Universidade Estadual de Londrina

SUELEN SANTOS DA SILVA

PROPRIEDADES BIOLÓGICAS DA PRÓPOLIS E DA CONCANAVALINA-A E NOVAS PERSPECTIVAS PARA O USO DO GLUCANTIME EM ESTUDOS COM *Leishmania amazonensis*

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Tese de doutorado apresentada ao Programa de Pós-Graduação em Patologia Experimental da Universidade Estadual de Londrina, como requisito parcial à obtenção do título de Doutor em Patologia Experimental.

Orientadora: Prof[a]. Dr[a]. Ivete Conchon Costa
Co-orientadora: Prof[a]. Dr[a]. Ionice Felipe

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"Toda a nossa ciência, comparada com a realidade, é primitiva e infantil - e, no entanto, é a coisa mais preciosa que temos."

Albert Einstein

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RESUMO

A leishmaniose é uma zoonose característica de regiões tropicais e subtropicais do mundo. Causada por protozoários do gênero *Leishmania* spp. a doença pode acometer diversos animais e o homem. A manifestação clínica apresentada pelos pacientes é complexa e envolve as diferentes espécies de agente etiológico e vetores relacionados com a transmissão da doença, bem como o tipo de resposta imune desenvolvida pelo hospedeiro. Os fármacos de primeira escolha utilizados para o tratamento da doença são os antimoniais pentavalentes, comercializado no Brasil por nome comercial Glucantime® (antimoniato de N-metil-glucamina). Este fármaco apresenta elevada toxicidade e restrições ao seu uso por conta dos efeitos colaterais, sendo seu mecanismo de ação não totalmente compreendido. As dificuldades na prevenção e tratamento da doença justificam o desenvolvimento de estudos para a busca por fármacos mais eficazes e a melhor compreensão dos mecanismos de ação do antimoniato de N-metil-glucamina. Desta forma, o objetivo deste estudo foi avaliar o efeito leishmanicida e anti-inflamatório do tratamento com extrato de própolis e, o efeito imunomodulador e leishmanicida da Concanavalina-A em modelos de infecção experimental com *Leishmania amazonensis*. Além disso, tivemos como objetivo verificar o efeito do antimoniato de N-metilglucamina em modelo de dor inflamatória induzida por infecção com *Leishmania amazonensis.* Quando investigada a utilização de extrato de própolis em modelos de infecção *in vitro* e *in vivo* com *L. amazonensis,* verificamos que a amostra de própolis testada apresentou ação leishmanicida reduzindo a proliferação de formas promastigotas e redução de carga parasitária do baço em camundongos susceptíveis a infecção. Além disso, em outro modelo, a mesma amostra de própolis apresentou efeito anti-inflamatório em fígado de camundongos BALB/c após infecção *in vivo*, reduzindo parâmetros inflamatórios como atividade de MPO e NAG, deposição de fibras de colágeno, produção de citocinas pró-inflamatórias e dos níveis de AST e ALT, onde consequentemente reverteu o quadro de hepatoesplenomegalia causado pela infecção. Desta forma, os resultados aqui apresentados evidenciaram o efeito leishmanicida e anti-inflamatório da amostra de própolis coletada na região de São Paulo. O efeito da Concanavalina-A foi avaliado *in vitro* em modelo de pré-tratamento intraperitoneal de camundongos BALB/c para posterior infecção de macrófagos peritoneais com *L. amazonensis*. Os resultados evidenciaram que o pré-tratamento (250µg/250µL, i.p.) com esta lectina foi capaz de imunomodular a resposta de leucócitos totais do peritônio, bem como potencializar a ação leishmanicida de macrófagos *in vitro* via produção de IL-1β e espécies reativas de oxigênio. Além destes achados, realizou-se um estudo para verificar a atividade do antimoniato de N-metil-glucamina na dor inflamatória frente à infecção experimental com *L. amazonensis*. O antimoniato de N-metil-glucamina (10mg/kg, i.p.) mostrou-se eficaz na redução da hiperalgesia em modelo de dor crônica induzido por infecção com *L. amazonensis* e estímulo com CFA. A redução da hiperalgesia foi acompanhada de redução de migração leucocitária (avaliada por MPO e NAG), bem

como na redução da produção de IL-1β e IL-6. Os dados aqui apresentados consolidam o efeito leishmanicida e anti-inflamatório do extrato de própolis e novas perspectivas para o uso da Concanavalina-A e do medicamento antimoniato de N-metil-glucamina. Estes dados incentivam novos estudos para o entendimento de outros mecanismos envolvidos.

Palavras-chave: *Leishmania amazonensis*. Própolis. Concanavalina-A. Glucantime. Hiperalgesia.

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ABSTRACT

Leishmaniasis is a zoonotic disease characteristic of tropical and subtropical regions of the world. Caused by protozoa of the genus *Leishmania* spp. the disease can affect various animals and human. The clinical manifestation apresented by patients is complex and involves different specie of etiologic agents and vectors related to the transmission of the disease and the type of immune response developed by the host. The first choice drugs used to treat the disease are the pentavalent antimonial compounds, marketed by Glucantime in Brazil (antimoniate N-methyl-glucamine). This drug has high toxicity and restrictions on their use because of side effects. The mechanism of action is not well understood presenting several hypotheses. The difficulties in the prevention and treatment of this disease justify the development of studies to search for more effective drugs and better understanding of the mechanisms of action antimoniate N-methyl-glucamine. Thus, the aim of this study was to evaluate the leishmanicidal and anti-inflammatory effect of treatment with propolis extract and the immunomodulatory and leishmanicidal effect of Concanavalin-A in experimental infection model with *Leishmania amazonensis*. In addition, we aimed to evaluate the effect of antimoniate N-methyl-glucamine me in model of inflammatory pain induced by infection with *Leishmania amazonensis*. When investigated the use of propolis extract in models of infection *in vitro* and *in vivo* with *L. amazonensis*, we found that the propolis samples tested showed leishmanicidal activity reducing the proliferation of promastigotes and reduction of parasite load of spleen in mice susceptible to infection. Furthermore, in another model, the same propolis sample showed anti-inflammatory effect on BALB/c mice liver after *in vivo* infection, reducing inflammatory parameters such as MPO and NAG activity, deposition of collagen fibers, production of pro-inflammatory cytokines and AST and ALT levels, that consequently reversed the hepatosplenomegaly condiction caused by infection. Thus, the results presented here showed the leishmanicidal and anti-inflammatory effect of propolis sample from São Paulo region. The effect of Concanavalin-A was evaluated in peritoneal macrophages a model of intraperitoneal pretreatment in BALB/c mice with subsequent *in vitro* infection with *L. amazonensis.* The results showed that the pre-treatment (250μg/250μL, i.p.) with this lectin was able to immunomodulate the total peritoneal leukocytes response and enhance the leishmanicide action of macrophages *in vitro* via IL-1β and reactive species oxygen. In addition to these findings, we performed a study to determine the activity of the inflammatory front glucantime pain to experimental infection with *L. amazonensis*. The antimoniate N-methy-lglucamine (10mg/kg) was effective in reducing hyperalgesia in a model of chronic pain induced by *L. amazonensis* infection and CFA stimulus. The decrease in hyperalgesia was followed in the reduction of leukocyte migration (assessed by MPO and NAG), as well as, the reduction of IL-1β and IL-6 production. The present data consolidate the leishmanicidal and anti-inflammatory effect of propolis extract and reported a new perspective to use of the Glucantime. However, is necessary more further research to understand the mechanisms involved.

Keywords: *Leishmania amazonensis*. Propolis. Glucantime. Hiperalgesia.

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LISTA DE ABREVIATURAS E SIGLAS

ALT	Alanina aminotransferase (transaminase)
AST	Aspartato aminotransferase (transaminase)
BALB/c	Linhagem de camundongos isogênicos
Células Th1	Células T helper tipo 1
Células Th2	Células T helper tipo 2
C57BL/6	Linhagem de Camundongos isogênicos
CFA	Adjuvante completo de Freund
CO_2	Gás carbônico
CR	Receptor de Complemento
ERN	Espécies Reativas de Nitrogênio
ERO	Espécies Reativas de oxigênio
GM-CSF	Fator Estimulador de Colônias de Granulócitos e Macrófagos
H2O2	Peróxido de hidrogênio
IFN-γ	Interferon gama
IL	Interleucina
IL-1	Interleucina 1
IL-6	Interleucina 6
IL-8	Interleucina 8
LLa	*Leishmania (Leishmania) amazonensis*
LTA	Leishmaniose Tegumentar Americana
LV	Leishmaniose Visceral
MPO	Mieloperoxidase
NAG	N-acetil-glicosaminidase
SbIII	Antimônio trivalente
SbV	Antimônio pentavalente
TNF-α	Fator de Necrose Tumoral alfa

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1 INTRODUÇÃO

1.1 DESCRIÇÃO DO PROBLEMA DE PESQUISA

As leishmanioses constituem um complexo de doenças que apresentam diferentes manifestações clínicas. Causada por protozoários do gênero *Leishmania* e transmitida através da picada de insetos flebotomíneos, podem apresentar-se clinicamente sob a forma visceral e cutânea (HANDMAN, 1999; KAYE; SCOTT 2011; MURRAY, 2001).

A leishmaniose visceral (LV) é uma doença sistêmica, na qual os parasitos possuem tropismo pelo sistema mononuclear fagocitário do baço, fígado, medula óssea e tecidos linfóides. As manifestações clínicas se caracterizam por febre prolongada, perda de peso, hepatoesplenomegalia, diarreia, tosse e pancitopenia. Quando não tratada a LV pode levar o hospedeiro a morte (MCCALL; ZHANG; MATLASHEWSKI, 2013).

Por outro lado, a leishmaniose tegumentar americana (LTA) caracteriza-se por apresentar inflamações crônicas que afetam a pele e/ou mucosas, podendo assim, se manifestar clinicamente em formas cutânea, cutânea difusa ou mucocutânea (REITHINGER et al., 2007).

A forma cutânea da doença, apresenta-se classicamente por pápulas que evoluem para úlceras com bordas elevadas e fundo granuloso, que podem ser únicas ou múltiplas. A forma difusa, caracteriza-se por placas nodulares, papulosas, que se apresentam de forma localizada ou difusa. Já a forma mucosa, secundária ou não à lesão cutânea, caracteriza-se por infiltração, ulceração e destruição de tecidos como os da cavidade nasal, faringe e laringe (BITTENCOURT; BARRAL, 1991; GREVELINK; LERNER,1996).

Existe associação entre a espécie de *Leishmania* envolvida e a forma clinica apresentada pelo hospedeiro, no entanto, uma mesma espécie pode produzir diversas manifestações clínicas. Desta forma, o amplo espectro de manifestações clinicas não está ligado apenas às características próprias do agente etiológico, como tropismo, capacidade invasiva e patogenicidade mas também está ligado à resposta imunológica desenvolvida pelo hospedeiro (BARRAL et al., 1991; NYLÉN; GAUTAM, 2010; CHANG; MCGWIRE, 2002).

Dentre as espécies circulantes no Brasil, as mais amplamente distribuídas responsáveis pela forma cutânea e mucocutânea são *L. amazonensis* e

L. braziliensis, enquanto que a espécie *L. chagasi* é responsável pela forma visceral (SILVEIRA et al.,1990; GRIMALDi et al., 1987; LUZ et al., 2000).

Esta parasitose apresenta distribuição mundial em regiões temperadas, tropicais e subtropicais, constituindo um grave problema de saúde pública, com registros em todos os estados brasileiros. No Paraná a LTA possui caráter endêmico com notificação principalmente nas regiões norte e oeste do Estado (LIMA et al., 2002).

No ano de 2013 o Brasil apresentou 21.792 casos notificados de leishmanioses, sendo destes, 3.117 referentes a forma visceral (LV). Dos 18.674 casos referentes a LTA, 1.066 apresentavam a forma clínica mucosa e 17.608 casos apresentavam a forma cutânea (SINAN - Sistema de Informação de Agravos de Notificação, 2015).

Neste mesmo ano, no estado do Paraná nenhum caso de LV foi notificado, enquanto que 302 casos de LTA foram notificados. De todos os casos confirmados 260 apresentavam a forma cutânea enquanto que 41 apresentavam a forma mucosa. No município de Londrina houveram 16 casos de LTA, 14 na forma cutânea e dois casos na forma mucocutanea nesta mesma época (SINAN - Sistema de Informação de Agravos de Notificação, 2015).

A notificação de leishmaniose é obrigatória no Brasil, o que auxilia a vigilância epidemiológica para o diagnóstico e tratamento precoce. No entanto, fatores relacionados a complexidade epidemiológica da doença (diferentes espécies de agentes etiológico, vetores e reservatórios) dificulta a prevenção e controle desta doença (ROBERTS, 2005).

Outro fator limitante no controle da leishmaniose está nos fármacos disponíveis para o tratamento. Os fármacos utilizados são principalmente os antimoniais pentavalentes como o estibogluconato de sódio (Pentostan®) e Antimoniato de N-metilglucamina (Glucantime®), bem como, anfotericina B e pentamidina (MURRAY et al., 2005; RATH et al., 2003, MONTE-NETO et al., 2011; SUNDAR et al., 2014).

A Organização Mundial da Saúde e o Ministério da Saúde do Brasil recomendam os antimoniais pentavalentes como medicamento de primeira escolha. No entanto, apresentam contraindicações ao uso dos antimoniais: gestantes e portadores de cardiopatias, nefropatias e hepatopatias. Além disso, o medicamento apresenta importantes efeitos colaterais (FUNASA, 2000; MEDEIROS et al., 2005).

Existem relatos de resistência dos parasitos a estes fármacos em áreas endêmicas. Isto, aliado a relatos da cura clínica não ser acompanhada de cura parasitológica de indivíduos após tratamento permite nos inferir que o tratamento para esta doença representa um grande desafio (LUCUMI et al., 1998; BAPTISTA et al., 2015; GOMES et al., 2015).

A recidiva, a resistência ao tratamento e a falta de vacinas eficazes são fatores que motivam a busca por fármacos alternativos, uma vez que o controle da doença por prevenção é de grande complexidade (FALQUETO; SESSA, 2005).

1.2 INTERAÇÃO PARASITO-HOSPEDEIRO

O ciclo biológico dos parasitos do genêro *Leishmania* envolve duas formas evolutivas distintas, a promastigota, que é flagelada e de vida extracelular, encontrada no sistema digestório do vetor e a forma amastigota que não possui mobilidade e é encontrada no interior das células do sistema imunitário do hospedeiro vertebrado (MICHALICK, 2005).

Quando inoculadas na derme do hospedeiro, as formas promastigotas metacíclicas sofrem interação, neste microambiente, com proteínas do sistema complemento do soro, saliva e fluidos digestivos do inseto. Ocorre então, o recrutamento de neutrófilos e captação de parasitos por neutrófilos, células dendrítica e macrófagos (BEATTIE; KAYE, 2011).

A interação com as células fagocitícas ocorre via receptores, onde promastigotas metacíclicos deixam-se captar via complemento (CR1 e 3), receptores tipo Toll (TLRs) 2 e 4 assim como receptores de manose-fucose e dectin (UENO; WILSON 2012; VÁZQUEZ-MENDOZA et al., 2013; CECÍLIO et al., 2014).

De qualquer maneira, parasitos do genero *Leishmania* podem modular ou interferir no reconhecimento de padrões mediados por receptores e podem utilizá-los como um mecanismo de escape da resposta imune do hospedeiro suprimindo as vias que desencadeiam respostas inflamatórias (DE VEER et al., 2003; NETEA et al., 2004).

Os fagócitos têm sido descritos como a primeira linha de defesa na leishmaniose experimental. A própria saliva do inseto induz uma rápida infiltração de neutrófilos e um recrutamento substancial de macrófagos para o local da picada.

Estas células além da sua capacidade fagocítica são produtoras de quimiocinas e citocinas que influenciam a resposta imune à infecção por estes parasitos (SCAPINI et al., 2001; PETERS et al., 2008).

Quando ativados, macrófagos aumentam a produção de espécies reativas de oxigênio (ERO) e espécies reativas de nitrogênio (ERN), aumentando a atividade microbicida, além de apresentar um aumento na produção de citocinas, como as interleucinas IL-1, IL-6 e IL-8, fator estimulador de colônias de macrófagos e granulócitos (GM-CSF) e fator de necrose tumoral α (TNF-α) que podem estimular outras células ou os próprios macrófagos em uma atividade autócrina (LUSTER et al., 2005).

Apesar dos neutrófilos serem uma das primeiras células do sistema imunológico natural inato a infiltrar para o tecido, seu papel na leishmaniose tem sido descrito nos casos de susceptibilidade a doença, visto a participação destas células como veículo de disseminação dos parasitos *Leishmania major* no organismo hospedeiro. Este mecanismo foi proposto como “Cavalo de Tróia”, que postula que a captação de neutrófilos infectados por macrófagos é um mecanismo de “entrada silenciosa” do parasito em macrófagos (CECÍLIO et al., 2014).

Neste contexto, é importante entender que os protozoários do gênero *Leishmania* possuem mecanismos que lhes permitem escapar da resposta imune inicial, de maneira que consiga evitar a ativação dos principais mecanismos microbicidas formados pelas células fagocíticas, conseguindo assim se estabelecer e se multiplicar no vacúolo parasitóforo (NYLÉN; GAUTAM, 2010).

Além do parasito modular a apoptose em netrófilos, como citado acima, outros mecanismos de evasão são descritos como a capacidade em alterar o processo de maturação do fagolisossomo, modular a produção de citocinas e quimiocinas pelas células hospedeiras (CECÍLIO et al., 2014).

Assim, alguns estudos têm evidenciado que apenas neutrófilos e macrófagos ativados são capazes de eliminar os parasitos intracelulares (PEARSON; STEIGBIGEL, 1981; NYLÉN; GAUTAM, 2010).

É importante entender que a ativação dos macrófagos confere ao hospedeiro maior resistência à infecção, seja pelo aumento de receptores em sua superfície ou pelo aumento de sua capacidade de eliminar o patógeno por meio da produção de substâncias de ação microbicida. Desta forma, estas células são consideradas como principais efetoras em infecções por patógenos intracelulares,

como as formas amastigotas de *Leishmania* spp.

1.3 RESPOSTA IMUNE EM INFECÇÃO EXPERIMENTAL: SUSCEPTIBILIDADE E RESISTÊNCIA À DOENÇA

Inúmeros estudos têm conseguido explicar os fatores responsáveis pelos fenótipos de resistência e susceptibilidade à infecção por parasitos do gênero *Leishmania*. Estes estudos envolvem os mecanismos de resposta imune nas leishmanioses e são realizados, normalmente, em modelos murinos (LAUNOIS et al., 1996).

O principal modelo utilizado para propor a relação entre o tipo de resposta imune desenvolvida pelo hospedeiro, susceptibilidade e resistência à doença tem sido por meio da utilização de infecções experimental com *L. major* em diferentes linhagens de camundongos. Assim, conseguiu-se verificar que camundongos BALB/c são geneticamente susceptíveis a infecção por *L. major*, pois desenvolvem lesões cutâneas no sítio de inoculação e não conseguem desenvolver uma resposta imune ao parasito mediada por células. No entanto, camundongos resistentes a infecção por *L. major* como, por exemplo: C57BL/6 apresentam cura espontânea, graças a uma forte resposta imune celular, e mostram-se resistentes a novas reinfecções (LOCKSLEY et al., 1987; AWASTHI et al., 2004).

Desta forma, tem-se estabelecido que a susceptibilidade e a resistência a esta doença é determinada pela distinta expressão de células do tipo Th1 ou Th2. No caso de estimulação de células Th1, as principais citocinas produzidas (IL-2, IL-12, INF-γ e TNF-α) logo no início da infecção, irão promover a ativação de macrófagos e, consequentemente, a eliminação do parasito. Já no caso de estimulação do tipo Th2, serão produzidas citocinas (IL-4, IL-5, IL-10 e IL-13) que inibirão a ativação de macrófagos, contribuindo para a sobrevivência do parasito (SACKS et al., 2002; HEINZEL et al., 1989).

Apesar do modelo proposto, podem existir diferenças significativas quanto à resposta das diferentes linhagens de camundongos às distintas espécies de *Leishmania*. Assim, camundongos BALB/c são susceptíveis a infecção por *L. major* e *Leishmania (Leishmania) amazonensis* (XIN et al., 2011). No entanto, este mesmo camundongo apresenta-se resistentes à infecção por *L. (Viannia) braziliensis* (ROCHA et al., 2007).

Em um estudo envolvendo a infecção das espécies *L. amazonensis* (MHOM/BR/1989/166MJO) e *Leishmania braziliensis* (MHOM/BR/1987/M11272) em camundongos BALB/c. Os autores observaram que a infecção por *L. amazonensis* estimulou com maior intensidade a migração de células em relação à infecção por *L. braziliensis*, com predomínio de neutrófilos, seguido de macrófagos. Já os animais infectados com *L. (V.) braziliensis* apresentavam predomínio de eosinófilos (WAKIMOTO et al., 2010).

As diferentes respostas de migração celular, podem se correlacionar com a informação de que camundongos BALB/c apresentam susceptibilidade a infecção por *L. amazonensis* e resistência a infecção por *L. braziliensis*. Isto, aliado a estudos que evidenciam a importância das quimiocinas produzidas na fase inicial da infecção, bem como a interferencia dos parasitos nas vias de sinalização por receptores de membrana pode esclarecer a manifestação clínica da doença por meio dos diferentes estabelecimento da resposta imune (CAMPANELLI et al., 2010; OGHUMU et al., 2010).

Desta forma, é importante perceber que a fase inicial de resposta é de extrema importância para o resultado da infecção por parasitos do gênero *Leishmania*. O resultado clínico torna-se dependente do recrutamento celular estabelecido pelas condições de imunidade do hospedeiro no momento inicial da lesão (CAMPANELLI et al., 2010; OGHUMU et al., 2010; GIUDICE et al., 2012).

Assim, existe complexidade das interações entre protozoários *Leishmania*, células hospedeiras e resposta imune, de modo que o curso da infecção dependerá tanto das características imunogenéticas associadas à resposta de células T do hospedeiro, como da virulência da espécie de *Leishmania* infectante (REIS et al., 2006; SCHRIEFER, 2005).

1.4 CARACTERIZAÇÃO DAS ATIVIDADES DO ANTIMONIATO DE N-METIL-GLUCAMINA

Antimoniato de N-metil-glucamina conhecido comercialmente por Glucantime®, tem sido utilizado como fármaco de primeira escolha para o tratamento das leishmanioses desde 1912 (GENARO; REIS, 2005).

Os primeiros relatos de seu uso foram na forma trivalente (antimônio trivalente – Sb^{3+}), o chamado tártaro emético (tartarato de potássio e antimônio)

(Bezerra et al. 2004). No entanto, essa formulação apresentava graves efeitos colaterais aos pacientes devido à alta toxicidade. Assim, a fim de diminuir tais efeitos, introduziu-se a utilização de outra formulação, onde o antimônio apresenta-se na forma pentavalente ($Sb5+$) por estibogluconato de sódio (Pentostam®) e o antimoniato de N-metil-glucamina (Glucantime®), reduzindo-se assim alguns dos efeitos colaterais e toxicidade (SUNDAR; OLLIARO, 2007; MURRAY et al., 2005; RATH et al., 2003, MONTE-NETO et al., 2011; SUNDAR et al., 2014).

As dificuldades relacionadas ao tratamento devem-se ao fato de que estes fármacos se apresentam na forma injetável, onde pode ser infundido via endovenosa, intramuscular ou parenteral, o que dificulta o tratamento, já que o paciente para ser tratado, precisa comparecer ao hospital, ou em alguns casos, deve ser internado para as aplicações. Os tratamentos na maioria das vezes seguem aplicação diária em ciclos de 28 dias (MEDEIROS et al., 2005).

Em geral, os tratamentos com os antimoniais pentavalentes são bem aceitos, porém podem apresentar algumas reações adversas tais como, fadiga, disfunção gastrintestinal, anormalidades no eletrocardiograma, dores musculares difusas, enrijecimento das articulações, arritmias, pancreatite e nefrotoxicidade (BALLOU et al., 1987; HERWALDT; BERMAN, 1992; DESJEUX, 1996; BLUM et al., 2004; LAWN et al., 2006; MONTE-NETO et al., 2011; SUNDAR et al., 2014). Podem ocorrer ainda, elevação dos níveis das transaminases hepáticas (AST e ALT), artralgia, mialgia, cefaleia e adinamia (TRACY; WEBSTER, 1996; GASSER et al., 1994).

Desta forma, devido às dificuldades apresentadas quanto à administração e a duração do tratamento com os fármacos pentavalentes, bem como os efeitos colaterais apresentados pelos mesmos, alguns pesquisadores vêm buscando novas formulações para este fármaco. Existe a sugestão do encapsulamento deste fármaco, que levaria a uma redução de seus efeitos colaterais, assim como melhoraria o direcionando do fármaco para o sitio de ação, controle da concentração e a velocidade de liberação do fármaco no órgão-alvo (TRACY; WEBSTER, 1996; SOARES-BEZERRA; LEON; GENESTRA, 2004).

O mecanismo de ação destes compostos ainda é pouco compreendido, porém alguns autores relatam que este fármaco atua na inibição das enzimas da via glicolítica e da β-oxidação de ácidos graxos em formas amastigotas, contudo, devido a sua constituição ser composta por metal pesado, acredita-se que

interfira em outras vias metabólicas de *Leishmania* spp., bem como, com algumas vias do hospedeiro (HERERWALDT, 1999; DAVIDSON, 1998, ROBERTS, 2005; SERENO et al., 1997; AÏT-OUDHIA et al., 2011).

Além destes mecanismos apresentados anteriormente, alguns autores demonstraram que fármacos pentavalente possuem a capacidade de inibir a enzima topoisomerase I e II, inibindo a síntese proteica. Além disso, este fármaco pode inibir a enzima fosfofrutoquinase, envolvida na síntese de nucleotídeos trifosfatados em diferentes espécies de *Leishmania* (CHAKRABORTY; MAJUNDER, 1988; LUCUMI et al., 1998; BERMAN et al., 1985; MURRAY et al., 2005; RATH et al., 2003, MONTE-NETO et al., 2011; SUNDAR et al., 2014).

Outra hipótese compreende a possível conversão do antimonial pentavalente para a forma trivalente (SbIII), atuando como pró-fármaco (SERENO et al., 1997; HAIMEUR et al., 2000; KOTHARI et al., 2007; LIMA et al., 2007; MURRAY et al., 2005; RATH et al., 2003, MONTE-NETO et al., 2011; SUNDAR et al., 2014). Os autores sustentam esta ideia, acreditando que conversão do SbV a SbIII é realizada por meio de uma enzima específica ao parasito *Leishmania*, enzima redutase dependente de tiol (TDR1), que utiliza glutationa (GSH) como redutor (Denton, Mcgregor et al., 2004). Por outro lado, alguns autores sugerem que cisteína (Cys) e cisteína-glicina (Cys-Gly), principais tióis dos compartimentos ácidos de células de mamíferos e a tripanotiona (tiol específico do parasito leishmania-T(SH)2), seriam os principais responsáveis pela redução de SbV a SbIII e não o GSH (glutationa) e, ainda, que tal conversão seria favorecida em pH ácido, similar aos fagolisossomos de macrófagos, local onde as amastigotas residem (DOS SANTOS; MARTINS, 2003; OLIVEIRA et al., 2006).

Além dos aspectos abordados, existem relatos de recidiva após o tratamento com os antimoniais pentavalentes. Desta forma, fármacos alternativos podem ser utilizados, especialmente nos casos de intolerância, contra-indicação ou resistência aos antimoniais. Nestes casos podem ser utilizados a anfotericina B, pentamidina, miltefosine e imunoterapia (MEDEIROS et al., 2005; MURRAY et al., 2005; RATH et al., 2003, MONTE-NETO et al., 2011; SUNDAR et al., 2014; SEBLOVA et al., 2014).

1.5 CARACTERIZAÇÃO DAS ATIVIDADES DA PRÓPOLIS

A produção da própolis, por abelhas, é realizada por envolvimento de diversas substâncias como: secreções salivares produzidas pela abelhas, ceras e produto resultante da digestão do pólen por abelhas, além da coleta de flores, pólen, brotos e exsudatos de plantas (PEREIRA et al., 2002; GHISALBERTI, 1979).

Desta forma, a composição química da própolis é dependente da biodiversidade de cada região visitada pelas abelhas, podendo ainda sofrer interferência pela espécie de abelha, época de coleta e substância (solvente) utilizada para a sua extração (WATSON et al., 2006; CHEN et al., 2003).

As atividades biológicas da própolis relatadas na literatura incluem atividades antibacteriana (SFORCIN et al., 2000), antiviral (VYNOGRAD et al., 2000), anti-inflamatória (KHAYYAL et al., 2003) e imunomodulatória (SFORCIN et al., 2002; SÁ-NUNES et al., 2003, DA SILVA et al., 2013).

Os efeitos leishmanicida da própolis foram evidenciados em estudos que demonstraram que própolis coletada em diferentes regiões foram efetivas em reduzir a proliferação de formas promastigota, amastigotas, ou ainda auxiliar na resolução de lesões provenientes de infecções experimentais *in vivo* (AYRES; MARCUCCI; GIORGIO, 2007; MACHADO et al., 2007; PONTIN et al., 2008; OZBILGE et al., 2010; DA SILVA et al., 2013; MIRANDA et al., 2015).

Além dos efeitos leishmanicidas alguns estudos tem relatado também a ação de extratos de própolis na cicatrização de lesões subcutâneas induzidas em diferentes modelos inflamatórios (BARBOSA et al. 2009; KHORASGANI; KARIMI; NAZEM, 2010; IKEDA et al., 2011; OLCZYK et al., 2013a; OLCZYK et al., 2013b; BÚFALO et al., 2013).

A própolis também pode exercer efeito hepatoprotetor (SEO et al. 2003). Os mecanismos de ação envolvido nos efeitos anti-inflamatórios deste apiterápico envolve a capacidade de inibir a ativação de células T, afetando principalmente as atividades de NF-κB, MAP cinases e STAT 3 (OKAMOTO et al., 2012; BÚFALO et al., 2013). Alguns estudos reportaram a diminuição das atividades de mieloperoxidase (MPO) e NADPH-oxidase (FRENKEL et al., 1993; VOLPERT; ELSTNER, 1996) e portanto exercendo ação anti-inflamatória.

1.6 CARACTERIZAÇÃO DAS ATIVIDADES DA CONCANAVALINA- A

Concanavalina-A (Con-A) é uma lectina extraída da semente de *Canavalia ensiformis*, leguminosa conhecida popularmente por feijão- de- porco, uma planta tropical, da família Fabaceae, cultivada em países tropicais (SUMNER; HOWELL, 1936).

De maneira geral, as lectinas apresentam afinidade por polissacarídeos e monossacarídeos terminais presentes em glicoconjugados (LIS; SHARON, 1998). De acordo com Etzler (1985), lectinas são proteínas não pertencentes ao sistema imunológico, porém capazes de reconhecer sítios específicos em moléculas e ligar-se reversivelmente a carboidratos, sem alterar a estrutura covalente das ligações glicosídicas destes sítios.

Assim, a Con-A pode se ligar diretamente às porções de carboidratos do MHC de células apresentadoras de antígenos (APC) e no receptor de linfócito T (TCR), induzindo a resposta celular pela oligomerização de TCRs na membrana plasmática, com consequente indução de resposta celular (KEREN; BERKE, 1984) como ativação policlonal dos linfócitos, estimula, a liberação de IFN-γ (OKAMOTO; KOBAYASHI 1997) e aumento de níveis de IL-1β RNAm bem como, níveis de IL-2 e IL-2R (OKAMOTO; KOBAYASHI, 1997).

Desta forma, Con-A tem sido descrita pela capacidade em estimular a mitose celular, e tem sido amplamente utilizada em pesquisas por sua capacidade em influenciar na resposta imune por estimular linfócitos T e células do sistema imune na indução e síntese de citocinas (DWYER; JOHNSON, 1981; SODHI; KESHERWANI, 2007; BAO et al., 2011; SODHI; TARANG; KESHERWANI, 2007; CARVALHO et al., 2012).

Alguns autores conseguiram evidenciar também a participação desta lectina no aumento de expressão de receptores do tipo Toll 2-9 em macrófagos de camundongos BALB/c (SODHI; TARANG; KESHERWANI, 2007) e aumento da expressão de receptores de manose em macrófagos de camundongos (LOYOLA et al., 2002).

A expressão aumentada de receptores de manose, associado ao aumento de citocinas como IFN-γ, IL-1β, IL-17 e IL-6 tem melhorado a capacidade fagocítica de macrófagos em modelos de candidíase experimental promovendo o controle da infecção e a eliminação deste parasita (KARBASSI et al., 1987;

MARODI; JOHNSTON,1993; GERALDINO et al., 2010; FELIPE et al., 1995 ; MORESCO et al., 2002; SODHI; TARANG; KESHERWANI, 2007; CARVALHO et al., 2012).

Alguns estudos evidenciaram que esta lectina poderia ser hepatotoxica. No entanto, Conchon-Costa et al., 2007 conseguiram estabelecer uma dose cujo efeito foi hepatoprotetor, promovendo efeito candidacida em macrófagos além de promover a sobrevida de camundongos infectados com *Candida albicans* (CONCHON-COSTA et al., 2007).

Assim, baseado nas evidências experimentais encontradas em outros modelos, acreditamos que a utilização como pré-tratamento e/ou tratamento na fase inicial da infecção por *Leishmania* possa apresentar atividade leishmanicida por meio da ativação celular, promovendo fenótipo de resistência a camundongos BALB/c.

1.7 DOR INFLAMATÓRIA E MODELOS DE DOR NA LEISHMANIOSE EXPERIMENTAL

O processo inflamatório é decorrente de resposta imune à estimulos lesivos, e envolve a participação de componentes vasculares, celulares e uma diversidade de substâncias solúveis. Os sinais clínicos característicos deste processo são: rubor, calor, edema e dor, podendo ou não haver prejuízo funcional (YOON; BAEK, 2005).

De forma geral, a inflamação é marcada pela migração de leucócitos para o local de injuria, evento este, decorrente de estímulos pró-inflamatórios como a produção de quimiocinas, citocinas e diversos mediadores inflamatórios (OLIVEIRA et al., 2011).

Desta forma, os sinais emitidos por mediadores inflamatórios, como quimiocinas e citocinas são fundamentais para a ativação de celulas efetoras e linfócitos para o início da resposta imune, destacando-se neste quadro as citocinas IL-1, IL-6 e TNF-α que são liberadas nos estágios iniciais (AKIRA et al.,1990).

O processo inflamatório pode ser dividido nos estágios de inflamação aguda e crônica. A inflamação aguda, estágio inicial, é marcado pela ativação das células do sistema imunitário, leucócitos, macrófagos e linfócitos a partir de uma fase vascular que tem início imediatamente após o dano, envolvendo

basicamente a microcirculação. Este tipo de inflamação persiste por um curto período de tempo e é, normalmente, benéfica ao organismo (NATHAN, 2012).

Na inflamação crônica ocorre a persistência do processo inflamatório caracterizadas por mudança progressiva nos elementos celulares e solúveis que infiltram o tecido. Ocorre maior envolvimento da imunidade adquirida, de células imunocompetentes, macrófagos e fibroblastos aumentando as probabilidades de que a reconstituição tecidual não seja completa (RICKLIN; LAMBRIS, 2013).

A dor, importante sinal clínico durante os processos inflamatórios, pode ser descrita como uma experiência sensorial e emocional desagradável decorrente de lesões teciduais já existentes ou potenciais, definição da Associação Internacional para o Estudo da Dor (AIED) (IASP, 2012).

O termo nocicepção está relacionado com o reconhecimento de sinais dolorosos pelo sistema nervoso e consiste dos processos de transdução, transmissão e modulação de sinais neurais gerados em resposta a um estímulo nocivo externo (MESSLINGER,1997). A hiperalgesia primária é caracterizada por acentuação da dor decorrente de estímulos de calor ou mecanicos relacionados à sensibilização periférica (ALI; MEYER ; CAMPBELL, 1996).

Diversos mediadores inflamatórios tem sido identificados com potencial hiperalgésicos, incluindo as interleucinas IL-1β, IL-8, IL-6 e TNF-α, fator de crescimento do nervo (NGF), prostaglandinas, leucotrienos, bradicinina, serotonina, adenosina, histamina e substância P (neurotransmissor SP) (RANG et al., 1994; KRAYCHETE et al., 2006; CARVALHO; LEMÔNICA, 1998; FERREIRA et al., 1993).

A atuação destes mediadores pode ser direta, atuando no nociceptor e promovendo uma redução no limiar de sensibilidade, ou ainda de ação indireta, onde a hiperalgesia ocorre por consequência da ação em outros tipos de em neurônios para posterior liberação de mediadores hiperalgésicos (CARVALHO; LEMÔNICA, 1998).

Desta forma, é possível entender que o processo de fisiopatologia da dor inflamatória é um processo complexo, envolvendo a participação de mediadores inflamatórios específicos, provenientes do tecido lesionado bem como de neurônios sensoriais nociceptivos periféricos. Além destes componentes, é importante mencionar o envolvimento de fibras nervosas, que sofrem modificações funcionais de sua excitabilidade neuronal após o contato com mediadores inflamatórios que são os estímulos recebidos pelos nociceptores localizados nas

fibras nervosas aferentes (FERREIRA, 1993; RIBEIRO et al., 2000).

Neste sentido, a dor pode estar associada a diversas doenças, provocando resposta sensorial e motora de proteção, benéfica por alertar que algo está prejudicando o organismo, um sintoma de alarme contra danos teciduais desenvolvido ao longo do processo evolutivo.

Diversos estudos apontam que a infecção por parasitos do gênero *Leishmania* interfere na percepção da dor, uma vez que as lesões decorrentes de leishmaniose cutânea em humanos são indolores (STIROPOULOS; WILBUR, 2001; MORRIS-JONES; WEBER, 2004; DABOUL, 2008). Os estudos de nocicepção na leishmaniose são escassos e controversos (KANAAN et al., 2000; SEITZ et al., 1994; CUNHA et al., 2005). E na maioria das vezes são realizados com espécies do parasito que atinge somente a Europa, como *L. major* (CANGUSSU et al., 2013; AHMED et al., 1998).

As infecções experimentais por *Leishmania major,* nos principais estudos de nocicepção, são realizados em diferentes linhagens de camundongos e os resultados apontam diferentes respostas a estímulos dolorosos, diferenças na densidade da inervação e nos níveis de produção das citocinas proinflamatórias locais (KANAAN et al., 2000; SEITZ et al., 1994; CANGUSSU et al., 2013; AHMED et al., 1998).

De qualquer forma os estudos apontam a participação de IL-1β, IL-6 e TNF-α na sensibilização dos nociceptores e, por sua vez, nas variações de dor. As diferenças observadas podem estar relacionadas às variações no grau de lesão dos nervos periféricos, que é influenciado pela quantidade de inóculo e cepa da *Leishmania* utilizada no estudo (KANAAN et al., 2000; CANGUSSU et al., 2013).

2 OBJETIVOS

2.1 Objetivos Gerais

Avaliar o tratamento com extrato de própolis, Concanavalina-A e do medicamento antimoniato de N-metil-glucamina em diferentes modelos de estudo experimental *in vitro* e *in vivo* com *Leishmania amazonensis*.

2.2 Objetivos Específicos

Verificar a atividade leishmanicida do extrato de própolis brasileiro em promastigotas de *L. amazonensis in vitro* e em carga parasitária de baço de camundongos BALB/c após infecção *in vivo*.

Avaliar a atividade anti-inflamatória do extrato de própolis brasileiro em fígado de camundongos BALB/c após infecção *in vivo* com *L.amazonensis.*

Estudar a atividade leishmanicida de Concanavalina-A em modelo de pré-tratamento intraperitoneal e infecção *in vitro*.

Verificar a atividade do antimoniato N-metil-glucamine na dor inflamatória frente a infecção experimental com *L. amazonensis*.

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3 PRODUÇÃO CIENTÍFICA

ARTIGO 1- Leishmanicidal activity of Brazilian propolis hydroalcoholic extract in *Leishmania amazonensis*

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Leishmanicidal activity of brazilian propolis hydroalcoholic extract in Leishmania amazonensis

Atividade leishmanicida de extrato hidroalcoólico de própolis brasileira em Leishmania amazonensis

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Abstract

As leishmanioses são consideradas doenças negligenciadas devido às altas incidências, ampla distribuição geográfica e dificuldade no tratamento sendo incluídas na relação de doenças prioritárias pela Organização Mundial da Saúde. Os tratamentos disponíveis para estas doenças apresentam elevada toxicidade, justificando a busca por fármacos alternativos. Estudos prévios com própolis, resina produzida por abelhas, demonstraram sua atividade antiparasitária e imunomoduladora em diversos modelos experimentais. O objetivo deste trabalho foi avaliar o efeito *in vitro* do extrato hidroalcoólico de própolis brasileira, coletado na cidade de Botucatu no estado de São Paulo, sobre formas promastigotas de *Leishmania amazonensis,* bem como analisar seu efeito *in vivo* sobre a carga parasitária em baço de camundongos susceptíveis à infecção. Assim, formas promastigotas tratadas com extrato hidroalcoólico de própolis brasileira nas concentrações 5, 10, 25, 50 ou 100 µg/mL apresentaram efeito inibitório sobre a proliferação desses parasitos nos tempos de 24, 96 e 168 h. No entanto, as concentrações de 50 e 100 µg/mL mostraram-se mais eficazes quando comparadas ao controle e às demais concentrações em todos os tempos avaliados. Em relação à carga parasitária, após 30 dias de infecção com *L. amazonensis,* camundongos BALB/c foram tratados diariamente com a própolis (5mg/kg), via oral ou intraperitoneal, durante 60 dias. Posteriormente, o baço destes animais foi coletado para análise da carga parasitária. O tratamento por via oral reduziu 40% da carga parasitária. Desta forma, a amostra de própolis brasileira testada apresentou ação leishmanicida sobre *L. amazonensis* em cultura e em camundongos infectados com este protozoário.

Keywods: Leishmaniose cutânea. Própolis. *Leishmania.*

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Resumo

Leishmaniosis are considered neglected diseases due to its high incidence, widespread and difficulty in treatment being included in the list of priority diseases by the World Health Organization. Available treatments for these diseases have high toxicity, which explains the search for more effective drugs. Previous studies with propolis - a resinous substance produced by bees - demonstrated immunomodulatory and anti-parasitic activity in several experimental models. The objective of this study was to evaluate the effect *in vitro* of Brazilian propolis hydroalcoholic extract, collected in the city of Botucatu in São Paulo State, on promastigotes forms of *Leishmania amazonensis* as well as its effect on the parasite load in the spleen of infected mice. Thus, promastigote forms treated with 5, 10, 25, 50 or 100 µg/mL of Brazilian propolis hydroalcoholic extract at 24, 96 and 168 hours showed inhibitory effect on the spread of these pararasite at all indicated times. However, the concentrations of 50 and 100 µg/mL were more effective, reducing the parasite spread when compared to the control and other concentrations at all times. Regarding parasitic load, after 30 days of infection with *L. amazonensis*, BALB/c mice were treated on a daily basis with propolis (5mg/kg) orally or intraperitoneally for 60 days. Further, the spleen was collected for parasite load analysis. Oral treatment reduced 40% of the parasitic load. Thus, the tested Brazilian propolis sample showed antileishmanial activity on *L. amazonensis* in culture and in parasite- infected mice.

Palavras-chave: Cutaneous leishmaniasis. Propolis, *Leishmania*.

Introduction

Leishmaniosis are infectious parasitic diseases showing worldwide distribution in temperate, tropical and subtropical regions. They constitute a serious public health problem and are present in most Brazilian states (HEPBURN, 2000).

The etiological agents of leishmaniosis are protozoans of Trypanosomatidae family and *Leishmania* genus that are transmitted by females of phlebotomine sandflies. The life cycle of these parasites involves two distinct developmental forms: promastigote- extracellular and flagellated found in the gut of the insect vector and the amastigote form- which lacks mobility and is found inside the cells of the immune system of the vertebrate host (NYLÉN; GAUTAM, 2010).

The disease can present a wide spectrum of clinical manifestations in different clinical forms as the appearing of self-healing skin lesions to severe visceral involvement of multiple organs (MICHALICK; RIBEIRO, 2011).

Despite the existence of an association between *Leishmania* species and clinical forms presented by the host, it has already been reported that the same species can produce different clinical manifestations related to the interaction of the host immune response and the characteristics of the etiological agent, as tropism and invasive capacity (BARRAL et al., 1991; NYLÉN; GAUTAM, 2010).

Therefore, it is important to report that *Leishmania amazonensis* has high pathogenic potential, since this species has been reported in cases of cutaneous, diffuse and visceral leishmaniosis (BARRAL et al., 1986; BARRAL et al., 1991).

The available drugs for the treatment of patients affected by this disease are pentavalent antimonials as Meglumine antimonate (commercialized in Brazil as Glucantime) and sodium stibogluconate (commercialized in Europe as Pentostam). Besides antimonials, amphotericin B, pentamidine and miltefosine can be used as therapeutic alternatives (DE VRIES; REEDIJK; SCHALLIG, 2015). However, the treatment of leishmaniasis patients is still a challenge, since these drugs have toxic side effects. Furthermore,

there are cases of post-treatment recurrence with antimonials, showing that clinical cure is not accompanied by parasitological cure (MEDEIROS; NASCIMENTO; HINRICHSEN, 2005).

In this sense, various natural or synthetic substances with antileishmanial, immunomodulatory and anti-inflammatory capacity have been investigated as an alternative to conventional treatment of this disease (ROCHA et al., 2005).

It is known that propolis is a natural product produced by bees which presents antibacterial (SFORCIN et al., 2000), antiviral (VYNOGRAD; VYNOGRAD; SOSNOWSKI, 2000), anti-inflammatory (KHAYYAL et al., 2003) and immunomodulatory activities (SFORCIN; KANENO; FUNARI, 2002).

In relation to the parasites of *Leishmania* genus, the results showed the leishmanicidal effect of propolis collected in different regions of Brazil and other countries against promastigote and amastigote forms of different species of this protozoan (AYRES; MARCUCCI; GIORGIO, 2007; DA SILVA et al., 2013; DURAN et al., 2008; MACHADO; LEON; CASTRO, 2007; PONTIN et al., 2008; OZBILGE et al., 2010).

Despite the evidences already presented in the literature, it is known that the chemical composition of propolis is dependent on the biodiversity of each area visited by bees. Thus, various propolis extracts may present variations in color, consistence and amount of biologically active substances, depending on the plant visited, bee species, age of collection and the substance used for extracting (WATSON et al., 2006).

In this context, this paper presents results concerning the leishmanicidal action, both *in vitro* and *in vivo*, of the hydroalcoholic extract of Brazilian propolis collected in Botucatu/ São Paulo (SP) on promastigote forms of *L. amazonensis*.

Materials and Methods

Leishmania amazonensis

L. amazonensis promastigote forms (MHOM/BR/1989/166MJO) maintained in culture medium 199 (GIBCO Invitrogen®, Grand Island, USA) supplemented with 10% fetal bovine serum-FBS (GIBCO Invitrogen®, Grand Island, USA), 1M of HEPES biological buffer (AMRESCO®, Solon, USA), 1% human urine, 1% L-glutamine (Synth®, Diadema, Brazil), penicillin and streptomycin (10 U/mL-10 µg/mL, GIBCO Invitrogen®, Grand Island, USA) and 10% sodium bicarbonate (Synth®, Diadema, Brazil). The cell cultures were grown in a BOD type incubator at 25°C in 25 cm^2 culture flask.

Hydroalcoholic extract of Brazilian propolis

The propolis sample used in this study was collected at *Fazenda Lageado* apiculture section, *Universidade Estadual Paulista (UNESP)*, Campus Botucatu, SP, from colonies of *Apis mellifera* bees. The extraction method, as well as the chemical composition of this sample have been documented in previous studies in which chemical analysis showed that major constituents of this sample are phenolic compounds such as flavonoids, aromatic acids and benzopyrans as well, di and tri terpenes and essential oils (SFORCIN, 2007). The final concentration of ethanol solvent in the experiments did not exceed 0.1%.

Proliferation Kinetics

L. amazonensis promastigote forms (10^6/mL) were treated with different concentrations of the hydroalcoholic extract of Brazilian propolis (5, 10, 25, 50, or 100 µg/mL) diluted in culture medium 199 supplemented and maintained at 25°C. Treatments were maintained for seven days, on which the promastigote forms were

counted in a Neubauer chamber after 24, 96, and 168 hours for the establishment of kinetics spread. As a means of control, promastigotes were used without treatment.

Animals

BALB/c mice weighing 25-30 g, 6-8 weeks old, from the central biotery at the State University of Maringá (UEM), Maringá, Paraná (PR) were used. The animals were kept in a biotery of parasitology at the State University of Londrina (UEL), Londrina, PR, with water and *ad libitum* fed, controlled luminosity (light-dark cycle, 12/12 h) and temperature (23° C ± 1° C). The project was approved by the Ethics Committee on Animal Experiments of UEL, under the registration number 09/2011 and Circular Letter N° 24/2011.

Experimental infection and treatment

The animals were divided into 4 groups (n= 6/ group): control without infection (animals that did not receive infection or treatment), control (infected but not treated animals), Propolis p.o. (infected animals orally treated with propolis), Propolis i.p. (infected animals treated with propolis intraperitoneally).

Animals from control groups, Propolis i.p. and Propolis p.o. were subcutaneously infected in the right hind paw with promastigote forms of *L. amazonensis* (10^7/20μL). After 30 days of infection, daily treatment with propolis (5 mg/kg) was initiated orally (p.o.) or intraperitoneally (i.p.) for 60 days. Infected mice used as controls received only phosphate buffer vehicle (PBS) (i.p. and p.o.). At the end of the experiment (30 days of infection and 60 days of treatment) the animals were euthanized and their spleens collected for weighing and parasite load analysis. Every effort was made to minimize the number of animals used and their suffering.

Parasite load

L. amazonensis parasite load in the spleen of BALB/c mice was performed according to microtiter methodology described by Buffet et al. (1995), with modifications. The spleen of each animal was removed aseptically, weighed and homogenized in culture medium 199 supplemented. The suspensions were serially diluted in 96-wells that were subsequently incubated at 25°C. Every week, during thirty days, plaques were observed in inverted light microscope. Each well was examined with 200x magnification, looking for *L. amazonensis* promastigotes. The highest dilution in which at least one parasite was found was defined as titer. The following formula was carried out in order to determine the parasite load:

Parasitic Load = (geometric average of the titer of each triplicate / body mass (mg) x 400, considering 400 as the fraction of homogenized organ and placed in the first well before dilution. The results were expressed in $\log_{10}$.

Statistical analysis

Data were analyzed using GraphPad PRISM statistical software (Graph-Pad Software Inc., USA, 5.00). Significant differences between treatments were determined by analysis of variance (ANOVA) followed by Tukey's multiple comparison test. Statistical significance was accepted at $p < 0.05$.

Results

Extract of Brazilian propolis presents leishmanicidal effect on promastigote forms of L. amazonensis

The promastigote forms of *L. amazonensis* were treated with different concentrations of propolis (5, 10, 25, 50 or 100 μg/mL) for 24 h (Figure 1A), 96 h (Figure 1B) and 168 h (Figure

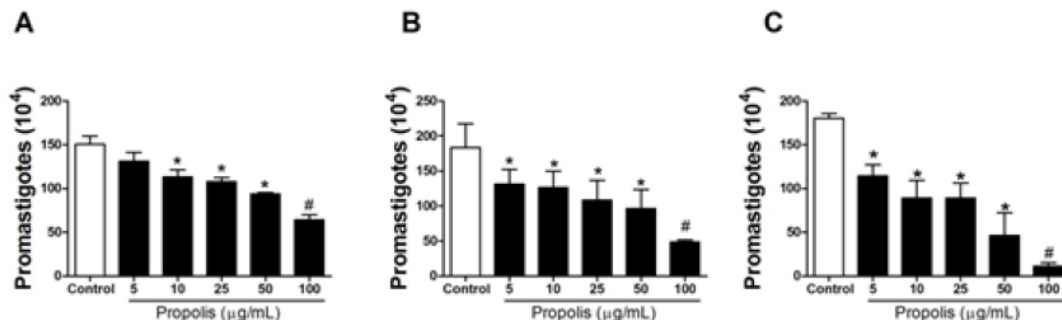
1C). Through counting in a Neubauer chamber, it was observed that treatment with Brazilian propolis reduced the spread of promastigotes at all evaluated times. The concentrations of 10, 25, 50 and 100 µg/mL reduced spread by 25, 29, 38 and 57% respectively after 24 h of treatment. On the other hand, the propolis concentration of 5 µg/mL inhibited the spread of *L. amazonensis* in 13% with no significant difference compared to the control.

After 96h treatment, the percentages of reduction reached 29, 31, 41, 48 and 74% for Brazilian propolis treatments with 5, 10, 25, 50 and 100 µg/mL, respectively (Figure 1B). After 168 h of incubation with the treatment, the reduction of parasites spread was 37, 51, 51, 74 and 94% for propolis treatments with 5, 10, 25, 50 and 100 µg/mL, respectively (Figure 1C).

Propolis extract can reduce the parasitic load on the secondary lymphoid organ (spleen) of BALB/c mice infected with L. amazonensis

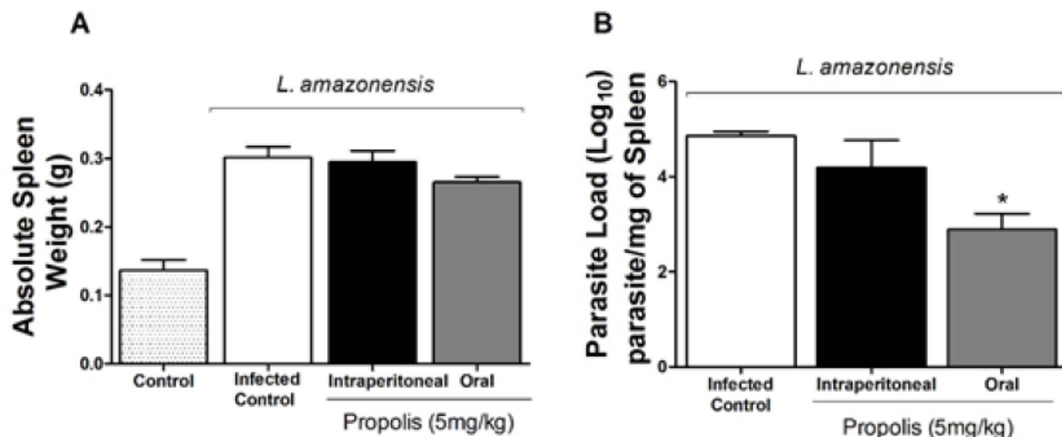
The obtained results considering the spleen weight and parasite load are shown in Figures 2A and 2B, respectively. Data showed that *L. amazonensis* was able to migrate to secondary organs, such as the spleen. Treatment with a dose of 5 mg/kg, p.o., of Brazilian propolis extract was able to reduce 40% the parasitic load in the spleen when compared to infected control. Yet, the treatment via i.p. promoted 13% of reduction in parasite load, showing no differences with the infected control (Figure 2B). Moreover, both treatments did not reduce organ weight (spleen) as compared to organ weight of the animals in the infected control group (Figure 2A).

Figure 1- Antileishmanial activity of Brazilian propolis extract in the kinetics of spread of *Leishmania amazonensis* promastigote forms treated with different concentrations of Brazilian propolis extract (5, 10, 25, 50, or 100 µg/mL) for 24 hours (A), 96 hours (B) and 168 hours (C). As a control, we used *L. amazonensis* promastigotes maintained in culture medium 199 without treatment. One-way ANOVA followed by Tukey test. Results represent the mean ± SEM of five independent experiments. * Significantly different from control ($p < 0.05$). # Significantly different of treatments with concentrations 5, 10 and 25 µg/mL of propolis ($p < 0.05$).



Fonte: authors

Figure 2 - Leishmanicidal activity of Brazilian propolis extract in secondary lymphoid organ of BALB/c mice. BALB/c mice infected with *Leishmania amazonensis* promastigote form (10^7) subcutaneously in the right hind paw. After 30 days of infection it was begun daily treatment with propolis (5 mg/ kg) by oral or intraperitoneal route for 60 days. The spleens of animals were collected and evaluated as the mass, expressed in g (A) and the parasitic load expressed in log_{10}/mg spleen (B) by means of serial dilutions of spleen homogenate culture. One-way ANOVA followed by Tukey test. Results represent the mean ± SEM of quadruplicate 6 animals per experimental group. * Significantly different from infected control ($p < 0.05$).



Fonte: authors

Discussion

This study evaluated the leishmanicidal activity of the hydroalcoholic extract of propolis collected in SP region on promastigote forms of *L. amazonensis*. We used models *in vitro* and *in vivo*, in which the apitherapic sample used demonstrated direct activity decreasing the proliferation of promastigote forms and indirect activity reducing the parasite load in the spleen of mice after infection.

Previous studies in experimental models *in vitro* and *in vivo* have demonstrated that propolis extracts collected in different regions of Brazil have leishmanicidal activity in promastigote and amastigote forms of *L. amazonensis, L. braziliensis, L. major* and *L. chagasi* (AYRES et al., 2007; DA SILVA et al., 2013; MACHADO; LEON; CASTRO, 2007; MIRANDA et al., 2015; PONTIN et al., 2008).

It is known that the propolis samples may exhibit different chemical composition or different concentrations of these components (WATSON et al., 2006). The sample used in this study presents a greater amount of phenolic compounds, di and tri terpenes and essential oils (SFORCIN, 2007). According to Cunha et al. (2011), these constituents have been previously reported to be associated with antileishmanial activity against promastigote and amastigote forms of various species of this protozoan.

Although the mentioned studies have demonstrated the leishmanicidal activity of various propolis extracts, it is known that the susceptibility of different species and parasite strains of the genus *Leishmania* when subjected to the action of various compounds is not homogenous and varies considerably between species/strains tested (MORAIS-TEIXEIRA et al., 2011; VILA-NOVA et al., 2013).

The propolis sample used in this study was previously conducted *in vitro* against *L. braziliensis* presenting leishmanicidal and immunomodulatory action, and in *in vivo* model against *L. amazonensis* where therapeutic potential associated with a nitric oxide donor was evaluated(DA SILVA et al, 2013;. MIRANDA et al., 2015). In the present study, we demonstrate that this Brazilian propolis extract was also effective in inhibiting *in vitro* spread of *L. amazonensis* promastigote forms in dependent dose manner, consolidating the leishmanicidal effect of this compound.

Among the tested concentrations, 5, 10 and 25 µg/mL have inhibitory effect on the proliferation of these parasites. However, the concentrations of 50 and 100 µg/mL were more effective over time when compared to the control and other concentrations.

When the action of Brazilian propolis extract on *in vivo* model was evaluated, the parasites were quantified in the spleen of BALB/c mice infected with *L. amazonensis* by microtiter technique in culture, after 60 days of treatment, a total of 90 days of infection. The presence of *L. amazonensis* promastigote forms was detected in the spleen of all infected animals. Thus, we can affirm that this protozoan species associated with the cutaneous form of the disease are able to visceralize in animal infection model. In addition, we confirm the susceptibility of BALB/c mice to infection by *L. amazonensis*.

These data corroborate the reported findings of Barral and colleagues (1986 and 1991) that *L. amazonensis* as the etiologic agent of visceral leishmaniasis in humans. Furthermore, in animal models, some authors reported that infection with *L. amazonensis* and other species associated with cutaneous disease, are able to visceralize in an animal infection model (ABREU-SILVA et al., 2004; MAGILL et al., 1993; ROBERTS; ALEXANDER; BLACKWELL, 1989; SOLIMAN, 2006; WALTON; INTERMILL; HAJDUK, 1977).

As for the different routes of propolis extract administration, most studies only show their chemical composition and pharmacological activities, not showing the peculiarities of pharmacokinetics (LUSTOSA et al., 2008). The few studies on the pharmacokinetics are made only with specific chemical compounds isolated from the extracts (MESBAH; SAMIA, 2011; METZNER et al., 1979; PAULINO et al., 2009).

We found that only p.o. treatment decreased the parasite load, reducing at 40% the number of parasites in the assessed body. This parasite burden reduction in secondary organs is extremely important in resolving the infection in the host.

Propolis extract therapeutic potential has been shown in several studies, including models using this apitherapic in combination with Glucantime, a drug already used for the treatment of leishmaniases (AYRES et al., 2011; FERREIRA et al., 2014). Thus, the presented data justify further study of the use of this natural compound in leishmaniosis treatment support.

Conclusion

This study demonstrated that Brazilian propolis hydroalcoholic extract collected in SP region presents leishmanicidal activity in *L. amazonensis in vitro* and *in vivo* models. These results represent an encouragement for research and mechanisms description of action and general pharmacology in using this apitherapic as a support in the therapy of leishmaniosis.

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ARTIGO 2- Propolis reduces *Leishmania amazonensis*-induced inflammation in the liver of BALB/c mice

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ORIGINAL PAPER

Propolis reduces *Leishmania amazonensis*-induced inflammation in the liver of BALB/c mice

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Abstract Experimental models of mouse paw infection with *L. amazonensis* show an induction of a strong inflammatory response in the skin, and parasitic migration may occur to secondary organs with consequent tissue injury. There are few studies focusing on the resolution of damage in secondary organs caused by *Leishmania* species-related cutaneous leishmaniasis. We investigated the propolis treatment effect on liver inflammation induced by *Leishmania amazonensis* infection in the mouse paw. BALB/c mice were infected in the hind paw with *L. amazonensis* (10^7) promastigote forms. After 15 days, animals were treated daily with propolis (5 mg/kg), Glucantime (10 mg/kg), or with propolis plus Glucantime combined. After 60 days, mice were euthanized and livers were collected for inflammatory process analysis. Liver microscopic analysis showed that propolis reduced the inflammatory process compared to untreated infected control. There was a decrease of liver myeloperoxidase and N-acetyl-β-glucosaminidase activity levels, collagen fiber deposition, pro-inflammatory cytokine production, and plasma aspartate transaminase and alanine transaminase levels. Furthermore, propolis treatment enhanced anti-inflammatory cytokine levels and reversed hepatosplenomegaly. Our data demonstrated that daily low doses of Brazilian propolis reduced the secondary chronic inflammatory process in the liver caused by *L. amazonensis* subcutaneous infection in a susceptible mice strain.

Keywords *Leishmania amazonensis* · Propolis · Liver · Glucantime · Inflammation

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Introduction

Protozoa of the genus *Leishmania* are the causative agent of leishmaniasis, a neglected disease with high morbidity, mortality, and therapeutic failure, constituting a public health problem, and can cause skin lesions or visceral involvement (Grevelink and Lerner 1996; Desjeux 2004). American cutaneous leishmaniasis is characterized by ulcerative skin lesions, localized or mucosal, and disseminated lesions (nonulcerated nodules) (Reithinger et al. 2007).

Leishmania amazonensis is one of the main etiologic agents responsible for cutaneous leishmaniasis in Brazil. This parasite may cause the localized or diffuse clinical forms of the disease, depending on the host immune response and parasitic virulence (Barral et al. 1991; Jones et al. 2000; Ji et al. 2003).

The classic lesions in cutaneous leishmaniasis are most often ulcerated lesions in the skin, with a granular base and raised borders (Bittencourt and Barral 1991). However, in experimental infections with *L. braziliensis*, *L. tropica*, *L. mexicana*, *L. major*, and *L. amazonensis*, cases of migration to secondary organs differing from the site of infection have been described. These species were initially described as parasites with a unique tropism for skin and mucosa (Walton et al.

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1977; Barral et al. 1986; Magill et al. 1993; Mohareb et al. 1996; Abreu-Silva et al. 2004; Wilson et al. 2005; Soliman 2006; Ribeiro-Romão et al. 2014). In addition, *L. amazonensis* has been reported as an etiologic agent of human visceral leishmaniasis (Roberts et al. 1989; Barral et al. 1991).

Visceralization due to hematogenous dissemination via phagocytic cells such as monocytes has resulted to histological damage to lymph nodes, liver, spleen, and bone marrow (Duarte and Corbett 1987). Splenomegaly is usually observed, with many macrophages parasitized by amastigotes. Liver disorders result from hypertrophy and hyperplasia of Kupffer cells, as well as intracellular fibrosis, and complications of fulminant hepatitis (Engwerda and Kaye 2000; Baranwal et al. 2007).

The host immune response is essential for disease control and elimination of the parasite, but an uncontrolled inflammatory response is a common mechanism involved in the majority of cases of clinical visceral leishmaniasis, resulting to secondary tissue damage with granulomatous changes and fibrosis (Gutierrez et al. 1984; Grevelink and Lerner 1996; Leite and Croft 1996; Nylén and Gautam 2010; Gupta et al. 2013).

Propolis has been widely used in popular medical practice and has shown promising results in a range of experimental models, including activity against some trypanosomatids of medical importance. For instance, propolis kills promastigote and amastigote forms of varied *Leishmania* species (Machado et al. 2007; Ayres et al. 2007; Duran et al. 2008; Pontin et al. 2008; Ozbilge et al. 2010; Ayres et al. 2011; da Silva et al. 2013).

In addition, several studies have shown that propolis has anti-inflammatory properties and accelerates tissue regeneration as well as exhibits an antimicrobial action and shortens healing time (Barbosa et al. 2009; Khorasgani et al. 2010; Ikeda et al. 2011; Olczyk et al. 2013a, b).

Studies have also demonstrated that the ethanolic extract of propolis has anti-inflammatory properties in both chronic and acute inflammation and exerts protective effects against hepatotoxicity (Seo et al. 2003; Batista et al. 2012). The molecular mechanisms involved in the immunomodulatory and anti-inflammatory activities of this natural compound include the capacity of inhibiting T cell activation by affecting mainly IL-2, NF-κB, MAP, STAT 3, and IL-6 (Okamoto et al. 2012; Búfalo et al. 2013). Other studies have also reported a decrease in myeloperoxidase (MPO) and NADPH-oxidase activities (Frenkel et al. 1993; Volpert and Elstner 1996) and ornithine decarboxylase, tyrosine protein kinase, and hyaluronidase activities (Miyataka et al. 1997).

Considering the antimicrobial and anti-inflammatory activity of propolis, the aim of the present study was to evaluate the effect of low dose propolis on liver inflammation in an experimental model of subcutaneous infection in a susceptible murine strain with *L. amazonensis*.

Materials and methods

L. amazonensis

L. amazonensis (MHOM/BR/1989/166MJO) was obtained from homogenate of popliteal lymph nodes of infected BALB/c mice. The promastigote forms were cultured in 199 medium (Invitrogen-GIBCO) and supplemented with 10 % fetal bovine serum (Invitrogen-GIBCO), 1 M Hepes, 0.1 % human urine, 0.1 % L-glutamine, 1 % penicillin/streptomycin solution (Invitrogen-GIBCO), and 10 % sodium bicarbonate. Cultures were incubated at 25 °C in 25-cm^2 flasks. Promastigote forms, in stationary the growth phase (5 culture days), were used for experimental infection of the animals.

Propolis extract

The propolis sample was collected in the Beekeeping Section of Lageado Farm, UNESP, Botucatu Campus, Brazil, from honeybee (*Apis mellifera L.*) colonies. The method of extraction as well as the chemical composition has already been documented in previous studies, where propolis was analyzed by gas chromatography (GC), gas chromatography–mass spectrometry (GC-MS), and thin layer chromatography (TLC) (Sforcin 2007). The final concentration of the ethanol solvent in the experiments did not exceed 0.1 %. It is noteworthy that we used the same batch of propolis extract in all experiments to avoid differences in the active products and solvents.

Animals and experimental infection

Male BALB/c mice (20–25 g), 4–6 weeks old, were obtained from the Fundação Osvaldo Cruz, FIOCRUZ, Curitiba, Brazil. Mice were kept under pathogen-free conditions and used according to protocols approved by the Ethics Committee of the Universidade Estadual de Londrina (protocol No. 09/11).

Mice were divided into five groups with eight animals each. The groups were (1) control group (without infection and without treatment); (2) infected control group (with infection and without treatment); (3) propolis group (with infection and treatment with propolis); (4) Glucantime group (received infection and treatment with Glucantime); (5) propolis+ Glucantime group (received infection and treatment with propolis and Glucantime combined).

Mice were infected subcutaneously in the right hind paw with *L. amazonensis* (MHO/BR/1989/166MJO) promastigote forms (10^7/20 μL). Daily treatment with propolis (5 mg/kg, orally [p.o.]) or Glucantime® (10 mg/kg, intraperitoneally [i.p.]), or propolis (5 mg/kg, orally [p.o.]) plus Glucantime (10 mg/kg, intraperitoneally [i.p.]) combined started 15 days after infection. After 60 days of treatment, mice were

euthanized, and blood was collected for biochemical tests and liver for histological and immunological analysis.

AST and ALT levels

The levels of aspartate aminotransferase (AST) and alanine aminotransferase (ALT) were used as markers of hepatocellular damage and determined in blood plasma by a colorimetric assay using a diagnostic kit from Labtest Diagnóstica™ (Lagoa Santa, MG, Brazil) (Hohmann et al. 2013).

Liver histological analysis

The liver of each animal was removed, perfused with saline, and sectioned into standardized fragments. One of the fragments was fixed in Bouin's solution for 48 h. Subsequently, the tissue was subjected to routine histological processing to obtain 4-μm sections, which were stained with hematoxylin-eosin and examined by light microscopy (Olympus, Miami, FL, USA). The analysis was performed according to quality and quantitative parameters of inflammation.

Histological variation was classified according to the level of lesions found by the following criteria: no histological alteration (−); isolated inflammatory foci, presence of up to 1 intralobular granuloma, and up to 5 Kupffer cells per microscopic field (+); isolated or coalescent area of histological changes including inflammation, 2–4 intralobular granulomas, and 5–10 Kupffer cells per field (++); disseminated histological changes including inflammation, over 4 intralobular granulomas, and 10 Kupffer cells per field (+++).

Hepatic fibrosis analysis

Collagen quantification was determined in Sirius red-stained liver sections under polarized light using a photomicroscope (CARL ZEISS Axio imager A1) with a camera (HBO 100) coupled to a computer using AxioVision software, at a final magnification of 200×. Eight images of four sections from each mouse were considered for the study and analyzed by Image Pro Plus (version 4.5). The results were expressed as the mean of area with presence of total collagen and percentage of area with type I and III collagen.

Myeloperoxidase activity and N-acetylglucosaminidase activity

Neutrophil migration to the liver was evaluated by the MPO kinetic-colorimetric assay. N-acetylglucosaminidase (NAG) assay was used for evaluating the infiltration of macrophages in the liver. Samples were collected in 50 mM K_2PO_4 buffer (pH 6.0) containing 0.5 % HTAB and were homogenized using a Polytron® (PT3100).

After the homogenates were centrifuged (16,100 × *g*, 2 min, 4 °C), the resulting supernatant was assayed spectrophotometrically for MPO or NAG activity at 450 nm (Spectra max) with three readings within 1 min. MPO activity of the samples was compared with a standard curve of neutrophils, and MPO activity results were presented as the number of neutrophils $\times 10^4$/mg of tissue. NAG activity in the samples was compared with a standard curve of macrophages, and NAG activity results were presented as the number of macrophages $\times 10^3$/mg of tissue) (Hohmann et al. 2013).

Cytokine measurement

Cytokines present in liver fragments were analyzed by enzyme-linked immunosorbent assay (ELISA). Samples were homogenized in 500 μL of buffer containing protease inhibitors (1 mM Phenylmethanesulfonyl fluoride, Sigma Aldrich), and IFN-γ, TNF-α, IL-17, IL-6, IL-12, IL-10, TGF-β, and IL-13 levels were determined by ELISA, according to the manufacturer's instructions (eBiosciences®, USA). Plates were read at 450 nm, using an ELISA plate reader (Thermo Plate—TP-Reader). Results were expressed as pg cytokine/mg tissue.

Statistic analysis

Data were analyzed using GraphPad Prism statistical software (GraphPad Software, Inc., USA-500.288, version 5.0). Significant differences between treatments were determined by ANOVA, followed by Tukey's test for multiple comparisons. Statistical significance was accepted when $P < 0.05$.

Results

Effect of propolis extract on blood plasma levels of aspartate aminotransferase (AST) and alanine aminotransferase (ALT) and weight of liver and spleen

Blood samples were collected after 60 days of treatment for the assessment of AST/ALT levels. The infection with *L. amazonensis* induced a significant increase in AST (Fig. 1a) and ALT (Fig. 1b) levels. These enzyme levels were reduced by propolis or by its combination with Glucantime by 85 and 87 %, respectively.

Liver and spleen weights increased after 75 days of infection (Fig. 2a, b). Data showed that the liver and spleen of animals treated with propolis alone or combined with Glucantime did not differ when compared with the uninfected group (Fig. 2a, b). However, the weight of the liver and spleen of animals treated with propolis or propolis and Glucantime combined decreased when compared to infected control (Fig. 2a, b).

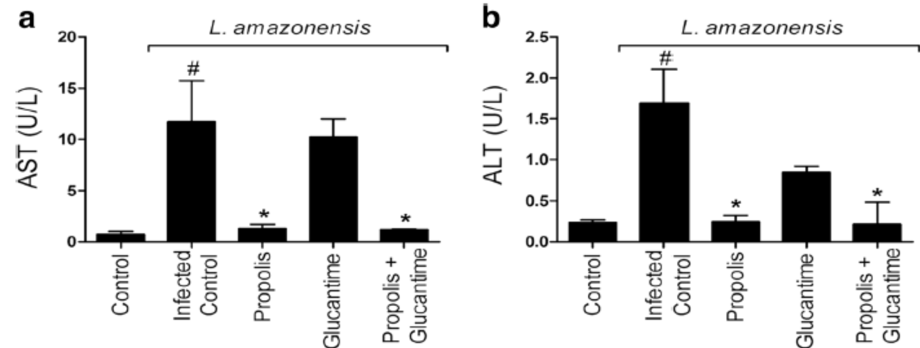

Fig. 1 Effect of propolis extract on blood plasma levels of aspartate aminotransferase (AST) and alanine aminotransferase (ALT). Blood samples were collected for the determination of AST (**a**) and ALT (**b**) levels. (*Number sign*) Significantly different from control. (*Asterisk*) Significantly different from infected control ($P < 0.05$)

The liver and spleen weights of animals treated with Glucantime alone were significantly increased compared to infected control animals (Fig. 2a, b).

Propolis promoted an anti-inflammatory effect in liver reducing granuloma formation and deposition of type I and III collagen fibers

The liver sample collected for histological analysis showed that *L. amazonensis* infection (10^7/20 μL/paw, s.c injection) induced histological changes. These alterations included increased infiltration of Kupffer cells, intralobular granuloma formation, and inflammation in the portal tracts (Fig. 3a–c). These changes were classified as disseminated histological changes (+++), which were reduced after treatment with 5 mg/kg propolis, classified as isolated inflammatory foci (+). Glucantime also reduced the changes in liver tissue to a level classified as isolated or coalescent area of histological changes (++). Glucantime in combination with propolis resulted in isolated inflammatory foci (+) (Table 1).

Infection induced the deposition of collagen fibers type I and III (Fig. 4a). Treatment with propolis, Glucantime, and the two combined reduced the deposition of total collagen fibers when compared with the infected control group (Fig. 4a).

The prevalence of type III collagen fibers was seen in all groups (Fig. 4b). Type I collagen fibers appeared less than 15.53 % in the control group without infection and in groups treated with propolis and propolis combined with Glucantime (Fig. 4b). The percentage of this type collagen fiber in infected control and Glucantime-treated groups was 35.38 and 28.18 %, respectively (Fig. 4b).

Propolis reduced *L. amazonensis*-induced myeloperoxidase and n-acetylglucosaminidase activities in liver tissue

After 60 days of treatment, the liver samples were collected for evaluation of MPO and NAG activities. *L. amazonensis* infection induced a significant increase in MPO (Fig. 5a) and NAG (Fig. 5b) compared to control. Treatment with propolis decreased MPO activity by 27 % and NAG activity by 31 % compared with infected control. Combined treatment also produced similar changes in enzyme activity.

Propolis reduced *L. amazonensis*-induced IFN-γ, TNF-α, IL-17, and IL-6 production and increased production of anti-inflammatory cytokines in the liver

After 60 days of treatment, liver samples were collected for the assessment of cytokine production. *L. amazonensis* infection increased the production of IFN-γ, TNF-α, IL-17, and IL-6 in liver (Fig. 6a–d). However, these levels were reduced by treatment with 5 mg/kg propolis (Fig. 6a–d). On the other hand, propolis increased IL-10, TGF-β, and IL-13 production

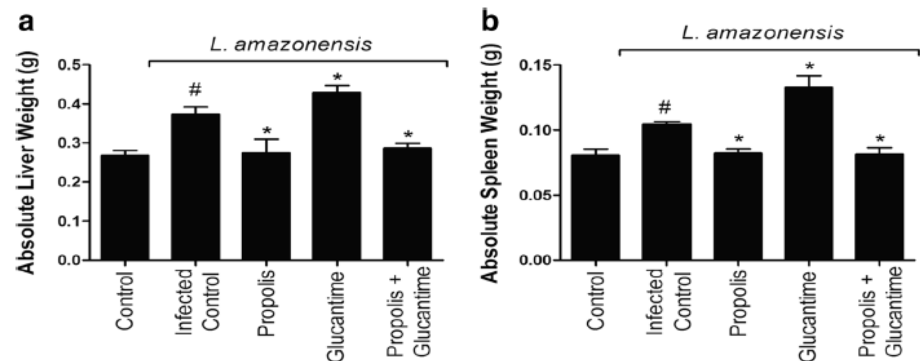

Fig. 2 Effect of propolis extract on weight of liver and spleen. Absolute weight of liver (**a**) and spleen (**b**) was evaluated as markers of hepatosplenomegaly. (*Number sign*) Significantly different from control. (*Asterisk*) Significantly different from infected control ($P < 0.05$)

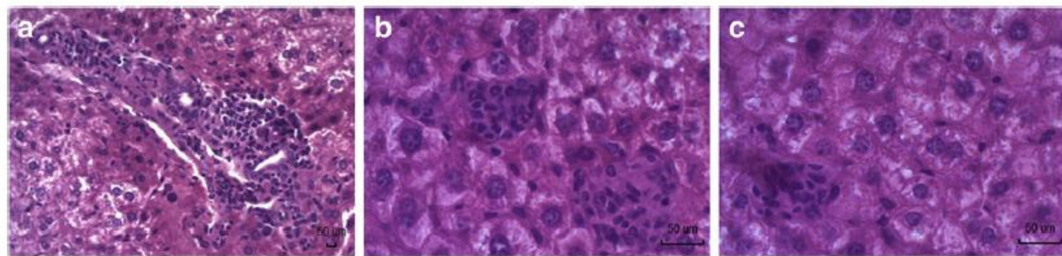

Fig. 3 Photomicrograph showing the main histological changes found in the liver of animals infected with *L. amazonensis*. Liver fragment samples were collected for analysis of the inflammatory process. Portal tract inflammation (**a**). Intralobular granuloma (**b**). Hyperplasia and hypertrophy of Kupffer cells (**c**). Histological sections (4 μm) were analyzed by optical microscope (magnification of ×100 for panel **a** and ×400 for panels **b** and **c**) (Olympus, Miami, FL, USA) after hematoxylin and eosin staining

(Fig. 7a–c) and did not affect IL-12 production (Fig. 6e). Furthermore, propolis combined with Glucantime also enhanced the production of IL-10 and IL-13 (Fig. 7a, c) and reduced TNF-α, IL-17, and IL-6 production when compared to infected control (Fig. 6b–d).

Discussion

In this study, *L. amazonensis* infection in the paw spreads to other tissues such as the liver. There are histological changes in the liver including inflammation of the portal tract, hyperplasia, and hypertrophy of Kupffer cells and fibrosis. These histological changes were corroborated by the increase in MPO and NAG activities as well as by increased levels of the pro-inflammatory cytokines IFN-γ, TNF-α, IL-17, and IL-6 in the liver. Moreover, liver injury was associated with the classic increase in AST and ALT levels in the blood and hepatosplenomegaly. Importantly, treatment with propolis in a therapeutic protocol reduced all liver inflammatory responses induced by *L. amazonensis* paw infection.

Similar to the present murine model, there are morphological changes in the liver during human visceral leishmaniasis, characterized mainly by hypertrophy and hyperplasia of Kupffer cells with granuloma formation and intralobular portal and diffuse intralobular fibrosis (Murray 2001).

It is known that wound healing response accounts for liver fibrosis in varied acute and chronic conditions. Liver fibrosis may occur due to increased synthesis and deposition of collagen (Friedman 2008).

Few studies have demonstrated the importance of the liver fibrosis process in murine models of experimental infection with *Leishmania*. However, it is known that infection with *L. donovani* in BALB/c causes an increase in the deposition of collagen, particularly type III, in granuloma formation regions (Leite and Croft 1996).

Liver fibrosis involves qualitative and quantitative changes in the composition of the extracellular matrix in the portal and sinusoidal space and is characterized by substantial deposition of fibrillar type I and III collagen, proteoglycans, fibronectin, and hyaluronic acid in the scar regions (George and Chandrakasan 1996; George et al. 2004; Zeisberg et al. 2006).

Table 1 Liver histological analysis (hematoxylin-eosin staining) of control mice and *L. amazonensis* experimental infected mice

Liver histologic analysis	Experimental groups				
	Control	Infected control	Propolis	Glucantime	Propolis + Glucantime
Portal tract inflammation	0	3	3	0	0
Intralobular granuloma (1)	0	0	4	1	1
Intralobular granuloma (2–4)	0	4	0	1	0
Intralobular granuloma (>4)	0	1	0	0	0
Kupffer cells (4)	0	1	4	3	3
Kupffer cells (5–10)	0	1	0	0	0
Kupffer cells (>10)	0	3	0	0	0
Total score	−	+++	+	++	+

Results are the number of animals in each group with lesions. No histological alteration (−); isolated inflammatory foci, presence of up to 1 intralobular granuloma, and up to 5 Kupffer cells per microscopic field (+); isolated or coalescent area of histological changes including inflammation, 2–4 intralobular granulomas, and 5–10 Kupffer cells per field (++); disseminated histological changes including inflammation, over 4 intralobular granulomas, and 10 Kupffer cells per field (+++). The number of animals in each group that presented histological change was also taken into consideration for the classification score (400× magnification)

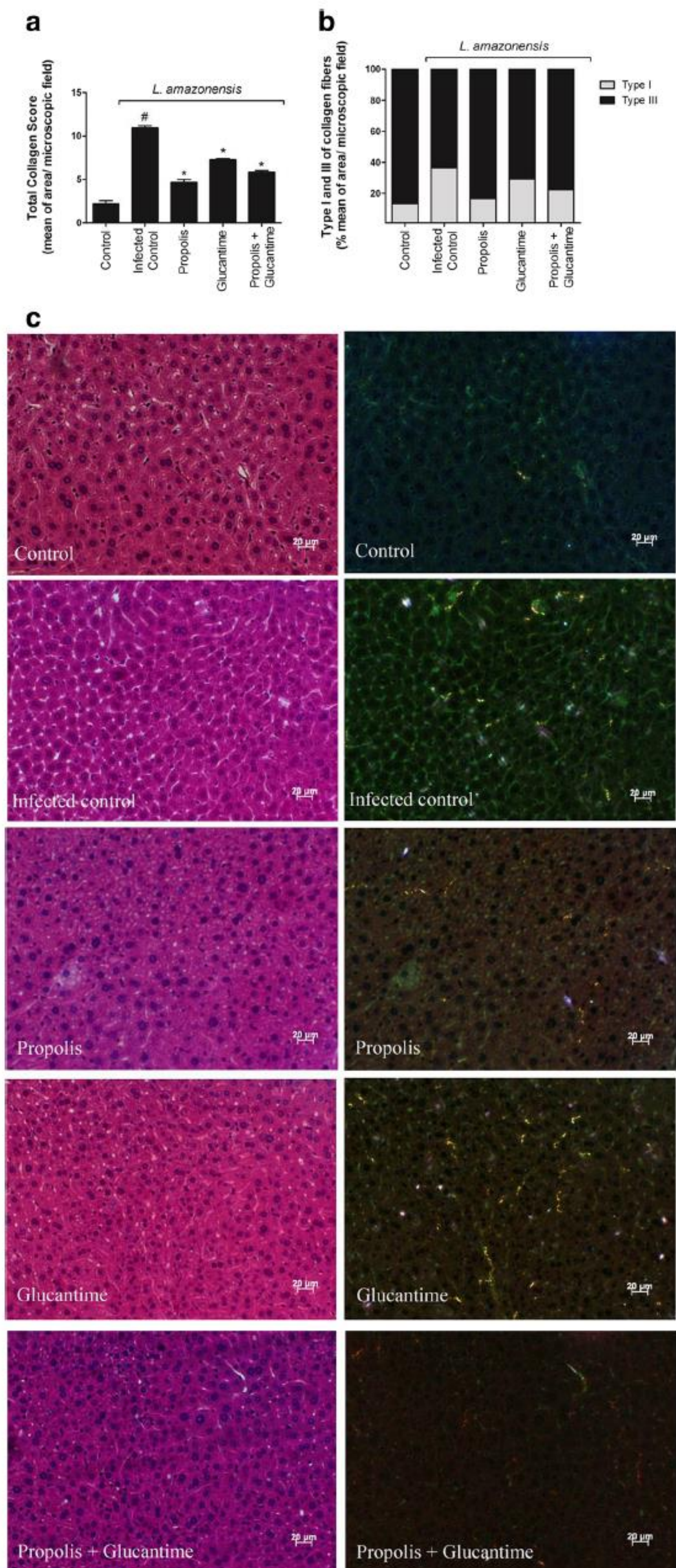

Fig. 4 Propolis reduced the deposition of type I and III collagen fibers. Liver fragment samples were collected for analysis of total collagen score (**a**) and percentage of Type I and III fiber collagen (**b**). Photomicrograph of hepatic histological sections with Sirius red staining in light and polarized microscopy (**c**). (*Number sign*) Significantly different from control. (*Asterisk*) Significantly different from infected control ($P<0.05$)

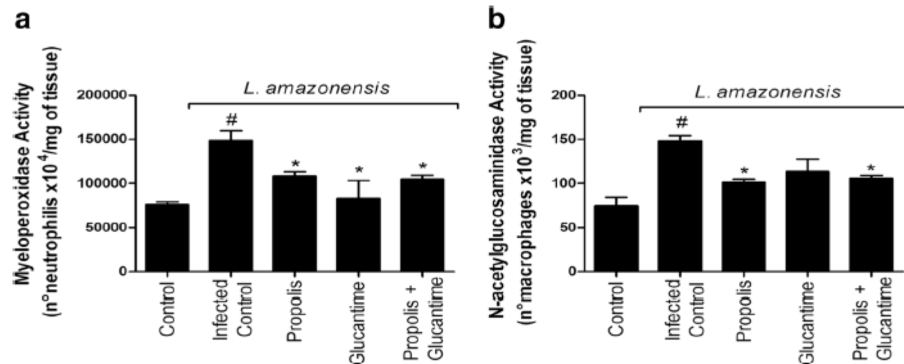

Fig. 5 Propolis reduced *L. amazonensis*-induced myeloperoxidase activity (MPO) and n-acetylglucosaminidase (NAG) on liver tissue. MPO (**a**) and NAG (**b**) were evaluated as markers of the inflammatory infiltrate on liver. BALB/c mice were infected with *L. amazonensis* (10^7) promastigote forms by subcutaneous (s.c.) injection in the hind paw. After 15 days, animals were treated daily with propolis (5 mg/kg, p.o) or Glucantime (10 mg/kg, i.p.) or combination of propolis plus Glucantime for 60 days. (*Number sign*) Significantly different from control. (*Asterisk*) Significantly different from infected control ($P<0.05$)

Interleukin-13 is associated with inducing fibrosis in chronic infectious and autoimmune diseases like schistosomiasis and chronic asthma. However, the pathogenesis of fibrous tissue involves IL-13 associated with a cascade of events that involve an increase of TGF-β and TNF-α, regulating collagen synthesis and collagen catabolism (Wynn 2004; Fichtner-Feigl et al. 2006).

In our study, the Sirius red staining method with polarized light microscopy was used, allowing the characterization of type I and III of collagen in tissues. Three colors can be distinguished: green, characteristic of thin collagen fibers and reticular type III, and the yellow to red spectrum, indicating dense type I fibers (Montes and Junqueira 1991). Thus, it was possible to evaluate the total collagen

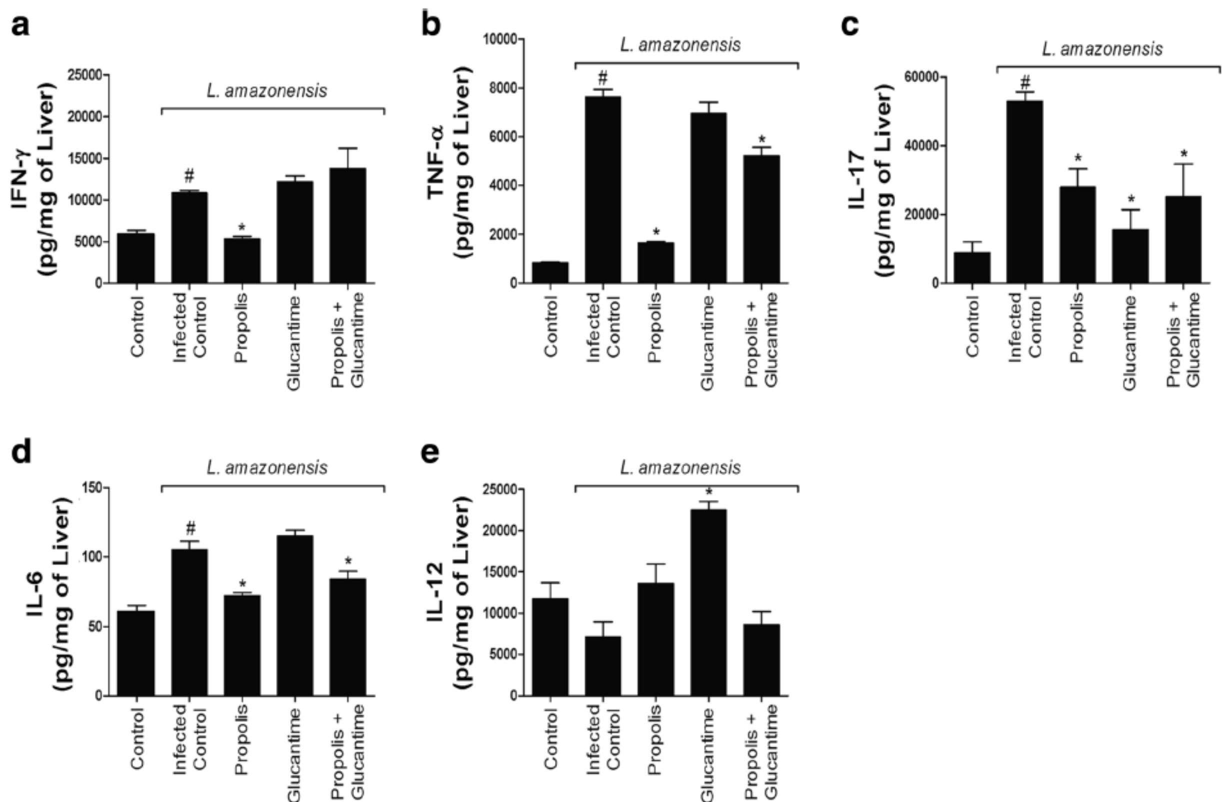

Fig. 6 Propolis reduced *L. amazonensis*-induced pro-inflammatory cytokine production in liver. IFN-γ (**a**), TNF-α (**b**), IL-17 (**c**), IL-6 (**d**), and IL-12 (**e**) were evaluated in homogenate liver. (*Number sign*) Significantly different from control. (*Asterisk*) Significantly different from infected control ($P<0.05$)

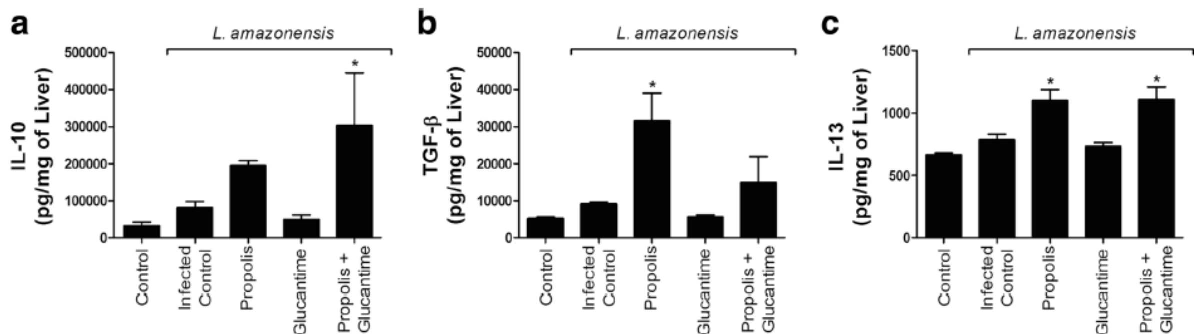

Fig. 7 Propolis increased anti-inflammatory cytokine production in liver. IL-10 (**a**), TGF-β (**b**), and IL-13 (**c**) were evaluated in homogenate liver. (*Asterisk*) Significantly different from infected control ($P < 0.05$)

score as well as the percentage of deposition of fibrillar type I and III collagen.

Some authors suggest that oxidative stress also participates in the process of fibrosis, which can progress to necrosis or apoptosis of hepatocytes. The increase in pro-fibrogenic response with consequent increased expression and deposition of type I collagen fibers may be evidenced by reduction of antioxidant defenses such as glutathione (GSH), catalase, or superoxide dismutase (SOD), along with an increase in lipid peroxidation (George 2003; Bataller and Brenner 2005; Nieto 2006).

Propolis-treated groups showed reduced chronic inflammation, with a decrease in the deposition of type I and III collagen; lower levels of the pro-inflammatory cytokines TNF-α, IL-17, and IL-6; decreased AST and ALT levels; and reduction in liver and spleen weights.

Hepatosplenomegaly is another important factor observed as a clinical sign in visceral leishmaniasis. Thus, to evaluate possible changes resulting from infection or treatment, the liver and spleen weights of the animals were evaluated. *L. amazonensis* infection increased liver and spleen weights. However, the treatment with propolis with or without Glucantime reversed this process.

Previous studies with different propolis extracts have shown antiprotozoal activity, as well as immunomodulatory and anti-inflammatory effects (Dimov et al. 1992; Ramos and Miranda 2007; Ayres et al. 2007; Sforcin 2007; da Silva et al. 2013). In addition, the wound healing activity of propolis has attracted attention (Olczyk et al. 2013a, b).

The sample of Brazilian propolis used in our experiments has exhibited leishmanicidal, fungicidal, antimicrobial, and immunomodulatory effects in different experimental models (Orsi et al. 2000, 2012; Murad et al. 2002; Sforcin 2007; Missima and Sforcin 2008; da Silva et al. 2013; Miranda et al. 2015). Some studies have suggested that the immunomodulatory action of propolis may occur through the inhibition of T cell activation by inhibiting mainly IL-2, NF-κB, MAPK, STAT 3, and IL-6. In addition, other studies have shown a decrease in MPO and NADPH-oxidase activities (Frenkel et al. 1993; Volpert and Elstner 1996; Okamoto et al. 2012; Búfalo et al. 2013).

The available drug in Brazil for the treatment of leishmaniasis is an antimonial, a complex of $Sb^{(V)}$ with N-methyl-D-glucamine (meglumine antimoniate or Glucantime®). However, this drug has serious side effects and limitations in its use and shows therapeutic failures (Sundar and Chakravarty 2013).

Here, we found that animals treated with Glucantime did not show an improvement of inflammatory responses induced by infection with *L. amazonensis*. However, when Glucantime was combined with propolis, the anti-inflammatory effects became more evident. Furthermore, studies have suggested the use of propolis combined with Glucantime to decrease the side effects of Glucantime in the host (Ayres et al. 2011; Ferreira et al. 2014).

Considering the antioxidant and anti-inflammatory role that propolis has shown in several models, our data indicated that the daily treatment in mice susceptible to infection with *L. amazonensis* is able to prevent the progression of lesions in the liver. This effect may be due to immunomodulatory effects with consequent reduction of inflammatory infiltrate, granuloma formation, and fibrosis in liver tissue.

Conclusion

Our study demonstrated the anti-inflammatory effect of propolis in the liver, when given at low daily doses. Propolis promoted immunomodulation with efficacy by reducing cellular recruitment, which prevented inflammatory processes in the liver due to infection with *L. amazonensis*. Furthermore, these data encourage further studies to determine the value of combining this apitherapeutic agent with Glucantime to increase the treatment efficacy in leishmaniasis and reduce the side effects of Glucantime.

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ARTIGO 3- Concanavalin-A displays leishmanicidal activity in macrophages of BALB/c mice by IL-1β and reactive oxygen species production

- **A ser enviado para Revista: Parasite Immunology**

ORIGINAL PAPER

Concanavalin-A displays leishmanicidal activity in macrophages of BALB/c mice by IL-1β and reactive oxygen species production

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SUMMARY

Previous study verified that pretreatment with concanavalin-A (Con-A) improve the phagocytic and candidacidal activities of peritoneal neutrophils and macrophages from Swiss mice. However, the immunomodulatory activity of this lectin has not been demonstrated in experimental leishmaniasis model. In this way, our aim was to investigate the effect of Con-A on the activation of BALB/c peritoneal macrophages *in vivo* with subsequent infection *in vitro* with *Leishmania amazonensis.* The analysis of pretreatment after 2 and 72h with Con-A (250µg/250µL; i.p.) increase the total leukocyte recruitment, cytokine production and nitric oxide levels (NO/peroxynitrite) in peritoneal exudate when compared to untreated control. Phagocytosis analysis showed that pretreatment with Con-A (250µg/250µL; i.p.) after 2 and 72h were able to induce an immunomodulatory response in peritoneal macrophages increasing the phagocytosis and killing *L. amazonensis* through IL-1β and ROS production. Our data suggest that this lectin works in immunomodulation and which consequently improves the leishmanicidal activity of peritoneal macrophages from susceptible mice strain in model of experimental infection with *L. amazonensis*.

Keywords: *Leishmania amazonensis* infection; Concanavalin A immune response, Leishmanicidal activity, Phagocytosis

INTRODUCTION

The lectin concanavalin-A (Con-A), isolated from *Canavalia ensiformis*, has been used in several experimental models as stimuli for immune response due their role as potent activator of T lymphocytes and consequently activation of effectors cells of immune response (1–4).

The Con-A has specific sugar binding sites, whose ligands are α-D-mannoside, methyl α-D-mannopyranoside, α-D-glucose, and methyl-α-D-glucose (5). Therefore, Con-A directly binds to carbohydrates in the constant region of MHC molecule and TCR receptor on T helper cells and induce polyclonal activation of lymphocytes, stimulating release of several cytokines (3,5).

Furthermore, the involvement of this lectin in increased expression of Toll-like receptor 2 to 9 in macrophages from BALB/c mice was demonstrated *in vitro* and the activation of these receptors occur by MyD88 pathway, IRAK1, TRAF6 and IRF3 molecules (6,8). In another study, analysis by immunofluorescence showed increase of mannose receptor expression in macrophages of Swiss mice after pretreatment *in vivo* with Con-A (9). The higher expression of receptors associated with the increase of cytokines production such as IFN-γ, IL-17 and IL-1β has been involved with enhanced the phagocytic capacity of macrophages (10,11).

Some studies evidenced in experimental candidiasis model that pretreated macrophages with Con-A (250µg/250µL, i.p.) induced an improved phagocytic and candidacidal activity with production of IL-12, IFN-γ, IL-1β, IL-6, TNF-α and IL-17 (12–14). In addition, Conchon-Costa et al., 2007 (15) demonstrated that intraperitoneal administration of low dose of Con-A presented hepatoprotective effect, promoted candidacidal effect of macrophages and the survival of mice infected with lethal dose of *Candida albicans* (15,16).

The American Tegumentary Leishmaniasis (ATL) is a disease characterized by chronic inflammation of skin and mucosa, wherein the pathogenesis is related to immune response developed by patient, as well as the species of *Leishmania* involved in infection (17–19).

Resistance or susceptibility to disease was established in murine models. In experimental infection with *L. major*, C57BL/6 mice strain develops a Th-1 polarization leading to an efficient immune response and self-limited lesions, while BALB/c develops a Th-2 polarization with higher levels of IL-4, culminating in progression of the disease and large lesions (20, 21).

Leishmania amazonensis, one of species involved in cutaneous leishmaniasis in South America, causes a wide spectrum of clinical manifestations with high potential pathogenic involved in cutaneous, diffuse and visceral leishmaniasis infections (20–22). Until now there are no drugs used effectively to treatment without induce a severe side effects and infection relapses (23).

Considering the potential of Con-A to activate a Th-1 polarization which is associated with protection and limited disease in mouse models, the aim of this study was to evaluate the immunomodulatory effect of pretreatment with Con-A and the leishmanicidal activity of macrophages from susceptible BALB/c mice strain.

MATERIALS AND METHODS

Animals and treatment

Male BALB/c mice (20–25 g), 4–6 weeks old, were obtained from the Fundação Osvaldo Cruz, FIOCRUZ, Curitiba, Brazil. Mice were kept under conditions according to protocols approved by the Ethics Committee of the Universidade Estadual de Londrina (process number 1677.2013.33).

Mice were pretreated with 250μg/250μL, i.p. route of Con-A (Sigma-Aldrich). Control groups received only phosphate buffer saline-vehicle (PBS) (250μL, i.p.) by 2 or 72h. The treatment with lipopolysaccharides (LPS from *Escherichia coli*- Sigma Aldrich) (200ng/200μL, i.p.) by 2h, 6h or 72h was used as positive control (24).

After this period the animals were sacrificed and peritoneal exudate was collected and used to determinate total leukocytes, cytokines and NO/peroxynitrite by real time and phagocytic assay.

Total leukocytes from peritoneal exudate

The total leukocytes were determined from exudate peritoneal in a Neubauer chamber after treatment of Con-A (250μg/250μL, i.p.) in 2 or 72h. The controls received only phosphate buffer saline-vehicle (PBS) (250μL, i.p.) or lipopolysaccharides (LPS) (200ng/200μL, i.p.) by 2 or 72h.

Real-time detection of NO/peroxynitrite by high sensitivity chemiluminescence

NO production was evaluated employing a highly sensitive NO detection system described by Kikuchi et al. (1993) (25), with some modifications. In this method, NO/peroxynitrite reacts with hydrogen peroxide, which in the presence of luminol produces triplet oxygen, which decays to singlet oxygen and emits photons, detected by a luminometer system coupled to a computer. The peritoneal exudate cells were diluted 1:1 in fresh sterile 2 mM Na_2CO_3 buffer, pH 8.5, previously degassed with bubbling N_2 for 20 min to eliminate the presence of molecular oxygen and oxidation of NO to nitrite/nitrate. The final reaction volume was 500μL of macerated paw plus 500μL of Na_2CO_3 buffer. The starting reagent was prepared by mixing equal volumes of luminol solution (4.39 μM dissolved in 1 M KOH) diluted 1:10 in 36.58 μM desferrioxamine and 2.44 μM H_2O_2, with 3 parts degassed Na_2CO_3

buffer. This mixture was vortexed for 5 min before use. All solutions were sterile and kept at 25°C in covered tubes, protected from light. Finally, the luminometer chamber was injected with 50 µL of starting reagent and the reaction was performed in a Glomax luminometer (Promega), with automatic reagent injector, employing a kinetic protocol which allowed following the reaction at 10 readings per second. The treatment with lipopolysaccharides (LPS from Escherichia coli) (200ng/200µL, i.p.) by 6h was used as positive control (24). All chemicals were purchased from Sigma.

Leishmania amazonensis

L. amazonensis (MHOM/BR/1989/166MJO) was obtained from homogenate of popliteal lymph nodes of infected BALB/c mice. The promastigote forms were cultured in 199 medium (Gibco® Life Technologies, Grand Island, NY, USA) and supplemented with 10% heat-inactivated fetal bovine serum (Gibco® Life Technologies, Grand Island, NY, USA), 10mM of HEPES biological buffer (AMRESCO®, Solon, USA), 0.1 % human urine, 0.1 % L-glutamine (Synth®, Diadema, Brazil), 1 % penicillin/streptomycin solution (Gibco® Life Technologies, Grand Island, NY, USA) and 10 % sodium bicarbonate (Synth®, Diadema, Brazil). Cultures were incubated at 25°C in 25-cm^2 flasks. Infective stage metacyclic promastigote forms of *L. amazonensis* were isolated from stationary cultures (5 culture days), were used for experimental infection of macrophages.

Phagocytosis assay

After 2 or 72h of treatment with Con-A (250µg/250µL, i.p.) macrophages (5×10^5/mL) obtained from the peritoneal cavity by the injection of 2 mL of RPMI 1640 culture medium (Gibco® Life Technologies, Grand Island, NY, USA) supplemented with 10% fetal bovine

serum (Gibco® Life Technologies, Grand Island, NY, USA) were incubated during 1h at 37°C with 5% of CO_2 on 24-well plates containing 13 mm diameter glass coverslips.

Adherent macrophages were incubated with promastigote forms of *L. amazonensis* (5:1) for 2h for phagocytic analysis. After 2h the cells were washed to remove extracellular promastigotes. Subsequently these cells were maintained in culture for 6 and 18h for leishmanicidal analysis. After 2, 6 and 18h of infection, the cells adhered to the cover slips were stained with May Grunwald and Giemsa (Laborclin®, Pinhais, Paraná, Brasil) to establish the phagocytic index of infection (by percentage). Supernatants was collected and utilized to measure cytokine and nitrite levels.

In another protocol, peritoneal macrophages (5×10^5/mL) after treatment with Con-A (250µg/250µL, i.p.) at 2 or 72h were cultured on 6-well plates at 37°C and 5% CO_2. After 1h for adherence, macrophages were washed and infected with promastigotes forms (5:1) for 2h. The culture was washed to remove extracellular parasites and incubated with 199 culture medium at 24°C. Recovered promastigotes were counted in a Neubauer chamber 3 days after the infection. Controls no received treatment.

Cytokine Determination

The IFN-γ, TNF-α, IL-12, IL-17, IL-1β, IL-10 and TGF-β levels was determined in the peritoneal exudate of BALB/c mice pre-treated with Con-A (250µg/250µL, i.p.) for 2 or 72h. Moreover, the supernatants obtained from phagocytic assay of 18h were used for the measurement of IL-1β, TNF-α, IL-12 and TGF-β levels. The technique of capture Enzyme-Linked Immune Sorbent Assay (ELISA) kit from Bioscience (USA) was used as the instructions. The concentration of cytokines was determined by reference to standard curve for serial dilutions and the optical absorbance measured at 450nm.

Determination of Nitrite Levels

The nitrite determination of supernatants collected from phagocytic assay were used as estimates of the concentrations of nitric oxide (NO) by Griess reagent accordingly to Panis et al. (2012) (26) with some modifications. Briefly, supernatant aliquots were deproteinized by adding 50 µL of $ZnSO_4$ 75 mM solution and 70µL of NaOH and centrifuged at 10000 rpm, 5 minutes, 25º C. The limpid supernatants were recovered and diluted in glycine buffer solution (45 g/L pH 9.7). Cadmium granules previously activated with $CuSO_4$ 5 mM solution was added to the samples for 10 minutes. Aliquots of 50µL were recovered into 96-wells plates for determination of nitrite and the same volume of Griess reagent was added. After 10 min incubation at room temperature, the absorbance was determined at 550 nm in a microplate reader. Calibration curve was prepared by dilution of $NaNO_2$.

Determination of Reactive Oxygen Species by florescence (ROS)

In order to evaluate the ROS production, macrophage cultures as phagocytic assay were established in 96-well black plate containing $5x10^5$ cells/mL and incubated for 1h at 37°C and 5% CO_2. After this period, the infection was carried out with 10^6 promastigotes/mL by 2h, and after the culture was washed to remove non-infected promastigotes. Subsequently these cells were maintained in culture with a total 18h for phagocytosis. After this time the cells were washed with PBS and treated with 2µM diacetate 2', 7'-dichlorofluorescein (H_2DCFDA) (Sigma-Aldrich) diluted in DMSO, incubate in the dark for 30 minutes at 37° C and 5% CO_2. N-Acetyl-L-Cysteine (NAC) (Sigma Aldrich,StLouis, MO) 5mM diluted in Tris-HCl 1M was used to inhibit ROS production. The exposition of H_2O_2 for 30min was used a positive control. Fluorescence intensity was measured on a plate reader Victor™ Multilabel X3 (Perkin Elmer Inc., Waltham, MA, USA) with issue fee 525nm and excitation 490nm.

Statistical procedures

Data were analyzed using GraphPad PRISM statistical software (Graph-Pad Software Inc., USA, 5.00). Significant differences between treatments were determined by analysis of variance (ANOVA) followed by Tukey's multiple comparison test. Statistical significance was accepted at $p < 0.05$.

RESULTS

Immunomodulatory effect of Concanavalin-A in peritoneal exudate of BALB/c mice

Peritoneal exudate samples were collected after 2 and 72h of Con-A treatment (250µg/250µL; i.p.) for the assessment of total leukocytes (Figure 1), cytokine production (Figure 2) and NO/peroxynitrite levels (Figure 3).

The groups of BALB/c mice were treated with Con-A (250ug/250uL, i.p.) or stimulated with LPS (200ng/200uL, i.p.) for 2 and 72h. Both stimulus were able to induce a significant increase of total leukocytes recruitment (Figure 1) as well IFN-γ, TNF-α and IL-12 production in both times of treatment (Figure 2 A-C).

However, the IL-17 and IL-1β was increased only after 2 hours of treatment with Con-A and LPS stimuli (Figure 2 D and E). On the other hand, the production of IL-10 and TGF-β were not affected (Figure 2 F and G).

The Con-A treatment in 2 and 72h induced an increase of NO levels when compared to control, as well the 6h of LPS stimuli (Figure 3).

Leishmanicidal and immunomodulatory activity of Con-A of peritoneal macrophages infected *in vitro*

As shown in Figure 4A, after 2 hours of exposure to the parasite, there was no significant differences in the phagocytic capacity between infected cells pretreated with 2h of Con-A compared to infected non-treated control. However, the pre-treatment with 2h of Con-A was able to reduce significantly the percentage of infected macrophages after 6 and 18h of culture (Figure 4B). Furthermore, in another model it was found that from the third day after infection the amount of recovered promastigotes was reduced compared to the untreated control (Figure 4C).

When analyzed the pretreatment of 72h, the phagocytic capacity of cells was higher than the infected non-treated control (Figure 5A), moreover, the percentage of infected macrophages after 6 and 18h of infection was decreased when compared to the untreated control (Figure 5B). The Con-A pretreatment at 72h promoted a reduction in recovered promastigotes in macrophages from the third day after infection (Figure 5C).

Cytokine production in macrophages pretreat with Con-A and infected *in vitro*

Pretreatment with Con-A at 2 and 72h induce the production of IL-1β in infection and no infection conditions (Figure 6A). The TNF-α production was increased in supernatants of non-infected cells 72h after treatment with Con-A (Figure 6B), while IL-12 production was increased in 2 and 72h of pretreatment of non-infected macrophages (Figure 6C). On the other hand, treatment and infection did not affect TGF-β production by peritoneal macrophages compared to control (Figure 6D).

***L. amazonensis* infection inhibits NO production induced by Con-A**

Concerning NO levels, our results showed that macrophages pretreated with Con-A at 2 or 72h increased the NO levels under not infection condition when compared to the control.

However, the infection with *L. amazonensis* displayed decreased of NO levels, even in pretreated cells (Figure 7).

Con-A-induced Reactive Oxygen Species in macrophages infected *in vitro*

ROS production by pretreated macrophages with Con-A (250µg/250µL) for 2 and 72h showed no statistical difference with pretreated macrophages in same conditions that received infection (data not showed). In the presence of the antioxidant NAC the results showed that the pretreatment with Con-A(250µg/250µL) for 2 and 72h induces ROS production when compared with control that also received NAC and no shows difference with the positive control (Figure 8).

DISCUSSION

Experimental infections with parasites of the genus *Leishmania* spp. demonstrate that the immune response which promotes the control of infection is dependent of recruitment of immune effector cells to the site of infection, as well as the proinflammatory cytokines production and microbicidal molecules production such as NO and ROS by de host (17,27,28).

This study shows that Con-A (250µg/250µL) administration by i.p. route in BALB/c mice for 2 or 72h promoted an increase of total recruitment leukocytes, cytokine production such as IFN-γ, TNF-α, IL-12, IL-17 and IL-1β and NO levels in peritoneal exudate, similarly to intraperitoneal challenge with LPS.

Therefore, we studied the *in vitro* infection with *L. amazonensis* in macrophages collected from peritoneal exudate after Con-A treatment. For *in vitro* phagocytosis, we consider 2h the time required for the process of internalization of promastigotes, from this period was possible to evaluate the leishmanicidal effect. Thus, our results showed a decrease

in the percentage of infected macrophages after 6 and 18h. Furthermore, there was a decrease of recovered promastigotes when evaluated the third, fourth and fifth day of infection. This way the leishmanicidal activity of Con-A was verified that both times of pretreatment (2 and 72h).

Macrophages activated increase the production proinflammatory cytokines, as well as the production of reactive oxygen species (ROS) and reactive nitrogen species (RNS) increasing the microbicidal activity to intracellular parasites (17,18,27). However, *Leishmania* parasites can modulate or interfering in some components of immune response of their host as a way to escape, permitting progression of infection (29–33).

In our phagocytic assay the Con-A pretreatment presented immunomodulation action, with increasing the synthesis of IL-1β, TNF-α and IL-12. However, when the cells were exposed to the parasite, only the IL-1β levels remained elevated.

With regarding of NO levels, we found that stimulation with Con-A promotes the NO production, however, infection with *L. amazonensis* inhibits this production. Nonetheless, infection with *L. amazonensis* is not able to inhibit the ROS production induced by stimulation with Con-A.

As reported previously some studies suggest that *Leishmania* spp. can modulate or interfere in immune response of host through alteration of cytokine and chemokine signaling and production (34) and may even change the levels of IL-1β, NO and ROS production (35–38). However, in our model can be verified that the production of IL-1β and ROS by pretreatment is not affected by the presence of the parasite which justifies the leishmanicidal effect.

Interleukin-1β (IL-1β) is an inflammatory cytokine that assists the activation of macrophages and stimulated the intracellular ROS production (39). In Leishmaniasis has been

reported that Inflammasome-driven IL-1β production facilitated host resistance to infection via NO and ROS production (37-39).

The immunomodulation of IL-1β and ROS production mediated by Con-A in our model did not suffer interference of *L. amazonensis* and use of antioxidant NAC. Moreover another models of Con-A study, showed that this lectin induces the production of IL-1β and ROS via p38/MAPK Pathway (40), reinforcing our findings.

The stimulation with concanavalin-A to induce strong immune modulation has been used in models of autoimmune hepatitis (16,40). However the dose tested in our model already was validated models of candidiasis (9,12–15).

Taken together, our data demonstrated that Con-A has immunomodulatory action on peritoneal exudate cells, and that the stimuli with Con-A in peritoneal macrophages of susceptible mice making these cells active, promoting a leishmanicidal effect through of IL-1β cytokine and ROS production.

Although far the use of concanavalin-A in clinic, by autoimmune response induction, these data provide incentive for exploration of future experimental studies to understand the parasite-host interaction and involvement in the immune response stimulation by Con-A in models of infection.

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FIGURES and LEGENDS

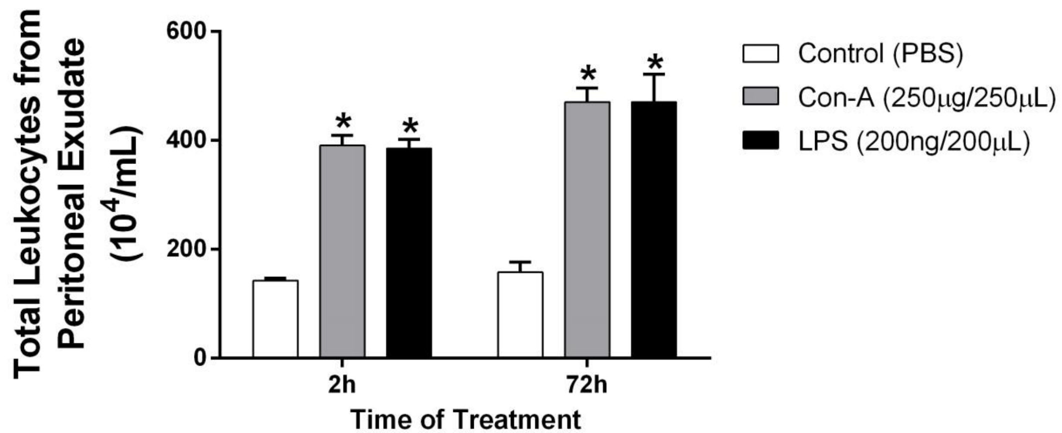

Figure 1-Effect of treatment with Con-A on total leukocyte recruitment of peritoneal exudate after intraperitoneal treatment with Con-A (250µg/250µL). The cells were counted in neubauer chamber. Data represent the mean ± SEM of six animals per group. *Significant difference relative to the control.

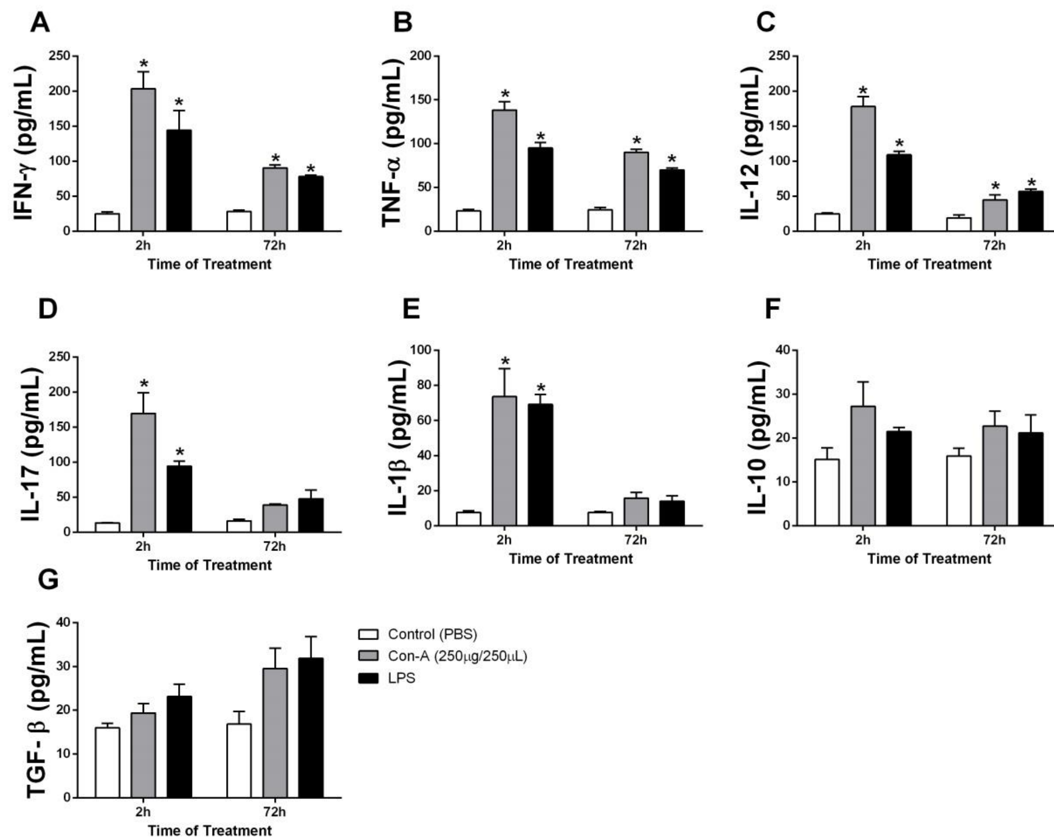

Figure 2- Cytokine production from peritoneal exudate of BALB/c mice after intraperitoneal treatment with Con-A (250μg/250μL) by 2 and 72h. The result was expressed as the mean ± SEM of six animals per group. *Significant difference relative to the control.

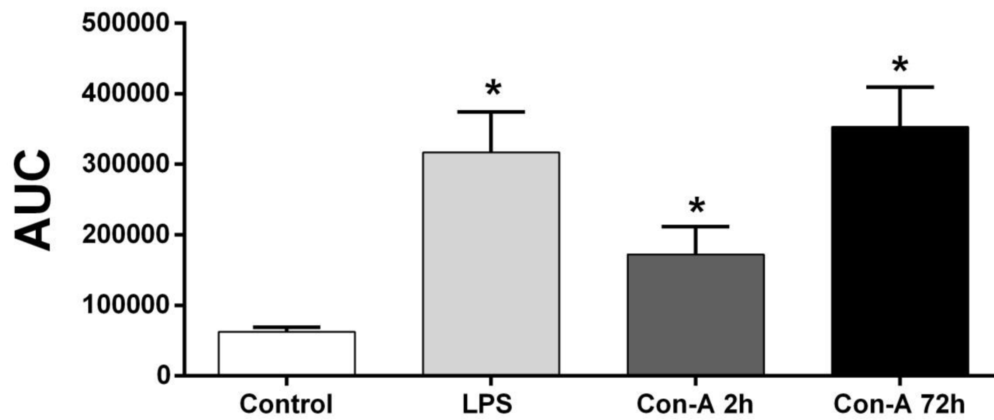

Figure 3- NO/peroxynitrite estimated by chemiluminescence in the peritoneal exudate after treatment with Con-A (250μg/250μL) at 2 and 72h. The result was expressed as the mean ± SEM of six animals per group. (AUC: area under the curve). *Significant difference relative to the control.

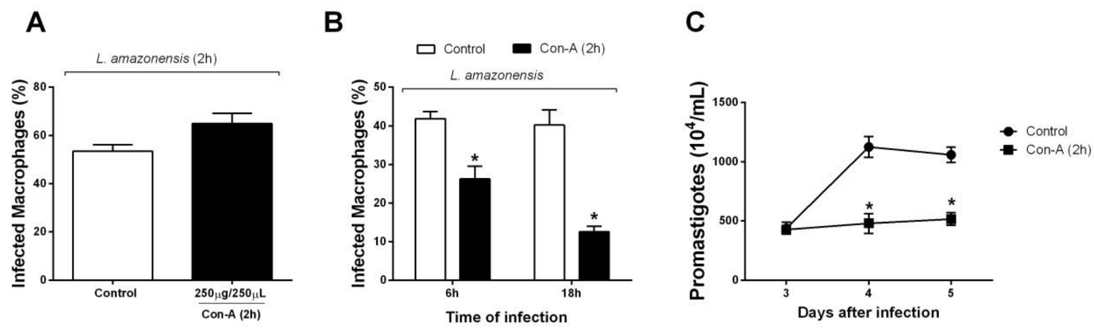

Figure 4- Phagocytic assay with *in vitro* infection of *L. amazonensis* in macrophages of peritoneal exudate pretreated with con-A (250µg/250µL) for 2 hours of infection (A) 6 and 18h of infection (B). Kinetics of recovered promastigotes from infected macrophages *in vitro* with *L. amazonensis* (C). The result was expressed as the mean ± SEM of three independent experiments in duplicate. *Significant difference relative to the control.

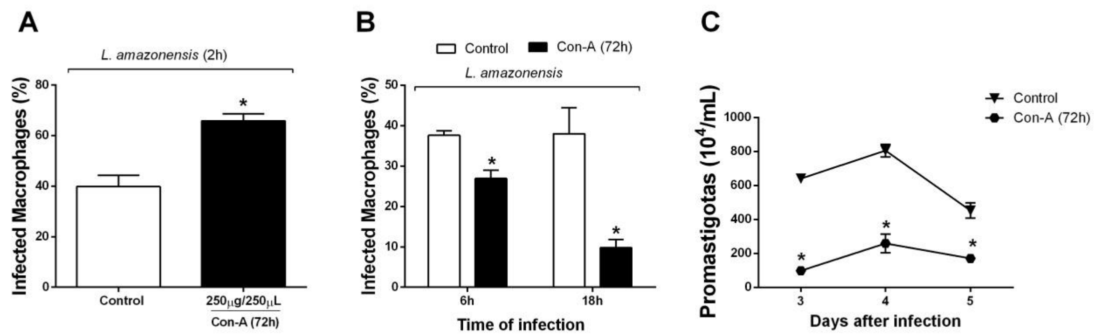

Figure 5- Phagocytic assay with *in vitro* infection of *L. amazonensis* in macrophages of peritoneal exudate pretreated with con-A (250µg/250µL) for 72 hours. 2h of infection (A) 6 and 18h of infection (B). kinetics of recovered promastigotes from infected macrophages *in vitro* with *L. amazonensis* 18h (C). The result was expressed as the mean ± SEM of three independent experiments in duplicate. *Significant difference relative to the control.

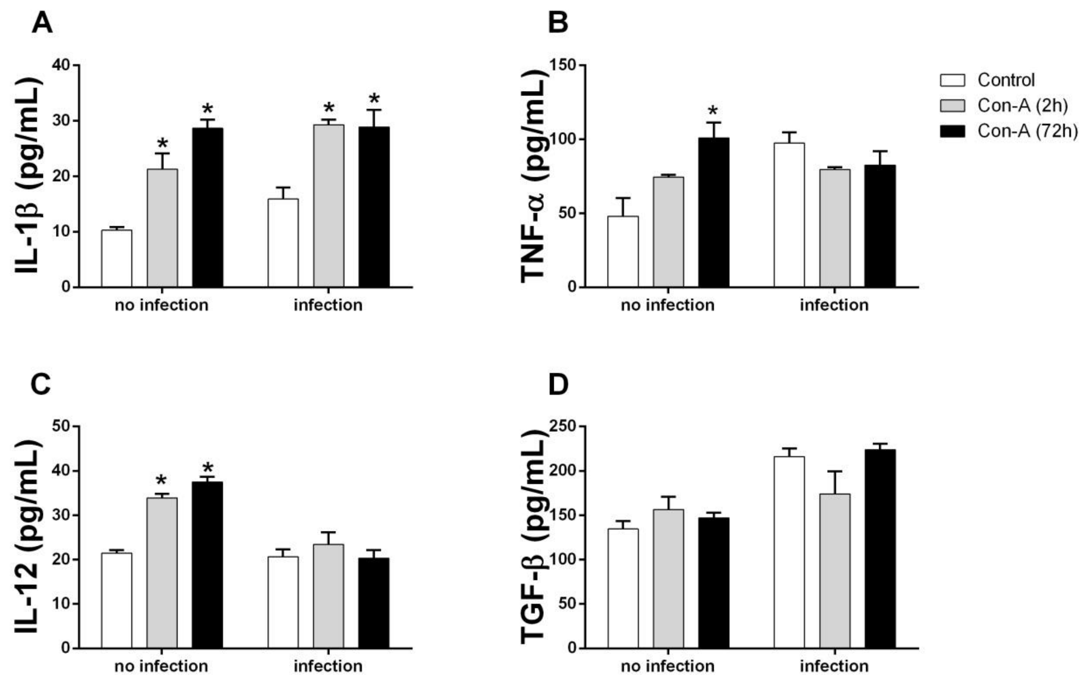

Figure 6- Cytokine production of supernatant of phagocytic assay after 18h with infection. The result was expressed as the mean ± SEM of three independent experiments in duplicate. *Significant difference relative to the control.

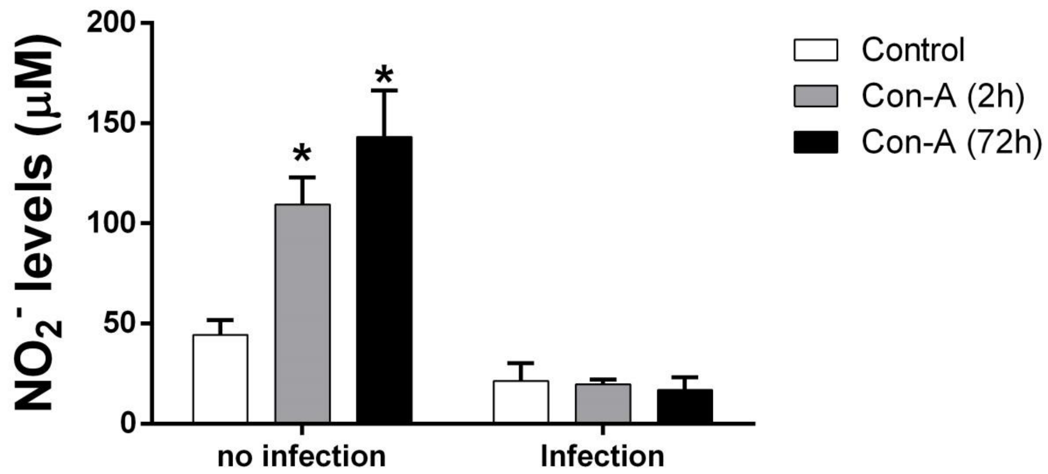

Figure 7- Nitric oxide estimate by nitrite levels of supernatant of phagocytic assay after 18h with infection. The result was expressed as the mean ± SEM of three independent experiments in duplicate. *Significant difference relative to the control.

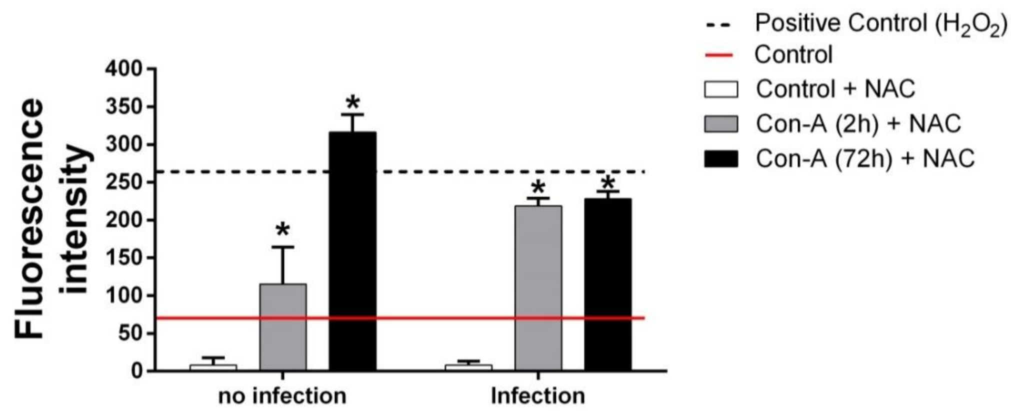

Figure 8- Reactive oxygen species production was measured by fluorescence intensity of H_2DCF-DA. Macrophages from BALB/c pretreated with Con-A (250µg/250µL, i.p.) 2 and 72h, and *in vitro* infected with promastigote of *L. amazonensis* at 18h. The result was expressed as the mean ± SEM of three independent experiments in duplicate. *Significant difference relative to the control+ NAC.

ARTIGO 4- Antimoniate N-methyl-glucamine reduces mechanical hyperalgesia in cutaneous leishmaniasis and Complete Freund´s Adjuvant model of chronic inflammatory pain

- **A ser enviado para Revista: Experimental Parasitology**

Full Length Article

Antimoniate N-methyl-glucamine **reduces mechanical hyperalgesia in cutaneous leishmaniasis and Complete Freund´s Adjuvant model of chronic inflammatory pain**

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ABSTRACT

There are no evidences that antimoniate N-methylglucamine treatment acts on inflammatory pain up to the present days. Furthermore, the studies about nociception in leishmaniasis are scarce, controversial and majority of approach are performed in *L. major*. In order to understand the possible analgesic effect of antimoniate N-methylglucamine in paw *in situ* chronic lesions from *Leishmania amazonensis* infection in susceptive BALB/c mice, we used cutaneous leishmaniasis and Complete Freund´s Adjuvant (CFA) in prolonged inflammatory pain model. *L. amazonensis*-induced chronic inflammation with significant increase of mechanical hyperalgesia, myeloperoxidase (MPO) and N-acetyl-β-glucosaminidase (NAG) activity, and also increase IL-1β, TNF- α and IL-6 levels, which were reversed by antimoniate N-methyl-glucamine treatment at dose 10mg/kg, i.p.. The inflammatory pain induced by CFA stimulus, the animals were treated with antimoniate N-methyl-glucamine (10mg/kg) during 7 days, which was able to reduces mechanical hyperalgesia, MPO and NAG activity and IL-1β, TNF-α, IL-6 and IFN-γ production. Moreover, antimoniate N-methyl-glucamine treatment increases anti-inflammatory cytokine IL-10. Our data demonstrated that antimoniate N-methylglucamine reduced the chronic inflammatory pain induced by *L. amazonensis* and CFA stimuli by acting on the inhibition of mechanical hyperalgesia, leukocytes migration and IL-1β and IL-6.

Key words: Antimoniate N-methyl-glucamine **Hyperalgesia; *Leishmania amazonensis*; CFA**

1. Introduction

The pentavalent antimonial (antimoniate N-methyl meglumine), in Brazil commercialized by Glucantime®, is the drug used for the treatment for leishmaniasis in several countries such as Brazil, France and Spain (Berman 1988).

Leishmaniasis is a complex disease caused by protozoa of the genus *Leishmania* spp., which can infect humans and several animals through the insect vector. The disease can manifest promoting skin or visceral lesions (Desjeux 2004).

The antimoniate N-methyl-glucamine was reported originally by Dr. Gaspar Vianna in 1912, its form trivalent (trivalent antimony - SbIII), called tartar emetic (potassium tartrate and antimony), however, this formulation showed high toxicity (Sundar and Chakravarty 2013). Therefore, others drugs were introduced to treat this illness, sodium stibogluconate (Pentostan®) and N-methyl-D-glucamine (Glucantime®), pentavalent antimonials, which antimony is pentavalent form (SbV), which showed less toxicity and side effects (Sundar and Olliaro 2007; Sundar and Chakravarty 2013).

Even though a drug employed a long time in treating this disease the mechanism of action of this compound is still controversial (Haimeur et al. 2000; Demicheli et al. 2002; Sundar and Chatterjee 2006).

There are several hypotheses about mechanism of action. Some authors identified the inhibition of oxidative and glycolytic activity of fatty acid on amastigote forms of parasite. Other assumptions include the possible conversion of pentavalent antimony species to a prodrug, the trivalent form (SbIII), which actively counteracts amastigotes acting on the reduction potential of thiols, as well as the possibility of occurring the immunomodulatory effect, increasing phagocytic activity of macrophages *in vitro* (Pathak e Yi 2001; Muniz-Junqueira and de Paula-Coelho 2008) .

The treatment with this drug requires different routes such as intravenous or intramuscular administration in clinical and laboratory follow-up, as it may cause adverse effects, such as pancreatic, liver, kidney and heart toxicity, and may also occur: chemical pancreatitis, arthralgia, myalgia, headache, asthenia and pain the injection site (Sundar and Chakravarty 2013; Saldanha et al. 2000).

Several studies suggest that infection with *Leishmania* parasites interfere in pain perception, since patients with cutaneous lesions reports no pain according to most authors, while some can report this sensation (Sotiropoulos and Wilbur 2001; Morris-Jones and Weber 2004; Daboul 2008).

Experimental infection with *L. major* in different models with pain can cause painful stimulation and production of proinflammatory cytokines (Karan et al. 2006; Kanaan et al. 2000). Moreover, most often of studies are realized with only *L. major*, specie that reaches Europe (Ahmed et al. 1998; Cangussú et al. 2013a).

Until this moment, no reports of performance of this antimoniate N-methyl-glucamine in analgesia. Thusly, the aim of this study was verify the pain reduction mechanism by intraperitoneal treatment with antimoniate N-methyl-glucamine in BALB/c mice in different inflammatory pain models.

2. Materials and Methods

2.1 *Leishmania amazonensis*

L. amazonensis (MHOM/BR/1989/166MJO) maintained in infected BALB/c mice was obtained from homogenate of popliteal lymph nodes and used in promastigote forms, on stationary growth phase (5 days culture), for infection of animals. The promastigote forms were cultured in 199 medium (Invitrogen-GIBCO®), and supplemented with 10% fetal bovine serum (Invitrogen-GIBCO®), 10mM Hepes, 0.1% human urine, 0.1% L-glutamine, 10µg/mL penicillin and streptomycin (Invitrogen- GIBCO®), and 10% sodium bicarbonate. Promastigote cultures were incubated at 25°C in 25cm^2 flasks.

2.2 Experimental infection, inflammatory stimulus and Treatment

Male BALB/c mice (20-25g) with aged 4-6 week-old were obtained from the Fundação Osvaldo Cruz, FIOCRUZ, Curitiba, Brazil. Mice were kept under conditions according to protocols approved by protocols approved by the Ethics Committee of the Universidade Estadual de Londrina (process number 3209.2015.24).

In the first set of experiments, the animals were infected in the right hind paw with *L. amazonensis* promastigote forms (10^7). After 15 days started daily treatment with antimoniate N-methyl-glucamine (10 mg/kg, i.p.) for 60 days.

The hyperalgesia was measured weekly during the treatment using an electronic version of von Frey filaments. After 60 days of treatment, the animals were euthanized and samples of paw tissue were collected for analysis of MPO, NAG activity and cytokines production.

In other model of chronic inflammatory pain the animals received injection of Complete Freund´s Adjuvant (CFA- Sigma F5881) (10µL i.pl) in the right hind paw. For

seven days was performed treatment with antimoniate N-methyl-glucamine (10 mg/kg v.ip.). The mechanical hyperalgesia was measured daily during the treatment using an electronic version of von Frey filaments.

After 7 days of treatment, the animals were euthanized and samples of paw tissue were collected for analysis of MPO, NAG and cytokines.

2.3 Mechanical hyperalgesia test

Mechanical hyperalgesia was measured by an electronic version of von Frey filaments (Cunha et al. 2004). The test consisted of evoking a hind paw reflex with a hand-held force transducer (electronic anesthesiometer; Insight, Ribeirão Preto, SP, Brazil). After the paw withdrawal, the intensity of the pressure was recorded automatically, with values being averaged across three measurements. Mice were tested before (basal) and after stimulus injection. The results are expressed as delta (Δ) withdrawal threshold (in g), calculated by subtracting the basal mean measurements from the mean measurements obtained.

2.4 Myeloperoxidase activity (MPO)

The neutrophil migration to the paw tissue was evaluated by the MPO kinetic-colorimetric assay (Ruiz-Miyazawa, 2015). Samples paw skin lesions were collected in 50 mM K_2PO_4 buffer (pH 6.0) containing 0.5% HTAB, and were homogenized using a Polytron® (PT3100).

After the homogenates were centrifuged at 16,100 g for 2 min, the resulting supernatant was assayed spectrophotometrically for MPO activity determination at 450 nm (Spectra max) with three readings within 1 min. The MPO activity of the samples was compared with a standard curve of neutrophils. The results were presented as the MPO activity (number of neutrophils x 10^4/mg of tissue, using a standard curve of neutrophils).

2.5 N-acetylglucosaminidase activity (NAG)

To assess the infiltration of mononuclear cells, especially macrophages, in the paw the enzyme N-acetylglucosaminidase activity was used as proposed by Bailey (1988). Samples paw skin lesions were collected in 50 mM K2PO4 buffer (pH 6.0) containing 0.5% HTAB, and were homogenized using a Polytron® (PT3100). After the homogenates were centrifuged at 16,100 g for 2 min, the resulting supernatant was assayed spectrophotometrically for NAG activity determination at 450 nm (Spectra max) with three readings within 1 min. The MPO

activity of the samples was compared with a standard curve of macrophages. The results were presented as the NAG activity (number of macrophages x 10^4/mg of tissue).

2.6 Cytokine Measurement

Cytokines present in skin of paw fragments were analyzed by enzyme-linked immunosorbent assay (ELISA). Samples paw skin lesions were homogenized in 500 µL of buffer containing protease inhibitors (1 mM Phenylmethanesulfonyl fluoride, Sigma Aldrich), and IL1β, TNF-α, IL-6, IFN-γ and IL-10 levels were determined by ELISA, according to the manufacturer's instructions (eBiosciences®, USA). Plates were read at 450 nm, using an ELISA plate reader (Thermo Plate—TP-Reader). Results were expressed as pg cytokine/mg tissue.

2.7 Statistic analysis

Data were analyzed using Prism Graph Pad statistical software (Graph-Pad Software, Inc., USA-500.288). Significant differences between treatments were determined by ANOVA, followed by Tukey's t test for multiple comparisons. Statistical significance was accepted when $P < 0.05$.

3. Results

3.1 antimoniate N-methyl-glucamine reduces *L. amazonensis*- induced chronic infection hyperalgesia in BALB/c mice.

L. amazonensis induced significant increase of mechanical hyperalgesia, which was inhibited by antimoniate N-methyl-glucamine at 21-70 days at dose (10mg/kg, i.p.) (up to 74.4%) with significant different with infected group control (Fig 1).

3.2 Myeloperoxidase (MPO) and N-acetylglicosaminidase (NAG) activity on *L. amazonensis* chronic lesion.

In order to verify the antimoniate N-methyl-glucamine influence in cell migration, samples of paw skin lesions were collected for the assessment of MPO and NAG activity. The infection with *L. amazonensis* was able to induce significant increase of myeloperoxidase activity (MPO) (Fig2, A) and N-acetylglicosaminidase (NAG) activity (Fig2, B) compared with control. However, antimoniate N-methyl-glucamine treatments were not able to decrease the activities of MPO and NAG activity when compared with group infected control (Fig2 A and B).

3.3 Antimoniate N-methyl-glucamine reduced *L. amazonensis*-induced IL-1β and IL-6 production on the site of infection.

In order to understand if antimoniate N-methyl-glucamine might modulate cytokine production in *L. amazonensis* infection, the animals were infected with *L. amazonensis* (10^7/ 20µL, i.pl) and after 3h the last treatment with antimoniate N-methyl-glucamine (10mg/kg, i.p.) injection, intraplantar paw skin samples were collected to determination cytokines production by ELISA. The *L. amazonensis* induced significant increase of inflammatory cytokines IL-1 β, TNF-α and IL-6 without change the IFN-γ and IL-10 production when compared to control (Fig 3).

However, treatment with antimoniate N-methyl-glucamine was able to reduces the IL-1β and IL-6 production (45% and 78%, respectively) (Fig 3 A and C). On the other hand, treatment with antimoniate N-methyl-glucamine did not change the TNF-α, IFN-γ and IL-10 cytokine production when compared to infected control (Fig 3 C, D and E).

3.5 Post-treatment with antimoniate N-methyl-glucamine inhibited CFA-induced mechanical hiperalgesia, MPO and NAG activity.

The mice were stimulated with CFA (10µL/paw, i.pl) injection and 3 hours after treated daily with antimoniate N-methyl-glucamine (10 mg/kg, i.p.) during 7 days. Complete Freund's adjuvant was able to increase the mechanical hyperalgesia and MPO and NAG activity in paw skin sample (Fig 4 A, B and C). Antimoniate N-methyl-glucamine inhibited the CFA-induced mechanical hyperalgesia (up to 48%) at 3 hours-7 days tested (Fig 4 A). In the same schedule, MPO (Fig. 4B) and NAG (Fig. 4C) activity was inhibits (99% and 96%, respectively) by Glucantime treatment.

3.6 Post-treatment with antimoniate N-methyl-glucamine inhibited CFA-induced IL-1β, TNF-α, IL-6, IFN-γ and increase IL-10 production in mice.

Mice were treated with antimoniate N-methyl-glucamine (10 mg/kg, i.p.) 3 h after CFA (10µL/paw, i.pl.) injection. The animals were treated daily during 7 days. After 3 h the last treatment, samples of plantar paw skin was collected for IL-1β (Fig 5A), TNF-α (Fig 5B), IL-6 (Fig 5C), IFN-γ (Fig 5D) and IL-10 (Fig 5E) measurement. CFA induced significant increase production of inflammatory cytokines such as IL-1β, TNFα, IL-6 and IFN-γ and decrease IL-10 in plantar paw skin, which was inhibited by Glucantime (58, 53, 49, 31 and 37%, respectively).

4. Discussion

The inflammatory process, independent of the origin of inflammation is usually associated with pain. This is due to the inflammatory process involves the formation of inflammatory mediators leading to nociception sensitization of primary or second order neurons involved in the transmission of nociceptive impulse. Therefore, these processes lead to central sensitization and the sensation of pain perceived as hyperalgesia (Kidd and Urban 2001; Zhang and An 2007 Ren e Dubner 2010).

In the present study, we first conducted the infection by *L. amazonensis* promastigote forms. This is a chronic model widely used for the study of alternative treatment or parasite-host interaction. This disease is considered complex because there are several species etiologic agent and several species of vectors. Moreover, the clinical manifestation is dependent on the species of *Leishmania* and the host immune response (Reithinger et al. 2007; Nylén and Gautam 2010; Kaye and Scott 2011).

L. amazonensis infection was able to induce the inflammatory process by increase mechanical hiperalgesia, MPO and NAG activities as well as by increased levels of the pro-inflammatory cytokines IL-1β, TNF-α and IL-6. Importantly, treatment with antimoniate N-methyl-glucamine reduced the mechanical hyperalgesia and IL-1β and IL-6 production induced by *L. amazonensis* in paw infection.

Some studies in BALB/c model of *L. major* reported increased levels of IL-6 and IL-1β in the infected footpad. However, others studies demonstrated that TNF and IL-1β did not play a direct role in *L. major*-induced hyperalgesia (Kanaan et al. 2000; Karam et al. 2006; Karam et al. 2011; Karam et al. 2013).Thus the nociception studies in experimental leishmaniasis have been low explored, which are usually performed with *L. major*. Furthermore, the role of cytokines in the hyperalgesia of this model is controversial (Ahmed et al. 1998; Karam et al. 2006; Karam et al. 2011; Cangussú et al. 2013b).

In leishmaniasis, the resistance and susceptibility profile to disease is related to the response provided by the host. Studies demonstrated this process were conducted in animal models with *L. major*, where the infection in BALB/c (TH2 profile) had persistent lesions, whereas C57BL/6 mice (TH1 profile) had lesions that progressed to spontaneous healing and shown to be resistant to reinfection (Locksley et al.; Awasthi et al. 2004). However, *L. amazonensis* does not exhibit the same behavior that *L. major* against immune response developed by the host (Afonso e Scott 1993).

Some studies have shown that different cell migration response due the involvement of different chemokines produced in the early stage of infection in leishmaniasis may account for the clinical manifestation of the disease through the establishment of the immune response. In this case, *Leishmania* parasites have the ability to modify the chemokine profiles of their host facilitating establishment of progressive infection (Campanelli et al. 2010; Oghumu et al. 2010; Gupta et al. 2013).

Moreover, in experimental infection can occur the persistence of the parasite that can infer the inflammatory response profile and hamper discussion in advanced stage of disease. Thus, was used another model to mimic a chronic inflammatory state without the parasite persistence. Peripheral inflammation was induced by intraplantar injection of CFA for analysis of inflammatory parameter used in the previous model.

Thus, in this study we can demonstrate an anti-inflammatory and analgesic effect independent of the leishmanicidal effect.

CFA is one of the most commonly used agents to mimic long-term inflammation with tissue damage (Woolf et al. 1997; Leitl et al. 2014). Indeed, this stimulation was able to increase the mechanical hyperalgesia, MPO and NAG activities as well as IL-1β, TNF-α, IL-6 and IFN-γ. On the other hand, treatment with antimoniate N-methyl-glucamine reduced all inflammatory responses induced by CFA.

Some authors suggest the IL-1β, TNF-α and IL-6 play in hyperalgesia induced by different models of inflammatory pain (Watkins et al. 1995; Sommer et al. 1999; Sommer and Kress 2004; Ren and Dubner 2010).

The pentavalent antimony compounds have been the first choice for the treatment of leishmaniasis, but the precise mechanism of action of these compounds remains an enigma (Demicheli et al. 2002; Sundar and Chakravarty 2013). Among the hypotheses for this activity pharmacy, some authors demonstrated its immunomodulatory action *in vitro* (Muniz-Junqueira and de Paula-Coelho 2008; de Saldanha et al. 2012).

Through this evidence antimoniate N-methyl-glucamine may also act in some signaling pathway by inhibiting or decreasing the production of pro-inflammatory cytokines such as hyperalgesia and leukocytes migration. However, it will be important to further investigate to clarify the drug's mechanism of action and the mechanisms involved in pain by *L. amazonensis* infection.

5. Conclusion

In this study our data demonstrated the antimoniate N-methyl-glucamine reduce the mechanical hyperalgesia induced by *L. amazonensis* infection and CFA model. Antimoniate N-methyl-glucamine promoted immunomodulation, reducing pro-inflammatory cytokines and cellular recruitment, which prevented inflammatory process and hyperalgesia in the paw. Furthermore, these data encourage further studies to determine the mechanisms involved in the action of antimoniate N-methyl-glucamine in inflammatory pain model.

Acknowledgments

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Figures and Legends

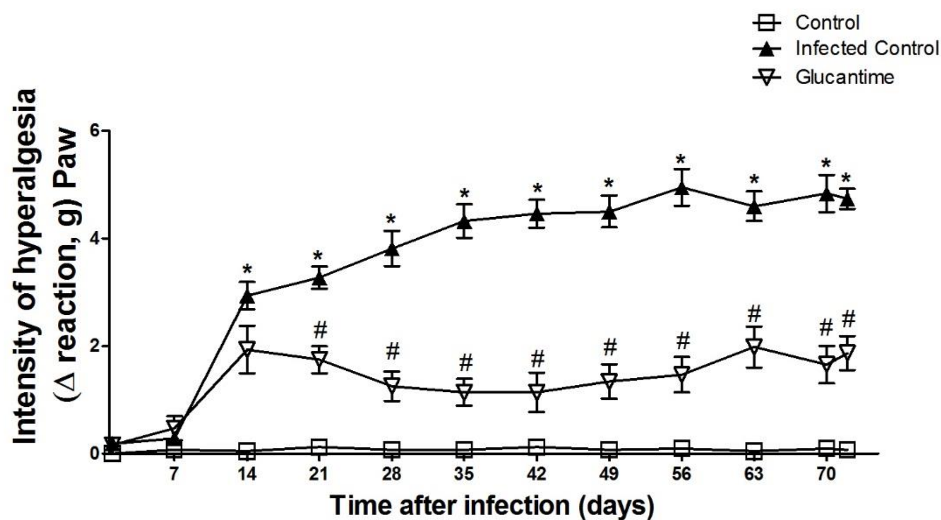

Figure 1- Glucantime reduce activity in the hyperalgesia *L. amazonensis* chronic course of infection in BALB/c mice. Mice were injected in the ride hind footpad with 10^7stationary-phase promastigotes of *L.amazonensis*. Dates represent the intensity of hyperalgesia. Each bar represents mean ± standard error of the mean for eight animals per group. *indicates a statistically significant difference ($P \leq 0.05$) compared with the control group and # indicates a statistically significant difference ($P \leq 0.05$) compared with the infected control group. . One-way ANOVA followed by Tukey's t test.

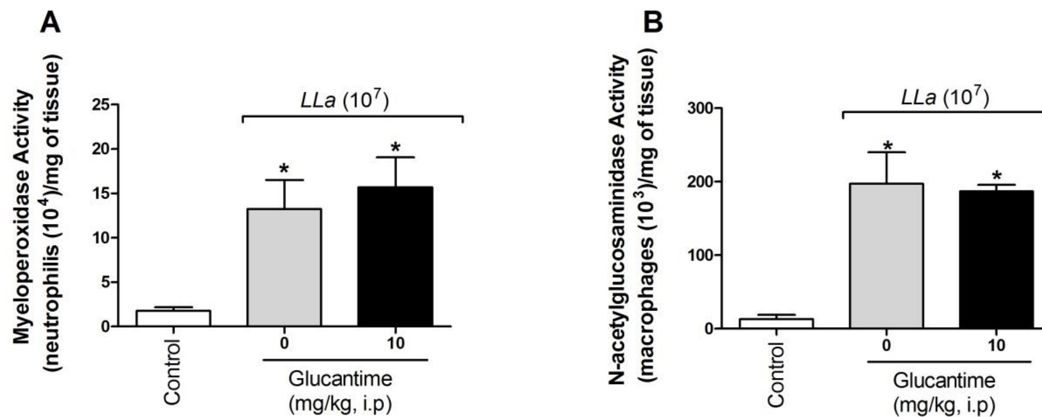

Figure 2- Myeloperoxidase (MPO) and N-acetylglicosaminidase (NAG) activity on *L. amazonensis* chronic lesion. Mice were injected in the ride hind footpad with 10^7stationary-phase promastigotes. A) represent the mean in myeloperoxidase activity in paw skin lesion sample after 70 days of infection. B) Represents the mean in n-acetylglicosaminidase activity in paw paw skin lesion sample after 70 days of infection. Each bar represents mean ± standard error of the mean for eight animals per group. *indicates a statistically significant difference ($P \leq 0.05$) compared with the control group. . One-way ANOVA followed by Tukey's t test.

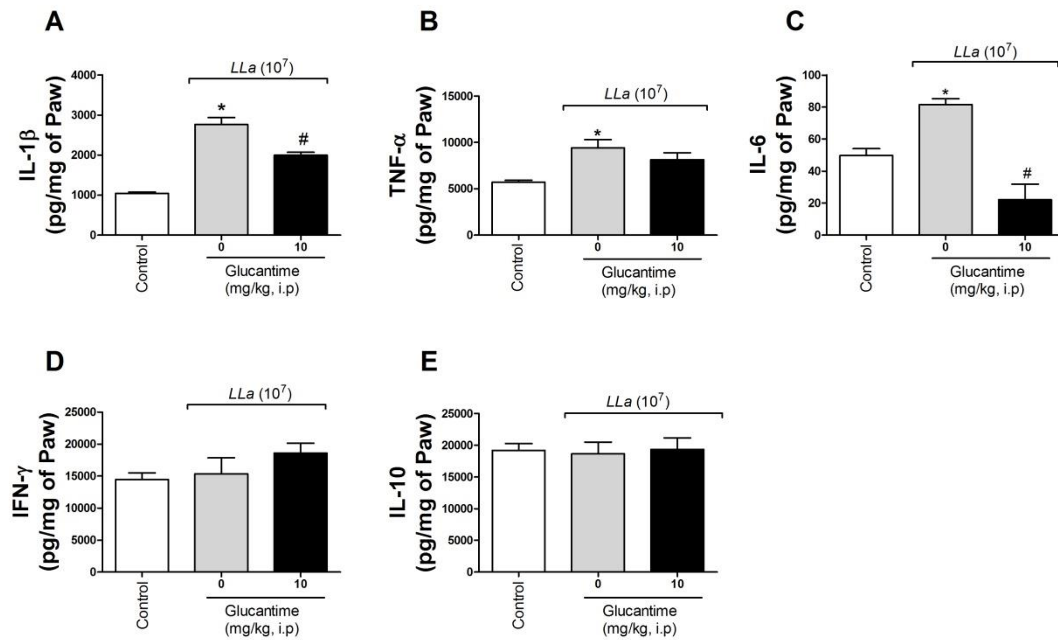

Figure 3- Post-treatment with Glucantime reduced *L.amazonensis*-induced IL-1β and IL-6 production. Mapping the cytokine profile produced on site of *L. amazonensis* infection by ELISA. Mice were injected in the ride hind footpad with 10^7stationary-phase promastigotes. After 15 days received treatment daily of Glucantime (10 mg/kg) i.p. by 60 days. A) IL-1β production, B) TNF-α production, C) IL-6 production, D) IFN-γ production and E) IL-10 production. Data represent the mean ± SEM of four animals. *indicates a statistically significant difference ($P \leq 0.05$) compared with the control group and # indicates a statistically significant difference ($P \leq 0.05$) compared with the infected control group. One-way ANOVA followed by Tukey's t test.

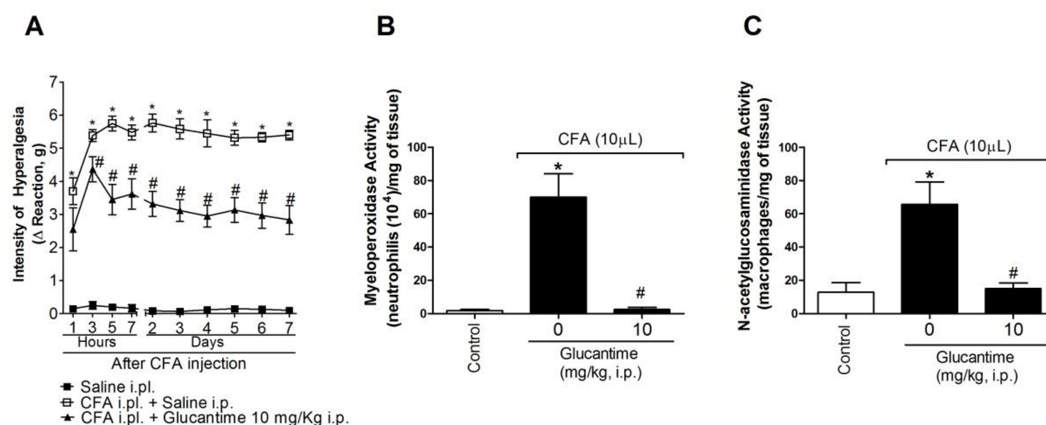

Figure 4- Post-treatment with Glucantime inhibits CFA-induced mechanical hyperalgesia, MPO and NAG activity. Mice were treated daily with Glucantime (10mg/kg, i.p) 3h after Complete Freund's Adjuvant CFA (10μL/paw, i.pl.) stimulus. Mechanical hyperalgesia (Panel A) with Glucantime at 1-7 days after CFA injection. The MPO and NAG activity (Panel B and C, respectively) were assessments 3 hours after the last treatment with Glucantime at 7th day. Results are presented as means ± SEM of 6 mice per group per experiment, and are representative of 2 separated experiments. *P < 0,05 compared to saline group, and #P < 0,05 compared to CFA + vehicle group. One-way ANOVA followed by Tukey's t test.

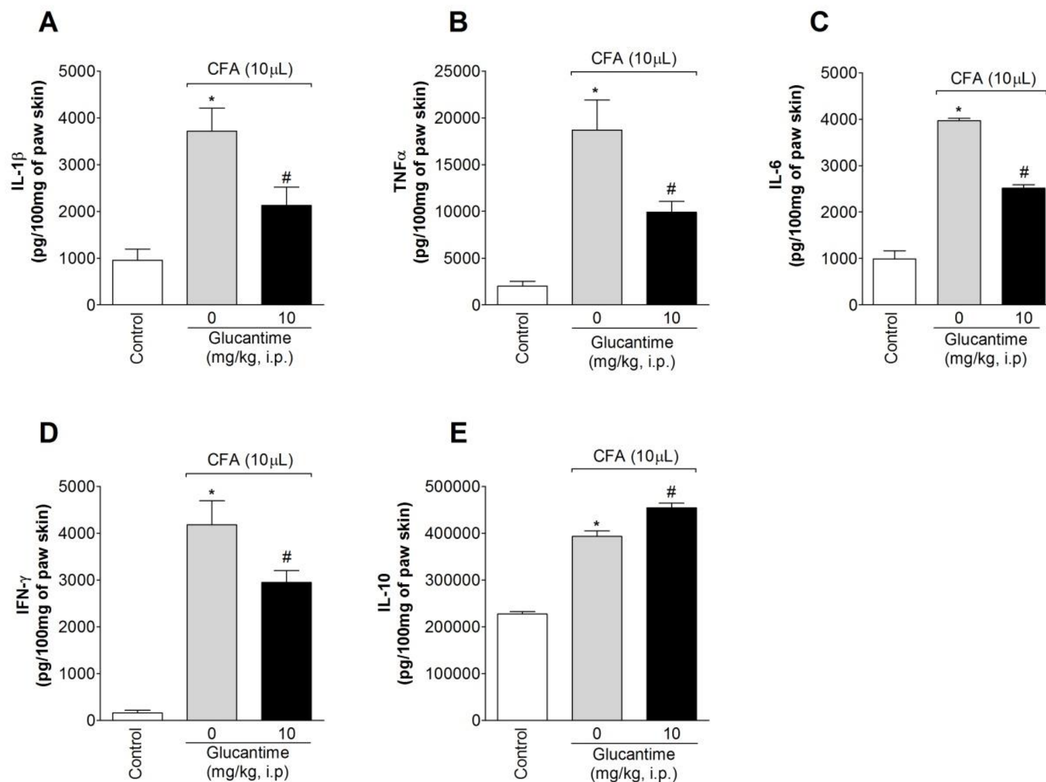

Figure 5- Post-treatment with Glucantime inhibits CFA-induced IL-1β, TNF-α, IL-6, IFN-γ and increase IL-10 production. Mice were treated daily with Glucantime (10mg/kg, i.p) 3h after Complete Freund's Adjuvant CFA (10μL/paw, i.pl.) stimulus. Three hours after the last treatment with Glucantime, plantar paw skin samples were collected for the determination of IL-1β, TNF-α, IL-6, IFN-γ and IL-10 production (Panels A, B, C, D and E, respectively) production. Results are presented as means ± SEM of 6 mice per group per experiment, and are representative of 2 separated experiments. *P < 0.05 compared to saline group, and #P < 0.05 compared to CFA + vehicle group. One-way ANOVA followed by Tukey's t test.

4 CONCLUSÃO GERAL

- A amostra de extrato de própolis, coletada na região de São Paulo, utilizada neste estudo apresentou atividade leishmanicida reduzindo à proliferação de formas promastigotas e redução na carga parasitária de baço em camundongos susceptíveis a infecção com *L. amazonensis.* Além destes achados o extrato de própolis foi eficaz em reduzir o processo inflamatório desencadeado no fígado de camundongos susceptíveis a infecção. Estes estudos evidenciaram o potencial patogênico de *L. amazonensis*, uma vez que os órgãos analisados eram secundários ao de origem da infecção e evidenciam a ação leishmanicida e anti-inflamatória da amostra de própolis testada.

- Concanavalina-A promove atividade leishmanicida em macrófagos de camundongos susceptíveis a infecção por *L. amazonensis* em modelo de pré-tratamento por 2 e 72 horas e infecção *in vitro.* Verificamos também a atividade imunomoduladora desta lectina em modelos de leishmaniose experimental com aumento na produção de IL-1β e produção de ROS.

- O composto antimoniato N-metil-glucamina mostrou-se eficaz na redução de hiperalgesia mecânica induzida por infecção com *L. amazonensis* e estímulo com CFA, reduzindo as atividades de MPO e NAG e, portanto a migração celular. Também verificamos redução na produção das citocinas IL-1β e IL-6 no local da infecção. Estes dados demonstram que o efeito anti-inflamatório e na analgesia são independentes da ação leishmanicida, uma vez que os dados foram observados nos dois modelos de estímulo de dor inflamatória.

APÊNDICE

APÊNDICE A- Aprovação no comitê de ética em experimentação animal para a análise da atividade da própolis na leishmaniose experimental

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COMITÊ DE ÉTICA EM EXPERIMENTAÇÃO ANIMAL

OF. CIRC. CEEA Nº 24/2011 Londrina, 05 de abril de 2011

Prezada Pesquisadora

O CEEA/UEL, reunido em 15 de março do ano corrente, avaliou o projeto de pesquisa intitulado **"Análise da atividade da própolis na leishmaniose"**, registrado no CEEA sob o nº 09/11, 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 310 camundongos BALB/c, machos, pesando entre 28 a 32 gramas, com procedência do Biotério Central de Maringá. Os animais serão divididos em 31 grupos com 05 animais cada. Os procedimentos experimentais serão: 1. Ensaio fagocítico - serão obtidas células do exsudato peritonial de camundongos BALB/c por inoculação de 2 ml de RPMI e mantidas em presença ou ausência de própolis nas concentrações de 5, 10, 25, 50 e 100 µg/well. 2. Atividade da própolis em células fagocíticas peritoniais - será estudada com subgrupos de 5 camundongos BALB/c que receberão 50 µg, 100 µg e 250 µg do extrato de própolis intraperitonial em 250 µg de PBS. Após 24 horas serão infectados i.p. com 10^7 formas promastigostas de Leishmania SP. (+ 01 grupo controle). 3. Atividade da própolis na infecção "in vivo" - subgrupos de 5 camundongos BALB/c receberão 10 µg, 25 µg e 50 µg do extrato de própolis em 50 µg de PBS, ou somente PBS no coxim plantar direito. Após 24 horas serão infectados com 2 X 10^6 formas promastigostas de Leishmania na fase estacionária em 50 µg de PBS livre de patógenos nas mesmas condições em que ocorreu o inoculo de parasitos. A evolução da doença nos animais infectados e no grupo controle será avaliada semanalmente, por um período de 10 semanas, pela medida do tamanho da lesão da pata infectada em comparação com medida da pata contralateral não infectada, utilizando-se um especímetro manual. Ao final da 10ª semana após a infecção, os animais serão sacrificados e removidos o coxim plantar e linfonodo regional poplíteo para análises. O estudo está previsto para ser desenvolvido entre abril de 2011 e março de 2013. Este comitê aprova a realização de duas repetições por tratamento e caso tenha necessidade de uma terceira repetição, o coordenador do projeto deverá solicitar ao autorização do CEEA-UEL.

Ilma. Sra.
Profa. Dra. Ivete Conchon Costa
Coordenadora do Projeto
Departamento de Ciências Patológicas
Centro de Ciências Biológicas

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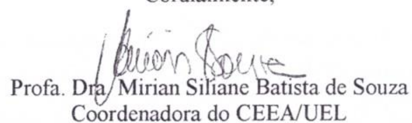
Cumpre orientar que caso se pretendam quaisquer alterações no protocolo experimental aprovado, deve-se submeter o novo protocolo à apreciação do CEEA/UEL anteriormente à execução das modificações.

Sem mais para o momento, subscrevo-me.

Cordialmente,



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

APÊNDICE B- Aprovação no comitê de ética em experimentação animal para a análise da atividade da Concanavalina-A na leishmaniose experimental

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COMISSÃO DE ÉTICA NO USO DE ANIMAIS

OF. CIRC. CEUA N° 056/2013 — Londrina, 19 de Março de 2013.

Prezada Pesquisadora,

A CEUA/UEL reunida em 19 de Fevereiro de 2013 avaliou o projeto de pesquisa intitulado **"Análise da atividade biológica da concanavalina-A na Leishmaniose experimental"**, processo CEUA nº **1677.2013.33**, 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 Conselho Nacional de Controle de Experimentação Animal estão respeitados.

Serão utilizados 198 camundongos Balb/c machos, com idade de 6 -8 semanas e peso aproximado a 25 gramas, procedentes do Biotério da Fiocruz de Curitiba-Pr. O projeto tem como objetivo avaliar a atividade biológica *in vitro* e *in vivo* da concavalina A na leishmaniose experimental. Os camundongos serão tratados previamente por via intraperitoneal ou não com concavalina A, e após 72 horas do tratamento os macrófagos da cavidade peritoneal serão coletadas e distribuídas em placas, até a aderência dos macrófagos. Será realizado a inoculação de formas promastigotas de *Leishmania amazonenses* na placa e após a infecção, o sobrenadante será coletado para dosagem de citocinas, quantificação de nitrito (NO), analise do índice fagocítico e do número de formas amastigotas por macrófagos, avaliação dos mecanismos de ação leishmanicidade da concavalina A, avaliação da migração celular, analise histológica e analise imunohistoquimica, dosagem de óxido nítrico, determinação da atividade da mieloperoxidase e Hepatotoxidade. O projeto está aprovado para execução em 36 meses após aprovação da CEUA.

Cumpre orientar que caso pretendam-se quaisquer alterações no protocolo de aula prática aprovado, deve-se submeter o novo protocolo à apreciação da CEUA/UEL anteriormente à execução das modificações. Sem mais para o momento, subscrevo-me.
Cordialmente,

Waldiceu Ap.° Verri Junior
Prof. Dr. Waldiceu Aparecido Verri Junior
Coordenador da CEUA/UEL

Ilma. Sra.
Profa. Dra. Ivete Conchon Costa
Coordenadora do Projeto
Departamento de Ciências Patológicas
Centro de Ciências Biológicas
Com copia para Sra Égle Maria de Sousa (Chefe da DCA/PROPPG) e Diretora do Centro de Ciências Biológicas.

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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
LONDRINA - PARANÁ - BRASIL

APÊNDICE C- Aprovação no comitê de ética em experimentação animal para a análise da atividade do Glucantime no modelo de dor inflamatória por infecção com *L. amazonensis* e Adjuvante Completo de freund's

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COMISSÃO DE ÉTICA NO USO DE ANIMAIS

OF. CIRC. CEUA Nº 055/2015 **Londrina, 13 de Abril de 2015.**

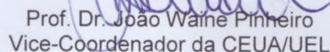
Prezada Pesquisadora,

A CEUA/UEL reunida em 31 de Março de 2015 avaliou o projeto de pesquisa intitulado **"Análise da atividade da própolis na Leishmaniose"**, registrado sob o processo CEUA **n°3209.2015.24**, como complementação do projeto **"Análise da atividade da própolis na Leishmaniose"** processo **n°09/11** com ofício circular **n°24/2011**, pesquisa do Centro de Ciências Biológicas, desenvolvido sob sua responsabilidade, julgando-o **aprovado** para execução entendendo-se que os princípios éticos postulados pelo Conselho Nacional de Controle de Experimentação Animal estão respeitados.

Serão utilizados 72 camundongos BALB/c machos com 6 a 8 semanas de idade, peso aproximado entre 20 e 25 g e provenientes do Biotério da Fiocruz de Curitiba - PR. O projeto tem como objetivo avaliar os efeitos do medicamento Glucantime® em modelos de dor inflamatória. Para tanto, os animais serão divididos em dois grupos experimentais para indução da resposta inflamatória com *L. amazonensis* ou Adjuvante Completo de Freund's (CFA). No primeiro experimento, será administrada *L. amazonensis* (10^7) via subcutânea na pata direita traseira, 15 dias antes do início do tratamento com o Glucantime® para avaliação de edema e hiperalgesia mecânica na pata infectada e, após o tratamento, os animais receberão anestesiam seguido de deslocamento cervical para retirada da pata. O tecido plantar será utilizado para análises de MPO, NAG, NO e citocinas do perfil de citocinas Th1, Th2 e Th17. No segundo experimento, será administrado CFA via intraplantar (i.pl., 10µl) na pata direita traseira, 7 horas antes do início do tratamento com Glucantime® e o tecido será outra vez utilizado para as análises anteriormente citadas. Além disso, será avaliado o mecanismo de ação deste medicamento, o qual ainda não foi demonstrado na literatura. Os protocolos experimentais estão aprovados com previsão para execução em 2 meses.

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

Coloco-me à disposição para quaisquer esclarecimentos que se fizerem necessária. Sem mais para o momento, subscrevo, cordialmente,



Prof. Dr. João Waine Pinheiro
Vice-Coordenador da CEUA/UEL

Ilma. Sra.
Profa. Dra. Ivete Conchon Costa
Coordenadora do Projeto
Departamento de Ciências Patológicas
Centro de Ciências Biológicas
Com cópia para Sra. Edilamar dos Anjos (Chefe da DCA/PROPPG), Chefe do Departamento de Ciências Patológicas e Diretor(a) do Centro de Ciências Biológicas.

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