenclamide, although these inhibitors alone did not alter the writhing score. Furthermore, pretreatment with L-cysteine, an HNO scavenger, confirmed that the antinociceptive effect of AS depends on HNO.
Conclusion: The present study demonstrates the efficacy of a nitroxyl donor and its analgesic mechanisms in overt pain-like behavior by activating the cGMP/PKG/ATP-sensitive potassium channel (K^+) signaling pathway.

Introduction

Nitric oxide (NO) is a gaseous substance that acts primarily as the endothelium-derived relaxing factor (EDRF) [1]. Nevertheless, NO also acts as a neuronal messenger in the central and peripheral nervous systems and is involved in various biological events such as synaptic plasticity and neurotransmission [2,3], vascular homeostasis, platelet aggregation, inflammation, angiogenesis, fibrinolysis [4], pain and antinociception [5]. NO is formed from L-arginine by action of three primary isoforms of NO synthase: neuronal (nNOS), endothelial (eNOS) and inducible (iNOS) [5–8]. The effects of NO are categorized as dependent or independent on activation of soluble guanylate cyclase (sGC), which subsequently induces the increase of the second messenger cyclic guanosine monophosphate (cGMP) signaling pathway [4].

Several lines of evidence have shown a dual role of NO in the nociceptive system. In fact, NO can either induce or reduce pain depending on the nociceptive system level (central or periphery) and the amount of NO [9,10]. Concerning the antinociceptive effect, use of NO donors has shown that NO induces analgesia [11,12]. NO also mediates the peripheral and central antinociceptive effect of analgesic compounds, such as opioids, some non-steroidal anti-inflammatory drugs (NSAIDs) such as diclofenac [13,14], dipyrone [15], natural products [16], statins [17], cannabinoids, the antiepileptic gabapentin, adenosine, PPAR-c agonists, the α2-adrenoceptor agonist xylazine, hormones such as

* Corresponding author.
E-mail addresses: waverri@uel.br, waldiceujr@yahoo.com.br (W.A. Verri Jr).

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estradiol and melatonin, bovine lactoferrin, the anesthetic gas nitrous oxide isosorbide dinitrate spray, hydrogen sulfide releasing drugs and phosphodiesterase inhibitors [5]. Importantly, these analgesic drugs are capable of blocking ongoing hyperalgesia. These data indicate that NO is likely the common denominator for the mode of action of peripheral analgesic drugs that directly block ongoing nociceptor sensitization. Therefore, the relevance of the antinociceptive effect of NO is already established. Furthermore, the antinociceptive effect of NO depends on formation of guanylate cyclase-derived cGMP [10], leading to the activation of cGMP-dependent protein kinase (PKG), which phosphorylates ATP-sensitive potassium channels, thereby diminishing nociceptive information transmission [9,15,18]

Until recently, most of the biological effects of NO have been attributed directly to NO itself. However, NO can be both oxidized (NO^+) and reduced (NO^-). Under physiological conditions, the species one-electron reduced from NO is protonated (HNO; nitroxyl) [19]. Unlike $NO^•$, HNO has yet to be shown to be endogenously biosynthesized. However, numerous chemical reactions have been described to suggest pathways for production of HNO under biological conditions [20]. For example, biochemical studies show that nitroxyl can be formed directly from NO synthase [21], particularly in the absence of tetrahydrobiopterin [21] or *via* oxidation of the NOS intermediates N^G-hydroxy-L-arginine [21,22] or hydroxylamine [23]. On the other hand, nitroxyl can also be formed *via* reduction of $NO^•$ by mitochondrial cytochrome c, xanthine oxidase, ubiquinol, hemoglobin or manganese superoxide dismutase (SOD) [19,21].

Nitroxyl donors displays vasorelaxant and cardioprotective effects in experimental models [19,24], which are mediated predominantly by activation of sGC, production of intracellular cGMP and consequently leading to interaction with voltage-gated potassium channels [25]. Importantly, *in vitro* organ bath and contractility studies demonstrated that nitroxyl has a prominent role as an EDRF in mesenteric arteries. Additionally, nitroxyl functions as a hyperpolarizing agent in resistance arteries [26]. Together, this suggests the importance of nitroxyl as a signaling molecule and HNO donors as pharmacological tools. The best known and well-studied nitroxyl donor is trioxodinitrate ($Na_2N_2O_3$) or Angeli's salt (AS) [27], which has been pivotal in the analysis of the biology/pharmacology of nitroxyl.

Recently, our laboratory demonstrated that AS inhibits mechanical hyperalgesia in rats *via* activation of sGC/cGMP/PKG/ATP-sensitive potassium channel signaling pathway and inhibition of cytokine production [28]. Distinct from mechanical hyperalgesia or thermal external stimuli, models of inflammatory overt pain are characterized by an almost immediate behavior such as abdominal contortions (writhing) and paw flinch and/or licking. These behaviors occur through direct or indirect (*via* release of inflammatory mediators) activation of the primary nociceptive neurons by chemical (phenyl-p-benzoquinone – PBQ; acetic acid; formalin) and biological (zymosan; CFA) stimuli [29–31].

In the present study, we investigated whether the Angeli's salt reduces the overt pain-like behavior induced by PBQ, acetic acid or formalin and if its antinociceptive mechanism depends on the activation of the cGMP/PKG/ATP-sensitive potassium channels signaling pathway.

Materials and methods

Chemicals

The following materials were obtained from the sources indicated: acetic acid from Mallinckrodt Baker, S.A (Mexico City, Mexico); glybenclamide, KT5823 (2,3,9,10,11,12-hexahydro-10R-methoxy-2,9-dimethyl-1-oxo-9S,12R-epoxy-1H diindolo [1,2,3-fg:3′,2′, 1′-kl] pyrrol [3,4-i][1,6]benzodiazocine-10-carboxylic acid, methyl ester) and L-cysteine were obtained from Sigma–Aldrich (St. Louis, MO, USA). ODQ (1H-[1,2,4]oxadiazolo[4,3-a]quinoxalin-1-one) was obtained from Calbiochem (San Diego, CA, USA). Angeli's salt ($Na_2N_2O_3$, sodium trioxodinitrate) was synthesized and utilized as previously described [32] The stability of stock solutions prepared in 10 mM NaOH and stored at −20 °C, was determined from the extinction coefficients at 250 nm (ε of 8000 M^{-1} cm^{-1} for Angeli's salt) [33]. The selected doses of drugs were chosen based on pilot studies and previous data of our laboratory. We detected that the doses of drugs used do not alter the nociceptive response per se, and inhibit the respective standard stimulus [16,18,34–37]

Animals

Male Swiss mice (25–30 g) from the Universidade Estadual de Londrina, Paraná, Brazil, were used in this study. Mice were housed in standard clear plastic cages with free access to food and water and a light/dark cycle of 12:12 h and kept at 21 °C. Different investigators prepared the solutions, treated the mice, injected the stimulus and quantified the nociceptive behaviors. All behavioral testing was performed between 9 am and 5 pm in a temperature controlled room. Animal care and handling procedures were in accordance with the International Association for the Study of Pain (IASP) guidelines and with the approval of the Ethics Committee of the Universidade Estadual de Londrina, which follows the National Council of Science of Animal Research. All efforts were made to minimize the number of animals used and their suffering.

Experimental protocols

Mice were treated with Angeli's salt (0.03–10 mg/kg/*sc*, diluted in 10 mM NaOH) 40 min before *ip* injection of PBQ, acetic acid or formalin. In another sets of experiments, the mice were pre-treated with the inhibitors ODQ (0.1–1 mg/kg/*ip*, diluted in saline, 30 min), KT5823 (0.17 or 0.5 μg/mouse/*ip*, diluted in 5% DMSO in saline, 5 min), glybenclamide (0.03–1 mg/kg/*po*, diluted in 20% Tween 80 in saline, 45 min) before treatment with Angeli's salt (3 mg/kg/*sc*, diluted in 10 mM NaOH) with the exception that only one dose of ODQ (1 mg/kg), KT5823 (0.5 μg/mouse) or glybenclamide (1 mg/kg) was tested in the formalin model. After an additional 40 min, mice received *ip* injection of PBQ, acetic acid or formalin. As control groups, mice received ODQ (1 mg/kg), KT5823 (0.5 μg/mouse) or glybenclamide (1 mg/kg) before PBQ, acetic acid and formalin injection. In another set of experiments, mice were pre-treated with the HNO scavenger (L-cysteine, 0.3–1 mg/kg, *ip*, diluted in saline, 30 min), before treatment with Angeli's salt (3 mg/kg/*sc*, diluted in 10 mM NaOH) with the exception that only one dose of L-cysteine (1 mg/kg) was tested in the PBQ acetic acid or formalin models. After an additional 40 min mice received injection of PBQ, acetic acid or formalin stimulus.

Writhing response tests

The PBQ [38] or acetic acid [29]-induced writhing model was performed as previously described [34,35]. PBQ diluted in 2% DMSO/saline (1890 μg/kg), acetic acid (0.8% v/v, saline, 10 ml/kg) or vehicle was injected into the mouse peritoneal cavity. After PBQ or acetic acid *ip* injection, each mouse was placed in a large glass cylinder and the intensity of nociceptive behavior was quantified by counting the total number of writhes occurring between 0 and 20 min after stimulus injection. The writhing response consists of a contraction of the abdominal muscle together with a stretching of

hind limbs. The intensity of the writhing response was expressed as the cumulative writhing score over 20 min.

Formalin test

The number of paw flinches were determined between 0 and 30 min after intraplantar (*ipl*) injection of 25 μl of 2.5% formalin, as previously [30]. The period was divided in intervals of 5 min and clearly demonstrated the presence of the first and second phases, which are characteristic of the method [16,30,39]. Results were obtained for both the first (0–5 min) and second (10–30 min) phases.

Statistical analyses

Statistical analyses were performed using GraphPad Prism 4.0 (La Jolla, 5 CA). Results are presented as means ± SEM of two independent experiments. The *n* in the legends refers to the number of mice per group in each experiment. The differences between the experimental groups were compared by one-way ANOVA and individual comparisons were subsequently made by Tukey's post hoc test. The level of significance was set at $P < 0.05$ and are represented by symbols (*; **, # or *f*; please see legends) in case of significance, otherwise a non-significant statistical difference was indicated.

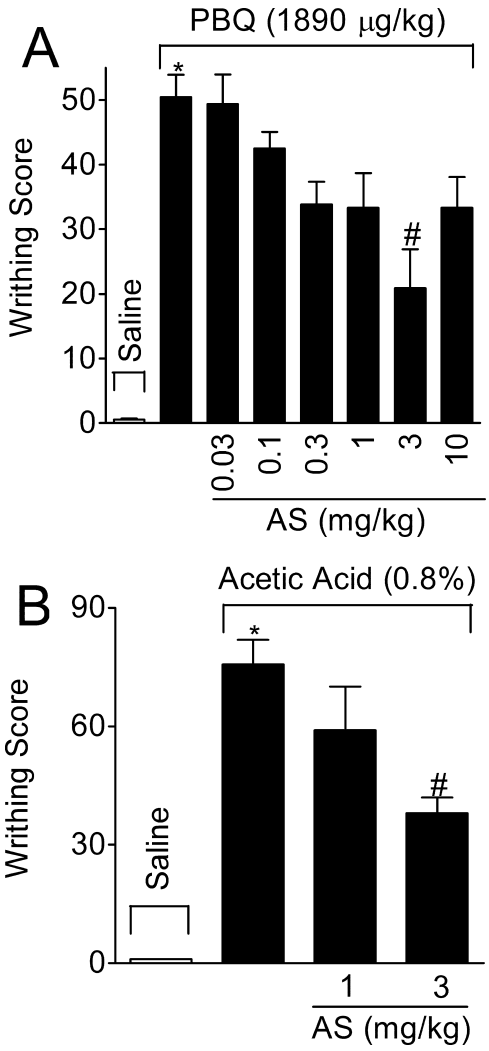

Fig. 1. Angeli's salt inhibits the writhing response. Mice were treated with AS (0.03–10 mg/kg, *sc.*, 150 μl) or vehicle (0.20 μl of NaOH 10 mM plus saline to complete 150 μl) 40 min before phenyl-*p*-benzoquinone (PBQ, 1890 μg/kg, Panel A) or acetic acid (0.8%, 10 ml/kg, Panel B) injection. The writhing response was evaluated over 20 min. $^{*}p < 0.05$ compared to the saline group (Panels A and B); $^{\#}p < 0.05$ compared to the PBQ or acetic acid group. One-way ANOVA followed by Tukey's test ($n = 6$).

Results

Angeli's salt inhibits the phenyl-p-benzoquinone or acetic acid-induced writhing response

Mice were treated with AS (0.03–10 mg/kg or 1–3 mg/kg, *sc*), or vehicle (19.35 μl of 10 mM NaOH plus saline to complete 150 μl) 40 min before PBQ or acetic acid *ip* injection, respectively. The writhing response was determined over 20 min after stimulus injection (Fig. 1). AS inhibited the PBQ-(Fig. 1A) or acetic acid-(Fig. 1B) induced writhing response. In the PBQ test, a U shaped curve was observed with a larger effect observed 3 mg/kg AS, but without difference with the lower doses of AS. The doses between

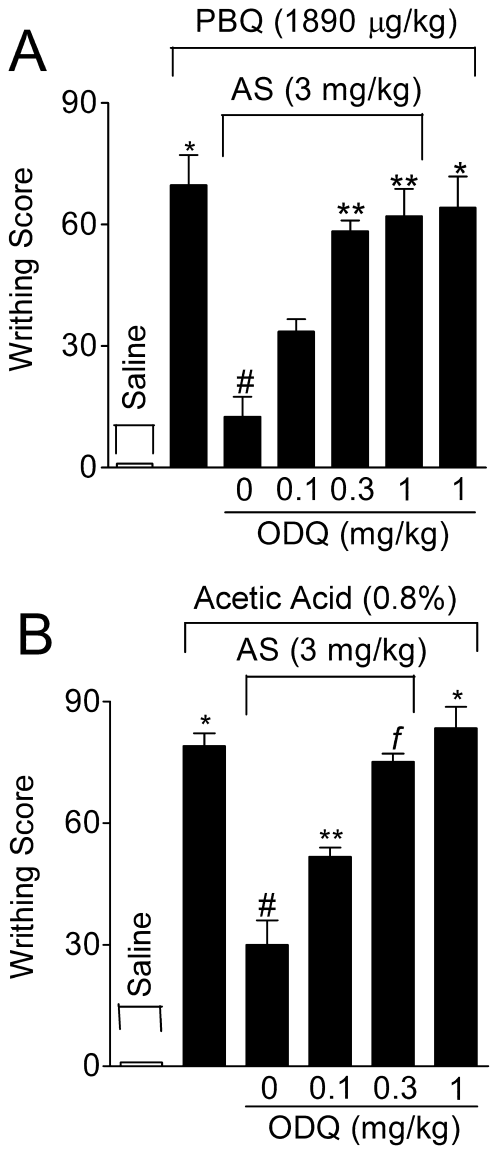

Fig. 2. Role of soluble guanylate cyclase in nitroxyl-induced antinociception. Mice were pre-treated with ODQ (0.1–1.0 mg/kg, *ip*, 200 μl) 30 min before AS (3 mg/kg, *sc*, 150 μl) or vehicle (1.075 μl of NaOH 10 mM plus saline to complete 200 μl) treatment. A group received ODQ (1 mg/kg, *ip*) without AS treatment. After additional 40 min all of the animals received the PBQ (Panel A) or acetic acid (Panel B) *ip* injection. The number of writhing was determined over 20 min. $^{*}p < 0.05$ compared to saline group; $^{\#}p < 0.05$ compared to PBQ or acetic acid group; $^{**}p < 0.05$ compared to AS group and $^{f}p < 0.05$ compared to AS group and dose of 0.1 mg/kg of ODQ (Panel B). One-way ANOVA followed by Tukey's test ($n = 6$).

0.03 and 1 mg/kg and 10 mg/kg of AS did not significantly reduce the PBQ-induced writhing response (Fig. 1A). In the acetic acid test, the dose of AS of 1 mg/kg did not reduce the nociception, and significant antinociception was observed only with a 3 mg/kg dose of AS (Fig. 1B). Thus, a dose of AS of 3 mg/kg was selected for the next experiments.

Pre-treatment with ODQ, an inhibitor of soluble guanylate cyclase, reverses the antinociceptive effect of AS in PBQ- and acetic acid-induced writhing response in mice

Mice were pre-treated with ODQ (0.1–1 mg/kg, *ip* - an inhibitor of soluble guanylate cyclase) or vehicle (saline) 30 min before administration of AS (3 mg/kg, *sc*) and after additional 40 min mice received *ip* stimulus with PBQ (Fig. 2A) or acetic acid (Fig. 2B). ODQ inhibited in a dose-dependent manner the antinociceptive effect of AS in the PBQ-induced writhing response (Fig. 2A). The dose of ODQ of 0.1 mg/kg was ineffective while the doses of 0.3 and 1 mg/kg prevented the antinociceptive effect of AS. In the acetic acid model, the dose of ODQ of 0.1 mg/kg present significant inhibition of the antinociceptive effect of AS, and the inhibition by ODQ was even increased with the dose of 0.3 mg/kg (Fig. 2B). ODQ (1 mg/kg, *ip*) alone did not affect the writhing response induced by PBQ or acetic acid (Fig. 2A and B). These results show that the antinociceptive effect of nitroxyl depends on activation of soluble guanylate cyclase.

Pre-treatment with KT5823, a selective inhibitor of cGMP-dependent protein kinase (PKG), reverses the antinociceptive effect of AS in PBQ and acetic acid-induced writhing response in mice

Mice were pre-treated with KT5823 (0.17 or 0.5 μg/mouse – an inhibitor of PKG) or vehicle (5% DMSO in saline) 30 min before administration of AS (3 mg/kg, *sc*) and after additional 40 min mice received *ip* stimulus with PBQ (Fig. 3A) or acetic acid (Fig. 3B).

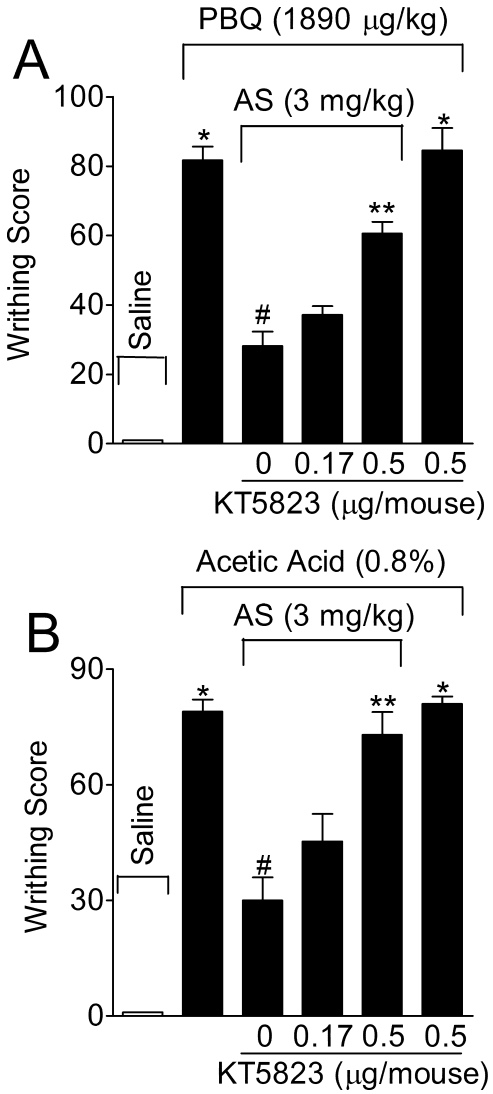

Fig. 3. Role of cGMP-dependent protein kinase (PKG) in Angeli's salt-induced-antinociception. Mice were pre-treated with KT5823 (PKG inhibitor, 0.17 or 0.5 μg/mouse, *ip*, 200 μl) 5 min before AS (3 mg/kg, *sc*, 150 μl) or vehicle (as in Fig. 2) treatment. A group received KT5823 (0.5 μg/mouse, *ip*) without AS treatment. After an additional 40 min all of the animals received the PBQ (Panel A) or acetic acid (Panel B) *ip* injection. The number of writhing was determined over 20 min. $^{*}p < 0.05$ compared to saline group; $^{\#}p < 0.05$ compared to PBQ group or acetic acid group; $^{**}p < 0.05$ compared to AS group. One-way ANOVA followed by Tukey's test ($n = 6$).

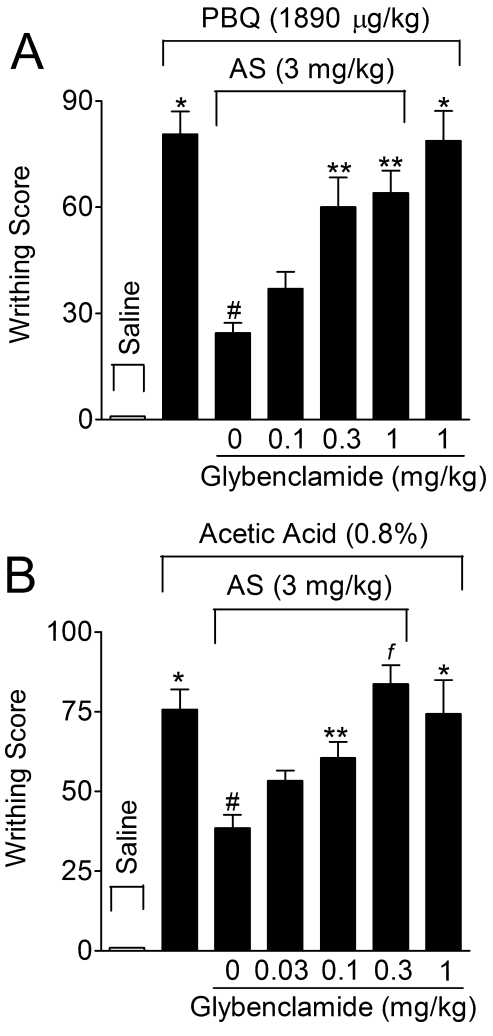

Fig. 4. Role of ATP-sensitive potassium channels in Angeli's salt induced-antinociception. Mice were pre-treated with glybenclamide (0.03–1 mg/kg, *po*, 150 μl) 45 min before AS (3 mg/kg, *sc*, 150 μl) or vehicle (as in Fig. 2) treatment. A group received glybenclamide (1 mg/kg, *ip*) without AS treatment. After an additional 40 min all of the animals received the PBQ (Panel A) or acetic acid (Panel B) *ip* injection. The number of writhing was determined over 20 min. $^{*}p < 0.05$ compared to saline group; $^{\#}p < 0.05$ compared to PBQ or acetic acid group; $^{**}p < 0.05$ compared to AS group, and $^{f}p < 0.05$ compared to AS group plus glybenclamide at dose of 0.1 mg/kg (Panel B). One-way ANOVA followed by Tukey's test ($n = 6$).

There was a tendency of reduction of the antinociceptive effect of AS by the treatment with KT5823 at the dose of 0.17 μg/mouse and significant inhibition with the dose of 0.5 μg/mouse in the PBQ (Fig. 3A) and acetic acid (Fig. 3B) models. The treatment with KT5823 (0.5 μg/mouse, *ip*) did not affect the nociceptive response induced by PBQ and acetic acid per se (Fig. 3A and B, respectively). These data show that the antinociceptive effect of nitroxyl depends on activation of PKG.

Pre-treatment with glybenclamide, an inhibitor of ATP-sensitive potassium channels, prevents the antinociceptive effect of nitroxyl in the PBQ or acetic acid-induced writhing response in mice

Mice were pre-treated with glybenclamide (0.03–1.0 mg/kg, *po* – an inhibitor of ATP-sensitive potassium channels) or vehicle (20% Tween 80 in saline) 45 min before administration of AS (3 mg/kg, *sc*) and after additional 40 min mice received *ip* stimulus with PBQ (Fig. 4A) or acetic acid (Fig. 4B). The dose of glybenclamide of 0.1 mg/kg induced a tendency of reduction of the antinociceptive effect of AS, but statistically significant inhibition was observed only with doses of glybenclamide of 0.3 and 1 mg/kg in the PBQ model (Fig. 4A). In the acetic acid model, 0.1 and 0.3 mg/kg doses of glybenclamide significantly inhibited the antinociceptive effect of AS, and a dose of 0.3 mg/kg completely reversed the antinociceptive effect of AS with statistical significance compared to 0.1 mg/kg (Fig. 4B). A 0.03 mg/kg dose of glybenclamide did not inhibit the antinociceptive effect of AS in the acetic acid model. Glybenclamide per se (1 mg/kg, *po*) did not affect the writhing response induced by PBQ (Fig. 4A) or acetic acid (Fig. 4B). These results show that the antinociceptive effect of nitroxyl depends on opening of ATP-sensitive potassium channels.

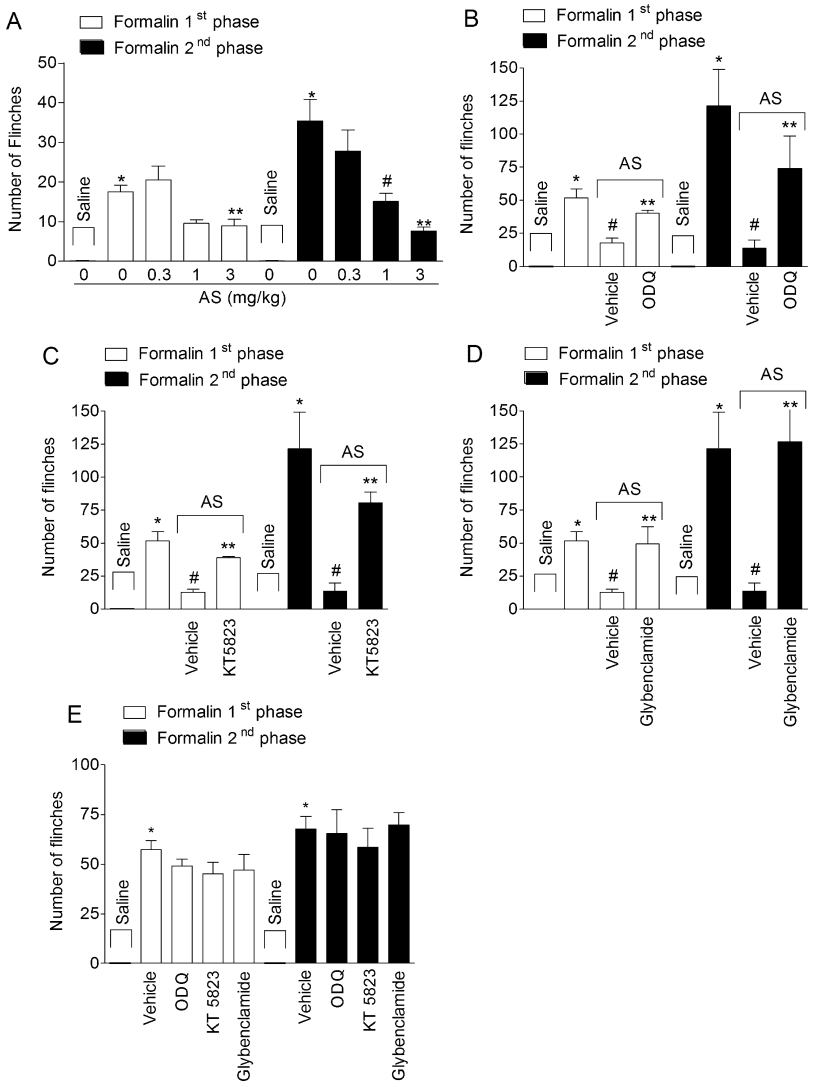

Fig. 5. Nitroxyl inhibits formalin-induced overt pain-like behavior by activating the cGMP/PKG/ATP-sensitive potassium channel pathway. Panel A: mice were treated with AS (0.3–3 mg/kg, *sc*, 40 min) or vehicle (as in Fig. 2) before intraplantar (*ipl*) stimulus with formalin 2.5% (25 μl). Panel B, C and D (respectively): mice were treated with ODQ (1 mg/kg, 30 min), KT5823 (0.5 μg/mouse, 5 min) or glybenclamide (1 mg/kg, 45 min) before Angeli's salt (AS; 3 mg/kg) or vehicle (as in Fig. 2) treatment. Panel E: mice received ODQ (1 mg/kg, *ip*), KT5823 (0.5 μg/mouse, *ip*), glybenclamide (1 mg/kg, *po*) or KT5823 (0.5 μg/mouse, *ip*) without AS treatment. After, mice received formalin 2.5% (25 μl, *ipl*) stimulus. The intensity of overt pain-like behavior was evaluated by the number of flinches within 5 min (first phase) and between 10–30 min (second phase) after formalin injection. $^{*}p < 0.05$ compared with the saline group, $^{\#}p < 0.05$ compared with AS group. $^{**}p < 0.05$ compared with lower AS dose (0.3 mg/kg; Panel A) or AS + vehicle treatment (Panels B-D). One-way ANOVA followed by Tukey's t test ($n = 7$).

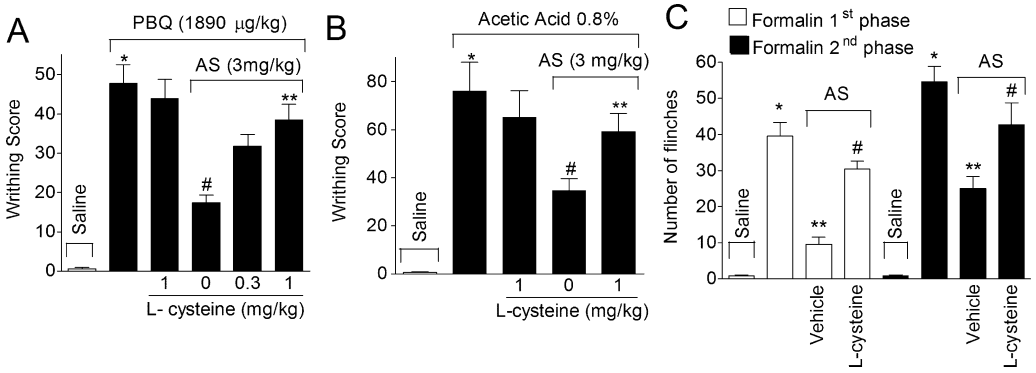

Fig. 6. L-cysteine prevents AS induced-antinociception. Mice were pre-treated with L-cysteine (0.3–1 mg/kg, *ip*, 200 μl) 30 min before Angeli's salt (AS, 3 mg/kg, *sc*, 150 μl) or vehicle (as in Fig. 2) treatment, and after an additional 40 min mice received PBQ (Panel A), acetic acid (Panel B) or formalin stimulus (Panel C). A group received L-cysteine (1 mg/kg) without AS treatment. The number of writhing was determined over 20 min and the number of flinches was determined over 30 min. $^{*}p < 0.05$ compared to saline group; $^{\#}p < 0.05$ compared to PBQ, acetic acid group or formalin group and $^{**}p < 0.05$ compared to AS group. One-way ANOVA followed by Tukey's test ($n = 6$).

Angeli's salt reduces formalin-induced overt pain-like behavior by activating the cGMP/PKG/ATP-sensitive potassium channels signaling pathway

Firstly, mice were treated with AS (0.3–3 mg/kg) or vehicle (19, 35 μl of 10 mM NaOH plus saline to complete 150 μl) 40 min before formalin (2.5%, *ipl*) administration (Fig. 5A). AS dose-dependently inhibited the first and second phases of formalin-induced overt pain-like behavior. In the first phase, a 0.3 mg/kg dose of AS had no effect while 1 mg/kg presented a tendency of inhibition although not significant. A dose of 3 mg/kg AS significantly inhibited formalin nociception compared to vehicle group and the lower dose of AS (0.3 mg/kg) (Fig. 5A). In the second phase of the formalin test, an AS dose of 0.3 mg/kg had no effect again, nevertheless, in this phase a dose of 1 mg/kg significantly inhibited the nociceptive response induced by formalin, and the inhibition by 3 mg/kg of AS was again significant compared to vehicle and 0.3 mg/kg AS. Therefore, a dose of 3 mg/kg AS was selected for the next experiments in which mice were treated with ODQ (1 mg/kg, *ip*, 30 min, Fig. 5B), KT5823 (0.5 μg/mouse, *ip*, 5 min, Fig. 5C) or glybenclamide (1 mg/kg, *po*, 45 min, Fig. 5D) before AS (3 mg/kg) or vehicle (1.6125 μl of 10 mM NaOH plus saline to complete 150 μl) treatment. After an additional 40 min, mice received formalin (2.5%, 25 μl) stimulus. All inhibitors (ODQ, KT5823 and glybenclamide) reversed AS antinociception in the formalin-induce flinch response in the first and second phases (Fig. 5B–D). These results indicate that AS presents the same antinociceptive mechanism in the PBQ or acetic acid writhing test and in the flinch response induced by formalin. The inhibitors ODQ (1 mg/kg), KT5823 (0.5 μg/mouse) and glybenclamide (1 mg/kg) did not affect the nociceptive response induced by formalin (Fig. 5E) [28,39].

Pre-treatment with L-cysteine, an HNO scavenger, prevents the antinociceptive effect of Angeli's salt in the overt pain-like behavior induced by PBQ, acetic acid or formalin

Mice were pre-treated with L-cysteine (0.3–1 mg/kg, *ip*) or vehicle (saline) 30 min before administration of AS (3 mg/kg, *sc*), and after an additional 40 min mice received PBQ (Fig. 6A), acetic acid (Fig. 6B) or formalin stimulus (Fig. 6C). There was tendency of reduction of AS antinociceptive effect in the PBQ model by treatment with L-cysteine at a dose of 0.3 mg/Kg while 1 mg/kg L-cysteine significantly inhibited the antinociceptive effect of AS over PBQ-induced writhings (Fig. 6A). Therefore, a dose of 1 mg/kg L-cysteine was selected for further experiments (Fig. 6B and C). L-cysteine inhibited the antinociceptive effect of AS in the acetic acid and formalin models. Furthermore, L-cysteine (1 mg/kg) did not affect PBQ, acetic acid or formalin-induced overt pain (Fig. 6A–C).

Discussion

The biological roles of nitroxyl have not been explored to a similar extent as of NO. The HNO donor Angelis Salt (AS) has been pivotal in the discovery of the biology/pharmacology of HNO [40]. In the present study, we demonstrated that AS reduced the overt pain-like behavior induced by phenyl-*p*-benzoquinone, acetic acid or formalin by triggering the cGMP/PKG/ATP-sensitive potassium channel antinociceptive signaling pathway in mice. The analgesic effect of AS was amenable by treatment with the HNO scavenger L-cysteine.

The acetic acid and PBQ models have similarities in their nociceptive mechanisms since both induce the release of many endogenous inflammatory mediators as prostanoids and cytokines such as TNF-α and IL-1β [34,35,41]. Acetic acid or PBQ intraperitoneal injection causes a stereotypical behavior in mice, which is characterized by abdominal contractions together with a stretching of the hind limbs [34,35,42]. In the formalin model, the overt pain-like behavior is characterized by first (0–5 min) and second (10–30 min) phases. The first phase involves histamine and serotonin release/action on nociceptors [16,43]. On the other hand, the second phase depends on inflammatory mediators produced in response to formalin stimulus comprising cytokines [44]. Hence, it is possible that nitroxyl inhibits mechanisms that are shared by the PBQ, acetic acid and formalin models such as cytokine production, since AS inhibited carrageenin-induced TNFα and IL-1β production [28].

The antinociceptive effect of AS in overt pain-like behavior models depends on activation of the cGMP/PKG/ATP-sensitive potassium channel pathway. These results line up well with the data on mechanical hyperalgesia in which AS also activated the cGMP/PKG/ATP-sensitive potassium channel pathway [28]. Furthermore, nitroxyl is capable of activating sGC to produce cGMP [19,45–47] and there are three possible lines of explanation for this activity. The first is that nitroxyl activates sGC *via* NO$^{\bullet}$ [19]. However, although it has been demonstrated that AS decomposes to NO$^{\bullet}$ in endothelial cells culture in the presence of superoxide dismutase [48], there is reduction of superoxide dismutase activity in inflammation [49] suggesting an inadequate environment for AS decomposition to NO$^{\bullet}$. Furthermore, AS decomposes to only 1–2% of NO$^{\bullet}$ comparing to 98–99% of HNO [48]. Therefore, these data [19,48,49] reduce the possible relevance of this first possible mechanism of nitroxyl activation of sGC. The second possibility suggests that nitroxyl could directly activate sGC by an NO$^{\bullet}$-independent mechanism [19]. Nitroxyl and NO$^{\bullet}$ present different

activities in *ex vivo* pharmacological and functional studies in isolated organ baths. Further supporting that $NO^{\bullet}$ and nitroxyl are relatively independent molecules, and that the effects of nitroxyl are not explained solely by simple conversion to $NO^{\bullet}$, nitroxyl is known for activating voltage-dependent K^+ (K_v) channels [25] in the same vascular bed that instead, $NO^{\bullet}$ activates calcium-activated K^+ channels (K_{Ca}) [50]. The third line of evidence demonstrated in anaerobic *in vitro* system that the HNO donors AS and NCTFA (1-nitrosocyclohexyl trifluoro acetate) inhibit isolated bovine lung sGC activity in the presence of $NO^{\bullet}$ scavenging compounds by interacting with the ferrous heme of sGC [50]. On the other hand, AS also inhibited sGC possibly by modifying enzyme cysteine thiol groups. Nevertheless, it is noteworthy to mention that AS achieved maximal activation of sGC at 10 μM while inhibition of sGC was observed at the supra-physiological concentration of 100 μM [50], indicating that such high doses are not recommended for inhibition of sGC studies with nitroxyl and that nitroxyl might present opposing roles depending on its concentration. Therefore, these data corroborate that AS activates sGC by an $NO^{\bullet}$-independent mechanism to reduce nociception.

In addition to these three lines of evidence and the present data demonstrating that the HNO scavenger L-cysteine inhibits the antinociceptive effect of AS, we have demonstrated that AS-induced DAF-2DA fluorescence in dorsal root ganglia neurons was sensitive to L-cysteine, which further corroborates AS is delivering nitroxyl to neurons [28]. It is important to mention that similar tools (ODQ, KT5823 and glybenclamide) were used in previous studies demonstrating the anti-hyperalgesic effect and mechanism of NO [9,15,35,39,51] and those inhibitors did not affect the nociceptive behavior induced by PBQ, acetic acid or formalin per se.

In addition to the therapeutic potential present herein, nitroxyl seems to offer considerable advantages over traditional nitrovasodilators (e.g., the NO donor DEA/NO) since tolerance to AS was not observed in rat isolated aortae [46]. Furthermore, nitroxyl presents a similar degree of antinociceptive action to NO donors such as SNP, SNAP or SIN-1 and drugs that act by an NO-dependent mechanism including morphine [5,9]. A possible disadvantage to be investigated is that several studies demonstrated the involvement of $NO^{\bullet}$ in the development of opioid tolerance in models of pain [52]. It remains to be determined whether nitroxyl is also involved in the development of morphine tolerance and further investigate the potential pharmacological applications of HNO and NO^-.

Concluding, we demonstrated that the nitroxyl donor Angeli's salt inhibits overt pain-like behavior by triggering the antinociceptive cGMP/PKG/ATP-sensitive potassium channel signaling pathway *in vivo*.

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Conflict of interest

There is no conflict of interest to declare.

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ANEXO B

Artigo científico a ser submetido na revista Free Radicals Biology and Medicine.

The nitroxyl donor Angeli's salt inhibits *Staphylococcus aureus*-induced septic arthritis in mice.

Larissa Staurengo-Ferrari[a], Kenji W. Ruiz-Miyazawa[a]; Sandra S. Mizokami[a], Talita P. Domiciano[a], Felipe A. Pinho-Ribeiro[a], Victor Fattori[a], Jacinta S. Pelayo[b], Rubia Casagrande[c], Katrina M. Miranda[d], Waldiceu A. Verri Jr[a,*].

[a]Departamento de Patologia, Centro de Ciências Biológicas, Universidade Estadual de Londrina, Rod. Celso Garcia Cid KM480 PR445, CEP 86057-970, Cx Postal 10011, Londrina, Paraná, Brazil

[b]Departamento de Microbiologia, Centro de Ciências Biológicas, Universidade Estadual de Londrina, Rod. Celso Garcia Cid KM480 PR445, CEP 86057-970, Cx Postal 10011, Londrina, Paraná, Brazil

[c]Department of Pharmaceutical Sciences, Healthy Sciences Centre, Londrina State University, Av. Robert Koch, 60, CEP 86038-350 Londrina, Parana, Brazil

[d]Department of Chemistry and Biochemistry, University of Arizona, Tucson, AZ 85721, USA

***Author for correspondence:** Prof. Waldiceu A. Verri Jr, PhD.Present address: Departamento de Patologia, Universidade Estadual de Londrina, Rod. Celso Garcia Cid KM480 PR445, CEP 86057-970, Cx Postal 10011, Londrina, Paraná, Brazil. Tel: + 55 43 3371 4979. Fax: + 55 43 3371 4387. E-mails: waverri@uel.br or waldiceujr@yahoo.com.br

Highlights

- AS inhibited *S. aureus*-induced inflammatory pain and articular inflammation in mice
- AS inhibited the *S. aureus*-induced cartilage damage and osteoclastogenesis
- AS inhibited *S. aureus*- induced of wide range of cytokines and NF-κB activation
- AS inhibited the *S. aureus*-induced oxidative estress
- AS played microbicidal effect against *S. aureus* and prevents systemic inflammatory response

Abstract

Septic arthritis is a severe and rapidly debilitating disease associated with severe joint pain, inflammation and oxidative stress. Nitroxyl (HNO) has become a nitrogen oxide of significant interest due to its pharmacological endpoints that are potentially favorable for treating of a variety of diseases. However, whether HNO also serves as bactericidal molecule to threat infectious diseases is currently unknown. The aim of this study was to investigate the effect of the HNO donor, Angeli's salt (AS), in the outcome of chronic *Staphylococcus aureus (S.aureus)*-induced septic arthritis in mice. Daily treatment with AS inhibited the mechanical hyperalgesia and the inflammatory responses (edema, leukocyte migration, cytokines release and NF-κB activation, and oxidative stress) resulting in preventing disease severity (clinical score, proteoglycan levels in the joints and osteoclastogenesis). In addition, AS decreased the number of *S. aureus* colony forming unities in synovial tissue and inhibited the worsening of systemic inflammatory response (leukocyte counts in the lung and systemic proinflammatory cytokine concentration). Our results suggest for the first time the therapeutic potential of AS in a model of septic arthritis by mechanisms involving microbicidal effects, anti-inflammatory actions and reduction of disease severity.

Keywords Nitroxyl, Angeli’s Salt, septic arthritis, pain, leukocytes, cytokines, oxidative stress, nitric oxide.

Introduction

Septic arthritis is a rapidly progressive and highly destructive joint disease in humans and is still a major medical challenge because of its high prevalence and poor prognosis regarding to joint functions[1]. The fatality rate for septic arthritis is high (5-15%) and permanent loss of joint functions due to cartilage damage and bone erosion develops in 25-50% of patients[2]. *Staphylococcus aureus* (*S. aureus*) is the major cause of all types of infectious arthritis. Patients with rheumatoid arthritis are susceptible to septic arthritis as result of immunosuppressive treatments, joint implant and the disease per se[3]. In this context, it is important to search for novel therapies for autoimmune diseases that do not damp the host innate response predisposing to septic arthritis.

The inflammatory response of the joint synovial membrane in the septic arthritis is conveyed by influx and activation of immune cells, and release of inflammatory mediators resulting in tissue destruction and dysfunction, leading to significant morbidity[1]. Neutrophils are highly motile phagocytic cells that constitute the first-line of innate immune system defense against infection[4] and they are abundant in the joints of septic arthritis patients during the course of the disease [5]. Neutrophils contribute to the local production of inflammatory mediators and to inflict joint damage, perpetuating the inflammatory response by recruiting macrophages and monocytes that are followed by T cells[5]. In fact, both Th1 and Th17 cells contribute to reduce *S. aureus* infection[6]. The majority of these mechanisms are coordinated by a number of cytokines and chemokines with the contribution of free radicals such as reactive oxygen species (ROS) and reactive nitrogen species (RNS)[7]. Thus, the influx of neutrophils and other immune cells into joints represent an important target for the development of new therapeutic strategies for septic

arthritis.

The therapeutic approaches of septic arthritis are relied on two main approaches: threating of infection with broad-spectrum antibiotic parentally prior to the knowledge, regardless to the type of microorganism and their resistance patterns, and threating the symptoms by using co-treatment with corticosteroid and antibiotic to prevent the neutrophils functions and down-regulate the severity of experimental septic arthritis[1]. Nevertheless, during the last decades no new major therapies have been developed to reduce the joint inflammation together with prevention of joint lesion and dysfunction in septic arthritis[3]. These facts have implications for identifying novel molecules that share microbicidal and anti-inflammatory properties.

Nitric oxide ($NO^{\bullet}$) is an abundant reactive radical that acts as an important oxidative biological signaling molecule in a wide variety of diverse physiological processes, including neurotransmission, blood pressure regulation, smooth muscle relaxation, pain and antinociception, immune regulation and defense mechanisms [8,9,10]. A critical role has been proposed for $NO^{\bullet}$ in septic arthritis regarding to the control of infection[11], but its redox sibling, nitroxyl (HNO), has not been described. This is of particular importance since the HNO shares signaling activities with $NO^{\bullet}$, and importantly, HNO also follows an entirely separate signaling and has unique biological activities when compared to $NO^{\bullet}$ supporting HNO and $NO^{\bullet}$ are different chemico-biological entities[12]. Such effects were demonstrated mainly in the cardiovascular system, including an ability of HNO to directly target thiols, it is resistant to be scavenging by superoxide, elevate plasma levels of calcitonin gene-related peptide (CGRP) and serve as a positive cardiac inotrope [13, 14, 15] properties that facilitate the use of HNO as a pharmacological agent without developing tolerance effects. Furthermore, another important pharmacological action of HNO is

its ability to elicit antioxidant effects[16], precondition against ischemia/reperfusion injury[17], induction of apoptosis and suppression of tumor angiogenesis [18,19]), and inhibition of alcohol metabolism[20]. All the analyses of these properties of HNO require the use of donor compounds due to the metastability as a result of irreversible dimerization. This dimerization reaction makes the quantitative endogenous detection of HNO a challenge. Therefore, HNO donors are crucial tools to study its biological effects. Angeli's salt (AS) is the most well studied and characterized HNO donor [21].

Recently our group demonstrated for the first time the analgesic effect of HNO[22,23]. In the first study, we demonstrated that the AS inhibited the mechanical hyperalgesia induced by carrageenan in rats via activation of sGC/cGMP/PGK/ATP-sensitive potassium channel signaling pathway and inhibition of cytokine production (e.g. TNF-α and IL-1β). Specifically, treatment with AS was capable of delivering HNO to dorsal root ganglia neurons[22]. Furthermore, AS also inhibited overt pain-like behavior by similar mechanisms activated in hyperalgesia models[23]. All these effects were mediated via HNO rather nitrite (the other metabolite of Angeli's salt in acid conditions) or extracellular oxidation of HNO to NO• since the L-cystein, HNO-selective scavenger, prevented the analgesic actions of AS[22, 23]. Despite these evidence suggested above, the effect of the HNO donor, AS, in other inflammatory conditions remains to be evaluated. The aim of the current study was to investigate the potential microbicide and anti-inflammatory and antioxidants effects of the treatment with AS in an experimental model of septic arthritis in mice.

Material and methods

Animals. Male Swiss mice (25 g), from the Londrina State University, Londrina, Parana, Brazil, were used in this study. Mice were housed in standard clear plastic cages with free access to water and food, and temperature of 23 °C ± 2. A 12/12 h

light/dark cycle was used with lights on at 6 h and off at 18 h. The behavioral tests were performed between 9 a.m. and 5 p.m. in a temperature-controlled room (23 °C ± 2). Animal care and handling procedures were in accordance with the International Association for Study of Pain (IASP) guidelines, and approved by the Ethics Committee of the Londrina State University (OF. CIRC. CEUA 84/10, process number 33358.2010.36).

Materials and Chemicals. The following materials were obtained from the sources indicated: NaCl 0.9% (Fresenius Kabi Brasil Ltda. Aquiraz, CE, Brazil), enzyme-linked immunosorbent assay (ELISA) Ready-SET-Go! (eBioscience, San Diego, CA, USA) and PathScan (Cell Signaling, Beverly, MA, USA) kits. SV Total RNA Isolation system (Promega), Platinum SyBR Green qPCR SuperMIX UDG (Invitrogen,USA). 2,2-azino-bis (3-ethylbenzothiazoline-6-sulfonate) (ABTS), Nitroblue Tetrazolium (NBT), Trizol, Sodium Hydroxide (NaOH), 1,9-Dimethyl-Methylene Blue zinc chloride double salt (DMMB), Chondroitin sulfate sodium salt from shark cartilage (Sigma–Aldrich, St. Louis, MO, USA).

Synthesis of nitroxyl donor, Angeli's Salt (AS). AS ($Na_2N_2O_3$, sodium trioxodinitrate) was synthesized by the condensation of hydroxylamine with an organic nitrate as previously described[24]. In brief, a solution of hydroxylamine hydrochloride was slowly treated with excess of sodium methoxide to yield the hydroxylamine and to prevent the saturation of the solution. After that, the formed mixture was diluted with cold methanol and filtered. The retained filtrated was diluted in another 2 equivalents of sodium methoxide in an ice slurry under stirring and mixed with the alkyl nitrate ($RONO_2$). The reaction was submitted to Argon to remove the oxygen for 3 hours and the final solution was diluted with a half volume of cold diethyl ether and filtrated

to retain the solid product. The solid product was washed successively with 3% of methoxide in methanol, just methanol and ether and dried for 10 minutes to obtain the final compound. To determine the stability of the compound, the UV absorption spectroscopy was used to determine the concentrations with coefficients at 250 nm (ε of 8000 M^{-1} cm) [25].

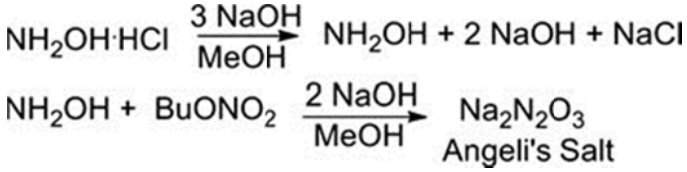

Figure 1. Synthesis and structure of Angeli's salt [26].

Mouse model of local S. aureus-induced arthritis. S. aureus was obtained from ATCC (American Type Culture Collection, U.S.A.) number 6538. Twenty-four hours before each experiment, samples of bacteria were cultured in blood agar medium at 37°C. After 24 hours, the plate was washed with sterile phosphate buffered saline (PBS) to obtain a bacterial suspension. The bacterial suspension was centrifuged and the pellet was re-suspended in sterile PBS to fit the McFarland 0,5 scale by visual comparison [27]. Septic arthritis was induced by local injection of $1x10^7$ CFU of *S. aureus* in 10 µl in sterile PBS into the right knee joints. Intra-articular (i.a.) injection of 10 µl of sterile PBS was used as negative control group. To evaluate the intensity of arthritis, a clinical scoring was carried out using a system where macroscopic inspection of the knee joints yielded a score of 0-4 for each limb (0 – normal, 1 – periarticular erythema, 2- articular erythema and edema, 3- function loss with difficult locomotion and articular extension, 4 – purulent process with abscess formation).

Electronic Pressure Meter Test. The electronic pressure meter test was utilized to evaluate the articular hyperalgesia as previously described [29,30]. It consists of a hand-

held force transducer fitted with a polypropylene tip (IITC 152 Inc., Life Science Instruments, Woodland Hills, CA, USA). For this model, a large tip (4.15mm^2) was adapted to the probe. An increasing 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 by paw withdrawal. A tilted mirror below the grid provided a clear view of the hind paw. The electronic pressure-meter apparatus automatically recorded the intensity of the force applied when the paw was withdrawn. The test was repeated until three subsequently consistent measurements (i.e. the variation among these measurements was less than 1 g) were obtained. The flexion-elicited mechanical threshold was expressed in grams (g).

Leukocyte migration to the knee joint and bacterial recovery. Mice received *S. aureus* or vehicle into articular cavity and were sacrificed at selected points after stimulus injection. The joint cavities were washed 3 times with 3,3 ul of sterile saline with 1 mM EDTA[30]. The total number of leukocytes was determined in a Neubauer chamber diluted in Turk's solution and the differential cell counts were determined by Roselfeld stained slices using a light microscope. The results were expressed as the number of total of leukocytes, neutrophils or mononuclear cells per cavity. Additionally, the same samples were plated in blood agar to determine the bacterial load in the knee joints and the results were expressed as colony-forming unity (CFU) per cavity.

Proteoglycan quantification assay. Proteoglycan contents were determined using the method described by Burkhardt et al. with modifications[31]. Patella were carefully collected from each animal and fixed with formaldehyde (4%) overnight using a shaker. They were then transferred into a solution of formic acid (5%) and incubated

for 4h using a shaker for decalcification. Each patella was then placed into 50 µl papain digestion buffer consisting of a papain suspension (5 mg/ml) in calcium and magnesium-free PBS with 5 mM cysteine and 10 mM EDTA, pH 7.4. The samples were sealed and incubated in a water bath at 60°C overnight. After reaching room temperature, the samples were centrifuged for 10 min at 1000 *g* to collect the condensation droplets. Next, 40 µl of the supernatants and of serial chondroitin sulfate solutions (standard curve; 50–1000 µg/ml) was placed into 96-well microtiter plates. The chondroitin sulfate STD solutions were also incubated with papain digestion buffer. Then, 300 µl of a 1,9-dimethylmethylene blue (DMMB; 50 mg/l) solution was added to each well, and the absorbance at 525 nm was measured immediately in a plate reader. The GAG content was calculated from the standard curve. The DMMB solution was prepared by dissolving 50 mg DMMB in 5 ml ethanol and diluting to a volume of 1000 ml with 0.2% (w/v) sodium formate buffer, pH 3.5.

Cytokine measurements. Mice were anesthetized and killed, and the knee joints from infected mice and noninfected mice were dissected out, frozen with liquid nitrogen, homogeneized in saline and centrifuged (3600 rpm x 4°C x 15 min) and the supernatants were used to determine the levels of TNF-α, IL-1β, IL-33, IL-17 and IFN-γ. The results were expressed as pg per 100 mg of tissue. Blood samples were collected from a peripheral vein and kept on ice. Plasma was obtained by centrifugation at 800 g for 15 minutes at 4°C, aliquoted, and stored at -80°C until the analysis day. All measurements were performed by ELISA kits following the manufacturer's instructions.

NF-κB activity. The assessment of total and phosphorylated NF- κB production was performed by ELISA using Cell Signaling Technology kits. Mice were anesthetized

and killed at selected points after ia *S. aureus* injection, and the knee joints were dissected out, frozen with liquid nitrogen and homogenized in lysis buffer. Lysates were centrifuged (3000 rpm x 10 min x 4 °C), and the supernatants were used to assess the levels of total and phosphorylated NF-κB following the manufacturer's instructions. The results were obtained by comparing the optical density of samples and the samples' weight and were expressed as ratio between total and phospho NF-κB absorbances per 100 mg of tissue.

Quantitative polymerase chain reaction (qPCR). qPCR was performed as previously described [30]. Samples were homogenized in Trizol reagent and mRNA was extracted by using SV Total RNA Isolation system. All reactions were performed in triplicate using cycling conditions: 50 °C for 2 min, 95 °C for 2 min, followed by 50 cycles of 95 °C for 15 s and 60 °C for 30 s. qPCR was performed in a LightCycler® Nano Instrument (Roche, Mississauga, ON, USA) and the sequence detection system by using the SYBR-green fluorescence. The primers used were: RANKL, sense: sense 5'-CCT GAG GCC CAG CCA TTT-3', antisense: 5'-CTT GGC CCA GCC TCG AT-3'; RANK, sense: 5'-CTA ATC CAG GGA AGC AAA T-3', antisense: 5'-GAC ACG GGC ATA GAG TCA GTT C-3', OPG system, sense: 5'-GGA ACC CCA GAG CGA AAC ACA-3', antisense: ANTISENSE 5'-CCT GAA GAA GGC CTC TTC ACA-3'; gp91phox, sense: 5'-AGCYAYGAGGYGGTGATGTTAGTGG-3, antisense: 5'-CACAATATTTGTACCAGACAGACTTGAG-3; Cox-2 (cicloxigenase-2), sense: 5'-GTGGAAAAACCTCGTACCAGA-3', antisense: 5'- GCTCGGCTTCCAGTATTGAG-3'; Gr, sense: 5'-TFCGTGAATGTTGGATGTGTACCC-3', antisense: 5'-CCGGCATTCTCCAGTTCCTCG-3; HO-1, sense: 5'-CCCAAAACTGGCCTGTAAAA-3', antisense: 5-CGTGGTCAGTCAACATGGAT-3; Gapdh, sense: 5'-CATACCAGGAAATGAGCTTG-3', antisense: 5'-ATGACATCAAGAAGGTGGTG-3.

The expression of GAPDH mRNA was used as a control for tissue integrity in all samples.

NBT Reduction. The superoxide anion production was determined by the reduction of the redox dye nitroblue tetrazolium (NBT) [32]. Frozen knee joint samples collected at selected points were homogenized with 500 µl of saline and 50 µl of the homogenate was placed in a 96-well plate, followed by the addition of 100 µL of NBT solution (1 mg/ml) and incubation for 1h at 37°C. The supernatant was carefully removed, and the formazan precipitated was then solubilized by adding 120 µl of 2M KOH and 140 µl of DMSO. The optical density was measured by microplate spectrophotometer reader (Multiskan GO Microplate Spectrophotometer, ThermoScientific, Vantaa, Finland) at 600 nm. The weight of samples was used for data normalization, and results were presented as NBT reduction (OD/mg of tissue).

Lipid peroxidation. Lipid peroxidation in the knee joints was assessed by determining the thiobarbituric acid reactive substances (TBARS) levels using a method previously described by Guedes et al., 2006 with modifications [32,33]. In brief, trichloroacetic acid (10%) was added to the homogenate to precipitate proteins. This mixture was then centrifuged (1000 g x 3 minutes x 4°C). The protein-free sample was extracted and thiobarbituric acid (0.67%) was added. The mixture was kept in water bath at 100 °C for 15 min. Malondialdehyde (MDA), an intermediate product of lipoperoxidation, was determined by difference between absorbances at 535 and 572 nm on a microplate spectrophotometer reader (Multiskan GO, Thermo Scientific) and the results were expressed as TBARS (nmol of MDA per mg of tissue).

GSH (reduced glutathione) levels. The samples from knee joint were collected at selected points after ia *S. aureus* injection and frozen at -80°C at least for 48 hours

until GSH assay. To determine the GSH levels were used a spectrophotometric method previously described by Ellman, 1959 with modifications[34]. In brief, the samples were homogenized with a polytron in ice-cold 0,02 M EDTA buffer solution. The homogenates were treated with 50% trichloroacetic acid. After 15 minutes, the homogenates were centrifuged at 1500g for 15 min and then 100 µl of supernatant was mixed with 200 µl of 0.4M Tris-HCl, pH 8,0 and 10 µl of 10 mM dithiobisnitrobenzoic acid (DTNB) in methanol solution. After 5 min, the measurements were performed in 412 nm (Shimadzu UV–Vis Spectrophotometer UV-1650, Shimadzu Corporation, Kyoto, Japan). The results are expressed as nmol of GSH per mg of tissue using a standard GSH curve (0.09–100 nmol).

ABTS and FRAP assays. The ability of samples to resist oxidative damage was determined by its free radical scavenging (ABTS assay) and ferric reducing (FRAP assay) properties. The tests were adapted to a 96-well microplate format from previously described assays[35]. In brief, samples from knee joint were collected at selected points after i.a. *S. aureus* injection and homogeized immediately with ice-cold 1,15% KCl in volume of 500 µl. The homogenates were centrifuged (200g x 10 minutes x 4°C), and the supernatants were used in both assays. Diluted ABTS solution (200 µl) was mixed with 10 µL of sample in each well. After 6 min of incubation (25 °C), the absorbance was measured at 730 nm (Multiskan GO Microplate Spectrophotometer, ThermoScientific, Vantaa, Finland). For FRAP assay, the supernatants (10 µl) were mixed with the freshly prepared FRAP reagent (150 µl). The reaction mixture was incubated at 37 °C for 30 min, and the absorbance was measured at 595 nm (Multiskan GO ThermoScientific). The results of ABTS and FRAP assays were equated against a standard Trolox curve (0.02–20 nmol) and expressed as µM of Trolox equivalent per mg f tissue.

Minimum bactericidal concentration (MBC) determination. The microbicidal effect of AS on viable *S. aureus* was determined by minimum bactericidal concentration (MIC) with adaptations. In brief, 50 µl of bacterial suspension in suitable growth medium (Mueller Hinton broth) was added to the wells of a sterile 96-well microtitre plate already containing 50 µL of two-fold serially diluted AS (initial concentration: 10 µM) or NaOH (10 mM) in proper growth medium (Muller Hinton broth). The final volume in each well was 100 µl. Control wells were prepared with culture medium only, bacterial suspension only, AS only and NaOH 10 mM only in amounts corresponding to the highest quantity present. Ciprofloxacin (50 ug), a broad-spectrum antibiotic, was utilized as positive control. The plate was incubated for 24 h and the supernatants was plated on blood agar for CFU counts. The results were expressed as the number of CFU per plate and compared with the positive and negative controls.

Lung analysis. Lungs were washed twice using 1 ml saline with 1 mM EDTA per animal. Each flush consisted of three slow up and down movements. The total number of leukocytes was determined in a Neubauer chamber diluted in Turk's solution and the differential cell counts were determined by Roselfeld stained slices using a light microscope. The results were expressed as the number of total of leukocytes, neutrophils or mononuclear cells in the BALF. Additionally, the same lungs were processed to measure the neutrophil accumulation by myeloperoxidase (MPO) assay. The absorbance of the tissue supernatant was compared with a standard curve of mouse peritoneal neutrophils, and the results as expressed as the number of neutrophils per mg of tissue.

Hepatic and Renal Toxicity. To investigate the potential of Angeli's Salt to cause

hepatic or renal toxicity, serum levels of aspartate aminotransferase (AST), alanine aminotransferase (ALT), creatinine and urea were determined**.** For this test, the blood samples from mice treated over 28 days was collected and then centrifuged (0,4g x 4°C x 20 min) to obtain the serum. After that, the levels of ALT, AST, creatinin and urea were determined using a diagnostic kit from Labtest according to the manufacturer's instructions.

Experimental Protocols. Mice (n = 6 per group per experiment, representative of two independent experiments) were treated with AS [3 mg/kg/subcutaneous (s.c.)] diluted in 10 mM NaOH and saline to complete 150 uL) or vehicle over 28 days starting in 1 hour after injection of *S. aureus* suspension. The dose of AS and the concentration of *S. aureus* were determined previously in our laboratory (Staurengo-Ferrari et al., 2013, unpublished data, [23]). Mechanical hyperalgesia, paw edema and clinical severity were evaluated on alternated days over 27 days. Leukocyte recruitment (total of leukocytes, neutrophils and mononuclear cells), proteoglycan content, cytokines (TNF-α, IL-1β, IL-17, IL-33 and IFN-γ) levels, NF-κB activity, qPCR (primers for $GP91^{phox}$, COX-2, HO-1, Gr, GPx1 and RANKL/RANK/OPG system), oxidative stress (NBT reduction, TBARS assay, FRAP assay, ABTS assay and GSH assay), bacterial recover and evaluation of systemic inflammatory parameters (leukocyte recruitment in lungs and cytokine levels in plasma) were evaluated 7, 14, 21 and 28 after *S. aureus* ($1x10^7$/10 µl) i.a. injection. The points were selected to determine the kinetic of events and the host-bacterium relationship involved in the model of septic arthritis and the modulation by the treatment with the nitroxyl donor, AS. Moreover, to determine to renal and hepatic toxicity of the chronic treatment with AS, serum samples were collected at 28 day and the levels of ALT, AST, creatinine and urea were determined. The MBC *in vitro* was tested to determine the lowest

concentration of AS was required to kill *S. aureus*. The vehicle data (10 mM NaOH plus saline) has no difference occurred in the results when they were compared by statistical analysis.

Statistical analysis. The statistical analyses were performed using GraphPad Prism 4.0 (La Jolla, 5 CA). Results are presented as means ± SEM of two independent experiments. The n in the legends refers to the number of mice per group in each experiment. The differences between the experimental groups were compared by t test or one-way ANOVA and individual comparisons were subsequently made by Tukey's post hoc test. The level of significance was set at $P < 0.05$ and are represented by symbols (* or #). Please, see legends in case of significance, otherwise a non-significant statistical difference was indicated.

Results

The nitroxyl donor, Angeli's salt (AS), inhibits *S. aureus*-induced articular inflammation and clinical severity. Previous results from our laboratory showed that the i.a. injection of *S. aureus* (10^5-10^7 CFU/10 µl induces dose-dependent mechanical hyperalgesia, joint edema and leukocyte recruitment, which peaked with 10^7 CFU per joint remaining up to 27 days (Staurengo-Ferrari et al., unpublished data). Thus, we used the concentration of $1x10^7$/10 µl/joint of *S. aureus* in all the experiments. Mice were daily treated with AS (3 mg/kg, sc, 150 µl) (Staurengo-Ferrari et al., 2014) or vehicle (19.35 µl of 10 mM NaOH plus saline to complete 150 µl) after the i.a. injection of *S. aureus* (10^7/10 µl/joint) and the intensity of mechanical hyperalgesia (Fig 2A), articular edema (Fig 2B) and clinical severity (Fig 2C) were determined at indicated points over 27 days. AS significantly inhibited the mechanical hyperalgesia and articular edema induced by *S. aureus* since the third day of

systemic treatment and its effect was kept up to the chronic phase of septic arthritis since both events peaked at the same time and they were inhibited in an intermediate phase of the septic arthritis development. Changes in the clinical severity of septic arthritis were noted after 5 days of treatment with AS. Additionally, considering the interdependence between the pro-nociceptive response (hyperalgesia) and leukocyte migration, we also investigate if AS modulates the leukocyte recruitment by the amounts of total leukocytes, neutrophils and mononuclear cells to the knee joint. In agreement, the treatment with AS was also effective in reducing the total of leukocytes, neutrophil and mononuclear cells recruited to the knee joints at 7, 14, 21 and 28 days after *S. aureus* stimulus. The treatment with vehicle had no effect on the evaluated parameters.

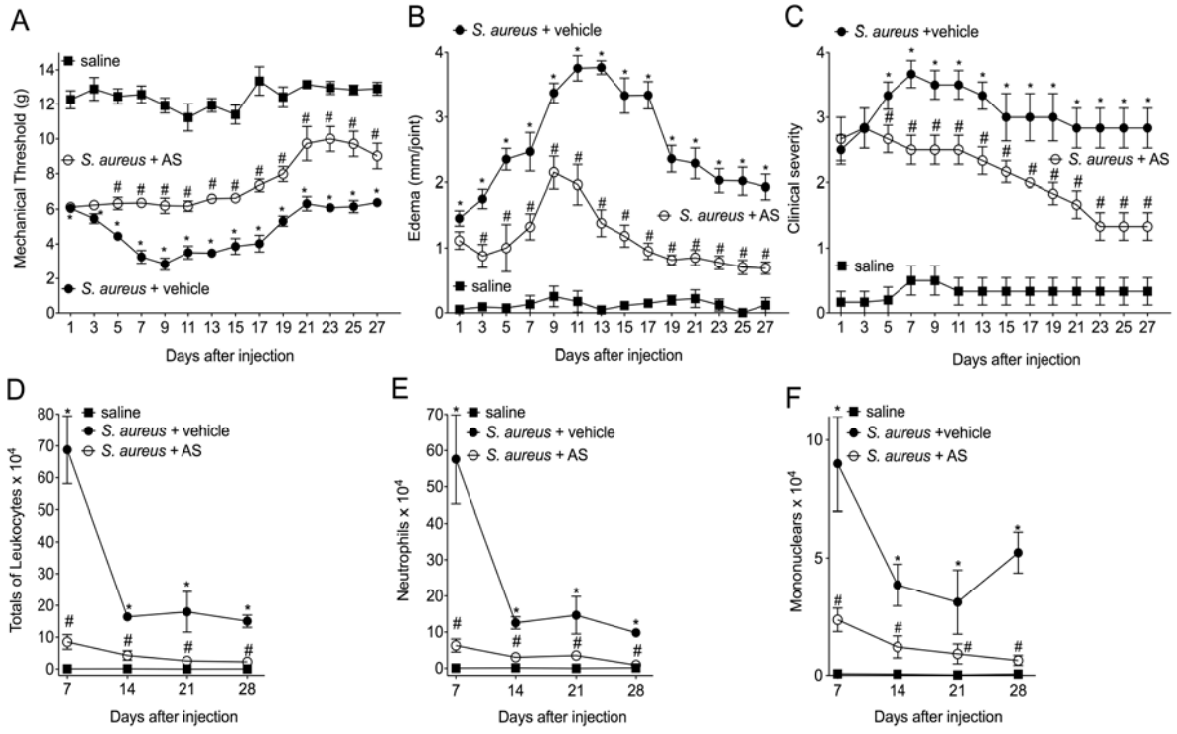

Figure 2. Angeli's salt (AS) reduces *S.aureus*-induced articular inflammation and clinical severity. Mice were treated with AS (3 mg/kg, s.c., 150 µl) or vehicle (19.35 µl

of NaOH 10 mM plus saline to complete 150 µl) over 28 days or until the selected day to specific analysis after i.a. injection of *S.aureus* (10^7 CFU/10 µl/joint). The mechanical hyperalgesia (panel A), articular edema (panel B) and the clinical severity (panel C) were evaluated over 27 days with eletronic pressure meter, analog caliper and visual score, respectively. At indicated points (7,14,21 and 28 days after *S. aureus* injection), the knee joints were harvested and the total leukocytes (panel D), neutrophils (panel E) and mononuclear cells (panel F) counts were determined using Neubauer chamber and Rosenfelt stained slices. n =6 per group per experiment, representative of two independent experiments. *P < 0.05 compared with the saline group, #P< 0.05 compared with the *S. aureus* + vehicle group. One-way ANOVA followed by Tukey's test.

AS inhibits *S. aureus*-induced proteoglycan content loss in patella samples. Mice were daily treated with AS (3 mg/kg, sc, 150 µl) or vehicle (19.35 µl of 10 mM NaOH plus saline to complete 150 µl) after the i.a. injection of *S. aureus* (10^7/10 µl/joint). Patella samples were collected at 7, 14, 21 and 28 days after the stimulus injection and used to determine the proteoglycan content (Fig 3). The i.a. injection of *S. aureus* induced a significant loss of proteoglycan since the acute phase (day 7) of the disease and it worsened in the chronic phase of septic arthritis (day 28). The treatment with AS prevented the progressive loss of proteoglycan at days 7, 14, 21 and 28 day.

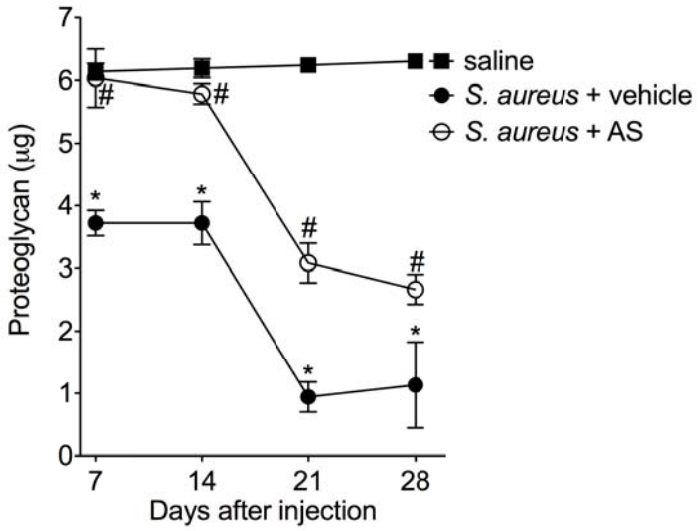

Figure 3. AS reduces *S.aureus*-induced proteoglycan content loss in patella samples. Mice were treated with AS (3 mg/kg, s.c., 150 µl) or vehicle (19.35 µl of NaOH 10 mM plus saline to complete 150 µl) over 28 days after i.a. *S.aureus* (10^7 CFU/10 µl/joint) injection. At indicated points (7,14,21 and 28 days after *S. aureus* injection), the knee joints were harvested and processed for analysis of proteoglycan content. N =6. *P< 0.05 compared with the saline group, #P<0.05 compared with the *S. aureus* + vehicle group. One-way ANOVA followed by Tukey's test.

AS inhibits *S. aureus*-induced mRNA expression of of receptor activator of nuclear factor kappa – ligand (RANKL)/receptor activator of nuclear factor kappa (RANK)/osteoprotegerin (OPG) system. Mice were daily treated with AS (3 mg/kg, sc, 150 µl) or vehicle (19.35 µl of 10 mM NaOH plus saline to complete 150 µl) after the i.a. injection of *S. aureus* (10^7/10 µl/joint). Knee joint samples were collected at 7, 14, 21 and 28 days after the stimulus injection and used to determine RANKL (Fig 4A), RANK (Fig 4B) and OPG (Fig 4C) mRNA expression. No preventive effect on RANKL and RANK mRNA expression was observed in the mice treated

with AS at day 7. On the other hand, AS inhibited the *S. aureus*-induced RANKL and RANK mRNA expression at days 14, 21 and 28. AS inhibited *S. aureus*-induced-OPG mRNA expression in all intervals evaluated. Thus, the treatment with AS prevented the mRNA expression of genes related to osteoclastogenesis in septic arthritis.

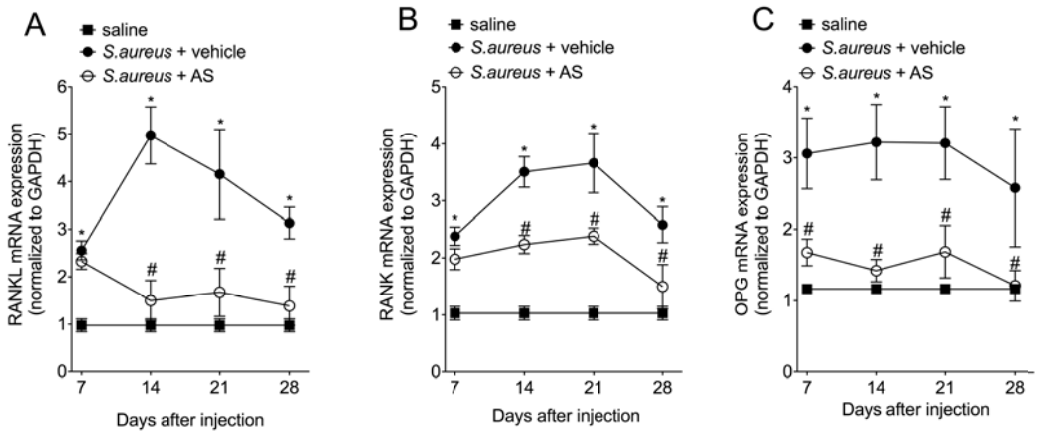

Figure 4. AS reduces *S. aureus*-induced mRNA RANK/ RANKL/ OPG system. Mice were treated with AS (3 mg/kg, s.c., 150 µl) or vehicle (19.35 µl of NaOH 10 mM plus saline to complete 150 µl) over 28 days after i.a. *S.aureus* (10^7 CFU/10 µl/joint) injection. At indicated points (7, 14, 21 and 28 days after *S. aureus* injection), the knee joints were harvested and processed to determine the mRNA expression of RANKL (panel A), RANK (panel B**),** OPG system (panel by real time PCR. N=5 per group per experiment, representative of two independent experiments. *P<0.05 compared with the saline group, #P<0.05 compared with the *S. aureus* + vehicle group. One-way ANOVA followed by Tukey's test.

AS inhibits *S. aureus*-induced cytokine production. Mice were daily treated with AS (3 mg/kg, sc, 150 µl) or vehicle (19.35 µl of 10 mM NaOH plus saline to complete

150 µl) after the i.a. injection of *S. aureus* (10^7/10 µl/joint). Knee joint samples were collected at days 7, 14, 21 and 28 after the stimulus injection and processed for cytokine level determination by ELISA. The systemic treatment with AS inhibited *S. aureus*-induced TNF-α (Fig 5A), IL-1β (Fig 5B), IL-33 (Fig 5C), IL-17 (Fig 5D) and IFN-γ (Fig 5E) production at all evaluated time points

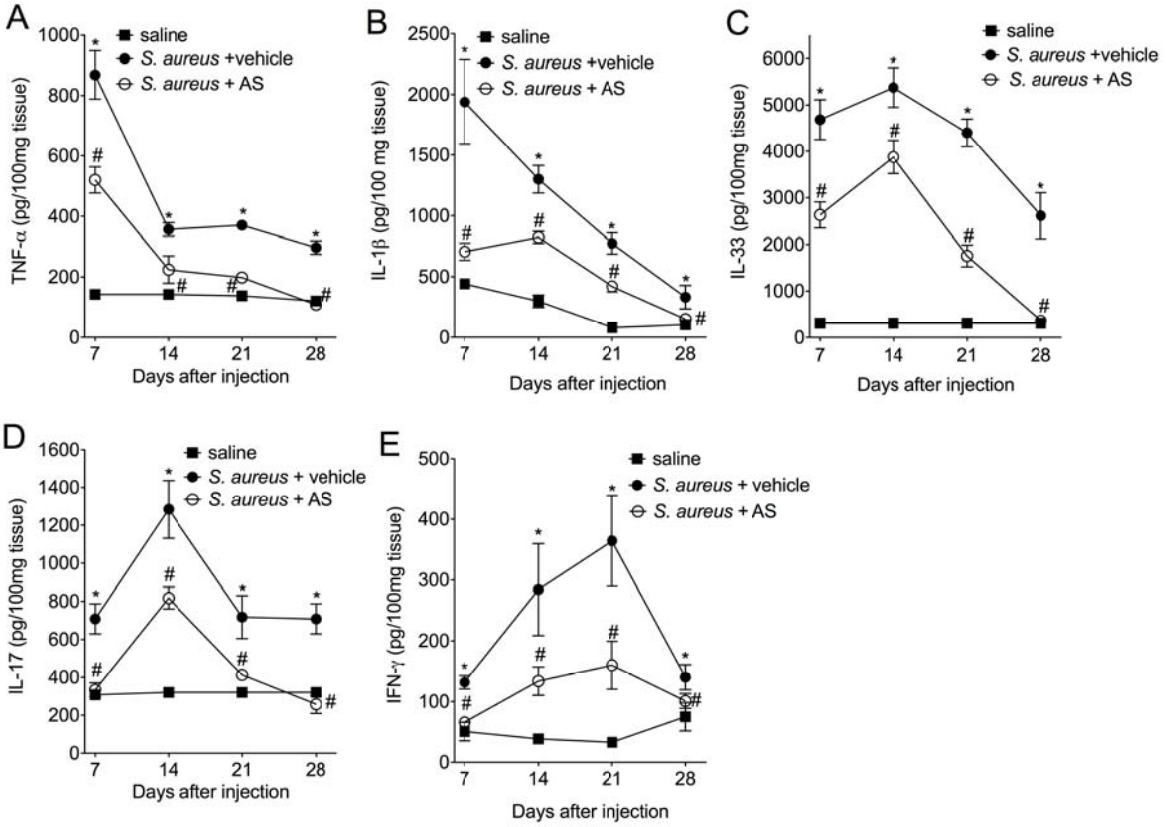

Figure 5. AS reduces *S. aureus*-induced cytokine production in the knee joint. Mice were treated with AS (3 mg/kg, s.c., 150 µl) or vehicle (19.35 µl of NaOH 10 mM plus saline to complete 150 µl) over 28 days after i.a. *S.aureus* (10^7 CFU/10 µl/joint) injection. At indicated points (7, 14, 21 and 28 days after *S. aureus* injection), the knee joints were harvested the knee and processed to determine the levels of TNF-α (panel A), IL-1β (panel B), IL-33 (panel C), IL-17 (panel D) and IFN-γ (panel E) by ELISA. N =6 per group per experiment, representative of two independent

experiments. *P < 0.05 compared with the saline group, #P< 0.05 compared with the *S. aureus* + vehicle group. One-way ANOVA followed by Tukey's test.

AS inhibits *S. aureus*-induced NF-κB activation. Mice were treated daily with AS (3 mg/kg, sc, 150 µl) or vehicle (19.35 µl of 10 mM NaOH plus saline to complete 150 µl) after the i.a. injection of *S. aureus* (10^7/10 µl/joint). Knee joint samples were collected at days 7, 14, 21 and 28 after the stimulus injection and processed to determine the activation of NF-κB by the levels of total and phosphorylated NF-κB p65 subunit (Fig 6). Corroborating with the inhibition of cytokine production by AS, it also significantly inhibited *S. aureus*-induced NF-κB p65 activation in all evaluated intervals. The inhibition was more pronounced on day 21 after stimulus injection.

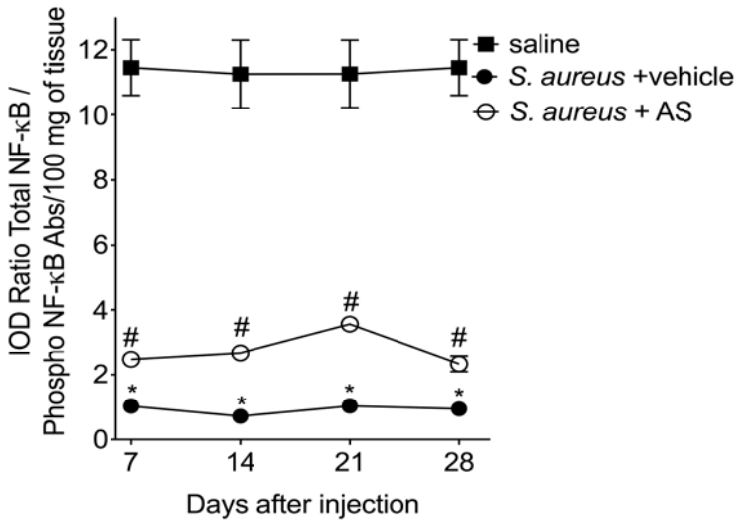

Figure 6. AS reduces *S. aureus*-induced NF-κB activation in the knee joint. Mice were treated with AS (3 mg/kg, s.c., 150 µl) or vehicle (19.35 µl of NaOH 10 mM plus saline to complete 150 µl) over 28 days after i.a. *S.aureus* (10^7 CFU/10 µl/joint) injection. At indicated points (7, 14, 21 and 28 days after *S. aureus* injection), the knee joints were harvested and processed to determine the activation of NF-κB p65 unit by ELISA. N =6 per group per experiment, representative of two independent

experiments. *P<0.05 compared with the saline group, #P<0.05 compared with the *S. aureus* + vehicle group. One-way ANOVA followed by Tukey's test.

AS inhibits *S. aureus* induced-mRNA expression of gp91^{phox}, COX-2 and induces heme oxygenase-1 (HO-1) and glutathione reductase (Gr) mRNA expression. Mice were daily treated with AS (3 mg/kg, sc, 150 µl) or vehicle (19.35 µl of 10 mM NaOH plus saline to complete 150 µl) after the i.a. injection of *S. aureus* (10^7/10 µl/joint). Knee joint samples were collected at days 7, 14, 21 and 28 after the stimulus injection and used to determine gp91phox (Fig 7A), COX-2 (Fig 7B), Gr1 (Fig 7C) and HO-1 (Fig 7D) mRNA expression. AS inhibited *S. aureus* induced-mRNA expression of gp91^{phox}, COX-2 mRNA expression in all evaluated intervals. The injection of *S. aureus* had no effect on Gr and HO-1 mRNA expression. On the other hand, AS induced the expression of Gr and HO-1 since the day 7 and 14, respectively, and the effect was kept over the next intervals. Hence, treatment with AS had an important role in redox biology involved in septic arthritis.

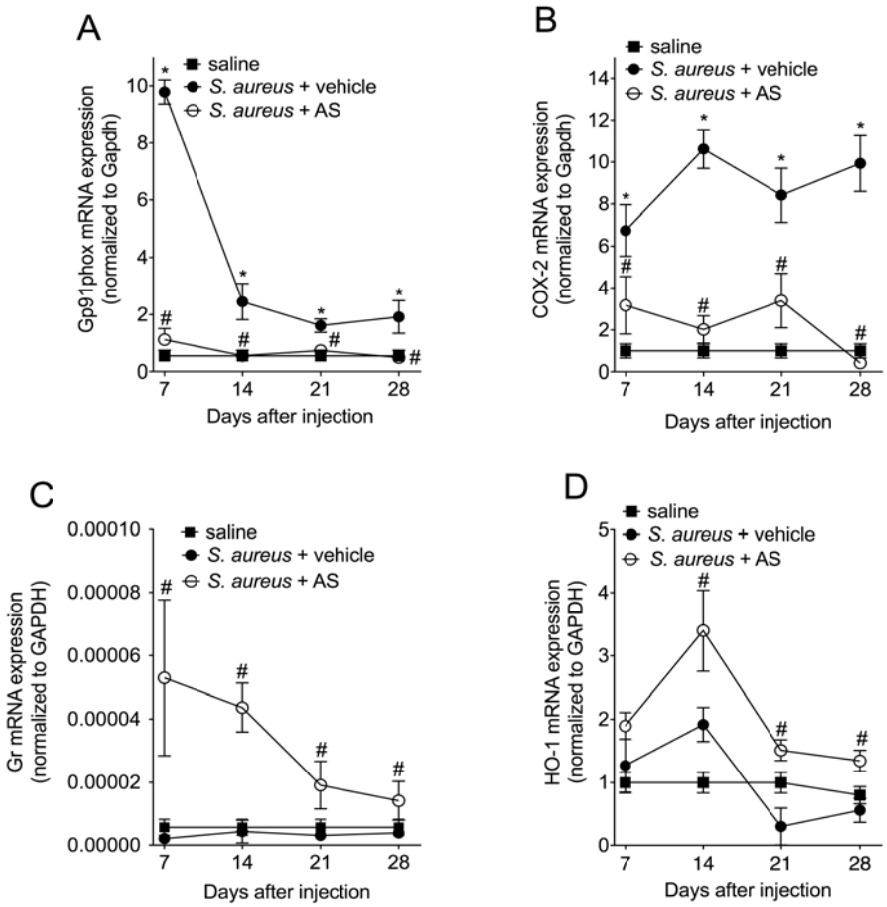

Figure 7. AS prevents *S. aureus*-induced expression of pro-oxidants enzymes and increases the antioxidants enzymes expression in the knee joint**.** Mice were treated with AS (3 mg/kg, s.c., 150 µl) or vehicle (19.35 µl of NaOH 10 mM plus saline to complete 150 µl) over 28 days after i.a. *S.aureus* (10^7 CFU/10 µl/joint) injection. At indicated points (7, 14, 21 and 28 days after *S. aureus* injection), the knee joints were harvested and processed to determine the mRNA expression of Gp91phox (panel A), COX-2 (panel B**),** Gr1 (panel C), HO-1 (panel D) by real time PCR. N=5 per group per experiment, representative of two independent experiments. *P<0.05 compared with the saline group, #P<0.05 compared with the *S. aureus* + vehicle group. One-way ANOVA followed by Tukey's test.

AS inhibits the *S. aureus*-induced oxidative stress. Mice were daily treated with

AS (3 mg/kg, sc, 150 µl) or vehicle (19.35 µl of 10 mM NaOH plus saline to complete 150 µl) after the i.a. injection of *S. aureus* (10^7/10 µl/joint). Knee joint samples were collected at days 7, 14, 21 and 28 after the stimulus injection and used to evaluate the oxidative stress. *S. aureus* injection increased the superoxide anion (Fig 8A) and MDA production (Fig 8B). These results are in line with the reduced levels of endogenous antioxidants as observed in GSH (Fig 8C), FRAP (Fig 8D) and ABTS free radical scavenging ability (Fig 8E) that were induced by *S. aureus* infection. AS inhibited significantly the superoxide anion and MDA production in all intervals evaluated and inhibited the oxidative stress by restoring the endogenous antioxidant capacity of the samples.

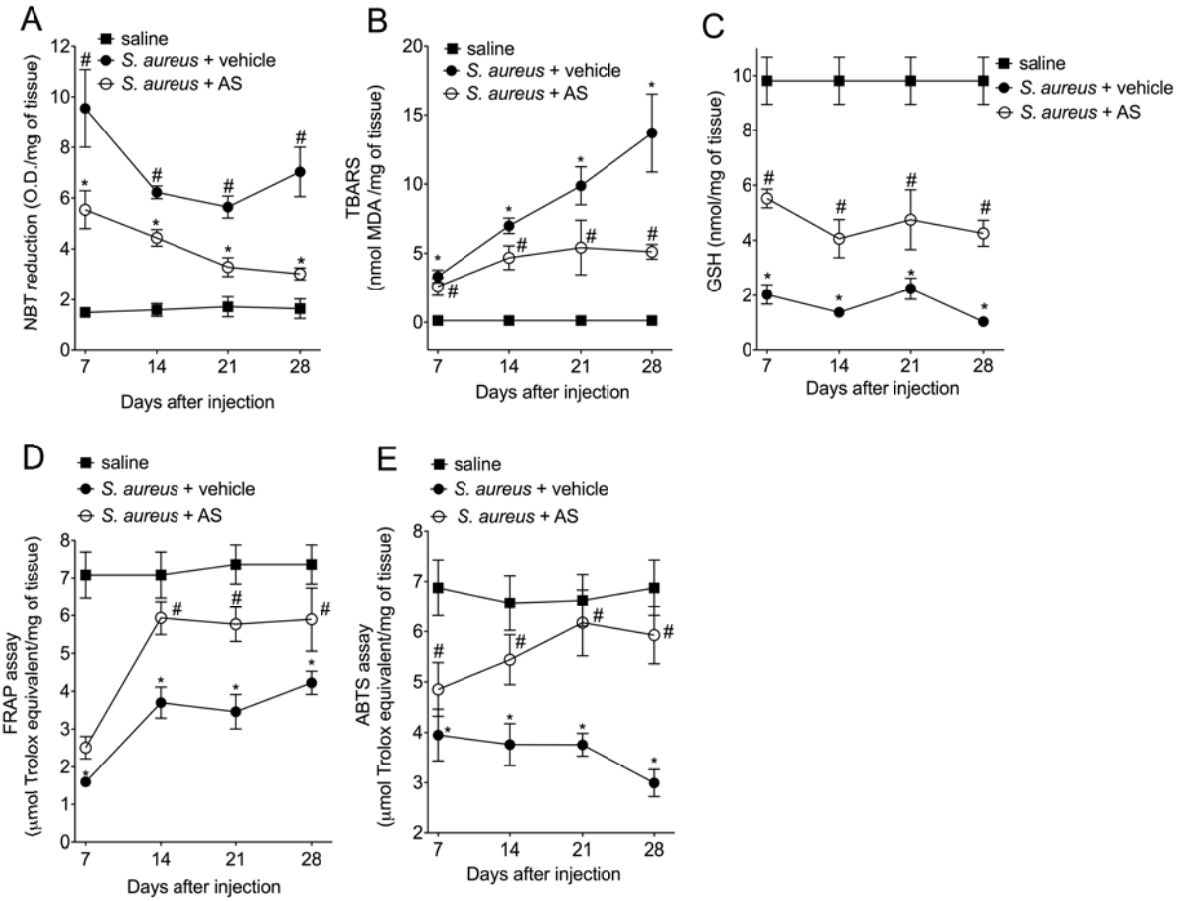

Figure 8. AS reduces *S.aureus*-induced oxidative stress in the knee joint**.** Mice were

treated with AS (3 mg/kg, s.c., 150 µl) or vehicle (19.35 µl of NaOH 10 mM plus saline to complete 150 µl) over 28 days after i.a. *S.aureus* (10^7 CFU/10 µl/joint) injection. At indicated points (7, 14, 21 and 28 days after *S. aureus* injection), the knee joints were harvested and the oxidative stress was assessed by determining the superoxide anion produced (panel A, NBT assay), lipidic peroxidation (panel B, TBARS assay), reduced GSH levels (panel C), ferric reducing antioxidant power (panel D, FRAP assay) and ABTS radical scavenging ability (panel E, ABTS assay). N =6 per group per experiment, representative of two independent experiments. *P < 0.05 compared with the saline group, #P< 0.05 compared with the *S. aureus* + vehicle group. One-way ANOVA followed by Tukey's test.

AS decreases *S. aureus* load into the knee joints and presents direct microbicidal effect '*in vitro*'. Mice were daily treated with AS (3 mg/kg, sc, 150 µl) or vehicle (19.35 µl of 10 mM NaOH plus saline to complete 150 µl) after the i.a. injection of *S. aureus* (10^7/10 µl/joint). Knee joint samples were collected at days 7, 14, 21 and 28 after the stimulus injection and spread on blood agar plates to determine the CFU number (Fig 9A). The treatment with AS inhibited significantly the CFU number in the knee joints since the acute phase of septic arthritis. Corroborating with the decrease of the bacterial load induced by the treatment with AS, it also significantly inhibited the *S. aureus* growth *'in vitro'* determined by MBC Fig 9B). The treatment in the suspension of *S. aureus* with vehicle in an equivalent concentration used *in vivo* did not alter the number of viable bacteria on agar plates. Ciprofloxacin, an antibiotic that belongs to second-generation fluoroquinolone, was used as positive control and inhibited the *S. aureus* growth. These results suggested the direct microbicidal effect of AS.

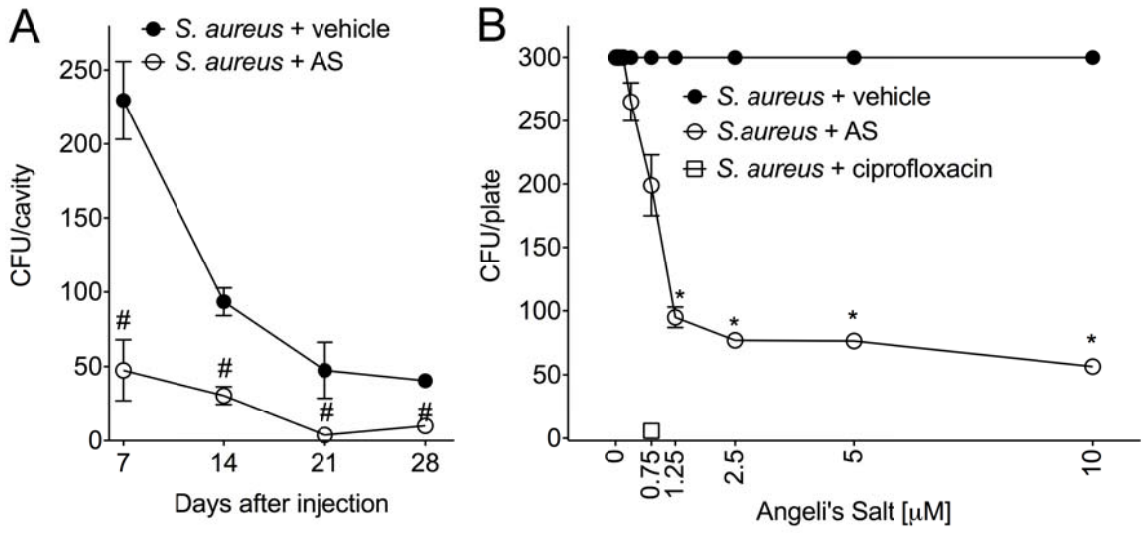

Figure 9. AS reduces *S. aureus* load into the knee joints and presents direct microbicidal effect '*in vitro*'**.** Mice were treated with AS (3 mg/kg, s.c., 150 µl) or vehicle (19.35 µl of NaOH 10 mM plus saline to complete 150 µl) over 28 days after i.a. *S.aureus* (10^7 CFU/10 µl/joint) injection. At indicated points (7, 14, 21 and 28 days after *S. aureus* injection), the knee joints were harvested and the bacterial count was determined (panel A). The microbicidal effect was determined by MBC and CFU counts on agar dishes (panel B). N=6 per group per experiment, representative of two independent experiments *P<0.05 compared with the saline group, #P<0.05 compared with the *S. aureus* + vehicle group. One-way ANOVA followed by Tukey's test.

AS inhibits *S. aureus*-induced pulmonar and systemic inflammatory response. The ability of AS to prevent systemic infection was investigated by inflammatory parameters of lungs dysfunction and levels of pro-inflammatory cytokines. Mice were daily treated with AS (3 mg/kg, sc, 150 µl) or vehicle (19.35 µl of 10 mM NaOH plus saline to complete 150 µl) after the i.a. injection of *S. aureus* (10^7/10 µl/joint). The bronchoalveolar lavage fluid (BALF), lungs and plasma samples were collected at

days 7, 14, 21 and 28 after *S. aureus* stimulus injection and used to determine the leukocyte recruitment, number the neutrophils and cytokine levels, respectivelly. The treatment with AS inhibited significantly the total of leukocytes (Fig 10A), neutrophil (Fig 10B) and mononuclear cells (Fig 10C) recruitment in the BALF at days 7, 14, 21 and 28 after *S. aureus* stimulus. Additionally, the treatment with AS prevented the neutrophil sequestration (MPO activity, Fig 10D) in the lungs and decreased systemic blood concentration of TNF-α (Fig 10E) and IL-1β (Fig 10F) in all time points evaluated in septic arthritis mice. These results suggest that AS prevented the worsening of local infection preventing the development of *S. aureus*-induced systemic infection.

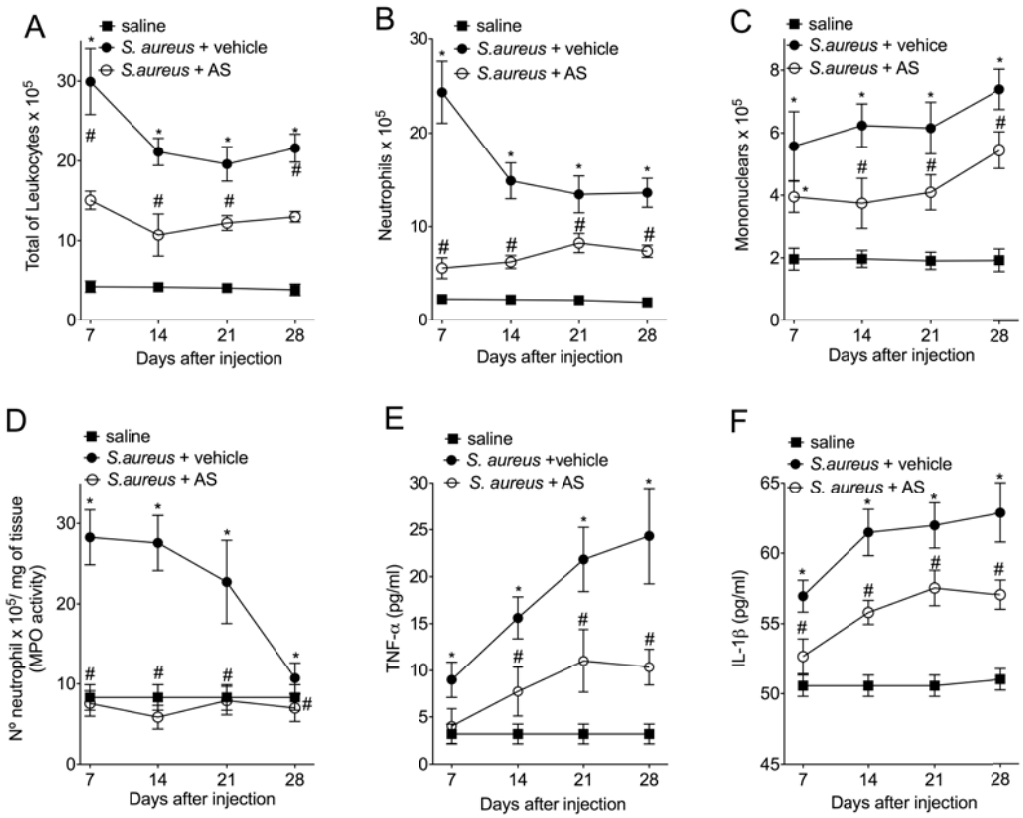

Figure 10. AS prevents *S.aureus*-induced pulmonar and systemic inflammatory response**.** Mice were treated with AS (3 mg/kg, s.c., 150 µl) or vehicle (19.35 µl of

NaOH 10 mM plus saline to complete 150 µl) over 28 days after intra-articular *S.aureus* (10^7 CFU/10 µl/joint) injection. At indicated points (7,14,21 and 28 days after *S. aureus* injection), the total leukocytes (panel A), neutrophils (panel B) and mononuclears cells (panel C) were determined in bronchoalveolar lavage fluid (BALF) samples using Neubauer chamber and Rosenfelt stained slices. The neutrophil recruitment in lungs (panel D) and cytokine production (TNF-α and IL-1β levels, panels E and F) was assesed by MPO activity and ELISA, respectively. N=6 per group per experiment, representative of two independent experiments. *P < 0.05 compared to saline group, #P<0.05 compared with the *S. aureus* + vehicle group. One-way ANOVA followed by Tukey's test.

Discussion

Pain, swelling and increased joint temperature are major local symptoms in patients affected by septic arthritis[36]. The reduction of these inflammatory symptoms is a main therapeutic goal for septic arthritis treatment as well as rapid control of infection. The major finding of this study is that pharmacological treatment with a HNO donor, Angeli's salt (AS), markedly decreased the mechanical hyperalgesia and the entire inflammatory repertoire produced by the i.a. stimulus with *S. aureus*, the most prevalent cause of septic arthritis. Importantly, HNO decreased the CFU number of *S. aureus* in articular space and presented microbicide effects, showing that AS controls the bacterial load, which is essential to induce and prolong the inflammatory process, tissue lesion and loss of joint function. In this sense, our findings add significant novelty to the pharmacological effects of HNO.

In studying the pain and inflammation triggering mechanisms in septic arthritis, the selection of an experimental model that mimics the pathophysiological septic

arthritis events is important. The *S. aureus*-induced arthritis mice model by the i.a. injection is a suitable and reproducible experimental system that exhibits several features involved in the inflammatory process within the joint cavity akin to those observed in humans, including the hyperalgesia. Septic arthritis occurs after sepsis in result of hematogenous seeding, systemic bacteremia, direct introduction or extension from a contiguous focus of infection (such as skin or bone infections) and therapeutic interventions[3]. Furthermore, there is an increasing case of septic arthritis following prosthetic surgeries indicating that not only blood stream dissemination, but also local delivery of bacteria is an important inducer of septic arthritis. Another important point is that septic arthritis is also induced by common non multi-resistant bacteria[37].

In the present study, the *S. aureus* injection into femur-tibial joint produced a chronic mechanical hyperalgesia similarly to previous studies of bacterial infection[38]. *S. aureus* can directly activate sensory nociceptive neurons by producing pore forming molecules and formylated peptides[39], which are bacterial virulence factors[40]. Other bacterial product such as LPS from gram-negative bacteria also induces mechanical hyperalgesia, but in this case, the nociceptor sensitization occurs indirectly by the activation of TLR4 in tissue resident immune cells, which produce cytokines, which activate their receptors expressed by nociceptors[41]. The treatment with AS inhibited inflammatory hyperalgesia induced by *S. aureus* in chronic and acute phases of septic arthritis. AS delivers HNO to DRG neurons inducing analgesic effect[22], thus, it is conceivable that in septic arthritis HNO also presents neuronal analgesic actions. However, it is likely that multiple contributing mechanisms of AS would jointly explain its action in septic arthritis rather that a sole mechanism of action.

In this sense, several papers have described the essential role of neutrophils in the induction of inflammatory hyperalgesia induced by varied stimuli[30,42]. Therefore, the AS inhibition of septic arthritis-induced mechanical hyperalgesia and leukocyte recruitment lined up as well. An important early step in the immune response against invading bacteria is the chemoattraction of neutrophils to kill the pathogen[4]. At the same time that they are involved to eradicate the infection, the cells are important sources of pro-inflammatory mediators, including cytokines, ROS-producing enzymes and proteases, presenting a critical role in initiating and maintaining the inflammatory process in septic arthritis[43]. Furthermore, the neutrophils are one of the most abundant cells present in the joints of septic arthritis patients, including synovial fluid and pannus/cartilage interface[44]. The data presented here provided the inhibitory effect of AS on neutrophil infiltration at the inflammatory infectious foci since the beginning of septic arthritis. The treatment with AS also inhibited the mononuclear infiltration, thus, the HNO prevented an exaggerated immune cells invasion in the articular joint that precedes the cartilage damage and bone destruction. Collectively, we provided compelling evidences that besides of the inhibitory effect on cell infiltration, the treatment with AS also reduced the cartilage destruction and osteoclastogenesis, relied on the quantification of proteoglycan in patella samples and modulation of RANKL/RANK/OPG system, respectively. Thus, our findings suggest that the therapeutic role of HNO is partially based on the impaired ability of leukocytes to massively migrate to infectious foci and inflict major damages over the cartilage and bone.

Cytokines are important molecules involved in the induction of inflammatory events of septic arthritis, including the recruitment and directing of leukocytes towards the infectious foci. The current idea is that the proinflammatory cytokines

(TNF-α, IL-1β, IL-33, IL-17 and IFN-γ) evaluated in this study have an important role against infection induced by *S. aureus*[6, 45, 46, 47, 48]. However, the exacerbated production at sites of inflamed joints that alters the balance between protective response and damage likely contribute to the progression of chronic joint infectious inflammation. The cytokine time-course of production suggests that these molecules are been released in a cascade-dependent manner and TNF-α, IL-1β and IL-33 seems to be the initiators. This hypothesis is also supported by the fact that the TNF-α and IL-1β, under its transcriptional control, keep the activation of the NF-κB, constituting a positive-feedback loop and perpetuating the deleterious effects involved in septic arthritis[49]. Additionaly, NF-κB activation contributes to the bacterial burden increase in septic arthritis[50]. In agreement with the AS reduction of cytokine production, AS also inhibited septic arthritis-induced activation of NF-κB in the knee joint tissue. Importantly, the activation of NF-κB is a communed-point of various signal transductions, including mitogen activated protein kinases superfamily (MAP-kinases), JNK, ERK ½ and p38 MAPK[49]. Recently, it has been shown the HNO inhibits p38 proteins phosphorylation[51]. Our results are in line with previous work demonstrating the inhibitory effect of AS on cytokine production in non-infectious inflammation[22].

There is a link between cytokines/NF-κB/leukocyte recruitment and oxidative stress in inflammatory response. For instance, TNF-α and IL-1β activate NADPH oxidase (nicotinamide adenine dinucleotide phosphate-oxidase) in leukocytes (neutrophil and macrophages), resulting in the production of superoxide anion. In turn, the superoxide anion activates the NF-κB and consequent production of cytokines and other genes expression, such as COX-2[52]. The role of ROS and, in particular, of superoxide in degradation of cartilage and bone is well documented.

Cartilage in sensitive to degradation by superoxide, and the treatment with SOD mimetic inhibited this degradation. The chondrocyte lipid peroxidation seems to be closely linked to cartilage oxidation/degradation[53, 54]. Indeed, we found increased levels of superoxide anion and lipoperoxidation in joint tissues associated with increased mRNA expression for gp91^{phox}, a subunit of NAPDH oxidase family. Recent reports have demonstrated that HNO can rapidly suppress the NADPH oxidative and consequently the superoxide production[51, 55]. In agreement, we found that the treatment with AS prevented the increase of superoxide anion production and gp91^{phox} mRNA expression since the starting of disease. In addition, AS inhibited the lipoperoxidation as well as inhibited the COX-2 mRNA expression in joint tissue, both effects closely dependent on NADPH oxidase activity. Corroborating with the results discussed above in which the AS treatment prevented the degradation of proteoglycan, the HNO plays an important role to prevent the cartilage damage and all events interconnected. This is important since the cartilage destruction in the intensity that occurs in septic arthritis is responsible for causing permanent joint movement debilitation.

The role of HNO in redox biology is still under examination and, like NO•, it seems to be capable of eliciting both pro- and antioxidant effects. However, regarding our interest in the antioxidants properties of HNO, it also has the potential to serve as a one-electron reducer, via donation of its hydrogen atom. Indeed, HNO induced an increase in the expression and activity of the antioxidant protein heme oxygenase (HO-1), which has an important role in the cellular response to the oxidative damage[56]. We also found that AS induces HO-1 expression during the course of septic arthritis. Interestingly, the infection limited the expression of HO-1 mRNA expression, which is consistent with AS effect of reducing gp91^{phox} and COX-

2 and increasing glutathione reductase mRNA expression as well as reducing superoxide anion production, TBARS, FRAP and ABTS assays and GSH levels.

Another aspect to be considered is that the HNO bioavailability may be augmented in the face of disease-associated thiol (SH) depletion[57] as it occurs in inflammatory conditions. Importantly, thiols are likely to be key scavengers of HNO in vivo due their high affinities and GSH, in particular, has been suggested to be a key scavenger of HNO due to high intracellular concentration (1-10 mM/L) [21]. In this sense, the bioavailability is greater under oxidative stress, decreased HNO scavenging due to thiol depletion and the resistance of HNO to scavenging. Furthermore, cellular thiols could compartmentalize the action of HNO such that HNO interacts more readily with membrane-bound-biomolecules where the concentrations of scavengers such as GSH are low[15]. Undoubtedly, such compartmentalization and differential reactivity with thiols contributes to the distinct pharmacology/biology of HNO versus NO•. As such, it may be anticipated that the HNO donor efficacy would be preserved and/or enhanced in diseases states associated with oxidative stress, such as septic arthritis.

We have previously shown that AS presents analgesic and anti-inflammatory effects in models of non-infectious inflammation[22, 23]. Therefore, it is conceivable that AS inhibits inflammation independently of any antimicrobial action. However, the inflammatory process has a protective role in infection and simply reducing inflammation would predispose to uncontrolled infection. Thereby, the anti-inflammatory actions of AS do not fully explain its overall beneficial therapeutic impact septic arthritis disease progression. There was an important tenuous balance between an effective elimination of infection without eliciting post infectious inflammatory tissue lesion sequels. AS presented antimicrobial effect against *S.*

aureus in vitro, reduced the bacterial load in the knee joints and also inhibited the progression of the local infection to systemic infection. In this sense, HNO seems to prevent the driving force behind the long-lasting joint inflammation.

As with all pharmacological agents, the therapeutic usefulness of AS as HNO donor depends on the ability to elicit beneficial response without inducing harmful side effects[58]. Corroborating with the potential applicability of Angeli's Salt, as HNO donor, we observed no alteration in the levels of AST/ALT and creatinin/urea levels, markers to toxicity (comple.

Concluding, this is the first study demonstrating that the nitroxyl donor, Angeli's salt, inhibits the pain and inflammation in *S. aureus*-induced septic arthritis by its microbicidal, analgesic and anti-inflammatory effects.

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Conflict of Interest

There is no conflict of interest to declare.

Abbreviations

Angeli's salt, AS; aspartate aminotransferase (AST); alanine transaminase (ALT); bronchoalveolar lavage fluid, BALF; calcitonin gene-related peptide, CGRP; cicloxigenase-2, COX-2; colony-forming unity (CFU); cyclic guanosine monophosphate, cGMP; cGMP-dependent protein kinase, PKG; dithiobisnitrobenzoic acid, DTNB; 1,9-Dimethyl-Methylene Blue zinc chloride double salt, DMMB; enzyme-linked immunosorbent assay, Ethilenediamine tetraacetic acid (EDTA); ELISA; glycosaminoglycan, GAG; heme-oxygenase, reduced gluthathione (GSH); HO-1; interferon-γ, intra-articular (i.a.); IFN-γ; interleukin-1β, IL-1β; interleukin-17, IL-17; interleukin-33, IL-33; Malondialdehyde, MDA; mitogen activated protein kinases superfamily, MAP-kinases; minimum bactericidal concentration (MBC); myeloperoxidase (MPO); nicotinamide adenine dinucleotide phosphate-oxidase, NADPH oxidase; nitroblue tetrazolium, NBT; nitric oxide, NO•; nitroxyl, HNO; nuclear factor κB (NF-κB); nuclear factor- κB ligand, RANKL; osteoprotegerin (OPG); Phosphate-buffered saline (PBS); Quantitative polymerase chain reaction, qPCR; reactive oxygen species, ROS; reactive nitrogen species, RNS; sodium hydroxide, NaOH; soluble guanilate cyclase, sGC; subcutaneous, s.c.; thiobarbituric acid reactive substances, TBARS; tumoral necrose factor-α, TNF-α.

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SUPPLEMENTARY MATERIAL

The nitroxyl donor Angeli's salt inhibits *Staphylococcus aureus*-induced septic arthritis in mice.

Larissa Staurengo-Ferrari[a], Kenji W. Ruiz-Miyazawa[a]; Sandra S. Mizokami[a], Talita P. Domiciano[a], Felipe A. Pinho-Ribeiro[a], Victor Fattori[a], Jacinta S. Pelayo[b], Rubia Casagrande[c], Katrina M. Miranda[d], Waldiceu A. Verri Jr[a,*].

Results

AS chronic treatment does not increase serum levels of alanine aminotransferase (ALT), aspartate aminotransferase (AST), creatinine and urea. In order to asses if chronic treatment with AS induces hepatic or renal toxicity, serum levels of AST, ALT, creatinine and urea were determined in the sera. Mice were daily treated with AS (3 mg/kg, sc, 150 µl) or vehicle (19.35 µl of 10 mM NaOH plus saline to complete 150 µl) after the i.a. injection of *S. aureus* (10^7/10 µl/joint). At the 28th days, blood samples were collected. No difference was observed in serum levels of ALT, AST, creatinine and urea between mice treated with AS or vehicle. Thus, these results indicate that 28 daily treatments with AS or vehicle does not induce hepatic or renal toxicity.

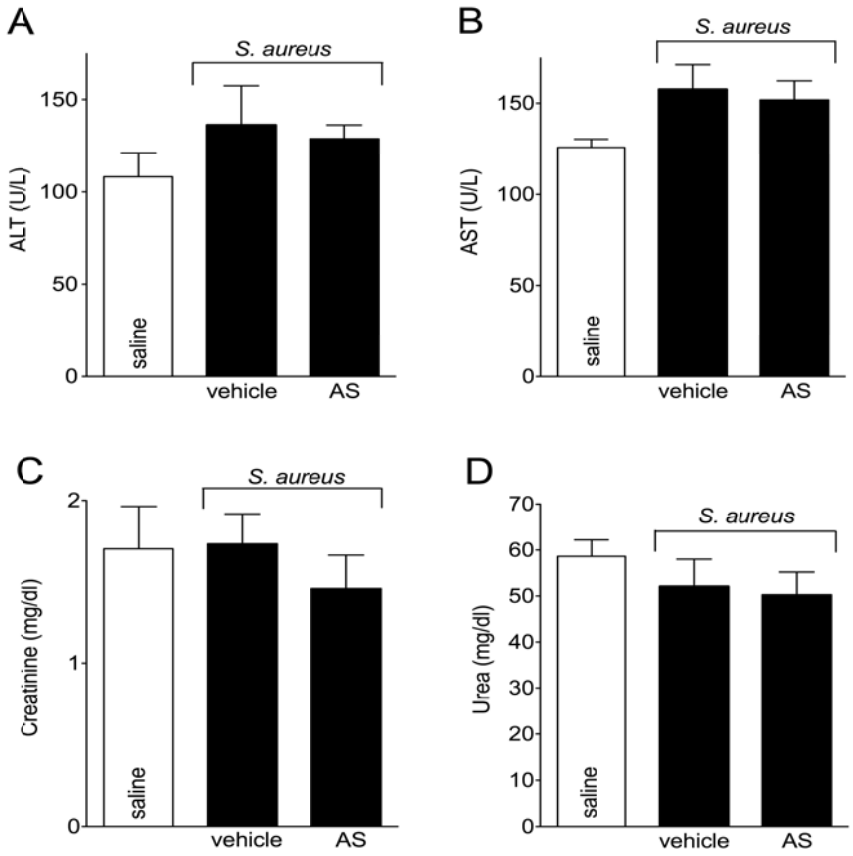


Figure S1. AS does not increase serum levels of aspartate aminotransferase (AST), alanine aminotransferase (ALT), creatinine and urea**.** Mice were treated with AS (3 mg/kg, s.c., 150 µl/joint) or vehicle (19.35 µl of NaOH 10 mM plus saline to complete 150 µl) over 28 days after i.a. *S.aureus* (10^7 CFU/10 µl) injection. After 28 days of treatment, mice were terminally anesthetized, and the blood were collected for ALT (panel A), AST (panel B), creatinine (panel C) and urea (panel D). N=6 per group per experiment, representative of two independent experiments. *P < 0.05 compared to saline group, # P < 0.05 compared to #P<0.05 compared with the *S. aureus* + vehicle group. One-way ANOVA followed by Tukey's test.

ANEXO C

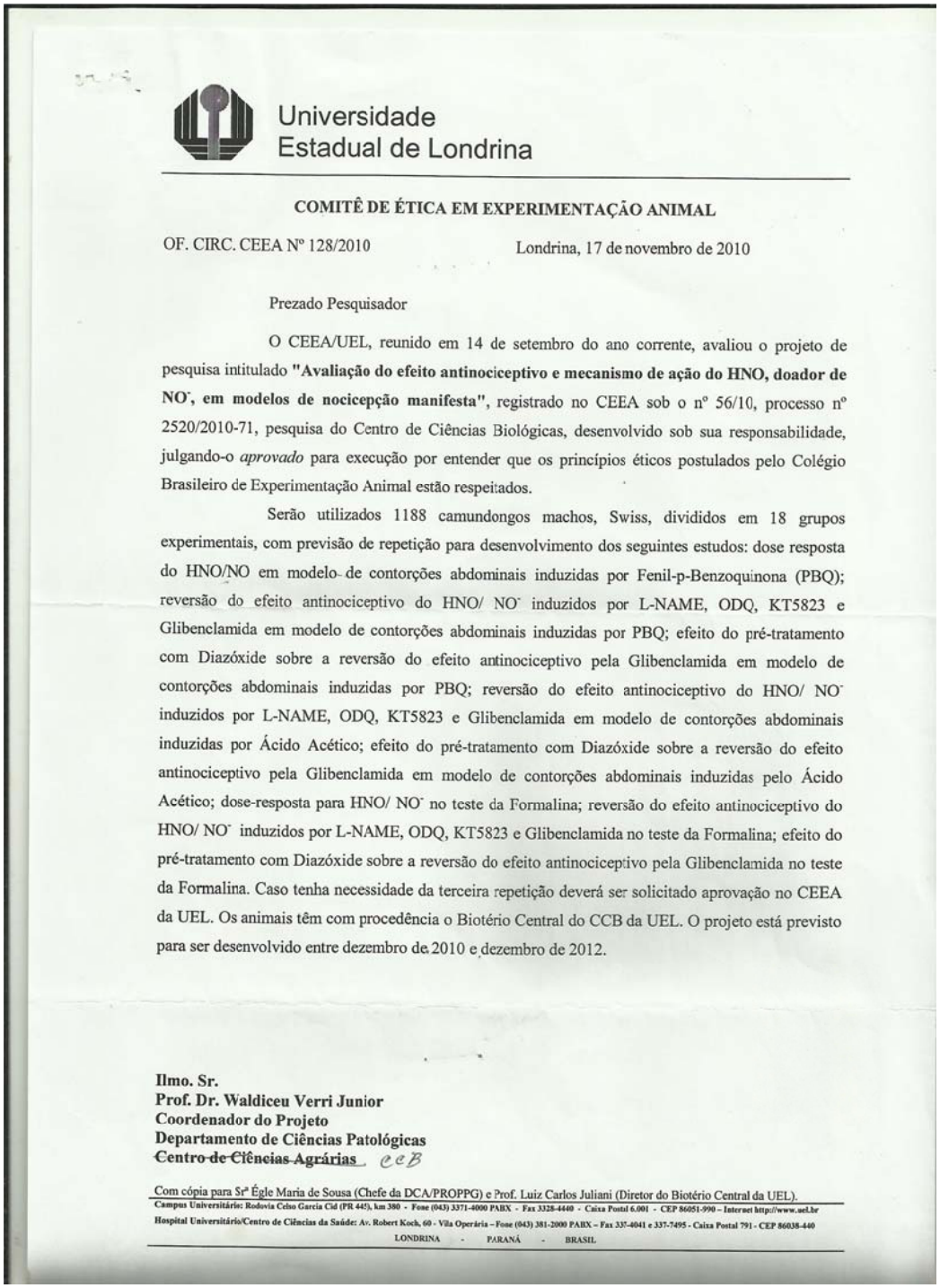
Endereço eletrônico do guia para autores das revistas científicas *Pharmacological Reports* e *Free Radicals Biology and Medicine*

Guia para autores da revista Pharmacological Reports. Disponível em: http://www.elsevier.com/journals/pharmacological-reports/1734-1140/guide-for-authors

Guia para autores da revista Free Radicals Biology and Medicine. Disponível em: http://www.elsevier.com/journals/free-radical-biology-and-medicine/0891-5849/guide-for-authors

ANEXO D

Pareceres das aprovações do Comitê de Ética em Experimentação Animal (CEUA) da Universidade Estadual de Londrina.



Universidade
Estadual de Londrina

COMITÊ DE ÉTICA EM EXPERIMENTAÇÃO ANIMAL

OF. CIRC. CEEA Nº 128/2010 Londrina, 17 de novembro de 2010

Prezado Pesquisador

O CEEA/UEL, reunido em 14 de setembro do ano corrente, avaliou o projeto de pesquisa intitulado **"Avaliação do efeito antinociceptivo e mecanismo de ação do HNO, doador de NO⁻, em modelos de nocicepção manifesta"**, registrado no CEEA sob o nº 56/10, processo nº 2520/2010-71, pesquisa do Centro de Ciências Biológicas, desenvolvido sob sua responsabilidade, julgando-o *aprovado* para execução por entender que os princípios éticos postulados pelo Colégio Brasileiro de Experimentação Animal estão respeitados.

Serão utilizados 1188 camundongos machos, Swiss, divididos em 18 grupos experimentais, com previsão de repetição para desenvolvimento dos seguintes estudos: dose resposta do HNO/NO em modelo de contorções abdominais induzidas por Fenil-p-Benzoquinona (PBQ); reversão do efeito antinociceptivo do HNO/ NO⁻ induzidos por L-NAME, ODQ, KT5823 e Glibenclamida em modelo de contorções abdominais induzidas por PBQ; efeito do pré-tratamento com Diazóxide sobre a reversão do efeito antinociceptivo pela Glibenclamida em modelo de contorções abdominais induzidas por PBQ; reversão do efeito antinociceptivo do HNO/ NO⁻ induzidos por L-NAME, ODQ, KT5823 e Glibenclamida em modelo de contorções abdominais induzidas por Ácido Acético; efeito do pré-tratamento com Diazóxide sobre a reversão do efeito antinociceptivo pela Glibenclamida em modelo de contorções abdominais induzidas pelo Ácido Acético; dose-resposta para HNO/ NO⁻ no teste da Formalina; reversão do efeito antinociceptivo do HNO/ NO⁻ induzidos por L-NAME, ODQ, KT5823 e Glibenclamida no teste da Formalina; efeito do pré-tratamento com Diazóxide sobre a reversão do efeito antinociceptivo pela Glibenclamida no teste da Formalina. Caso tenha necessidade da terceira repetição deverá ser solicitado aprovação no CEEA da UEL. Os animais têm com procedência o Biotério Central do CCB da UEL. O projeto está previsto para ser desenvolvido entre dezembro de 2010 e dezembro de 2012.

Ilmo. Sr.
Prof. Dr. Waldiceu Verri Junior
Coordenador do Projeto
Departamento de Ciências Patológicas
~~Centro de Ciências Agrárias~~ CCB

Com cópia para Srª Égle Maria de Sousa (Chefe da DCA/PROPPG) e Prof. Luiz Carlos Juliani (Diretor do Biotério Central da UEL).

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Hospital Universitário/Centro de Ciências da Saúde: Av. Robert Koch, 60 - Vila Operária – Fone (043) 381-2000 PABX – Fax 337-4041 e 337-7495 - Caixa Postal 791 - CEP 86038-440
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Cumpre orientar que caso se pretendam quaisquer alterações no protocolo experimental aprovado, deve-se submeter o novo protocolo à apreciação do CEEA/UEL anteriormente à execução das modificações.

Sem mais para o momento, subscrevo-me.

Cordialmente,

Profa. Dra. Mirian Siliane Batista de Souza
Coordenadora do CEEA/UEL

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COMITÊ DE ÉTICA EM EXPERIMENTAÇÃO ANIMAL

OF. CIRC. CEEA Nº 29/2011 Londrina, 09 de abril de 2011

Prezado Pesquisador

O CEEA/UEL, reunido em 14 de dezembro de 2010, avaliou o projeto de pesquisa intitulado **"Controle da artrite séptica pelo tratamento com um doador de nitroxil, o sal de Angeli: avaliação do efeito e mecanismos de ação na redução da dor inflamação e infecção"**, registrado no CEEA sob o nº 84/10, processo nº 33358.2010.36, pesquisa do Centro de Ciências Biológicas, desenvolvido sob sua responsabilidade. Esclarecidos os aspectos metodológicos solicitados, o projeto está *aprovado* para execução entendendo-se que os princípios éticos postulados pelo Colégio Brasileiro de Experimentação Animal estão respeitados.

Serão utilizados 696 camundongos Swiss, machos, pesando entre 20 e 25g, com procedência do Biotério Central do CCB-UEL. Os animais serão divididos em 216 grupos com 3 a 6 animais cada. Serão avaliadas a hiperalgesia mecânica e térmica, edema, avaliação da migração de leucócitos para a articulação, histologia, avaliação da produção de citocinas, extração do RNA, produção do DNAc e análise por PCR quantitativo, severidade clínica e teste de antioxidante. O projeto está previsto para ser executado entre abril de 2011 e fevereiro de 2013. Caso tenha necessidade da realização de mais uma repetição dos grupos experimentais, este deverá ser solicitada aprovação por este comitê.

Cumpre orientar que caso se pretendam quaisquer alterações no protocolo experimental aprovado, deve-se submeter o novo protocolo à apreciação do CEEA/UEL anteriormente à execução das modificações.

Sem mais para o momento, subscrevo-me.

Cordialmente,

Profa. Dra. Mirian Siliane Batista de Souza
Coordenadora do CEEA/UEL

Ilmo. Sr.
Prof. Dr. Waldiceu Aparecido Verri Junior
Coordenador do Projeto
Departamento de Ciências Patológicas
Centro de Ciências Biológicas

Com cópia para Srª Égle Maria de Sousa (Chefe da DCA/PROPPG) e Prof. Luiz Carlos Juliani (Diretor do Biotério Central da UEL).

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