DANIEL SILVA SENA BASTOS

AVALIAÇÃO DE DANOS TECIDUAIS APÓS VACINAÇÃO COM PROTEÍNA RECOMBINANTE LIPOFOSFOGLICANO-3 E REVISÃO SISTEMÁTICA DA ASSOCIAÇÃO DE DROGAS VISANDO O CONTROLE DA LEISHMANIOSE VISCERAL

Tese apresentada à Universidade Federal de Viçosa, como parte das exigências do Programa de Pós-Graduação em Biologia Celular e Estrutural para obtenção do título de *Doctor Scientiae*.

Orientador: Eduardo de Almeida Marques da Silva

Coorientadora: Eliziária Cardoso Santos

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RESUMO

BASTOS, Daniel Silva Sena, D.Sc., Universidade Federal de Viçosa, fevereiro de 2020. **Avaliação de danos teciduais após vacinação com proteína recombinante lipofosfoglicano-3 e revisão sistemática da associação de drogas visando o controle da leishmaniose visceral.** Orientador: Eduardo de Almeida Marques da Silva. Coorientadora: Eliziária Cardoso Santos.

Contextualização: Leishmaniose visceral (LV) é considerada uma doença tropical negligenciada causada por *Leishmania infantum* no norte da África, Europa e América Latina e no subcontinente da Índia e leste da África ocorre prevalência de *Leishmania donovani*. A doença é amplamente distribuída ao redor do mundo e se tornou um problema de saúde pública em 88 países. O tratamento atual para LV é limitado a quimioterapia utilizando antimônios pentavalentes seguido de pentamidina ou anfotericina B como segunda escolha. Entretanto estas drogas possuem efeitos adversos marcantes e apresentam baixa eficácia em alguns casos. Além disso, não existem vacinas disponíveis para programas de imunização em massa para o controle efetivo da doença. **Objetivos:** i) Avaliar efeito da infecção por *Leishmania infantum chagasi* no fígado de camundongos vacinados com a proteína recombinante lipofosfoglicano-3, considerando os parâmetros de dano tecidual e resposta inflamatório produzido pela vacina; ii) Verificar a efetividade da combinação de drogas para o tratamento da LV através de uma revisão sistemática. **Métodos:** i) Utilizamos a proteína recombinante LPG3 (rLPG3) como imunógeno em camundongos BALB/c antes do desafio com formas promastigotas de *L. infantum chagasi*. Os animais foram separados em 5 grupos: NI: animais não infectados; NV: não vacinados; SAP: imunizado com saponina; rLPG3: imunizado com rLPG3; rLPG3 + SAP: imunizado com rLPG3 mais SAP. A experimento foi conduzida em réplica e o protocolo de vacinação consistiu em três doses subcutâneas de rLPG3 (40 µg + dois reforços de 20 µg). Os camundongos foram desafiados três semanas após a última imunização; ii) As diretrizes do PRISMA (Principais Itens para Relatar Revisões sistemáticas e Meta-análises) foram adotadas para a realização da revisão sistemática. A plataforma SYRCLE's Risk of Bias foi utilizada para análise da qualidade metodológica dos estudos. **Resultados:** i) Nossos resultados demostraram que a imunização com rLPG3 + SAP reduziu o parasitismo em 99%, conferindo proteção imunológica ao fígado dos animais infectados. A imunização aumentou as defesas oxidantes, aumentando a atividade de CAT e GST enquanto reduziu os marcadores de dano oxidativo como H_2O_2 e NO_3/NO_2 e proteína carbonilada. Como consequência, a imunização com rLPG3 + SAP preservou integridade tecidual e reduziu a formação de granuloma, infiltrado inflamatório, e as

concentrações séricas de AST, ALT, ALP; ii) A combinação de drogas é efetiva, reduzindo o parasitismo, a dosagem e o tempo de tratamento fazendo com que o tratamento apresente menos efeitos adversos. **Conclusão:** Nossos resultados mostram que a vacina com rLPG3 confere proteção em camundongos desafiados com *L. infantum chagasi*, enquanto previne os efeitos prejudiciais causados pela vacina além da infecção. Nós acreditamos que a combinação de drogas para o tratamento da LV é eficiente, entretanto a heterogeneidade dos estudos torna difícil a obtenção de uma evidência clara.

Palavras-chave: LPG3. Leishmaniose Visceral. Morfologia do Fígado. Estresse Oxidativo. Vacina.

ABSTRACT

BASTOS, Daniel Silva Sena, D.Sc., Universidade Federal de Viçosa, February, 2020. **Evaluation of tissue damage after vaccination with recombinant lipophosphoglican-3 protein and systematic review of the drug association aiming the control of visceral leishmaniasis**. Adviser: Eduardo de Almeida Marques da Silva. Co-adviser: Eliziária Cardoso Santos.

Background: Visceral leishmaniasis (VL) is considered a neglected tropical disease caused by *Leishmania infantum* in North Africa, Europe and Latin America, and *Leishmania donovani* prevails in East Africa and the Indian subcontinent. This disease is widely distributed around the world and is a public health problem in 98 countries. The current treatment options for VL are limited by chemotherapy using pentavalent antimonies, followed by pentamidine or amphotericin B as second-choice. However, these drugs have a significant side effects and showed low efficacy in some cases. Besides, there is no suitable vaccine for mass immunization programs for the effective control of VL. **Objective:** i) The aim was to evaluate the effects of *Leishmania infantum chagasi* infection on the liver of vaccinated mice, with lipophosphoglican-3, considering parameters of tissue damage and the inflammatory response elicited by vaccination; ii) To verify the effectiveness of the combination of drugs for the treatment of VL through a systematic review. **Methods:** i) We used recombinant LPG3 protein (rLPG3) as immunogen in BALB/c mice before challenge with promastigote forms of *L. infantum chagasi*. The animals were separated into 5 groups: NI: non-infected animals; NV: non-vaccinated; SAP: immunized with saponin; rLPG3: immunized with rLPG3; rLPG3 + SAP: immunized with rLPG3 plus SAP. The experiment was conducted in replicate and the vaccination protocol consisted of three subcutaneous doses of rLPG3 (40 µg + two boosters of 20 µg). The mice were challenged three weeks after the last immunization;

ii) The PRISMA (Preferred Reporting Items for Systematic Reviews and Meta-analyses) statement was adopted for conducting the systematic review. The SYRCLE's Risk of Bias tool was used to analyze the methodological quality of the studies. **Results:** i) Our results showed that rLPG3 + SAP immunization decreased the parasite burden in 99%, conferring immunological protection in the liver of the infected animals. Moreover, the immunization improved the antioxidant defenses, increasing CAT and GST activity, while reduced the levels of oxidative stress markers, such as H2O2 and NO3/NO2, and carbonyl protein in the organ. As a consequence, rLPG3 + SAP immunization preserved tissue integrity and reduced the granuloma formation, inflammatory infiltrate and AST, ALT, ALP serum levels: ii) The combination of drugs is effective to reduce parasitism, dosage and treatment time, making the

treatment less toxic. **Conclusions:** i) Our results showed that rLPG3 vaccine confers liver protection in mice challenged with *L. infantum chagasi*, while maintaining the liver tissue protected against the harmful inflammatory effects caused by the vaccine followed by the infection; ii) Based on the results found in review, we believed that the combination of drugs for the treatment of VL is efficient, although heterogeneity between studies makes it difficult to obtain clear evidence.

Keywords: LPG3. Visceral Leishmaniasis. Liver Morphology. Oxidative Stress. Vaccine.

APRESENTAÇÃO

A presente tese foi elaborada de acordo com as normas estabelecidas pela Pró Reitoria de Pesquisa e Pós-Graduação da Universidade Federal de Viçosa – UFV. O corpo do trabalho compreende em uma introdução geral, e dois artigos. O primeiro artigo intitulado "Lipophosphoglycan-3 Recombinant Protein Vaccine Controls Hepatic Parasitism and Prevents Tissue Damage in Mice Infected by Leishmania infantum chagasi" foi pubicado na revista Biomedicine & Pharmacotherapy (DOI: 10.1016/j.biopha.2020.110097). O segundo artigo consiste em uma revisão sistemática intitulada Effectiveness of Drug Association in the Treatment Of Visceral Leishmaniasis in Animal Model - A Systematic Review que foi formatado de acordo com as normas da revista Parasitology, para a qual o artigo será submetido

SUMÁRIO

INTRODUÇÃO GERAL

As leishmanioses compreendem um grupo de doenças tropicais negligenciadas cujos causadores são parasitos intracelulares obrigatórios pertencentes ao reino Protista, classe Kinetoplastida, ordem Trypanosomatida, família Trypanosomatidae e gênero *Leishmania*. Os parasitos deste gênero possuem ciclo de vida digenético, ou seja, completam o ciclo de vida passando por diferentes tipos de hospedeiro, sofrendo alterações na motilidade e morfologia (Akhoundi et al., 2016). A forma promastigota, flagelada extracelular, é o estádio evolutivo infectante do sistema digestório do hospedeiro invertebrado (Dostálová & Volf, 2012). A forma amastigota, é infectante das células do sistema fagocitário dos hospedeiros vertebrados, possui morfologia ovoide e é imóvel, com flagelo interno (Sacks, 2001).

A transmissão da doença para o hospedeiro vertebrado ocorre pela picada de insetos flebotomíneos fêmeas infectadas (Ordem: Diptera; Família: Psychodidae; Subfamília: Phlebotominae), durante o repasto sanguíneo. No velho mundo, a transmissão da doença é feita por insetos do gênero *Phlebotomus*. O Novo Mundo tem como principal vetor insetos do gênero *Lutzomyia* (Dostálová & Volf, 2012). As fêmeas são infectadas durante o repasto sanguíneo feito em um hospedeiro vertebrado infectado, no qual formas amastigotas do parasito são ingeridas juntamente com o sangue. No intestino médio do vetor, as formas amastigotas sofrem alterações morfológicas, desenvolvem um pequeno flagelo e se transformam em formas promastigotas procíclicas (Sacks, 2001). Nesta fase ocorre o ciclo proliferativo do parasito: após 48 a 72 horas inicia-se um processo de redução de proliferação e alterações bioquímicas e morfológicas, dando origem à forma infectante do parasito conhecida como promastigota metacíclica. Esta forma apresenta flagelo bem desenvolvido e capacidade de reprodução limitada (Nieto et al., 2011; Freitas et al., 2012). Neste estádio o parasito migra para a válvula estomodeal do vetor e bloqueia a ingestão de sangue durante o repasto sanguíneo. Por isso, o parasito é regurgitado durante a picada (Sacks, 2001).

As formas promastigotas metacíclicas que infectam o hospedeiro vertebrado, ao serem introduzidas na epiderme, são fagocitadas por células do Sistema Fagocitário Mononuclear e neutrófilos. No interior destas células os parasitos, induzidos pelo baixo pH do vacúolo parasitóforo e pela elevada temperatura do hospedeiro vertebrado, se transformam na forma amastigota e se multiplicam intensamente, levando ao rompimento da célula infectada (Sacks, 2002; Dostálová & Volf, 2012). As formas amastigotas livres são capazes de infectar novas células, sobretudo em tecidos com grande número de células fagocíticas como no baço, órgãos linfoides, medula óssea e fígado. Os órgãos afetados pelo ciclo de infecção e lise

sofrem alterações morfológicas, enzimáticas e funcionais, resultando nas manifestações clínicas das leishmanioses, que variam de pequenas lesões cutâneas a lesões que acometem órgãos internos (Heidarpour et al., 2012; Souza et al., 2014 Moreira et al., 2017). A evolução da doença e as diferentes formas clínicas dependem da espécie do agente infecioso, da susceptibilidade genética do hospedeiro, idade, e estado nutricional e imunológico. As principais formas clínicas da doença são: leishmaniose cutânea (LC), caracterizada principalmente por lesões que se iniciam como pápulas e podem se tornar ulcerativas (WHO, 2016); leishmaniose mucocutânea (LMC), que ocorre após metástase do parasito para a mucosa oral e do trato respiratório (McGwire & Satoskar, 2014); e leishmaniose visceral (LV), considerada a forma mais grave da doença, visto que pode se tornar fatal quando não tratada (Freitas et al., 2016).

A LV é causada principalmente por parasitos das espécies *Leishmania infantum chagasi* no Novo Mundo e no Velho Mundo, e por *Leishmania donovani* no Velho Mundo. A doença é relatada em 98 países. Entretanto, aproximadamente 90% dos casos desta enfermidade são encontrados no Brasil, Etiópia, Sudão, Sudão do Sul e Índia (Alvar et al., 2012), afetando 300.000 pessoas em todo o mundo e chegando a 20.000 óbitos por ano. Estima-se que mais de 500 milhões de pessoas se encontram em áreas de risco de contrair a doença (WHO, 2016). A patologia da LV se caracteriza pela infecção dos órgãos internos como fígado, baço, medula óssea e linfonodos. Os sintomas clínicos são: febre, náusea, perda de peso, dores abdominais, esplenomegalia e hepatomegalia. O diagnóstico é também auxiliado por achados laboratoriais como neutropenia, hipoalbuminemia, hipergamaglobulinemia e aumento das concentrações séricas das transaminases hepáticas (Romero and Boelaert 2010; Serafim et al., 2010; Freitas et al., 2016).

O fígado é um dos principais órgãos acometidos pela LV. A infecção neste órgão causa diversas mudanças morfológicas teciduais afetando tanto o parênquima, células de Kupffer e hepatócitos, quanto o estroma, espaço porta, sinusoides e veias hepáticas (Giunchetti et al., 2008). Essas alterações levam também a distúrbios patológicos, como intenso infiltrado inflamatório, com presença de linfócitos e plasmócitos, além de hipertrofia e hiperplasia das células de Kupffer (Murray, 2001; Moreira 2010). A lesão do tecido hepático ocorre devido à presença do parasito e sua multiplicação e tem como outro fator agravante a resposta imune do hospedeiro. A resolução da infecção no fígado está associada ao padrão Th1 de resposta imune, que se inicia com a produção de IL-12 pelas células dendríticas e macrófagos ativados, fazendo com que as células Th0 se diferenciem em T CD4+ efetoras

com padrão Th1, que produzem citocinas pró-inflamatórias, interferon gama (INF-γ), fator de necrose tumoral (TNF) e interleucina 2 (IL-2), que são capazes de ativar os mecanismos microbicidas dos macrófagos, levando à explosão respiratória e produção de oxido nítrico (Faleiro et al., 2014; Freitas et al., 2016).

O aumento das espécies reativas de oxigênio (EROs) no fagolisossomo, durante a explosão respiratória, ocorre através das múltiplas subunidades de oxidases fagocíticas dependentes de NADPH que liberam grandes quantidades de elétrons dentro do fagolisossomo (Carneiro 2016). Esse influxo de elétrons reduz o oxigênio a ânion superóxido (O_2^-) e também podem gerar hidroxilas (OH^-) e peroxido de hidrogênio (H_2O_2). Como resposta a infecção, após a produção de EROS, ocorre a indução de oxido nítrico (NO) através da oxido nítrico sintase (iNOS ou NOS2) (Miao et al., 2009, Carneiro et al., 2016). Espécies reativas de nitrogênio só são produzidas dentro dos fagolisossomo após a ativação do macrófago via IFN-γ (Gantt et al 2001). O NO é derivado da oxidação do nitrogênio terminal da guanidina da L-arginina. No entanto, estes processos são lesivos às células e tecidos adjacentes (Heidarpour et al., 2012; Srivastav, 2014; Moreira et al., 2017), uma vez que as EROs podem escapar do vacúolo parasitóforo e agir sobre macromoléculas das células vizinhas. Além disso, o processo de fagocitose do parasito pode liberar hidrolases, metaloproteinases e glicosidases, que são capazes de lesar componentes da matriz extracelular (Filho, 2016).

A formação de granulomas é uma resposta tecidual à persistência da infeção nos órgãos e representa expressão do sucesso da resposta imune Th1 sobre o parasitismo tecidual. Em condições ótimas o granuloma se forma apresentando uma estrutura anatômica circunscrita, estruturalmente funcional, para limitar fisicamente a infecção, matar e remover o parasito, podendo, a partir daí, reparar qualquer lesão tecidual associada (Murray, 2001). O granuloma se inicia com a fusão de células de Kupffer infectadas e não infectadas, formando o granuloma inicial, seguindo para um granuloma complexo e maduro com a presença de linfócitos T e B e formando um microambiente que favorece a eliminação do parasito (Salguero et al., 2018). Entretanto, além de não garantir a total eliminação do parasito, a resolução do granuloma pode formar uma área de tecido conjuntivo não especializado, alterando a constituição do parênquima tecidual (Sanchez et al., 2004). Além disso, a ação do parasito associada às defesas antimicrobianas pode levar à injúria do tecido, a alterações morfofuncionais e ao desequilíbrio no balanço de microminerais (Souza., 2014; Moreira et al., 2017), tornando o órgão frágil a intervenções quimioterápicas. Frente a estes fatores, é

importante para o indivíduo infectado que se consiga limitar a infecção de modo a não sobrecarregar os tecidos afetados, principalmente nos casos em que o paciente tenha alguma outra patologia que envolva os órgãos acometidos pela infecção.

O tratamento para LV tem se limitado a quimioterapias utilizando antimoniais pentavalentes como droga de primeira escolha. A amphotericina B e pentamidina são as segundas opções (Martins et al., 2015, Ponte-Sucre et al., 2017; Moreira et al., 2017). Entretanto, essas drogas são muito tóxicas e necessitam de longos períodos de tratamento, com administração geralmente por intermédio de injeções intramusculares. A maioria dos medicamentos apresentam efeitos colaterais marcantes, como cardiotoxicidade, hepatotoxicidade, pancreatite, insuficiência renal, anemia, leucopenia, trombocitopenia, dor abdominal, náusea, vômitos e hipergamaglobulinemia. Além disso, a eficácia destes fármacos tem se reduzido significantemente, com o surgimento de cepas resistentes ao tratamento (Akbari & Oryan, 2017; Sundar & Chakravarty, 2010).

O uso de métodos profiláticos foi um avanço no controle de doenças infecciosas, já que anteriormente à implantação de programas vacinais a morbidade e a mortalidade por doenças passíveis de prevenção eram muito altas em comparação com o período pós-vacinal. Porém, a perda de confiança relacionada com o aparecimento de efeitos adversos pode levar ao ressurgimento de doenças anteriormente controladas pelos programas de imunização (WHO 2017). Ainda hoje, desde o início dos estudos em vacinologia, os exemplos de vacinas que apresentam maior eficácia, consideradas “padrões ouro”, correspondem a vacinas de organismos vivos atenuados, como é o caso da OPV para a poliomielite ou das vacinas contra a febre amarela, contra o sarampo ou contra rotavírus. Porém, o risco de reativação de virulência e mesmo de outros efeitos colaterais em indivíduos que recebem essas vacinas existe (Sharman et al., 2002; Vasconcelos et al., 2001; Novadzki & Rosario Filho, 2010). A partir dessa constatação, a preocupação com a segurança é um ponto de destaque no desenvolvimento das vacinas modernas, que se baseiam principalmente em uso de organismos inativados ou de suas partes imunogênicas, como proteínas ou polissacarídeos. Estas, por sua vez, apresentam geralmente menor imunogenicidade se comparadas com as vacinas padrão ouro. Por isso, é de extrema importância a busca por formulações vacinais que aumentem a capacidade imunogênica das vacinas, utilizando principalmente adjuvantes que direcionem ou fortaleçam a resposta imune induzida pelo imunógeno utilizado.

Diante dos desafios relacionados ao tratamento da LV, a busca por novos métodos de controle e prevenção da doença tem aumentado. Ainda não existe nenhuma vacina disponível

no mercado que possa ser usada em programas de imunização em massa, visando reduzir o número de casos. Moléculas relacionadas aos mecanismo de virulência da *Leishmania* têm sido alvo de estudo por diversos grupos de pesquisa, na tentativa de desvendar o papel destas moléculas e de avaliar sua efetividade como antígeno vacinal (Gonzalez-Aseguinolaza et al., 1999, Zhang & Matlashewski, 2001, Descoteaux et al., 2002, Marques-Da-Silva et al., 2008).

Na tentativa de encontrar novas proteínas com potencial vacinal, nosso grupo de pesquisa investigou a presença de proteína ligante de heparina em formas promastigotas de *L. infantum chagasi* utilizando técnica de purificação de proteínas por cromatografia de afinidade em coluna de heparina (Martins et al., 2015). Essa pesquisa se justifica principalmente por essa proteína funcionar como lectina, que pode ser importante fator de virulência do parasito, participando de processo de adesão dos mesmos se ligando a açúcares presentes em células do sistema imune. Neste trabalho foram descritos a localização celular e o papel da proteína ligante de heparina de *L. infantum chagasi* (HBP*Lc*) no processo de infecção das células de camundongos. HBP*Lc* é encontrada em toda a superfície externa do parasito e internamente, principalmente próxima ao kinetoplasto. Quando esta proteína foi bloqueada com heparina, a capacidade de internalização do parasito pelo macrófago foi parcialmente reduzida (Martins et al., 2015; Marques-Da-Silva & Martins, 2016).

Martins e colaboradores (2018) predisseram a HBP*Lc* como uma proteína tetramérica com massa molecular estimada em 348 kDa. Suas estruturas monométricas são compostas por α-hélices interpassadas por fitas β, conforme predição realizada *in silico*. Nesse trabalho foi descrito também que a proteína tem função de ATPase e sítio de ligação para heparina. Ao realizar o sequenciamento da HBP*Lc*, o gene da proteína foi identificado como lipofosfoglicano 3 (LPG3), e as novas funções descritas foram associadas à proteína.

Diante do quadro acima descrito e devido à necessidade de melhoria nos métodos de controle para LV, o objetivo deste trabalho foi investigar parâmetros de segurança relacionados com a aplicação da vacina produzida com a proteína recombinante LPG3 associada ao adjuvante saponina em camundongos frente ao desafio com *L. infantum chagasi*, em paralelo com a avaliação da eficácia protetora conferida pela imunização. Para este propósito, foram avaliados o padrão de resposta imune induzido pela proteína, a quantificação da carga parasitária do tecido hepático, a histopatologia e morfometria do fígado, assim como a atividade das enzimas antioxidantes e os níveis dos biomarcadores de lesão hepática e de estresse oxidativo e nitrosativo no órgão.

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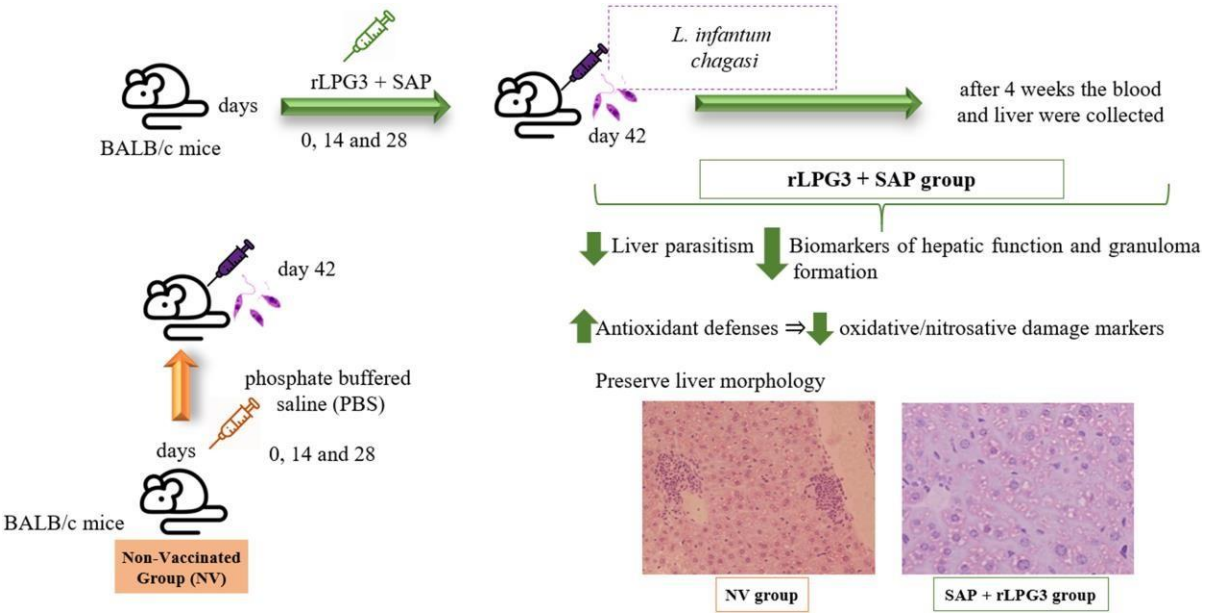
ARTIGO 1

LIPOPHOSPHOGLYCAN-3 RECOMBINANT PROTEIN VACCINE CONTROLS HEPATIC PARASITISM AND PREVENTS TISSUE DAMAGE IN MICE INFECTED BY *Leishmania infantum chagasi*

Graphical Abstract



Abstract

Aims: In this work, we aimed to evaluate the effects of the Leishmania infantum chagasi infection on the liver of vaccinated mice, considering parameters of tissue damage and the inflammatory response elicited by vaccination.

Main methods: We used recombinant LPG3 protein (rLPG3) as immunogen in BALB/c mice before challenge with promastigote forms of L. infantum chagasi. The animals were separated into five groups: NI: non-infected animals; NV: non-vaccinated; SAP: treated with saponin; rLPG3: immunized with rLPG3; rLPG3 + SAP: immunized with rLPG3 plus SAP. The experiment was conducted in replicate, and the vaccination protocol consisted of three subcutaneous doses of rLPG3 (40 μg+two boosters of 20 μg). The mice were challenged two weeks after the last immunization.

Key findings: Our results showed that rLPG3 + SAP immunization decreased the parasite burden in 99 %, conferring immunological protection in the liver of the infected animals. Moreover, the immunization improved the antioxidant defenses, increasing CAT and GST activity, while reducing the levels of oxidative stress markers, such as H2O2 and NO3/NO2, and carbonyl protein in the organ. As a consequence, rLPG3 + SAP immunization preserved tissue integrity and reduced the granuloma formation, inflammatory infiltrate and serum levels of AST, ALT, and ALP.

Significance: Taken together, these results showed that rLPG3 vaccine confers liver protection against L. infantum chagasi in mice, while maintaining the liver tissue protected against the harmful inflammatory effects caused by the vaccine followed by the infection.

Keywords: LPG3, Visceral leishmaniasis, Liver morphology, Oxidative stress, Vaccine

INTRODUCTION

Leishmaniasis is a neglected globally distributed tropical disease that remains as a public health problem worldwide [1]. Parasites of Leishmania genus are the causative agents of the disease, and, depending on the parasite species, clinical manifestations consistent with cutaneous or visceral lesions are evidenced [2–4]. The Visceral Leishmaniasis (VL) or kala-azar is the most severe clinical form of the disease, caused mainly by *Leishmania infantum chagasi* in the New and Old World and by *Leishmania donovani* in the Old World [5]. VL is characterized by a broad spectrum of clinical manifestations, such as chronic low-grade fever, weight loss and hepatosplenomegaly, associated with laboratory findings, which include pancytopenia, low albumin and hypergammaglobulinemia [2–4].

The liver, used as the focus of this work, is one of the primary target organs in VL. The pathogenesis and disease severity are largely related to the oxidative burst of macrophages, a typical response from a phagocytosis process [6–8]. The oxidative burst may induce overproduction of reactive oxygen metabolites and nitric oxide by macrophages [9,10], which, in turn, may react with host biomolecules and increase the parasitic infection [6,8,9]. Consequently, the disease can lead to hepatic dysfunction, characterized by high levels of serum liver specific enzymes and tissue damages [2,6,11].

The treatment for all kinds of leishmaniasis is limited by chemotherapy using pentavalent antimonies [8,12], followed by pentamidine or amphotericin B as second-choice drugs [12–14]. Although these chemotherapy treatments are recommended by the World Health Organization, the drugs are expensive, inefficient and induce marked side effects, which complicates the treatment [8,14]. Besides, there is no suitable vaccine for mass immunization programs for the effective control of VL [11]. These facts reinforce the need for searching for new prophylactic strategies, mainly by the use of vaccine antigens able to induce Th1 response, which can activate oxidative and NO burst in macrophage, limiting the infective potential of the parasite and preventing the disease [2,15,16].

Lipophosphoglycan-3 (LPG3) has been explored as a candidate to immunogen in vaccine tests against leishmaniasis (reviewed by [17]). Although its use as a DNA vaccine has not resulted in protection [18], the vaccine using recombinant LPG3 protein expressed by *Leishmania tarentolae* in combination with CpG oligodeoxynucleotides conferred partial protection to mice against *L. infantum chagasi* infection [19], indicating that further studies with other preparations are needed to improve the protective action of the vaccine.

Immunolabelling assay showed that this protein is extensively distributed internally and over the outer surface of the L. infantum chagasi plasma membrane. LPG3 from promastigote forms of L. infantum chagasi was characterized as a heparin-binding protein. When the parasite is treated with heparin, a partial reduction in its internalization by RAW macrophages is observed, in "in vitro" assays [13]. Therefore, LPG3 from *L. infantum chagasi* may be considered an important parasite virulence factor that affects mechanisms of adhesion, internalization, and survival of the parasite during the infectious process [13,20,21].

In this study, we used BALB/c mice that were protected against L. infantum chagasi infection by immunization with recombinant LPG3 (rLPG3) protein from *L. infantum chagasi* to evaluate the effects of the immunization over the hepatic tissue integrity, considering the profiles of toxicity, redox metabolism, and morphological remodeling of the tissue arising from the inflammatory process.

MATERIAL AND METHODS

2.1 Animals and ethics statement

Female BALB/c mice aged 5–8 weeks were obtained from the Central Animal Laboratory of the Center of Biosciences and Health of the Federal University of Viçosa (Brazil), and maintained under conditions of controlled temperature (25 ± 2 °C) and 12 h light/dark cycles. The animals received food and water ad libitum. The animal experimentation complied with the ethical principles of the Code of Professional Veterinarian, according to the opinion of the Ethics Committee for Animal Use (approved by CEUA/UFV – Research project – process number: 16/2016), based on the actual Brazilian Legislation (Law no. 11.794, October 08, 2008), the Normative Resolutions edited by CONCEA/MCTI, and the "Diretriz Brasileira de Prática para o Cuidado e a Utilizacão de Animais para Fins Científicos e Didáticos" (DBCA), following the guidelines for the practices of euthanasia commended by CONCEA/MCTI.

2.2 Parasite

Leishmania infantum chagasi promastigotes, M2682 strain (MHOM/BR/75/M2682), were cultured in Grace's insect medium (GIBCO BRL, Grand Island, NY, USA) supplemented with 10 % heat-inactivated fetal calf serum (FCS; LGC Biotecnologia, Cotia, SP, Brazil), 2

mmol× L^{-1} Lglutamine (GIBCO BRL), and 100 U × mL^{-1} penicillin G potassium (USB Corporation, Cleveland, OH, USA) at 26 °C (pH 6.5). Infectivity was maintained by passage through mice.

2.3 Experimental design

The vaccination protocol consisted of three doses (2 weeks apart between each). Two weeks after the last immunization (day 42), the mice were challenged with 1×10^7 promastigotes of L. infantum chagasi given intravenously through the lateral tail vein. The animals were euthanized two weeks after the challenge (day 70) (Fig. 1).

The animals were randomly separated into five groups (four animals per group): non-challenged group NI (PBS treated); and challenged groups NV (non-vaccinated, PBS-treated); SAP (treated with saponin - 50 µg/subcutaneous dose); rLPG3 (immunized intraperitoneally with rLPG3 - 40 µg in the first dose, and two boosters of 20 µg); and SAP + rLPG (immunized subcutaneously with rLPG3 plus saponin in the same.

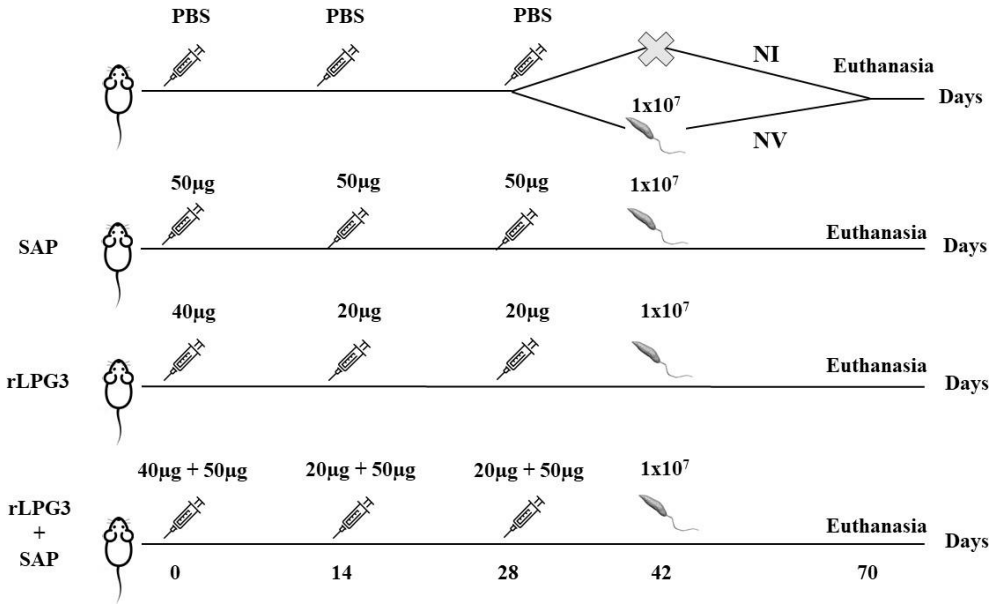


Fig. 1 - Vaccine protocol. rLPG3 was administered 40 µg in the first dose (day 1) and two boosters of 20 µg (days 14 and 28). Saponin was administered in three subcutaneous doses (50 µg/dose; days 1, 14 and 28). The other groups received only PBS. On day 42 the mice from groups NV, SAP, rLPG3 and rLPG3 + SAP were challenged with 1 × 10^7 promastigotes of *L. infantum chagasi*. Two weeks later the animals were euthanized (day 56).

2.4 Production of rLPG3

rLPG3 was obtained as described by Martins et al. (2018). Briefly, the LPG3 gene was synthesized in vitro. The insert was cloned in vector pUC19 and subcloned in pET 28a+, which was propagated in transformed Escherichia coli DH5α strain cells (GenOne, Rio de Janeiro, RJ, Brazil). The recombinant protein was expressed in E. coli Rosetta (DE3) strain cells transformed with pET 28 constructs. The cells were lysed by sonication, and the supernatant of soluble proteins was recovered and submitted to affinity chromatography on the Ni-NTA column, followed by affinity chromatography on heparin-agarose and a Superdex 200 10/ 300 G L column, the last two assembled in a liquid chromatography system (AKTA purifier; GE Healthcare Life Sciences, Freiburg, Germany). The purity of the eluate was assessed by Coomassie stained 12 % SDS-PAGE

2.5. Euthanasia and tissue collection

The animals were euthanized 28 days after the challenge by cervical dislocation. Blood samples from all mice were collected by cardiac puncture and centrifuged at 3000 × g for 10 min at room temperature. The serum was collected for the assessment of functional markers of hepatic damage and antibodies assay. After thoracotomy, the liver was removed and weighed. Liver fragments from each animal were randomly sectioned. One fragment was frozen in liquid nitrogen and stored in a freezer at −80 °C for enzymatic analysis. The second fragment was immersed in Karnovsky's fixative solution (2.5 % glutaraldehyde and 4% paraformaldehyde in 0.1 mmol × L^{-1} phosphate buffer, pH 7.2) for histopathological analysis. The third fragment was dehydrated in ethanol series for mineral microanalysis. One liver fragment was also immersed in Grace's insect medium for parasite load determination by quantitative limiting-dilution culture.

2.6 Parasite load

The liver parasite load was determined by quantitative limiting-dilution culture, as previously described by Marques-da-Silva et al. (50). Briefly, the liver fragment was homogenized in a tissue grinder, resuspended in 500 µL Grace's insect medium (GIBCO BRL) supplemented with 10 % heat-inactivated FCS (LGC Biotecnologia), 2 mmol × L^{-1} L glutamine (GIBCO BRL), and 100 U × mL^{-1} penicillin G potassium (USB Corporation), at 26 °C (pH 6.5), and plated onto 48-well flat bottom microtiter plates. Five-fold serial dilutions were performed, and, after two weeks of incubation at 25 °C, the plates were microscopically scored for parasite growth. The number of parasites was determined from the reciprocal of the

highest dilution at which promastigotes could be detected and expressed as parasites per milligram of tissue

2.7. Serological assay for antibodies

rLPG3-specific antibodies were detected in the serum by the technique described by Voller et al. [22], using enzyme-linked immunosorbent assay (ELISA). Polystyrene microplates (96 well) were sensitized overnight, at 4 °C, with 100 µL of LPG3 antigen (1.0 µg × mL^{-1}). Following washing steps with 0.05 % PBS and 20 % Tween 20 and blocking with PBS and 1% gelatin, 100 µL of the diluted serum (1:40) from all groups were added to the selected wells and the plate was incubated for 2 h, at room temperature. After 3 washes, 100 µL of peroxidase-conjugated goat anti-mouse IgG1 or IgG2a (dilution 1:1,000 and 1:2,000, respectively) (Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA) were added. The plate was incubated for 2 h, at room temperature, and washed again. O-phenylenediamine (OPD) solution in 0.15 mol × L−1 citrate-phosphate buffer (pH 5.0) and H_2O_2 were added, and the reaction was stopped using 1.5 mol × L−1 H2SO4. The optical density (OD) was determined using a 490 nm filter (SoftMax Pro 4.0, Life Sciences edition).

2.8. Biomarkers of hepatic function

The blood samples collected during euthanasia were centrifuged at 3000 × g for 10 min at room temperature, and the serum was immediately used for the assessment of aspartate aminotransferase (AST), alanine aminotransferase (ALT), and alkaline phosphatase (ALP), using biochemical kits (Bioclin Laboratories, Belo Horizonte, MG, Brazil), in accordance with the manufacturer's instructions.

2.9. Enzymatic activity assays

For the analysis of antioxidant enzymes, 100 mg of frozen liver was homogenized in ice-cold PBS and centrifuged at 10,000 × g for 10 min at 4 °C. The supernatant was used for the analysis of superoxide dismutase (SOD), catalase (CAT), and glutathione S-transferase (GST) activities. SOD activity was estimated by the pyrogallol method, based on the ability of this enzyme to catalyze the superoxide and hydrogen peroxide reactions [23]. The activity of this enzyme was calculated as units (U) per milligram of protein, where one U of SOD is defined as the amount that inhibited the rate of pyrogallol autoxidation by 50 %. CAT activity was evaluated by measuring the rate of H_2O_2 decomposition, according to Aebi [24]. The extinction coefficient of ε240 = 0.036 mmol × L^{-1} × cm^{-1} was used for calculations. One unit

of CAT activity was defined as the amount of the enzyme that decomposes one mmol H_2O_2 for 1 min. CAT activity was calculated as U per milligram of protein. GST activity was estimated spectrophotometrically as described by Habig et al. [25], and assayed according to the formation of glutathione-conjugated 2,4-dinitrochlorobenzene (CDNB). The molar extinction coefficient used for CDNB was ε340 = 9.6 mmol × L^{-1} × cm^{-1}. One unit of GST activity was defined as the amount of the enzyme that catalyzed the formation of one μmol of product × min^{-1} × mL^{-1}. GST activity was expressed as U per milligram of protein.

2.10. Markers of oxidative/nitrosative damage

Lipid peroxidation was assessed in the hepatic tissue by the analysis of the levels of MDA in the liver homogenate [26]. Protein oxidation was analyzed by the quantification of protein carbonyls in liver tissue pellets from the tissue homogenates, using the 2,4-dinitrophenylhydrazine (DNPH) method [27]. The results were expressed as nmol per mg of protein, based on the molar extinction coefficient of ε370 = 22 mmol × L^{-1} × cm^{-1}. The total protein level in the liver tissue was measured using the Bradford method [28]. The NO production was indirectly quantified by the nitrite content in the supernatants of the liver homogenate, using standard Griess reaction [29]. The H2O2 production was quantified in the supernatants of the liver homogenates [30]. Briefly, 50 μL of supernatants was incubated with 50 μL of OPD and an equal volume of peroxidase type II 15 mmol × L^{-1} for 60 min, at 37 °C. The reaction was stopped using 50 μL of 3 mol × L^{-1} H_2SO_4. The absorbance was measured at 490 nm, in a microplate scanning spectrophotometer (Multiskan GO). The conversion of absorbance into micromolar concentrations of H_2O_2 was calculated from a standard curve. The results were expressed as μmol × L^{-1}.

2.11. Mineral microanalysis

The liver mineral content was assessed by Energy Dispersive X-ray Spectroscopy (EDS), using a scanning electron microscope (SEM) (Leo 1430 V P, Carl Zeiss, Jena, Thuringia, Germany) with an attached x-ray detector system (Tracor TN5502, Middleton, WI, USA), according to Novaes et al. [31], as described below. Liver fragments were dehydrated in ethanol series, submitted to critical point drying (CPD 030, Bal-tec, Witten, North Rhine-Westphalia, Germany) and coated with evaporated carbon (Quorum Q150 T, East Grinstead, West Sussex, England, UK). The EDS microanalysis was performed at 150× magnification, 20 kV accelerating voltage and 19 mm working distance. The following chemical elements

were assessed: Se (Selenium), Zn (Zinc), Cu (Copper), Fe (Iron), Mn (Manganese), Ca (Calcium), K (Potassium), and Na (Sodium).

2.12. Histopathological and stereological analysis of the liver

After 24 h in Karnovsky's fixative solution, liver fragments were dehydrated in crescent ethanol series, and embedded in 2-hydroxyethyl methacrylate (Historesin®, Leica Microsystems, Nussloch, Germany). 3 μm-thick liver sections were obtained using a rotary microtome (RM 2255, Leica Biosystems, Nussloch, German) and stained with hematoxylin/eosin (HE) for histopathological and stereological analyses, and periodic acid–Schiff (PAS) for glycogen analysis. To avoid repetitive analyses of the same histological area, the sections were evaluated in semi-series, using a different one in each of the five sections. Digital images of hepatic tissue were obtained using a light microscope (Olympus BX-53, Tokyo, Japan) connected to a digital camera (Olympus DP73, Tokyo, Japan). Eighty photomicrographs from HE and PAS (20× objective) were randomly obtained for each group. For the stereological analysis, a test system of 266 points was used in a standard test area. In sections stained with HE, the points were recorded in liver components (Hepatocytes, sinusoidal capillaries, blood vessels, and macrophages), and inflammatory infiltrate [32]. In sections stained with PAS, the points were recorded in glycogen-containing cytoplasmic inclusions. The volumetric proportion of each component was calculated using the following formula: $V = PP/PT$, where PP is the number of points located on the interest structure, and PT is the total number of points in the histological area [33]. All stereological analyses were performed using the ImageJ software (National Institutes of Health). The number of granulomas was also evaluated in ten histological fields per animal, using the sections stained with HE, under light microscope at 200× magnification.

2.13. Statistical analysis

The statistical analysis was performed using the GraphPad Prism 7.0® statistical software (GraphPad Software, Inc., San Diego, CA, USA). The data were compared using one-way analysis of variance (ANOVA), followed by the Newman-Keuls test. Statistical significance was considered at $p < 0.05$.

3 Results

3.1. Vaccination with rLPG3 dramatically reduces liver parasitism and biochemical markers of hepatic damage, and alters IgG1/IgG2a ratio in L. infantum chagasi infected mice.

The parasitism was evaluated in the infected groups NV, SAP, rLPG3 and rLPG3 + SAP. The rLPG3 and rLPG3 + SAP groups presented a remarkable decrease of the parasitism, 93.82 % and 99.33 % respectively, compared to the NV group. SAP and NV presented a similar parasitism rate (Fig. 2A). The IgG1/IgG2a ratio in the infected groups was evaluated to verify the pattern of cellular immune response induced by the immunization. rLPG3, with or without SAP, reduced the IgG1/IgG2a ratio compared to the NV group (Fig. 2B). The infected groups NV, SAP, rLPG3 and rLPG3 + SAP presented a significant increase in the serum level of AST compared to the NI group. However, the rLPG3 + SAP group showed lower serum level of AST than the other infected groups (Fig. 3A). The NV group presented the highest serum level of ALT compared to the other experimental groups (Fig. 3B). Moreover, the levels of ALT and ALP showed a similar result, revealing an increase of these enzymes in the NV, SAP and rLPG3 groups in comparison with NI group. rLPG3 + SAP and NI presented similar levels of both hepatic damage markers (Fig. 3B and C).

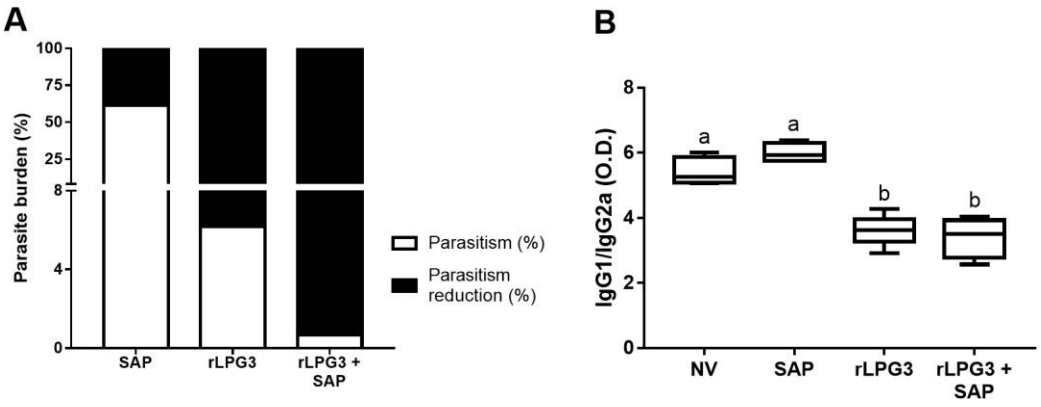


Figure 2. Liver parasitism and IgG1/IgG2a ratio of BALB/c mice submitted to rLPG3 vaccination and challenged with L. infantum chagasi. (A) Parasite burden changes. The changes in the hepatic parasite burden were obtained from the percentage of the average parasitism of each group in relation to the average of the NV control group parasitism. (B) IgG1/IgG2a ratio. NV: non-vaccinated; SAP: treated with saponin; rLPG3: immunized with rLPG3; rLPG3 + SAP: immunized with rLPG3 plus SAP. Different letters denote statistical difference among the groups ($p < 0.05$), determined by ANOVA, followed by the Student–Newman–Keuls test.

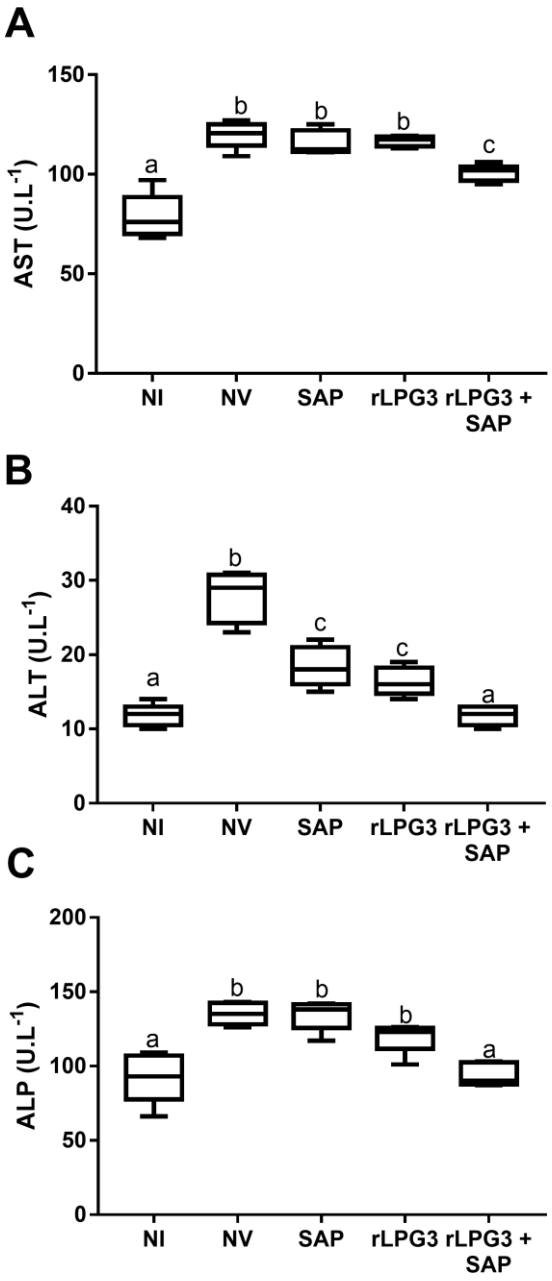


Figure 3. Serum levels of hepatic enzymes from BALB/c mice submitted to rLPG3 vaccination and challenged with L. infantum chagasi. (A) AST: aspartate aminotransferase; (B) ALT: alanine aminotransferase; (C) ALP: alkaline phosphatase. NI: non-infected animals; NV: non-vaccinated; SAP: treated with saponin; rLPG3: immunized with rLPG3; rLPG3 + SAP: immunized with rLPG3 plus SAP. The box refers to the interquartile interval with the median indicated as horizontal line. The whiskers refer to the superior and inferior quartiles. Different letters denote statistical difference among the groups ($p < 0.05$), determined by ANOVA followed by the Student–Newman–Keuls test.

3.2. Immunization with rLPG3 plus SAP increases antioxidant defenses in L. infantum chagasi infected hepatic tissue, which minimizes oxidative/ nitrosative damage markers

After the challenge with *L. infantum chagasi*, the enzymes of the hepatic endogenous antioxidant system, CAT and GST, showed decreased activity in all infected groups compared to the NI group. Nevertheless, in animals from the rLPG3 + SAP group, the activity of these enzymes was higher than that observed in the other infected groups (Fig. 4B and C). On the other hand, hepatic SOD enzymatic activity presented significant increase in infected animals compared to the NI group, but it was lower in rLPG3 + SAP group in comparison with the other infected groups (Fig. 4A). The levels of the product of lipid peroxidation malondialdehyde (MDA) measured in the hepatic tissue has remained higher in infected groups compared to the NI group (Fig. 5A). However, the immunization with rLPG3 + SAP adjuvant was effective to reduce the content of protein oxidation induced by the infection compared to the groups NI and NV, as observed in the reduced carbonyl protein (CP) levels (Fig. 5B). Moreover, the oxidative damage markers hydrogen peroxide and the nitrite/nitrate content were significantly higher in the NV, SAP, and rLPG3 groups compared to NI, but they were similar between the rLPG3 + SAP and NI groups (Fig. 5C and D).

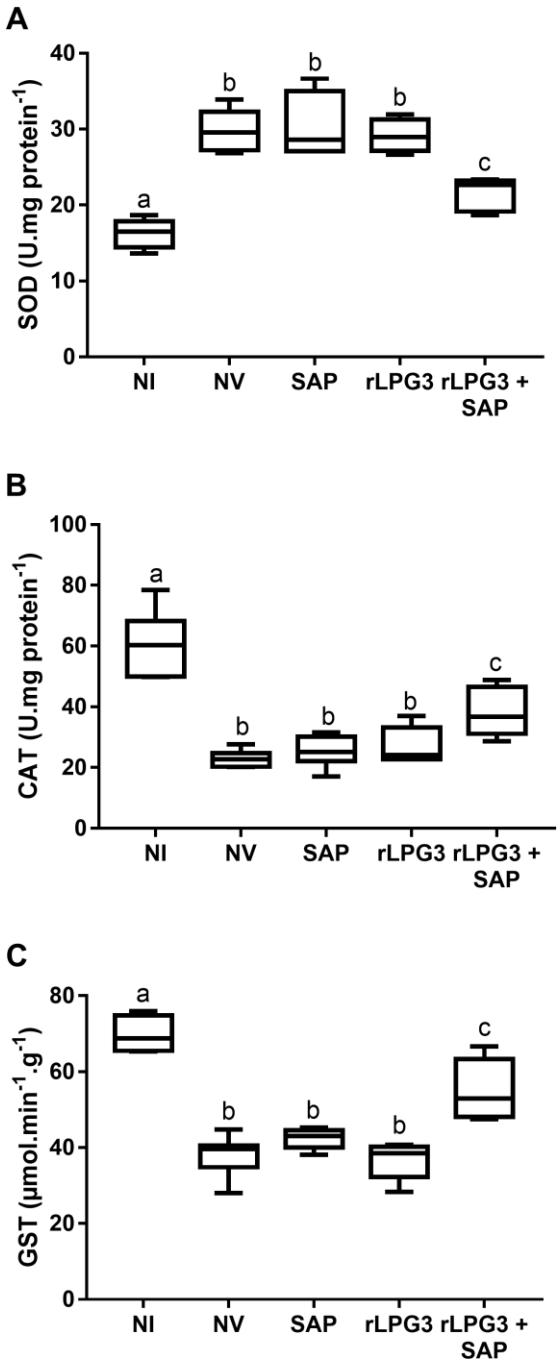


Figure 4. Antioxidant enzyme activities in the liver of BALB/c mice submitted to rLPG3 vaccination and challenged with L. infantum chagasi. (A) SOD: superoxide dismutase; (B) CAT: catalase; (C) GST: glutathione S-transferase. NI: noninfected animals; NV: non-vaccinated; SAP: treated with saponin; rLPG3: immunized with rLPG3; rLPG3 + SAP: immunized with rLPG3 plus SAP. The box refers to the interquartile interval with the median indicated as horizontal line. The whiskers refer to the superior and inferior quartiles. Different letters denote statistical difference among the groups ($p < 0.05$), determined by ANOVA, followed by the Student–Newman–Keuls test.

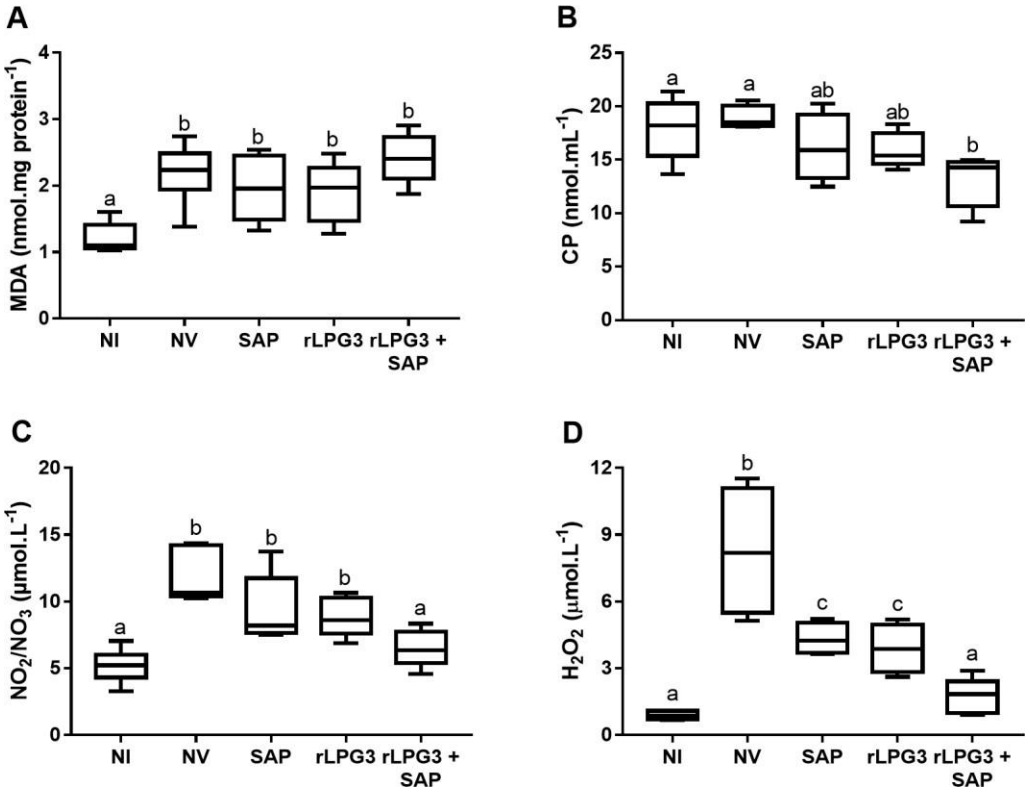


Figure 5. Oxidative/nitrosative stress markers in the liver tissue of BALB/c mice submitted to rLPG3 vaccination and challenged with L. infantum chagasi. (A) MDA: malondialdehyde; (B) CP: carbonyl protein; (C) NO2/NO3: nitrite and nitrate content; (D) H2O2: hydrogen peroxide production. NI: non-infected animals; NV: non-vaccinated; SAP: treated with saponin; rLPG3: immunized with rLPG3; rLPG3 + SAP: immunized with rLPG3 plus SAP. The box refers to the interquartile interval, with the median indicated as a horizontal line. The whiskers refer to the superior and inferior quartiles. Different letters denote statistical difference among the groups ($p < 0.05$), determined by ANOVA, followed by the Student–Newman–Keuls test.

3.3. Immunization with rLPG3 + SAP changes micromineral content in hepatic tissue in mice infected with L. infantum chagasi.

The analysis of mineral content in the liver tissue presented marked changes in the levels of the elements evaluated. The proportion of Zn and Fe decreased in the NV, SAP, and rLPG3 groups compared to the NI and rLPG3+SAP groups. The immunization with SAP or rLPG3+SAP increases the Cu levels in relation to the other treatments. The treatments did not promote any changes in the levels of Mn, Ca, and Na. The levels of Se were lower in the rLPG3 group in comparison with the other treatments. The group rLPG3 + SAP showed increased K concentration compared to the NV group (Fig. 6).

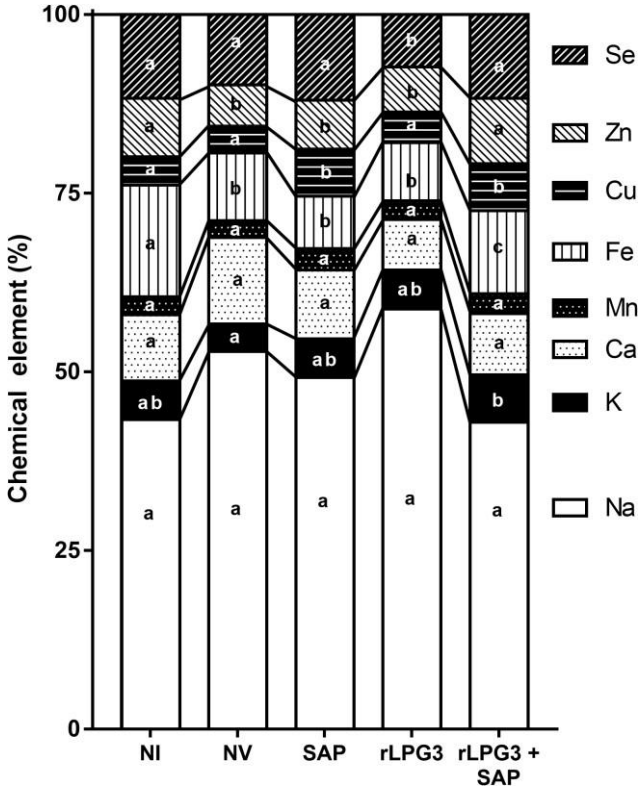


Figure 6. Mineral proportion in the liver of BALB/c mice submitted to rLPG3 vaccination and challenged with L. infantum chagasi. NI: non-infected animals; NV: non-vaccinated; SAP: treated with saponin; rLPG3: immunized with rLPG3; rLPG3 + SAP: immunized with rLPG3 plus SAP. Se (Selenium), Zn (Zinc), Cu (Copper), Fe (Iron), Mn (Manganese), Ca (Calcium), K (Potassium) and Na (Sodium). Each box refers to the percent of the element related to the total of mineral content. Different letters denote statistical difference among the groups ($p < 0.05$), determined by ANOVA, followed by the Student–Newman–Keuls test.

3.4. Immunization with rLPG3 alone or in the presence of SAP preserves liver morphology in L. infantum chagasi infected mice.

The stereological analysis of liver components showed marked remodeling of stroma and parenchyma components in animals of the NV group compared to non-infected animals, SAP, rLPG3 and rLPG3+SAP groups, presenting evident parenchymal loss, demonstrated by the decreased hepatocyte percentage, and proportional expansion of stromal elements, blood vessels and sinusoidal capillaries (Fig. 7A, B, and C). The histopathological and stereological analysis of the liver tissue showed an increase of the inflammatory infiltrate focus in animals challenged with L. infantum chagasi. Both inflamatory infiltrate focus and macrophage content were higher in the NV control group if compared with the groups of mice immunized with SAP and rLPG3, either alone or in association. The rLPG3 and rLPG3 + SAP groups clearly showed a reduced number of granulomas compared to the NV and SAP groups. The

animals from NV, SAP and rLPG3 groups presented lower proportion of glycogen-containing cytoplasmic inclusions in comparison with the NI and rLPG3 + SAP groups (Fig. 8).

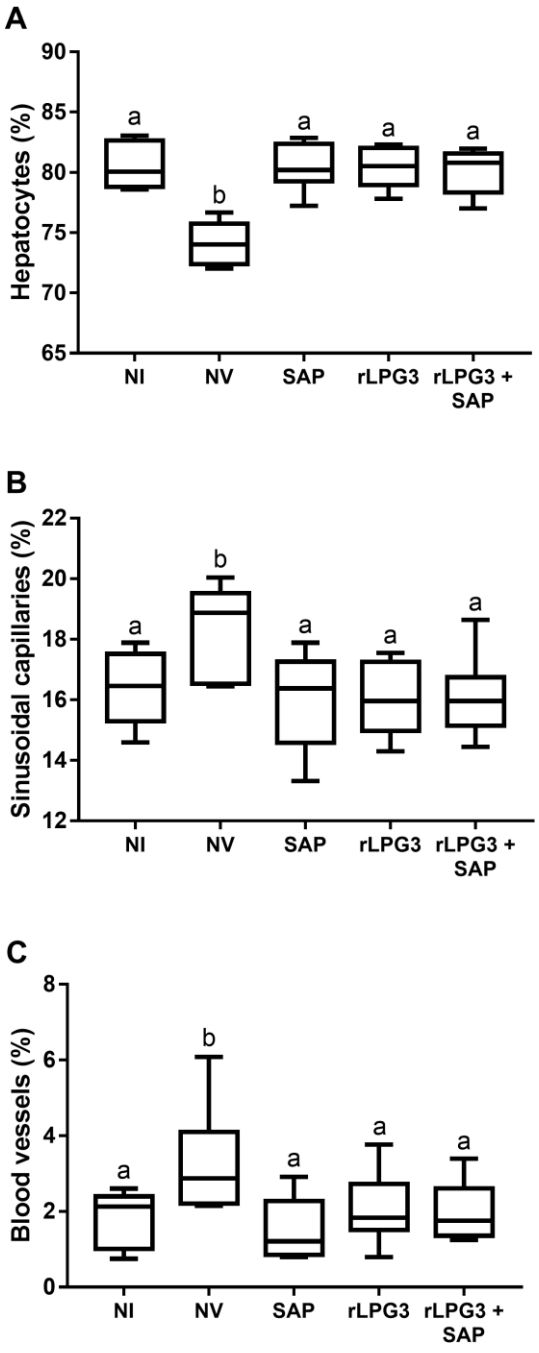


Figure 7. Liver stereological parameters in BALB/c mice submitted to rLPG3 vaccination and challenged with L. infantum chagasi. (A) Hepatocytes. (B) Sinusoidal capillaries. (C) Blood vessels. NI: non-infected animals; NV: nonvaccinated; SAP: treated with saponin; rLPG3: immunized with rLPG3; rLPG3 + SAP: immunized with rLPG3 plus SAP. The boxes refer to the interquartile interval with the median indicated as horizontal line. The whiskers refer to the superior and inferior quartiles. Different letters denote statistical difference among the groups ($p < 0.05$), determined by ANOVA, followed by the Student–Newman–Keuls test.

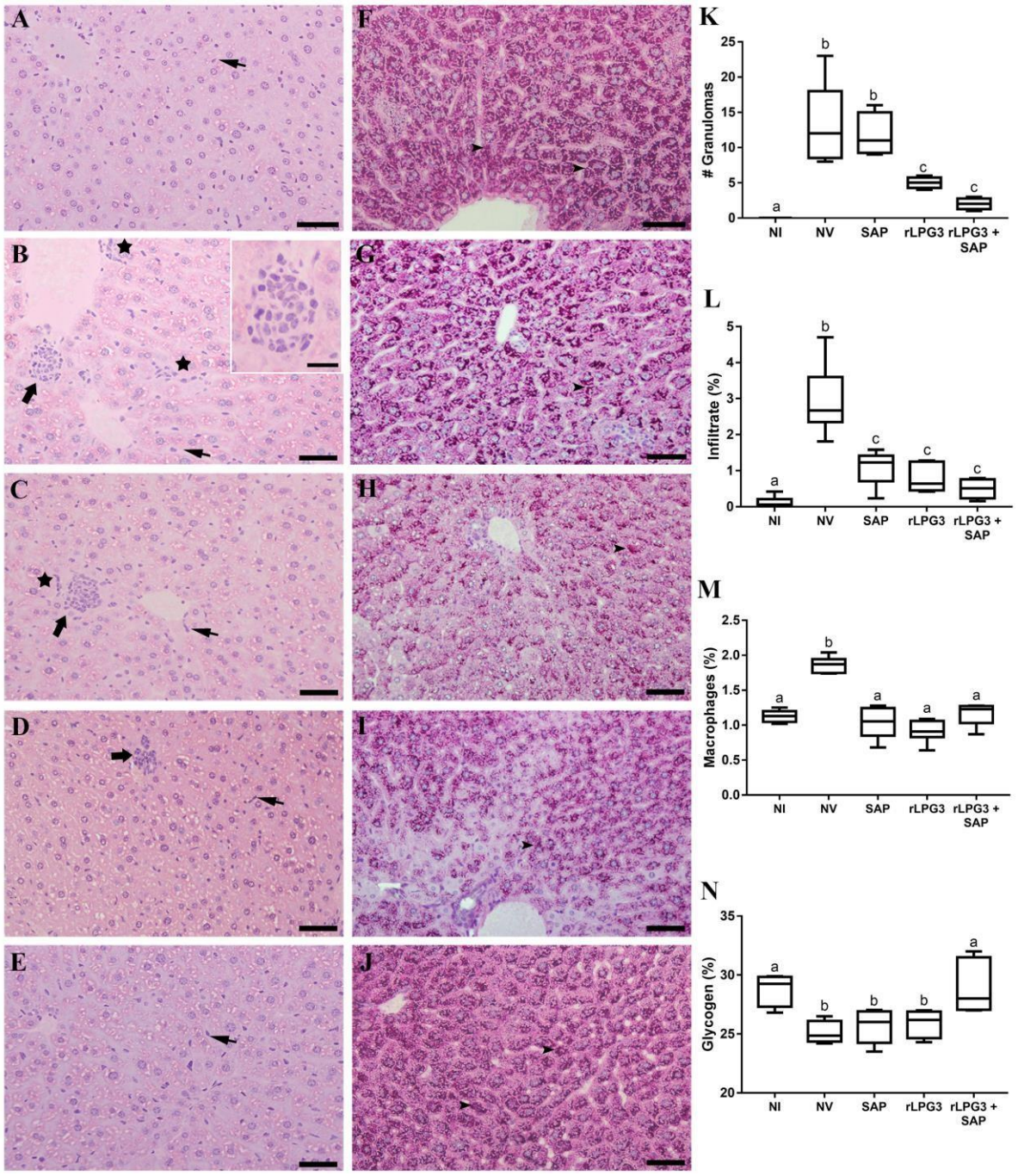


Figure 8. Representative photomicrographs, stained with HE (AeE) and PAS (FJe), and stereological parameters of liver pathologies (KeN) in BALB/c mice submitted to rLPG3 vaccination and challenged with L. infantum chagasi. NI: non-infected animals; NV: non-vaccinated; SAP: treated with saponin; rLPG3: immunized with rLPG3; rLPG3+SAP: immunized with rLPG3 plus SAP. A and F: non-infected group; B and G: non-vaccinated group; C and H: saponin group; D and I: rLPG3 group; E and J: rLPG3 + SAP group. Star: inflammatory infiltrate; Fat arrow: granulomas; Thin arrow: macrophages; Arrowhead: glycogen. Detail: granuloma. Bars = 50 μm. The graphs show the results of stereological analysis. The box refers to the interquartile interval with the median indicated as horizontal line. The whiskers refer to the superior and inferior quartiles. Different letters denote statistical difference among the groups ($p < 0.05$) determined by the Newman-Keuls test.

4. Discussion

The current study evaluated the effect of immunization of BALB/c mice using rLPG3 as immunogen, considering its effect on the parasitism, inflammatory profile, and parameters of tissue damage on the liver of the L. infantum chagasi challenged mice. Our results showed that vaccination with rLPG3, alone or associated with SAP, was efficient in reducing the parasitism. Interestingly, the cell function was preserved, with global improvement of antioxidant defenses and maintenance of hepatocyte morphology and integrity, remarkably in the rLPG3 + SAP vaccinated mice.

The immunogenic potential of the antigens used for immunoprophylaxis has been demonstrated by the use of SAP as an adjuvant, which is able to induce a strong pro-inflammatory response [15,34,35]. In our results, mice that received rLPG3 in association with SAP showed a decreased parasite burden (99.33 % of depletion) in the liver. Part of the resistance to VL may be associated with the development of parasite-specific cell-mediated immune responses, characterized by a polarization to the Th1 profile [2,21,36]. The lower ratio of IgG1/IgG2a antibodies in the serum of the animals, compared to the NV or SAP groups, suggests that rLPG3 + SAP immunization shifted the immune response toward a Th1 phenotype, which may have contributed to the reduction in the parasitism. The switch from class IgG1 to IgG2a, as a consequence of the pro-inflammatory response, is mainly mediated by interferon gamma (IFN-γ) production [37]. Miahipour et al. [38] demonstrated that rLPG3 could promote differentiation of CD4 + T lymphocytes toward a Th1 phenotype through up-regulation of Th1 lineage. Our study is focused especially on the assessment of the action of the vaccine on tissue liver integrity following infection with L. infantum chagasi. Experiments related to deeper immunological analysis are being prepared for further work that will specifically address this issue. However, it is known that IFN-γ has the fundamental role to further amplify cellular recruitment of macrophages, as well as activate infected cells, leading to the production of NO and other free radicals that kill the parasites [11,14,39,40]. We believe that the vaccination with rLPG3 was essential for this process of macrophage activation, since it leads to a decreased hepatic parasitism, even without the adjuvant.

It is well established that the spread of Leishmania infection in different organs occurs in part due to the infection of cells of the mononuclear phagocytic system [41,42]. In the liver, these infected cells contribute to the parasitic peak that occurs between 2 and 4 weeks after infection in the murine experimental model [11,43]. In infected dogs, a consequent increase of

free radicals and exacerbation of inflammatory response, which compromises both the structure and function of the hepatocytes, is observed [9]. In our investigation, the establishment of higher hepatic parasitism in the mice from NV, SAP or rLPG3 groups was accompanied by significant changes in the ability to respond to the increased reactive metabolites, compared to the group vaccinated with rLPG3 + SAP. In those first groups, the activity of hepatic CAT and GST, enzymes of the endogenous antioxidant system, remained significantly lower in comparison with the rLPG3 + SAP group. This enzymatic response pattern was accompanied by oxidative stress, considering the higher levels of carbonyl protein, nitrite and nitrate and H2O2 contents, compared to the rLPG3 + SAP group. Despite the decreased H2O2 in the SAP or rLPG3 groups, compared to the control group NV, the content of this oxidizing agent was significantly lower in the rLPG3 + SAP group. It demonstrates the potential of this association to protect against oxidative damage mediated by this free radical, which has the potential to compromise mitochondrial ATP synthesis (e.g. by inhibiting the ATPase-synthase complex), in addition to elevating the intracellular calcium, thus leading to the disruption of the cytoskeleton, also accelerating the disintegration of the plasma membrane, with consequent cell lysis [44].

In normal metabolic processes, free radicals are produced continuously [45]. However, their production rate increases during certain inflammatory processes or parasitic infections [6–9]. In the leishmaniasis, the macrophages, target of infection, when phagocytizing the parasites, respond with intensive formation of reactive species that can damage biomolecules, of which lipids are probably the most susceptible, in the absence of an appropriate antioxidant scavenging system [6,8,14]. In our investigation, the effects of those reactive metabolites on proteins have been prevented in the rLPG3 + SAP group, which shows reduced CP levels in comparison with the NI and NV groups. However, the lipids were at least preserved in all vaccinated or SAP treated mice, as observed in the constant levels of malondialdehyde, a product of lipid peroxidation, in these experimental groups compared to the NV group. We believe that the SOD enzyme may have contributed to this result, since SOD levels remained high in the infected groups, despite the reduced CAT and GST levels obtained.

The protective potential of the endogenous antioxidant enzymatic system depends on the maintenance of the micromineral dynamics [8,43], since the activity of their enzymes requires the adequate supply of these components (e.g. Cu-Zn is linked to SOD, CAT requires Fe for its activity, and GST requires Se to catalyze its enzymatic reactions) [9,46]. We

demonstrated that the vaccine with rLPG3 + SAP contributes to the higher liver levels of Zn, Fe, K and Cu compared to the NV group. The higher activity of CAT observed in rLPG3 + SAP in comparison to the other infected groups might be correlated to the increased levels of Fe found in this group. Altered values of trace elements have been recorded in L. infantum infection [9,21,47]. Kala-azar patients present electrolyte disorders, including global low levels of K [48]. Potassium plays an important role in the activation of inflammasome in infectious diseases [49,50]. Therefore, the higher levels of K in the liver may have contributed to parasitism depletion in the SAP + rLPG3 group. Weyenbergh et al. demonstrated that high levels of Zn were associated with an improvement of immune response against Leishmania infection, triggering an increase in IFN-γ production [51]. According to this, the higher levels of Zn observed in the SAP + rLPG3 compared to NV group may be contributing to the protective effects of the vaccine. The rLPG3 immunization associated to SAP, therefore, prevents the redistribution of trace elements in the liver, which increases the activity of antioxidant enzymes (CAT and GST), consequently reducing the tissue levels of H2O2, which prevents the peroxidation of macromolecules.

Associated with the imbalance of the reactive metabolites caused by L. infantum chagasi infection, the installation of a hepatic pro-inflammatory niche besides the damaged macromolecules, such as lipids and proteins, negatively affects the structure and function of hepatocytes [2,8,9]. Hepatic morpho-functional damage was confirmed by increased ALT, AST and ALP serum levels in the NV group compared to the NI group. Serum ALT and AST activities reflect damage to hepatocytes and are considered highly sensitive and fairly specific preclinical and clinical biomarkers of hepatotoxicity [52]. Thereby, the determination of hepatic transaminase levels has served as a good indicator of cellular injury [53,54], since the damage in hepatocyte membranes increases permeability, instability and enzyme release into blood vessels [55]. Our results revealed that the vaccination using rLPG3 associated with SAP prevented protein and membrane damages in hepatocytes and resolved infection effectively, as observed in the low serum concentrations of ALP and transaminases and reduced tissue parasitism in the liver.

The histopathological and morphometric data presented a remodeling of the parenchyma and stroma components of the hepatic tissue, with reduced inflammatory infiltrate and macrophage content, and increased glycogen content in rLPG3 + SAP group in comparison to the NV group, and reduced hepatocyte proportion in NV compared to the other

groups. This may result from higher levels of reactive metabolites, disturbances in mineral homeostasis, redox metabolism and damaged macromolecules, which culminates in cell death in this control group [9]. The evident loss of parenchyma was accompanied by an increased number of stromal components in this group. The higher number of macrophages and the inflammatory infiltrate focus present on liver tissue can be related to granuloma formation in the NV group. Kupffer cells (KCs) are at the "heart" of the hepatic granuloma, often fusing one with each other, as a result of the migration of monocytes from sinusoids [56]. The fused KCs recruit T, B and natural killer (NK) cells, monocytes and neutrophils, by the action of adhesion molecules, chemokines and cytokines [43,57,58]. The granuloma becomes mature from 4 weeks post-infection, where the antileishmanial response becomes sufficient to reduce the parasitism. The SAP, rLPG3 and rLPG3+ SAP groups have preserved tissue architecture with reduced inflammatory infiltrate, macrophages and number of granulomas. However, in the two first groups, no reduction and lower reduction were observed, respectively, in the parasitism compared to the rLPG3 + SAP group. A possibly inefficient or insufficient immune response may be the cause of the persistence of the parasitism in the SAP group. Moreover, the glycogen content was reduced in the NV, SAP and rLPG3 groups. Reduced glycogen content of hepatocytes may have resulted from a compensatory response to parasitic infection [59]. These data indicate that rLPG3 associated with SAP was able to maintain the reserve of cellular energy in the tissue.

5 Conclusion

Taken together, our findings showed that rLPG3 plus SAP immunization resulted in the protection of the liver tissue against L. infantum chagasi infection. The vaccine preserves the functional properties and architecture of the organ in mice challenged with promastigote forms of the parasite, while controlling the parasite load as result of the modulation of the immune response. Despite the need for additional studies to clarify the immunological mechanism triggered by rLPG3 plus SAP immunization, our results highlight the importance of the vaccination, not only as an anti-parasitic mechanism, but also as a factor that acts maintaining the integrity of the infected organ.

Declaration of Competing Interest

The authors declare that there are no conflicts of interest.

Acknowledgments

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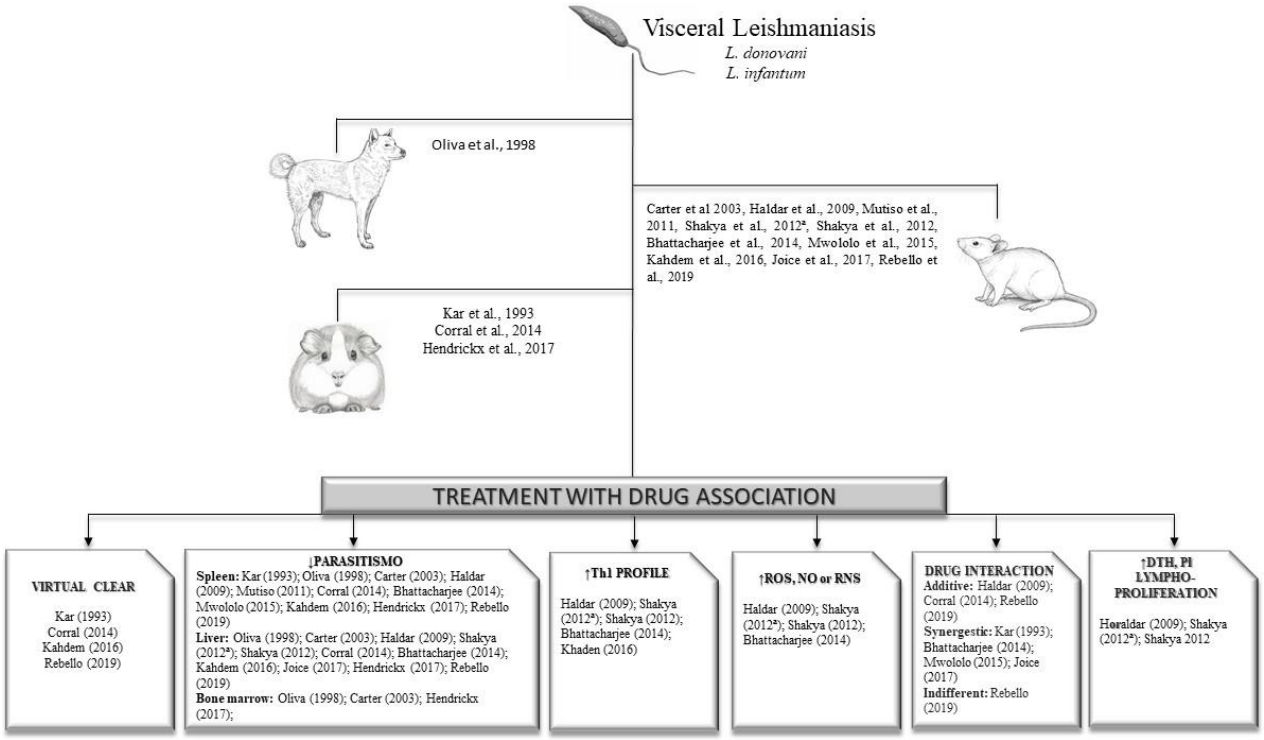
Artigo 2

EFFECTIVENESS OF DRUG ASSOCIATION IN THE TREATMENT OF VISCERAL LEISHMANIASIS IN ANIMAL MODEL - A SYSTEMATIC REVIEW

Graphical abstract



Abstract

Background: Visceral leishmaniasis (VL) is considered a neglected tropical disease caused by *Leishmania infantum* in North Africa, Europe and Latin America, and *Leishmania donovani* in East Africa and the Indian subcontinent. This disease is widely distributed around the world and is a public health problem in 88 countries. The current treatment options for VL are limited by chemotherapy using pentavalent antimonies, followed by pentamidine or amphotericin B as second-choice. However, these drugs have a significant side effects and showed low efficacy in some cases.

Objectives: Thus, the aim of this study was to evaluate the effectiveness of the drug association for the treatment of experimental VL in animal models.

Methods: For this, we performed a systematic review following the PRISMA (Preferred Reporting Items for Systematic Reviews and Meta-analyzes) guidelines to conduct the research. MedLine (PubMed), Scopus and Web of Science platforms were used to search for papers related to drug association in the treatment of experimental VL. The keywords used were based on filters constructed by three criteria: (i) drug association, (ii) visceral leishmaniasis and (iii) animal model. The studies included were limited to those that used drug association in the treatment of visceral leishmaniasis in animal model. Data extraction was categorized as follows: publication characteristics, characteristics of the animal model, infection parameters, *in vitro* studies, characteristics of the administered treatment and outcomes. Bias analysis and methodological quality assessment were examined through the SYRCLE's Risk of Bias toll.

Results: Fourteen papers were selected. The prevalent animal model was BALB / c mice (50%). The *Leishmania donovani* species was the most used for experimental infection of the animals (80.56%), mainly by intravenous route (41.67%). For treatment, 18 different drugs were used in association, with miltefosine being prevalent in most associations. The quantification of the parasite burden, activation of microbicide defenses and polarized Th1 response were the main evaluations raised. Analysis of methodological quality indicated that most items were not reported, resulting in a high risk of bias

Conclusions: Based on the results found, we believed that the combination of drugs for the treatment of VL is efficient, although the heterogeneity between the studies makes it difficult to obtain clear evidence.

Keywords: Viscera leishmaniasis, Drug Association, Chemotherapy

1. Introduction

Visceral Leishmaniasis (VL) or kala-azar is an infectious disease caused by the parasite protozoans *Leishmania infantum chagasic*, *Leishmania donovani* and *Leishmania infantum* (Freitas *et al.*, 2012). This is a severe and potentially deadly neglected disease closed correlated to poverty, precarious conditions of basic sanitation and hygiene (Freitas *et al.*, 2012; WHO, 2016). Although VL is endemic in more than 98 countries, around of 90% of the 500,000 cases reported annually by the World Health Organization (WHO) were associated to 5 countries, specifically Bangladesh, Brazil, Ethiopia, Sudan, and India (WHO, 2016). It is estimated that 50,000 deaths per year occur due to VL (Nieto *et al.*, 2011; Dostálová and Volf, 2012). While *Leishmania infantum* is responsible for the disease in North Africa, Europe and Latin America; *Leishmania donovani* prevails in the East Africa and Indian subcontinent (Hendrickx *et al.*, 2017).

The disease develops after the transmission of metacyclic forms of *Leishmania* spp. to vertebrate hosts by sand flies, mainly of the genders *Phlebotomus* spp. and *Lutzomyia* spp. (Nieto *et al.*, 2011; Dostálová and Volf, 2012). At the site of infection, the parasites are phagocytosed by macrophages, within which they survive and multiply by binary fission as amastigote forms (Dostálová and Volf, 2012; de Freitas *et al.*, 2016). A broad spectrum of unspecific clinical manifestations is detected during VL development, especially chronic low-grade fever, anorexia, weight loss, weakness and hepatosplenomegaly. In addition, laboratory findings such as pancytopenia, low plasma albumin, high aminotransferase levels and hypergammaglobulinemia are also often associated with VL (Serafim *et al.*, 2010; Mwololo *et al.*, 2015; WHO, 2016).

The specific treatment of VL is limited to pentavalent antimonies, followed by pentamidine or amphotericin B as a second-choice (Corral *et al.*, 2014; Joice *et al.*, 2017). New nanostructured lipid formulations of amphotericin B (amphotericin liposomal) have shown relevant results in preclinical (Corral *et al.*, 2014) and clinical (Sundar *et al.*, 2011) studies. However, this treatment is still expensive and often unavailable (Corral *et al.*, 2014; Ponte-Sucre *et al.*, 2017). Although all these drugs are recommended by the WHO as the reference chemotherapy, marked side effects (i.e., nausea, vomiting, arthralgia, cardiac dysrhythmias, hepatitis and pancreatitis), limited efficacy, high cost, as well as complex administration (parenteral) make VL chemotherapy a challenging task (Sundar *et al.*, 2011; Ponte-Sucre *et al.*, 2017; Hendrickx *et al.*, 2017). In addition, infections caused by parasites resistant to the reference chemotherapy represent a more recent and worrying barrier to the treatment of this

disease (Mwololo *et al.*, 2015; Ponte-Sucre *et al.*, 2017). In this sense, as suitable animal and human vaccines are not currently available (Corral *et al.*, 2014; Mwololo *et al.*, 2015), developing more effective and less toxic treatment protocols for VL is necessary and urgent (Khadem *et al.*, 2017; Joice *et al.*, 2017).

In the last years, the use of combination therapy has emerged as an alternative to VL treatment (Mwololo *et al.*, 2015; Joice *et al.*, 2017; Rebello *et al.*, 2019). As combined chemotherapy allows to increase drugs half-life, reduce medication dose, treatment time, systemic toxicity and side effects (van Griensven *et al.*, 2010; Corral *et al.*, 2014; Bhattacharjee *et al.*, 2015; Rebello *et al.*, 2019); greater adherence to the treatment protocol and better therapeutic outcomes can be obtained (van Griensven *et al.*, 2010). With the increase in therapeutic failures after the administration of antimonial drugs and miltefosine, combination chemotherapy is also relevant as a strategy to reduce *Leishmania* spp. resistance to treatment (Sundar *et al.*, 2012; Rijal *et al.*, 2013; Ponte-Sucre *et al.*, 2017). This approach has also emerged as an alternative for the treatment of complicated VL cases, such as patients co-infected with HIV, for which monotherapy does not achieve satisfactory results (Alvar *et al.*, 2008; van Griensven *et al.*, 2010; Rebello *et al.*, 2019).

Despite the potential benefits of combination chemotherapy, the current evidence is fragmented, making it difficult to establish a profile of the drugs and protocols administered, as well as to assess their therapeutic relevance for VL. Therefore, we use a systematic review framework to retrieve and analyze the preclinical evidence on the applicability and relevance of leishmanicidal or leishmaniostatic drugs combination for the chemotherapeutic management of VL. In addition to mapping the available drug combinations and their spectrum of effectiveness, the preclinical models and treatment protocols used, as well as risk of bias related to the studies that supports the evidence were critically analyzed. By charactering the rationale underlying the co-administration of different antileishmanial drugs, this systematic review may be relevant to support translational investigations.

2. Materials and Methods

2.1 Guiding questions

The main questions to be answered in this systematic review were: Are combinations of antileishmanial drugs effective in preclinical models of visceral leishmaniasis? What are the

main chemotherapy protocols and primary research outcomes used to determine treatment effectiveness?

2.2 Search strategy and selection of primary studies

The PRISMA (Preferred Reporting Items for Systematic Reviews and Meta-analyses) statement was adopted for conducting this systematic review (Hooijmans *et al.*, 2014). We performed an extensive literature search using two comprehensive databases: (i) PubMed (https://www.ncbi.nlm.nih.gov/pubmed) and (ii) Scopus (https://www.scopus.com/home.ur) Web of Science . The research strategy was based on search filters optimized for each database. The search filters were structured in three complementary levels as follows: (i) drug association, (ii) visceral leishmaniasis and (iii) animal model. A search filter was initially developed for PubMed according standardized descriptors obtained from the platform's thesaurus MeSH (Medical Subject Headings (https://www.ncbi.nlm.nih.gov/mesh). To expand the recovery of relevant indexed studies and those in the indexing process, the commands [MeSH Terms] and [TIAB] were combined. To detect all animal studies in PubMed, a standardized animal filter was applied (Pereira *et al.*, 2017). The same search filter used to diseases and drug association was adapted to Scopus and Web of Science. It was applied a search limit for animal model in Scopus search and language (English) for Scopus and Web of Science. No chronological restriction was applied in the article search. All studies identified and published until March 24, 2020 (09:12 p.m), were included in the systematic review. The complete research strategies applied in both databases can be consulted in the supplementary material (Table S1). The initial selection was independently performed by three investigators (DSSB, ACFS and AC), which screened the title and abstract of all recovered papers. Duplicate studies were removed by comparing the authors, title, year and journal of publication.

2.3 Inclusion and exclusion criteria

Only studies investigating effectiveness of drug combination in preclinical models of VL were included in the systematic review. After the initial screening, irrelevant studies were excluded (not related to the subject), all potentially relevant studies were recovered in full-text and evaluated for eligibility. Study exclusion was based on well-defined criteria as follows: (i) studies exclusively based *in vitro*, *ex-vivo* or *in silico* assays, (ii) studies evaluating cutaneous leishmaniasis or unrelated diseases, (iii) secondary studies (i.e., literature reviews, editorials, commentaries, short communication and letters to the editor), (iv) clinical studies, (v) without

monotherapy as control, (vi) non drugs (plant extract, cytokines, vaccines), (vii) without drug association and (viii) studies published in other language that not English. After identifying the relevant studies in the primary search, we included a secondary screening to enhance the recovery of research records on the subject investigated. Thus, the reference lists of all papers identified in electronic databases and included in the systematic review were manually screened for additional relevant studies. In both search levels, three researchers (DSSB, AC and RVG) independently analyzed the eligibility criteria, and disagreements were resolved by arbitration, consulting a fourth researcher (RDN or EAMS).

2.4 Study characteristics and data extraction

Qualitative data were extracted from all included articles. For this, standardized spreadsheets (data extraction masks) were built, indicating the essential information to be collected from the reading of the individual study chain. Thus, the information summarized in data extraction masks was categorized as follows: (i) publication characteristics: authors, years and country; (ii) characteristics of the animal models: species, lineage, sex, weight and age; (iii) infection parameters: *Leishmania* specie, strain, number of parasites inoculated, route of inoculation; (iv) treatment protocol: drugs co-administered, dose, frequency, route of administration (v) complementary *in vitro* assays: parasitological/toxicity tests for drug interaction assessment; and (vi) research outcomes: positive and negative results.

2.5 Reporting quality as a risk of bias

The risk of bias in preclinical animal studies was analyzed by SYRCLE'S (SYstematic Review Center for Laboratory Animal Experimentation) guidelines, which is based on the Cochrane Collaboration risk-of-bias tool for randomized trials (RoB) (Hooijmans *et al.*,2014). This instrument was adjusted for bias aspects that play a specific role in animal intervention studies. The objective is to establish consistency and avoid discrepancies in the evaluation of methodological quality in the field of animal experimentation. In order to increase transparency and enforceability, signaling issues have been formulated to facilitate judgment based on the following: 1. Random sequence generation. 2. Baseline characteristics. 3. Allocation concealment. 4. Random housing. 5. Blinding of participants and personnel. 6. Random outcome assessment. 7. Blinding of outcome assessment. 8. Incomplete outcome data. 9. Selective outcome reporting. 10. Other bias. The items in the RoB tool were scored with "yes,"

indicating low risk of bias; “no,” indicating high risk of bias; or “unclear,” indicating that the item was not reported, and therefore, the risk of bias was unknown.

3. Results

3.1 Research records retrieved

The initial search strategies recovered 2239 articles from PubMed, Scopus and Web of Science, of which 593 were duplicates. After title and abstract screening, 1646 studies were excluded due to inadequate research topic. Of these, 349 studies were based only *in vitro* parasite viability assays, 595 investigated the drug treatment without association, 89 studies evaluated other diseases, 386 studies corresponded to non-original papers (i.e., reviews, short communications, letters and clinical cases), 5 clinical studies and 23 studies were not written in English. Considering papers that investigated the drug association for treatment of visceral leishmaniasis, 40 studies were selected for full text evaluation. Of these 40 publications, 14 studies were included in this systematic review (Fig. 1).

3.2 Animal models of visceral leishmaniasis

The data about the animal model used in the studies for VL are showed in Table S2. The most of the studies were produced in India (35.71%, n = 5), followed by Kenya (14.29, n = 2). The remain studies were conducted in United States of America, Italy, United Kingdom, Spain, Canada, Belgium and Brazil, and together represent 50% of the studies with one study each one. Most of studies used BALB/c mice (71.43%, n = 10) as the animal model, followed by hamster (21.43%, n = 3), especially the lineage golden (14.29%, n = 2). One study did not describe the lineage of hamster. Just one work was performed using dogs, being carried out with animals belonging to different breeds.

The female gender was the most used in the studies (35.71% n = 5) as well as studies that used both genders (35.71% n = 5). The male gender was found in only one study (7.14%, n = 1). This parameter was underreported in 3 studies (21.43%). The age of the animals ranged from 4 to 8 weeks (50%, n = 7) for mice and hamster, while in studies using dogs the age ranged from 2 to 10 years old (7.14%, n = 1). This variable was neglected in 21.43% of the studies (n = 6). The animal’s weight ranged from 18-30 g for mice (28.57%, n = 4), and 80-100 g for hamster (14.29%, n = 2). The most of the studies did not report this data (57.14, n = 8).

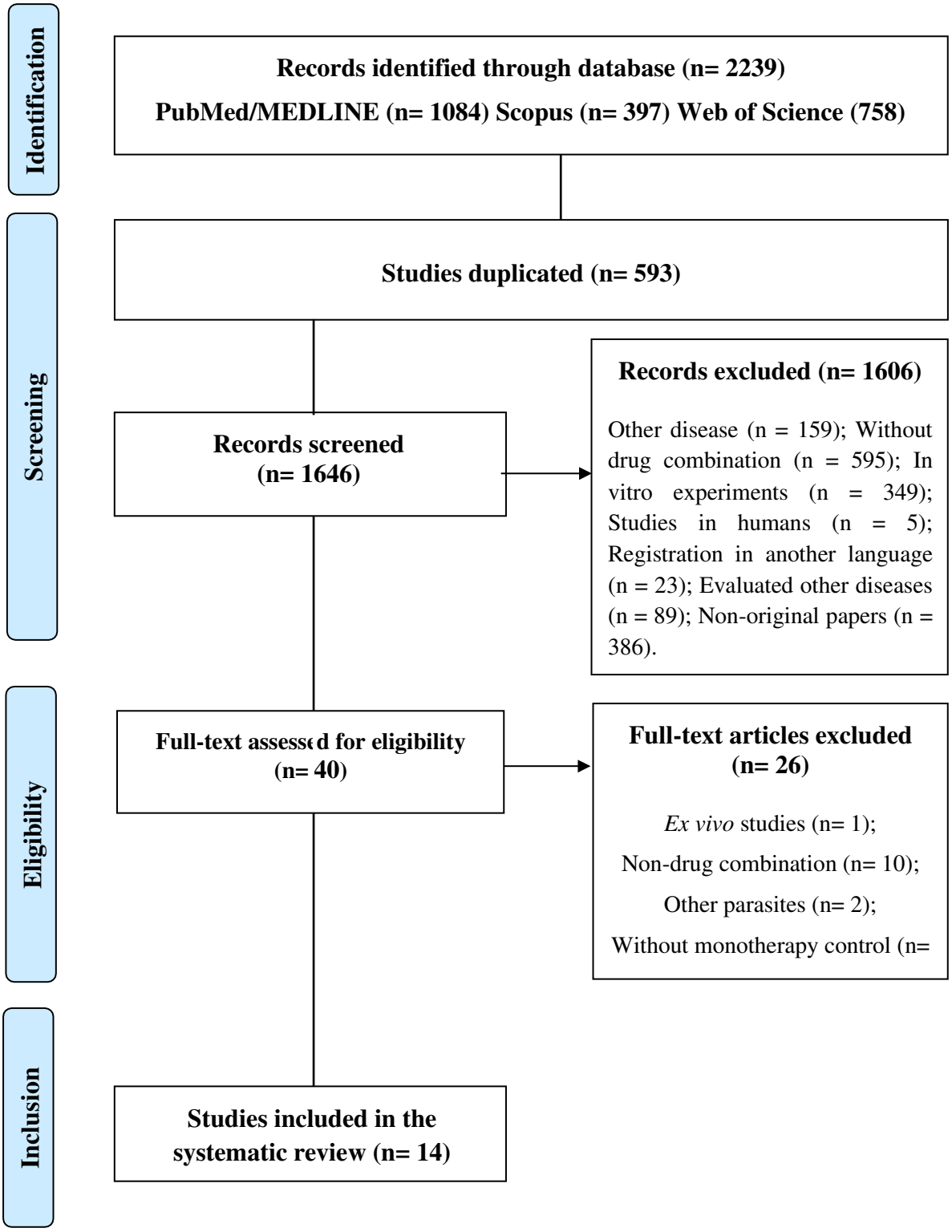


Fig. 1. Flow diagram of the systematic review literature search results. Based on PRISMA statement "Preferred Reporting Items for Systematic Reviews and Meta-Analyses". www.prisma-statement.org.

3.3 Visceral leishmaniasis characteristics

The most studies used *L. donovani* species (64.29%, n = 9), followed by *L. infatum* (28.57%, n = 4). In the study that used dogs, the animals were naturally infected and the leishmania species was not identified. Sixteen different strains were used in the studies included in this systematic review. The strains MHOM/KE/82/LRC-L445/NLB065 and MHOM/MA/67/ITMAP263 were used in 3 studies each one, while MHOM/IN/89/GE1F8R and MHOM/ET/67/HU3 strains were found in 2 studies each one. The remaining studies used 5 different strains. Only one study did not describe the strain used to infect the animals (6.25%) (Table S3).

The inoculation routes of parasites observed were intravenous and intraperitoneal, being used in 5 (35.71%) studies each one, followed by intracardiac route in 3 studies (21.43%). The study performed in dogs described the animals as naturally infected (7.148%). The inoculum size ranged from 1×10^6 to 1×10^8 parasites in studies using mice and hamsters. This data was not reported in dogs since the animals were naturally infected (Table S3).

3.4 General characteristic of the treatment protocols

Considering all 14 studies, 23 drugs were used in 17 different combinations (Table 1 and S4). In mice models, 17 different drugs were used in 11 combinations (64.71%). For mice, Miltefosine (MTF) was the most used drug (n = 3), followed by sodium stibogluconate (SSG), Diminazene (DIM) and DB766 compound, with 2 combination each one. Glycyrrhizic acid (GA), Sodium antimony gluconate (SAG), CAL-101 (CAL) and Amphotericin B (AMB) were also used in combination for VL treatment (Table 2 and S4).

For hamster models, 5 (29.41%) combinations were performed using 8 different drugs. Trans-aconitic acid was the most drug used (n = 3) in combination therapy (Table 1 and S4). The other drugs used were: SSG, Pentamidine (PET), Allopurinol (ALL), Allicin (ALI), AMB, Paromomycin (PAR) and MTF. The combined treatment for dogs was realized with Paromomycin (PAR) associated to Meglumine antimoniate (MEG).

The most commonly used routes for drug administration were intraperitoneal (48%, n = 12,) and per oral (24, n = 6). Intramuscular, subcutaneous and intravenous routs were reported in 24% of the studies. Only one study did not report this data. Most of the drugs were administered daily (55.56%, n = 10) or in a single dose (16.77%, n = 3). Two studies administered the drugs in alternate days (11.11%), and 2 more studies used the administration twice a week (11.11%).

Only one study chooses the administration drugs twice in a day (5.56%). To aim reduce the toxicity, adverse effects the most of studies (71.42%, n = 14) used sub doses in the therapeutic regimens tested (Table 2).

3.6 Main outcomes

The main outcomes were shown in Table 1, 2 and Figure 2. Preclinical studies demonstrated that the drug association interventions provide an effective therapeutic scheme for VL. In most studies, parasitism was used as a primary outcome to assess the success of antiparasitic chemotherapy. In the entire dataset with 14 studies, 100% reported a significant reduction in organ parasitism, especially spleen (n = 11, 44%), liver (n = 11, 44%) and bone marrow (n = 3, 12%).

An improved parasite clearance was often associated to: (i) induction of a Th1 immune cytokines by increasing IL-12, IL-6, TNFα, IFN-γ, IgG2 levels and consequent decrease in Th2 cytokines IL-10, IL-4 or TGFβ; and (ii) upregulation of reactive oxygen and nitrogen species (ROS, RNS) and increase in the levels of reduced and total glutathione. Improved cellular parameters such as macrophage phagocytic ability was also associated to a better parasitological control in animals treated with combination chemotherapy. Score and clinical sings as well as reduction in organs weigh, increase in body weight and survivorship were reported as good markers of success for the drug association. In addition, among 14 studies 8 (57.14%) evaluated the pharmacological interaction of the drugs. The pharmacological interaction is able to enhance the effect of drugs. Synergistic and additive interactions were found in 4 (28.57%) and 3 studies (21.42%), respectively. One combination had no effect, indifferent (7.14%).

Table 1. Drug combinations, pharmacological interaction and main chemotherapy outcomes identified in all studies reviewed

Animal model	Author	Combination chemotherapy	Organ	Parasitism suppression	Drug interaction*
Mice	(Carter *et al.*, 2003)	Buthionine sulfoximine (BSO) + Sodium stibogluconate (SSG)	liver/spleen/ bone-marrow	98/99/82 0/93/0	-
	(Haldar *et al.*, 2009)	Diperoxovanadate (PV6) + Sodium stibogluconate (SSG)	liver/ spleen	77.1/79.2	additive
	(Mutiso *et al.*, 2011)	Diminazene (DIM) + Artesunate (ART)	spleen	↓	-
	(Shakya *et al.*, 2012b)	PAM3Cys + Miltefosine (MTF)	liver	92.5	-
	(Shakya *et al.*, 2012a)	Tufisin (TUF) + Miltefosine (MTF)	liver	93	-
	(Bhattacharjee *et al.*, 2015)	Glycyrrhizic acid (GA) + Sodium antimony gluconato (SAG)	liver/ spleen	92.07/ 93.28	synergistic
	(Mwololo *et al.*, 2015)	Diminazene (DIM) + Chloroquine (CHQ)	spleen	↓	synergistic
	(Khadem *et al.*, 2017)	CAL-101 (CAL) + Amphotericin B (AMB)	liver/ spleen	100/100¨	-
	(Joice *et al.*, 2017)	DB766 + Posaconazole (POS) or Ketoconazole (KET)	liver	86	synergistic
	(Rebello *et al.*, 2019)	Miltefosine (MTF) + Lopinavir (LPV)	liver/ spleen	100/100¨	additive

Table 1. Drug combinations, pharmacological interaction and main chemotherapy outcomes identified in all studies reviewed

Animal model	Author	Combination chemotherapy	Organ	Parasitism suppression	Drug interaction*
Hamster	(Kar *et al.*, 1993)	*Trans*-aconitic acid (TAA) + Sodium stibogluconate (SSG) or Pentamidine (PET) or Allopurinol (ALL)	spleen	98.8/99.7/99	synergistic
	(Corral *et al.*, 2014)	Allicin (ALI) + Amphotericin B (AMB)	liver/ spleen	100¨/94.5	additive
	(Hendrickx *et al.*, 2017)	Paromomycin (PAR) + Miltefosine (MTF)	liver/spleen/ bone-marrow	99.6/99.6/ 98.1	indifferent
Dog	(Oliva *et al.*, 1998)	Paromomycin (PAR) + Meglumine antimoniate (MEG)	liver/spleen/ bone-marrow	↓¨/↓¨	-

* Drug interaction evaluated from *in vitro* or *in vivo* parasitological/cytotoxicity tests. ¨No parasite was detected.

Table 2 - Effective drug combinations able to induce an efficient parasite clearance.

Animal model	Combination chemotherapy (Dose)	Sub dose		Main effects	
Mice	MTF (1.92 to 20 mg/kg)	PAM3Cys (100 µg/animal)	y	↑ NO, ROS, H_2O_2, IL-12, TNFα, IFN-γ, IL-6, PhI,	↓ IL-10, Organ weight,
		TUF (60 µg/animal)	y		
		LPV (246.6, 493.2 mg/kg)	y		
	SAG (250 mg/kg)	GA (5 to 75 mg/kg)	y	↑ TNFα, IL-12 IFN-γ, NO, ROS; ↑ effectivity against Sb-resistant strain,	↓ antimony efflux ↓ TGFβ, IL-10, IL-4
		D-PV6 (0.5 µmol/30g bw)	n		
	Diminazene (12.5 mg/kg)	ART (12.5 mg/kg)	n	↑ Survivorship	↓Body weight, IgG antibody
		CHQ (12 mg/kg)	n		
	POS (7.5, 15, 30 mg/kg);	DB766 (19, 38, 75 mg/kg)	y	↑ Liver concentrations of DB766	
	SSG (50 to 282 mg of Sb^v/kg)	BSO-NIV (34 mg/kg)	y	↑ Reduced and total glutathione Complete cure*	↓ efficiently against resistant strain
	AMB (0.1 mg/kg)	CAL-101 (0.05 mg/animal)	y	↑ IFN- γ Complete cure*	↓ T-reg cell, ↓ treatment time

PhI: Phagocytic index. * No parasite was detected. **y**: yes; **n**: no. **BM**: Bone Marrow

Table 2 - Effective drug combinations able to induce an efficient parasite clearance.

Animal model	Combination chemotherapy (Dose)		Sub dose	Main effects	
Hamster	TAA (200 or 400 mg/kg)	SSG (50 mg of Sb^v/kg)	y	Complete cure* (1-month model); Inhibited *Leishmania* transformation and multiplication;	↑ liver and spleen weight
		PET (8 mg/kg)	y		
		ALL (15 mg/kg)	y		
	AMB (1 and 5 mg/kg)	Allicin (5 mg/kg)	y	↑ Drug concentration ↑ IgG2	
	MTF (20 mg/kg)	PAR (350 mg/kg)	y	No cross-resistance	↓ effectivity in sub dose and short treatment
Dog	MEG (20 Sb/kg)	PAR (3.5 mg/kg)	y	↑ Clinical sings, Complete cure* (BM)	↓ IgG, relapse after 180 days

PhI: Phagocytic index. * No parasite was detected. **y**: yes; **n**: no. **BM**: Bone Marrow

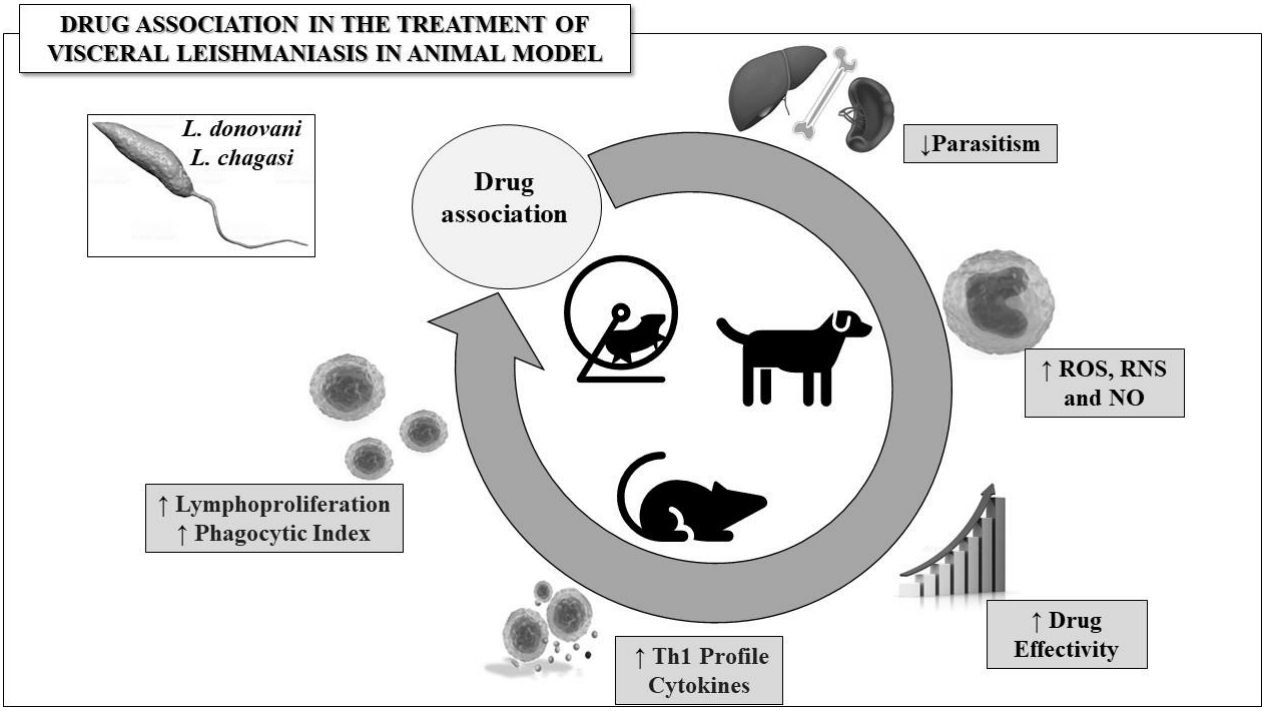


Fig. 2. Representative model of the main results obtained from drug combination used in the treatment of visceral leishmaniasis.

3.8 Risk of Bias

The risk of bias analyzed for all studies included in the systematic review is shown in Figure 3. None of the studies fulfilled all methodological criteria, and the mean quality score of all studies reviewed was 43.57±7.45%. Most items covered by the SYRCLE's toll were not reported, resulting in a marked risk of bias. The chronological analysis of all studies indicated that the risk of bias exhibited no time-dependent influence. Seven studies (50%) reached a below-average score (Figure 4).

Considering individually each criterion analyzed, none of the studies reported information such as experimental blindness (outcome assessment, participants and personnel), which resulted in high risk of bias. In addition, the randomization of housing and outcome of assessment were not accomplished by any work. Therefore, the random sequence generator was used in only one study (7.14%). Five studies did not report the method for randomization (35.71%) and 8 studies did not randomize the animal selection (57.14%). The selection bias criteria consist in conceal allocation and the fact that the animals have the same characteristics at the baseline. The baseline characteristics for animal model were reported in the major of the studies (78.57%, n = 11), while one study did not report this data (7.28%) and two studies do

not start the experiment with similar groups (14.28%), indicating a high risk of bias. On the other hand, the conceal allocation has not been fulfilled for none of studies. Incomplete outcome data was adequately addressed in 78.57% (n = 11) of the studies. Selected studies that are apparently free from other problems that could result in a high risk of bias accounted for 71.43%. Other potential sources of bias were detectable in 4 studies (28.57%), such as animals from different lineage, breeds, sex, weight and clinical condition.

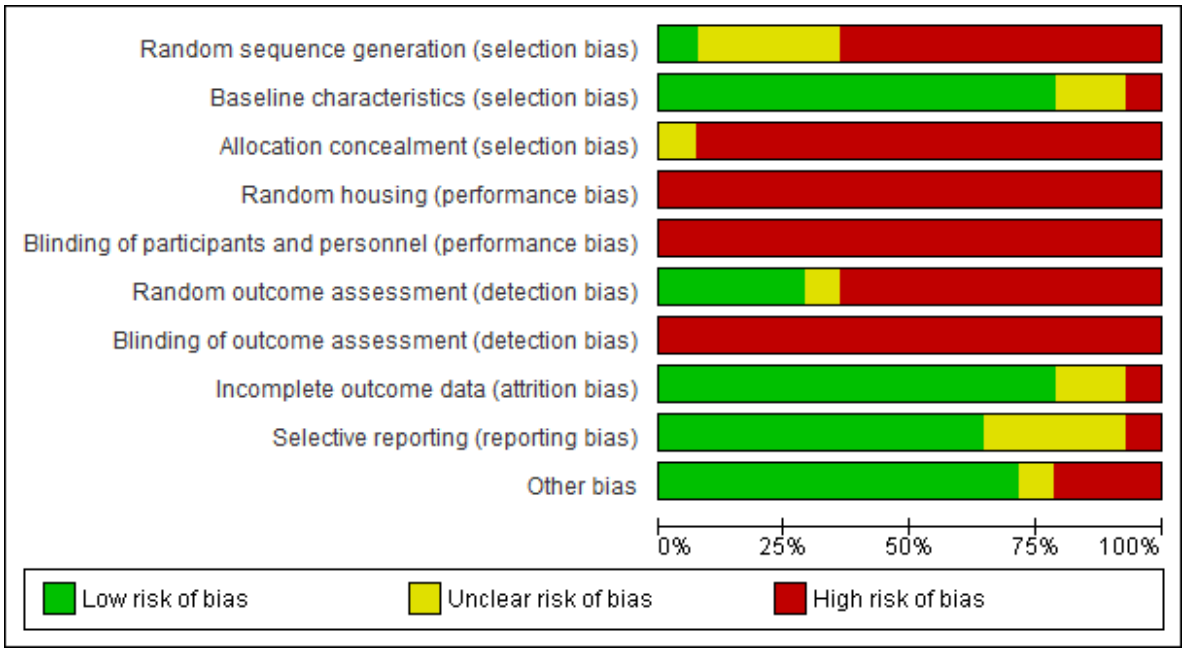


Fig. 3. Results for the risk of bias and methodological quality indicators for all studies included in this systematic review that evaluated the effect of associated drugs for treatment of visceral leishmaniasis. The items in the Systematic Review Centre for Laboratory Animal Experimentation (SYRCLE) Risk of Bias assessment were scored with “yes” indicating low risk of bias, “no” indicating high risk of bias, or “unclear” indicating that the item was not reported, resulting in an unknown risk of bias.

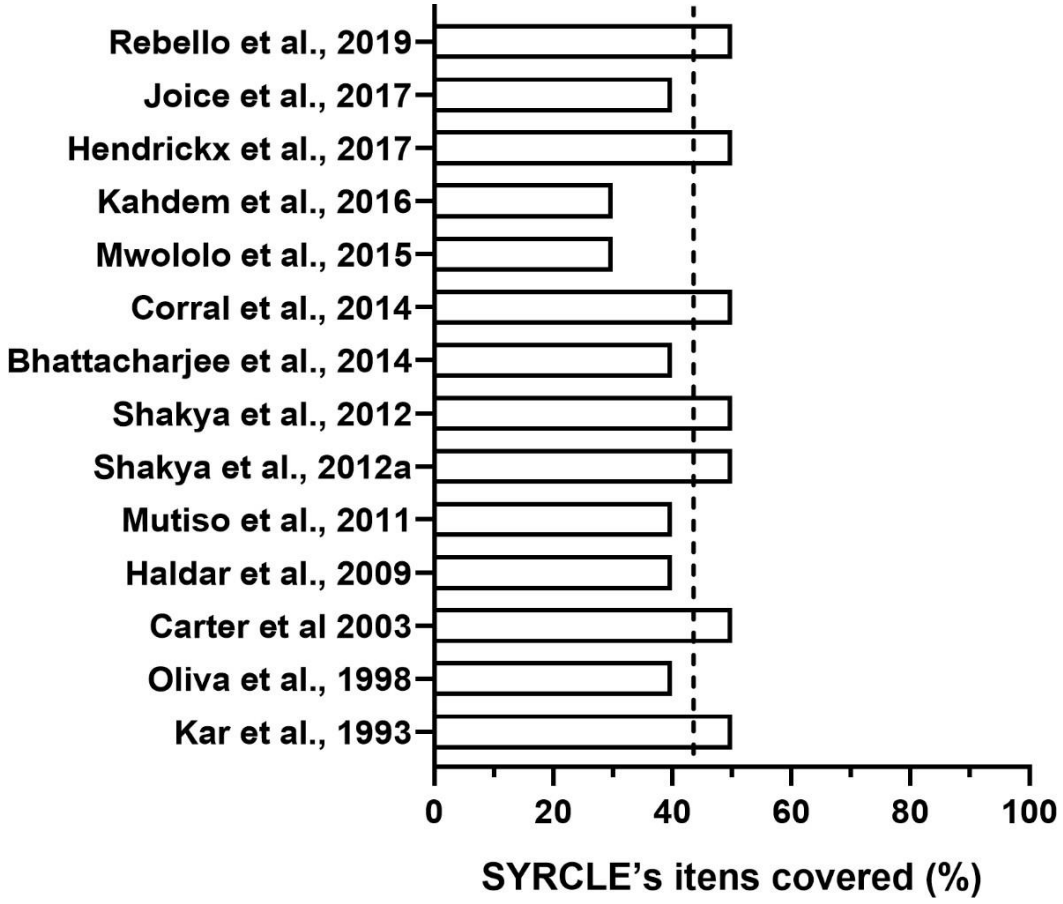


Fig. 4. Analysis of the risk of bias in each study included in the systematic review: Based on the SYRCLE's risk of bias tool for animal studies (Hooijmans *et al.*, 2014). The dotted line indicates the average score obtained for all studies reviewed.

4 Discussion

In absence of effective candidates aimed to immunoprophylaxis against different *Leishmania* strains, chemotherapeutics schemes (e.g. Pentavalent antimonials, amphotericin-B and its formulations, paromomycin and miltefosine, among others) remain the base for treatment, despite high toxicity and low organic tolerability at the host described with its use (Alvar *et al.*, 2006; van Griensven *et al.*, 2010; Shakya *et al.*, 2012a). Though none of these drugs had offer parasitological cure when were use isolatedly, therapeutic schemes of co-administration of antileishmanial drugs had pointed new perspectives of treatments (van Griensven *et al.*, 2010; Shakya *et al.*, 2012a; Rebello *et al.*, 2019). In this context, we conducted a systematic review to evaluate the effectiveness of the drug combination on treatment of

visceral leishmaniasis. In general, almost all studies analyzed provide evidences that the drug combination is a good strategy on treatment of VL, due effectiveness in reduces parasite burden associated at induction of appropriate immune response and consequent improves of the cellular activity, drug efficacy.

Our findings indicate that the studies investigating the impacts of drug association in the treatment of VL in animal model were concentrated in endemic areas. Among forms of leishmaniasis, the visceral or kala-azar, endemic in 98 countries, is the most serious form of the disease representing a major public health problem, with significant morbidity and mortality (Alvar *et al.*, 2012). Although this disease form to be characterized in different parts of world, more than 90% cases of VL are reported from Bangladesh, Brazil, Ethiopia, India, Sudan (Alvar *et al.*, 2012; WHO, 2016). Therefore, the most of studies are concentrated in India, and epidemiological data pointed that this country is responsible for the largest proportion of global VL cases (Alvar *et al.*, 2012). This occur mainly due the vector persistence favored by environmental factors such as humidity, temperature and rainfall, besides the social vulnerability (Singh *et al.*, 2016). These factors added the high incidence and prevalence of VL in these regions had force these countries to adopt measures of prevention and treatment with purpose to decrease the disease impact on population health. In this sense, the governments implementation of the kala-azar elimination program (WHO, 2015; Oryan and Akbari, 2016) with goal to reduce incidence of the disease to less than one case per 10,000 population, mainly in the endemic poor and vulnerable communities. The program covers both prevention and treatment of the disease including the association of drugs as therapy and in fact, progress has been noted in the decrease of the number of cases of VL (Oryan and Akbari, 2016).

Comparatively, VL was also widely distributed in other parts of the world and with the same impact and seriousness to public health (Alvar *et al.*, 2012). Considerably, South America has a large number of VL cases. Despite the high incidence of cases, among the studies selected for this review only one was carried out in South America. In other side of the world, specifically in countries from Europe (e.g. Italy, Belgium, Spain and United Kingdom) and North America (Canada, United States of America), VL is considered an emerging disease. This due mainly due to the introduction of exotic *Leishmania* species into Europe via traveling of humans infected and domestic dogs, besides that the natural spread of them from the Mediterranean region of Europe to neighboring countries (Pérez-Ayala *et al.*, 2009). In this way, studies that evaluated the combined therapy for VL were concentered in India and the other studies were widely distributed among eight different nationalities.

The choice of the animal model reflects the objective work. The most studies used preferably mice (BALB/c lineage) and hamster (golden lineage) as a second choice. Despite extensively used, BALB/c mice do not develop overt progressive disease (Loeuillet *et al.*, 2016). It is known that these animals are able to control Leishmania infection through the generation of NO (Loeuillet *et al.*, 2016). Despite the above data, the laboratory mice are extensively used as model for VL due to each strain are genetically identical, the simplicity of keeping, breeding and reproducing them (Loria-Cervera and Andrade-Narvaez, 2014). In addition, immunological data are easier to obtain using mice due to a large number of tools to investigate these mechanisms.

Interestingly, hamster is considered an excellent choice as animal model for VL and was used in 3 studies (Melby *et al.*, 2001). The choice of this animal model is due its ability to mimics the features of active human disease showing an uncontrolled parasite replication in the liver, spleen, and bone marrow despite a strong Th1-like cytokine. In addition, the clinicopathologic features and immunopathologic mechanisms of VL in the hamster model are remarkably similar to the human disease being unable to control parasite replication (Melby *et al.*, 2001; Loria-Cervera and Andrade-Narvaez, 2014). However, the use of hamster is still limited due to a lack of resources to investigate the role of the immune response in disease (Gupta and Nishi, 2011; Loria-Cervera and Andrade-Narvaez, 2014). Studies using dog model for VL led to better understanding of epidemiology, pathology and immunology of canine leishmaniasis and its genetic basis (Loria-Cervera and Andrade-Narvaez, 2014). Nevertheless, only one studies was found using this experimental model, probably due to high cost of maintenance in large animal facilities and ethical issues basis (Loria-Cervera and Andrade-Narvaez, 2014). Therefore, different experimental models have been used to simulate the *Leishmania* infection. Studies have pointed that a model capable to simulate the human infection (e.g. characterized by progressive increase in visceral parasite burden associated at chronic low-grade fever, weight loss, hepatosplenomegaly, and hypergammaglobulinemia), would be an ideal model to investigate the potentialities of therapeutic schemes in an attempt to minimize the broad spectrum of clinical manifestations of the disease (Loria-Cervera and Andrade-Narvaez, 2014; Saini *et al.*, 2020).

Through of analysis of different studies in this review we note that besides the model used, parameters such as sexes, age and weight were also capable to influence in parasitism of animals infected with *Leishmania*. In general, the most of studies used experimental animals of both sexes in a homogeneous proportion. However, sometimes sex information was not

reported, impairing studies reproducibility. Sex hormones such as androgens and estrogens can interact with cells of the immune system such as macrophages and lymphocytes altering the pattern of immune response for males and females. Testosterone and progesterone are immunosuppressive, reducing the NK cell activity and macrophage cytokines production as well as suppressing NFκB signal transduction. In addition, both hormones can induce a production of Th2-associated anti-inflammatory cytokines such as IL-10 and IL-4 (Piccinni *et al.*, 2000; Snider *et al.*, 2009). This pattern is associated with the susceptible and progression of the VL. Male hamsters infected with *L. donovani* are also more susceptible to disease and have higher parasite burdens than the female counterparts (Travi *et al.*, 2002; Lockard *et al.*, 2019). Treatment with exogenous testosterone enhances the disease in both genders, while castration reduces parasite levels, and ovariectomy promotes the disease (Anuradha *et al.*, 1990; D'Agostino *et al.*, 1999). Despite of this, the sex standardization has been a traditional strategy to control the impact of biological variability, being the use of both sexes extremely important due to the immunological differences between them.

In relation at age and weight, although can change the host response to parasitic infections and influence directly on resistance or susceptibility in VL, the most of studies neglected these parameters. Some authors reinforce that the younger animals are more susceptible to infection and the age-related higher prevalence of disease is most remarkable in visceral leishmaniasis (Müller *et al.*, 2008; Boldizsar *et al.*, 2010; Lockard *et al.*, 2019). The immune response has a curvilinear activation profile and effectiveness through the host life cycle, younger and older animals showed a higher susceptibility to disease. Animal from different ages may differ as the innate and acquired immune responses (Boldizsar *et al.*, 2010; Fuentes *et al.*, 2017). In most studies included in the review, this information was neglected. Thus, the use of different ages impairs the comparison between studies and the understanding to what extent the immune response may have influenced the results of the drug association.

Different species of *Leishmania* was related with a specific pattern of virulence mainly according with geographic region of each strain. However, although the species used to induce the disease are homogenous, in this review, the most of the studies use *L. dononvani*. The use of the strains may be related to the geographic position of the studies. In Old World, visceral leishmaniasis is caused by *L. donovani* and *L. chagasi* (Hendrickx *et al.*, 2017), and the most of the studies found are from the European and Asian continents. The parasite strains have different virulence, pathogenicity and respond to treatment differently (Samarasinghe *et al.*, 2018). In addition, parasite strain variability is an important determinant of disease outcome in

L. infantum infection, with relevant implications for studies on host-pathogen interaction and for leishmanicidal drug development (Loeuillet *et al.*, 2016; Samarasinghe *et al.*, 2018). As well as the *Leishmania* species, the most used strain was MHOM/IN/80/Dd8 and the other researchers used 9 different strains. The virulence factors of *Leishmania* strains greatly influence the course of infection (Samarasinghe *et al.*, 2018). This approach broadens the search for better treatments since several strain present resistance to the drugs commonly prescribed.

The induction of leishmaniasis in experimental animals and the consequent development of the disease do not depends only on the characteristics of the parasite and the susceptibility of the individual. Besides that, several other aspects must be analyzed and considered, since the route of inoculation and the parasite amount can influence on the parasite burden (Loeuillet *et al.*, 2016). In this review, the studies founded showed that doses between $1x10^6$ (medium dose) to $1x10^8$ (higher dose) were more used to induce a considerable degree of parasitism consistent with clinical disease manifestations (Rolão *et al.*, 2004; Oliveira *et al.*, 2012). Studies reinforce that minimal doses (10^3 to 10^4) can induce a strong polarization of the immune response profile to a Th1 pattern (INF-γ and NO production) and the consequent infection resolution (Kaur *et al.*, 2008; Oliveira *et al.*, 2012). However, high doses (above 10^7) led to high parasite burden with the immune response polarized to Th2 (IL-4, IL10 and TGFβ production) (Rolão *et al.*, 2004; Kaur *et al.*, 2008). Therefore, the use of high doses, seen in selected studies, are necessary to determine the effectiveness of new therapies.

The route of inoculation is also a determining factor in the progression of the disease. Kaur and colleges (Kaur *et al.*, 2008) evaluated the parasite load induced by several inoculation routes, and concluded that subcutaneous route is less efficient than intradermal, intraperitoneal and intracardiac routes, in term of liver parasitism. The intracardiac route is responsible for the development of susceptible Th2 immune response and the intraperitoneal promotes a moderate parasite burden and preferentially Th2 pattern (Mukherjee *et al.*, 2003). These two routes were used in 80% of the researches and, once again, these inoculation options are those that generate persistence infection and parasite load.

Once *Leishmania*, obligatory intramacrophagic parasite, penetrates in the organism and the infection is established, the immune system of host cannot fight the parasite and the wide spectrum of clinical manifestations begins to arise. In this infection stage, the determination of therapeutic schemes must be directed to decrease parasitemia and minimize damage at cells and organs of the host. The monotherapy for VL especially using pentavalent antimonials, amphotericin B (either as a deoxycholate or liposomal formulation), paromomycin, and

miltefosine has been shown to be increasingly inefficient, besides display significant toxicities and require long courses of administration (Corral *et al.*, 2014; Joice *et al.*, 2017). Due to low potential microbicide of drugs against *Leishmania* and lack perspective for rational design of new drugs, the combination therapeutic for treatment has emerged as a viable strategy (Khadem *et al.*, 2017). This strategy of combination of drugs for treatment has been successfully in other diseases such as tuberculosis, malaria, leprosy and in cases of co-infection by different pathogens (Nosten and Brasseur, 2002; van Griensven *et al.*, 2010; Ramón-García *et al.*, 2011).

In VL, the major chemotherapeutic associations found in the studies analyzed were using MTF and antimony-based drugs (SSG, SAG, PET). According to Olliaro (Olliaro *et al.*, 2005) this strategy of drug association initially showed as a viable approach mainly due three reasons: i) reduces the treatment time and cost; ii) decreases the emergence of parasite resistance as mode of action of drugs will be different; iii) increases the efficacy rate even in the case of co-infection. The formulation with heavy metal antimony, the SSG, though indicated as the first choice for leishmaniasis treatment by the WHO, presents a complex approach and possesses high cost. The drug is poorly absorbed by oral route and must give via intramuscular or intravenous injections, besides present marked toxicity (i.e. nephrotoxicity, cardiotoxicity, pancreatitis, and bioaccumulation) (Carter *et al.*, 2003; Haldar *et al.*, 2009; Alves *et al.*, 2018). The mechanism of action of pentavalent antimonial (SSG and MEG) still remains poorly understood (Shaked-Mishant *et al.*, 2001). However, Sb(V) would behave as a prodrug, reducing to SB (III) a more toxic form (Shaked-Mishant *et al.*, 2001; Frézard *et al.*, 2014). In addition, these drugs act increasing phagocytosis, production of superoxide anion and TNF-α (Muniz-Junqueira and de Paula-Coelho, 2008). The treatment with SSG in Africa was associated with gastrointestinal symptoms (diarrhea and/or vomiting), increasing risk of death during treatment (Collin *et al.*, 2004). Besides this symptomatology, studies have been pointed that some strains have resistance at the drug. In India, for example, since the late 1990s this has been a constant problem requiring recurrent monitoring (Kar *et al.*, 1993; Carter *et al.*, 2003).

MTF has been most used in India for the treatment of patients with leishmaniasis refractory to conventional treatment with Antimonial, presenting very promising results (Sindermann *et al.*, 2004; Hendrickx *et al.*, 2017). Miltefosine belongs to the class of alkylphosphocholine and is considered an inhibitor of Akt (Dorlo *et al.*, 2012; Srivastava *et al.*, 2017). Initially developed as an anticancer drug, for topical treatment of skin lesions from breast cancer and the oral formulation was used to solid tumors (Sindermann *et al.*, 2004)). The antileishmanial activity of MTF has not been fully described. However, some experiments

evidence that MTF act in lipid metabolism, inhibits transmembrane signals and the synthesis of the cellular membrane, and can triggers apoptosis in promastigotes forms (Rakotomanga *et al.*, 2007; Srivastava *et al.*, 2017).

MTF appears as the best alternative for the treatment of VL, since the drug can be administered orally, facilitating its use by the patient, and there is no need for hospitalization to complete the full dose of treatment (Dorlo *et al.*, 2012; Hendrickx *et al.*, 2017). As well as the SSG, MTF has significant side effects, as well as teratogenic effects and cannot be used by pregnant, besides provoking resistance from the infectious agent to these drugs (Sindermann *et al.*, 2004). Despite that, there are cases of resistance to drug (Rijal *et al.*, 2013).

In attempt of preventing, that Leishmania parasites developing resistance at chemotherapeutic agents and to minimize its toxicity profile at the host, a new therapeutic strategy based on combination treatment of drugs has been a reality (van Griensven *et al.*, 2010; Joice *et al.*, 2017). Many researchers have used this strategy associating sub-doses (i.e. lower than recommended doses) of the drugs commercially prescribed and indicated to VL, including both new as old formulations (Kar *et al.*, 1993; Oliva *et al.*, 1998; Carter *et al.*, 2003; Haldar *et al.*, 2009; Shakya *et al.*, 2012a; b; Corral *et al.*, 2014; Hendrickx *et al.*, 2017; Joice *et al.*, 2017; Rebello *et al.*, 2019).

In this review, eighteen drug formulations were used in seventeen therapeutic combination treatment. The combination aimed to reduce the negative effects of monotherapy such as the high cost, poor compliance and safety, treatment failure and drug resistance. Of all the therapeutic associations, the better results for hamster were showed by Hendrickx *et al.* (2017) with a combination of 20mg/kg MTF + 350 mg/kg PM. According these authors, this therapeutic combination using the fixed-ratio isobologram method, showed drug interaction when hamsters infected with *L. infantum* were treated. They reported cumulative efficacy in reducing parasite burdens in the liver, spleen and bone marrow of animals. In *vitro* results, when the intracellular amastigote stage was repeatedly exposed to the PM+MTF combination, no significant susceptibility decreases towards either drug was noted (Hendrickx *et al.*, 2017). This combination becomes valuable, since there are reports of resistance to treatments using drug associations (García-Hernández *et al.*, 2012). The emergence of drug resistance is a major hurdle in the control of the VL being this approach essential to design a new strategy for treatment.

For dogs, the combinations were not able to achieve clinical or parasitological cure. Nevertheless, the combination showed a second-line for treatment and could represent a good alternative to the current protocols in the treatment of canine leishmaniosis (Oliva *et al.*, 1998).

For studies using mice, the best results were reached by Rebello (2019) showed that LPV (493.2 and 246.6 mg/kg) plus MTF (7.7 mg/kg) virtually eliminated the parasite infection. In addition, in mice we found an immunological approach in the studies (Haldar *et al.*, 2009; Shakya *et al.*, 2012a; b; Bhattacharjee *et al.*, 2015; Khadem *et al.*, 2017). The drug combination was able to improve the immunological system resulting in improvement of leishmanicidal defenses. In addition, an effective immune response is required to support anti-leishmanial drugs, patients with comorbid or coinfection can be particularly hard to cure (García-Hernández *et al.*, 2012)

In the other combinations strategies, almost half of the studies totality presented the results of the therapeutic associations both *in vitro* and *in vivo*. Over half described the pharmacological interaction between the drugs used. *In vitro* studies are extremely important, bringing baseline approaches to animal models. In addition, this approach helps in the adjustment of doses, allowing amplification of possibilities and perspectives in minimize the drugs toxicological profile (Tegazzini *et al.*, 2016). The synergistic effect was the most seen, indicating a combined action of drugs without interferences. Moreover, some studies described the association as additive effect when evaluated *in vivo*, showing an improve leishimanicid effect in sub doses (Haldar *et al.*, 2009; Corral *et al.*, 2014; Rebello *et al.*, 2019) In general, in almost totality of reported cases of therapeutic combinations the results were consistent with increase of the treatment efficacy compared at monotherapy, improved of protection parameters against lipid peroxides, reduction the side effects drugs caused at the host, and decrease of effective cost and the treatment duration, facilitating the use by patient. Besides that, they showed improved of the effect leishmanicid with decrease parasitism.

Moreover, more than two thirds of the studies reported improvement in microbicide defense mechanisms such as the production of ROS and RNS, production of cytokines Th1 pattern (IFN-γ, IL-12), and still reported low toxicity of the new treatments. The success in treatment for VL is associated to a development of an effective immune response, Th1 pattern, able to activate macrophages to produce ROS and RNS to kill intracellular amastigotes (Kaur *et al.*, 2008; Khadem *et al.*, 2017). In this way, many researches try to induce a Th1 response as a therapeutic strategy (Haldar *et al.*, 2009; Mutiso *et al.*, 2011; Shakya *et al.*, 2012a; b; Bhattacharjee *et al.*, 2015; Khadem *et al.*, 2017).

Methodological limitations were objectively identified from the analysis of reporting quality. Even considering the specificities of each research design in the context of a bias analysis, no study fulfilled all methodological criteria, with an average of 43.57% fulfilled criteria. In addition, presenting variable methodological scores without a temporal influence (year of publication). Surprisingly, over half of the essential criteria to be reported in *in vivo* animal studies were neglected. There is no doubt that under-reported aspects such as randomization of animals, allocation concealment, blinding of the participants, personal and outcome assessment, and incomplete outcomes are serious limitations to the internal and external validity of the included studies, impairing experimental reproducibility and the reliability of results. The analysis of methodological bias corroborated the low reporting quality, showing a high or unknown risk of bias for most studies in the majority of categories evaluated. The same baseline characteristics are the best performed criteria by studies. Nevertheless, studies using dogs do not have animals with the same baseline. These animals are naturally infected and present different breed, sex, weight and age adding a methodological limitation.

Based on this systematic review, it was possible to conclude that the drug combination is the best strategy on treatment of VL, due to the effectiveness in reducing parasite burden associated with the induction of immune response polarized for the Th1 type pattern, and consequently improving the microbicide activity. In addition, this approach reduces the cost, adverse effects, time, and doses of treatment making the therapy more acceptable to the patient.

4. Conflict of Interest

The authors declare that they have no conflict of interest.

5. Acknowledgments

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Supplementary Material

Table S1. Search filters used in PUBMED/MEDLINE and SCOPUS databases.

PUBMED
Animal filter 1
["animal experimentation"[MeSH Terms] OR "models, animal"[MeSH Terms] OR "invertebrates"[MeSH Terms] OR "Animals"[Mesh:noexp] OR "animal population groups"[MeSH Terms] OR "chordata"[MeSH Terms:noexp] OR "chordata, nonvertebrate"[MeSH Terms] OR "vertebrates"[MeSH Terms:noexp] OR "amphibians"[MeSH Terms] OR "birds"[MeSH Terms] OR "fishes"[MeSH Terms] OR "reptiles"[MeSH Terms] OR "mammals"[MeSH Terms:noexp] OR "primates"[MeSH Terms:noexp] OR "artiodactyla"[MeSH Terms] OR "carnivora"[MeSH Terms] OR "cetacea"[MeSH Terms] OR "chiroptera"[MeSH Terms] OR "elephants"[MeSH Terms] OR "hyraxes"[MeSH Terms] OR "insectivora"[MeSH Terms] OR "lagomorpha"[MeSH Terms] OR "marsupialia"[MeSH Terms] OR "monotremata"[MeSH Terms] OR "perissodactyla"[MeSH Terms] OR "rodentia"[MeSH Terms] OR "scandentia"[MeSH Terms] OR "sirenia"[MeSH Terms] OR "xenarthra"[MeSH Terms] OR "haplorhini"[MeSH Terms:noexp] OR "strepsirhini"[MeSH Terms] OR "platyrrhini"[MeSH Terms] OR "tarsii"[MeSH Terms] OR "catarrhini"[MeSH Terms:noexp] OR "cercopithecidae"[MeSH Terms] OR "hylobatidae"[MeSH Terms] OR "hominidae"[MeSH Terms:noexp] OR "gorilla gorilla"[MeSH Terms] OR "pan paniscus"[MeSH Terms] OR "pan troglodytes"[MeSH Terms] OR "pongo pygmaeus"[MeSH Terms]]
Animal filter 2
[animals[tiab] OR animal[tiab] OR mice[Tiab] OR mus[Tiab] OR mouse[Tiab] OR murine[Tiab] OR woodmouse[tiab] OR rats[Tiab] OR rat[Tiab] OR murinae[Tiab] OR muridae[Tiab] OR cottonrat[tiab] OR cottonrats[tiab] OR hamster[tiab] OR hamsters[tiab] OR cricetinae[tiab] OR rodentia[Tiab] OR rodent[Tiab] OR rodents[Tiab] OR pigs[Tiab] OR pig[Tiab] OR swine[tiab] OR swines[tiab] OR piglets[tiab] OR piglet[tiab] OR boar[tiab] OR boars[tiab] OR "sus scrofa"[tiab] OR ferrets[tiab] OR ferret[tiab] OR polecat[tiab] OR polecats[tiab] OR "mustela putorius"[tiab] OR "guinea pigs"[Tiab] OR "guinea pig"[Tiab] OR cavia[Tiab] OR callithrix[Tiab] OR marmoset[Tiab] OR marmosets[Tiab] OR cebuella[Tiab] OR hapale[Tiab] OR octodon[Tiab] OR chinchilla[Tiab] OR chinchillas[Tiab] OR gerbillinae[Tiab] OR gerbil[Tiab] OR gerbils[Tiab] OR jird[Tiab] OR jirds[Tiab] OR merione[Tiab] OR meriones[Tiab] OR rabbits[Tiab] OR rabbit[Tiab] OR hares[Tiab] OR hare[Tiab] OR diptera[Tiab] OR flies[Tiab] OR fly[Tiab] OR dipteral[Tiab] OR drosphila[Tiab] OR drosophilidae[Tiab] OR cats[Tiab] OR cat[Tiab] OR carus[Tiab] OR felis[Tiab] OR nematoda[Tiab] OR nematode[Tiab] OR nematoda[Tiab] OR nematode[Tiab] OR nematodes[Tiab] OR sipunculida[Tiab] OR dogs[Tiab] OR dog[Tiab] OR canine[Tiab] OR canines[Tiab] OR canis[Tiab] OR sheep[Tiab] OR sheeps[Tiab] OR mouflon[Tiab] OR mouflons[Tiab] OR ovis[Tiab] OR goats[Tiab] OR goat[Tiab] OR capra[Tiab] OR capras[Tiab] OR rupicapra[Tiab] OR chamois[Tiab] OR haplorhini[Tiab] OR monkey[Tiab] OR monkeys[Tiab] OR anthropoidea[Tiab] OR anthropoids[Tiab] OR saguinus[Tiab] OR tamarin[Tiab] OR tamarins[Tiab] OR leontopithecus[Tiab] OR hominidae[Tiab] OR ape[Tiab] OR apes[Tiab] OR pan[Tiab] OR paniscus[Tiab] OR "pan paniscus"[Tiab] OR bonobo[Tiab] OR bonobos[Tiab] OR troglodytes[Tiab] OR "pan troglodytes"[Tiab] OR gibbon[Tiab] OR gibbons[Tiab] OR siamang[Tiab] OR siamangs[Tiab] OR nomascus[Tiab] OR symphalangus[Tiab] OR chimpanzee[Tiab] OR chimpanzees[Tiab] OR prosimians[Tiab] OR "bush baby"[Tiab] OR prosimian[Tiab] OR bush babies[Tiab] OR galagos[Tiab]

Table S1. Search filters used in PUBMED/MEDLINE and SCOPUS databases

PUBMED
Animal filter 2
Or galago[Tiab] OR pongidae[Tiab] OR gorilla[Tiab] OR gorillas[Tiab] OR pongo[Tiab] OR pygmaeus[Tiab] OR "pongo pygmaeus"[Tiab] OR orangutans[Tiab] OR pygmaeus[Tiab] OR lemur[Tiab] OR lemurs[Tiab] OR lemuridae[Tiab] OR horse[Tiab] OR horses[Tiab] OR pongo[Tiab] OR equus[Tiab] OR cow[Tiab] OR calf[Tiab] OR bull[Tiab] OR chicken[Tiab] OR chickens[Tiab] OR gallus[Tiab] OR quail[Tiab] OR bird[Tiab] OR birds[Tiab] OR quails[Tiab] OR poultry[Tiab] OR poultries[Tiab] OR fowl[Tiab] OR fowls[Tiab] OR reptile[Tiab] OR reptilia[Tiab] OR reptiles[Tiab] OR snakes[Tiab] OR snake[Tiab] OR lizard[Tiab] OR lizards[Tiab] OR alligator[Tiab] OR alligators[Tiab] OR crocodile[Tiab] OR crocodiles[Tiab] OR turtle[Tiab] OR turtles[Tiab] OR amphibian[Tiab] OR amphibians[Tiab] OR amphibia[Tiab] OR frog[Tiab] OR frogs[Tiab] OR bombina[Tiab] OR salientia[Tiab] OR toad[Tiab] OR toads[Tiab] OR "epidalea calamita"[Tiab] OR salamander[Tiab] OR salamanders[Tiab] OR eel[Tiab] OR eels[Tiab] OR fish[Tiab] OR fishes[Tiab] OR pisces[Tiab] OR catfish[Tiab] OR catfishes[Tiab] OR siluriformes[Tiab] OR arius[Tiab] OR heteropneustes[Tiab] OR sheatfish[Tiab] OR perch[Tiab] OR perches[Tiab] OR percidae[Tiab] OR perca[Tiab] OR trout[Tiab] OR trouts[Tiab] OR char[Tiab] OR chars[Tiab] OR salvelinus[Tiab] OR "fathead minnow"[Tiab] OR minnow[Tiab] OR cyprinidae[Tiab] OR carps[Tiab] OR carp[Tiab] OR zebrafish[Tiab] OR zebrafishes[Tiab] OR goldfish[Tiab] OR goldfishes[Tiab] OR guppy[Tiab] OR guppies[Tiab] OR chub[Tiab] OR chubs[Tiab] OR tinca[Tiab] OR barbels[Tiab] OR barbus[Tiab] OR pimephales[Tiab] OR promelas[Tiab] OR "poecilia reticulata"[Tiab] OR mullet[Tiab] OR mullets[Tiab] OR seahorse[Tiab] OR seahorses[Tiab] OR mugil curema[Tiab] OR atlantic cod[Tiab] OR shark[Tiab] OR sharks[Tiab] OR catshark[Tiab] OR anguilla[Tiab] OR salmonid[Tiab] OR salmonids[Tiab] OR whitefish[Tiab] OR whitefishes[Tiab] OR salmon[Tiab] OR salmons[Tiab] OR sole[Tiab] OR solea[Tiab] OR "sea lamprey"[Tiab] OR lamprey[Tiab] OR lampreys[Tiab] OR pumpkinseed[Tiab] OR sunfish[Tiab] OR sunfishes[Tiab] OR tilapia[Tiab] OR tilapias[Tiab] OR turbot[Tiab] OR turbots[Tiab] OR flatfish[Tiab] OR flatfishes[Tiab] OR sciuridae[Tiab] OR squirrel[Tiab] OR squirrels[Tiab] OR chipmunk[Tiab] OR chipmunks[Tiab] OR suslik[Tiab] OR susliks[Tiab] OR vole[Tiab] OR voles[Tiab] OR lemming[Tiab] OR lemmings[Tiab] OR muskrat[Tiab] OR muskrats[Tiab] OR lemmus[Tiab] OR otter[Tiab] OR otters[Tiab] OR marten[Tiab] OR martens[Tiab] OR martes[Tiab] OR weasel[Tiab] OR badger[Tiab] OR badgers[Tiab] OR ermine[Tiab] OR mink[Tiab] OR minks[Tiab] OR sable[Tiab] OR sables[Tiab] OR gulo[Tiab] OR gulos[Tiab] OR wolverine[Tiab] OR wolverines[Tiab] OR minks[Tiab] OR mustela[Tiab] OR llama[Tiab] OR llamas[Tiab] OR alpaca[Tiab] OR alpacas[Tiab] OR camelid[Tiab] OR camelids[Tiab] OR guanaco[Tiab] OR guanacos[Tiab] OR chiroptera[Tiab] OR chiropteras[Tiab] OR bat[Tiab] OR bats[Tiab] OR fox[Tiab] OR foxes[Tiab] OR iguana[Tiab] OR iguanas[Tiab] OR xenopus laevis[Tiab] OR parakeet[Tiab] OR parakeets[Tiab] OR parrot[Tiab] OR parrots[Tiab] OR donkey[Tiab] OR donkeys[Tiab] OR mule[Tiab] OR mules[Tiab] OR zebra[Tiab] OR zebras[Tiab] OR shrew[Tiab] OR shrews[Tiab] OR bison[Tiab] OR bisons[Tiab] OR buffalo[Tiab] OR buffaloes[Tiab] OR deer[Tiab] OR deers[Tiab] OR bear[Tiab] OR bears[Tiab] OR panda[Tiab] OR pandas[Tiab] OR "wild hog"[Tiab] OR "wild boar"[Tiab] OR fitchew[Tiab] OR fitch[Tiab] OR beaver[Tiab] OR beavers[Tiab] OR jerboa[Tiab] OR jerboas[Tiab] OR capybara[Tiab] OR capybaras[Tiab]] NOT medline[subset]]

Table S1. Continuation

PUBMED
Visceral leishmaniasis
["leishmaniasis, visceral"[mesh terms] or “visceral leishmaniasis” [tiab] or "leishmania infantum"[mesh terms] or "leishmania infantum"[tiab] or “leishmania infantum chagasi” [tiab] or "leishmania donovani"[mesh terms] or "leishmania donovani"[tiab]]

PUBMED
Drugs
[“amphotericin b” [mesh terms] or “amphotericin b” [tiab] or “pentavalent antimonials” [tiab] or “pentamidine” [mesh terms] or “pentamidine” [tiab] or “miltefosine” [tiab] or “paromomycin” [mesh terms] or “paromomycin” [tiab] or “antifungal agents” [mesh terms] or “antifungal agents” [tiab] or “liposomal amphotericin b” [tiab] or “azoles”[mesh terms] or “azoles” [tiab]

The search stratey used for Web of Science

Web of Science
TS=(Visceral AND leishmaniasis) OR TS=(Leishmania AND infantum) OR TS=(Leishmania AND infantum AND chagasi) OR TS=(Leishmania AND donovani)TS=(Animal) OR TS=(Animal model) OR TS=(Murine AND model) OR TS=(Animals) OR TS=(Rodent) OR TS=(Mice) OR TS=(Rat) OR TS=(Rats) OR TS=(Guinea AND pig) OR TS=(Hamster) OR TS=(Dog) OR TS=(Dogs) TS=(Amphotericin AND B) OR TS=(Pentavalent AND Antimonies) OR TS=(Pentamidine) OR TS=(Miltefosine) OR TS=(Paromomycin) OR TS=(Antifungal AND Agents) OR TS=(Liposomal AND Amphotericin AND B) OR TS=(azoles)

The search stratey used for Scopus

Scopus
(title-abs-key (amphotericin b) or title-abs-key (pentavalent antimonials) or title-abs-key (pentamidine) or title-abs-key miltefosine) or title-abs-key (paromomycin) or title-abs-key (antifungal agents) or title-abs-key (liposomal amphotericin b) or title-abs-key (azoles) and title-abs-key (leishmaniasis, visceral) or title-abs-key (leishmania infantum) or title-abs-key (leishmania infantum chagasi) or title-abs-key (leishmania donovani))

Table S2. General characteristics of the animal models used in all studies included in the systematic review.

Author	Country	Animal	Lineage	Sex	Age/Weight	Number animals	Control
Kar et al., 1993	IN	Hamster	Golden	♂	?/?	?	SSG/PEN /ALO
Oliva et al., 1998	IT	Domestic dogs	Various breeds	?	2-10 y/?	32	MEG/PAR
Carter et al 2003	UK	Mice	Balb/c	♀/♂	?/20-25g	?	SSG
Haldar et al., 2009	IN	Mice	Balb/c	?	4-6w/?	?	SSG
Mutiso et al., 2011	KE	Mice	Balb/c	♀/♂	6-8 w/?	30	AMB
Shakya et al., 2012a	IN	Mice	Balb/c	♀/♂	?/18-20g	?	MTF
Shakya et al., 2012	IN	Mice	Balb/c	♀/♂	?/18-20g	?	MTF
Bhattacharjee et al., 2014	IN	Mice	Balb/c	?	4-6w/?	20	SSG
Corral et al., 2014	ES	Hamster	?	♀	?/80-90g	35	AMB

ES: Spain; **IN**: India; **CA**: Canada; **US**: United States of America; **IT**: Italy **KE**: Kenya; **BE**: Belgium; **BR**: Brazil; **y**: years; **w**: week; ♀: female; ♂: male; **?**: data not reported; **POS**: Posaconazole; **SSG**: Sodium Stibogluconate; **MEG**: Meglumine Antimoniate; **PAR**: Paromomycin; **MTF**: Miltefosine; **AMB**: Amphotericin B; **MET**: Metronidazole; **KET**: Ketoconazole**.**

Table S2. General characteristics of the animal models used in all studies included in the systematic review.

Author	Country	Animal	Lineage	Sex	Age/Weight	Number animals	Control
Mwololo et al., 2015	KE	Mice	Balb/c	♀/♂	6-8 w/?	40	AMB
Kahdem et al., 2016	CA	Mice	Balb/c	♀	6-8w/20g	?	AMB
Hendrickx et al., 2017	BE	Hamster	Golden	♀	?/80-100g	45	MTF
Joice et al., 2017	US	Mice	Balb/c	♀	6-8w/?	?	POS
Rebello et al., 2019	BR	Mice	Balb/c	♀	6-8w/?	?	MTF

ES: Spain; **IN**: India; **CA**: Canada; **US**: United States of America; **IT**: Italy **KE**: Kenya; **BE**: Belgium; **BR**: Brazil; **y**: years; **w**: week; ♀: female; ♂: male; **?**: data not reported; **POS**: Posaconazole; **SSG**: Sodium Stibogluconate; **MEG**: Meglumine Antimoniate; **PAR**: Paromomycin; **MTF**: Miltefosine; **AMB**: Amphotericin B; **MET**: Metronidazole; **KET**: Ketoconazole**.**

Table S3. General characteristics of the infection models used in all studies included in the systematic review.

Author	*Leishmania spp.*	*Leishmania* strain	N° parasite inoculated	Inoculation route
Kar et al., 1993	*L. donovani*	MHOM/ET/67/HU3	$5x10^7$	intravenous
Oliva 1998	*?*	?	?	naturally infected
Carter et al 2003	*L. donovani*	200011 and 20016	$2x10^7$	intravenous
Haldar et al., 2009	*L. donovani*	MHOM/IN/83/AG83 and MHOM/IN/89/GE1F8R	$5x10^6$	intracardiac
Mutiso 2011	*L. donovani*	MHOM/KE/82/LRC-L445/NLB-065	$1x10^6$	intraperitoneal
Shakya et al., 2012a	*L. donovani*	MHOM/KE/82/LRC-L445/NLB-065	$1x10^6$	intraperitoneal
Shakya et al., 2012	*L. infantum*	MHOM/MA/67/ITMAP263	$2x10^7$	intracardiac
Bhattacharjee et al., 2014	*L. donovani*	MHOM/IN/89/GE1F8R	$1x10^7$	intravenous
Corral et al., 2014	*L. infantum*	MCAN/ES/97/10.445	$1x10^7$	intraperitoneal

?: data not reported

Table S3. General characteristics of the infection models used in all studies included in the systematic review.

Author	*Leishmania spp.*	*Leishmania* strain	N° parasite inoculated	Inoculation route
Mwololo et al., 2015	*L. donovani*	MHOM/KE/82/LRC-L445/NLB-065	1x106	intraperitoneal
Kahdem et al., 2016	*L. donovani*	MHOM/ET/67/HU3	5x107	intravenous
Hendrickx et al., 2017	*L. infantum*	MHOM/MA/67/ITMAP263	2x107	intracardiac
Joice et al., 2017	*L. donovani*	MHOM/ET/67:LV82	5x107	intravenous
Rebello 2019	*L. infantum*	MHOM/MA/67/ITMAP-263	1x108	intraperitoneal

?: data not reported

Table S4. General characteristic of the treatment protocols used in all studies included in the systematic review.

Author	Combination chemotherapy	Dose	Frequency	Route
Kar et al., 1993	*Trans*-aconitic acid (TAA) + Sodium stibogluconate (SSG) or Pentamidine (PET) or Allopurinol (ALL)	TAA (200 or 400 mg/kg); SSG (50 mg of Sb^v/kg); PET (8 mg/kg); ALL (15 mg/kg)	Daily	p.o. (TAA, ALL); ip. (SSG, PET)
Oliva 1998	Paromomycin (PAR) + Meglumine antimoniate (MEG)	PAR (3.5 mg/kg); MEG (20 Sb/kg)	PAR (Daily); MEG (bis in d.)	sc/im
Carter et al 2003	Buthionine sulfoximine (BSO) + Sodium stibogluconate (SSG)	BSO (34 mg/kg); SSG (70 or 282 mg of Sb^v/kg)	Once	iv/iv
Haldar et al., 2009	Diperoxovanadate (DPV6) + Sodium antimony gluconato (SAG)	PV6 (0.5 µmol/30g bw) SSG (50 mg/kg)	alt. d. (DPV6) bis in 7d. (SSG)	ip/im
Mutiso et al., 2011	Diminazene (DIM) + Artesunate (ART)	DIM (12.5 mg/kg); ART (12.5 mg/kg)	Daily	?
Shakya et al., 2012a	PAM3Cys + Miltefosine (MTF)	PAM3Cys (100 µg/animal) MTF (2.5, 5, 20 mg/kg)	Once (PAM3Cys) Daily (MTF)	ip/p.o.
Shakya et al., 2012	Tufisin (TUF) + Miltefosine (MTF)	TUF (60 µg/animal) MTF (2.5, 5, 20 mg/kg)	Once (TUF) Daily (MTF)	ip/p.o.
Bhattacharjee et al., 2014	Glycyrrhizic acid (GA) + Sodium antimony gluconato (SAG)	GA (5, 10, 25, 50, 75 mg/kg) SAG (250 mg/kg)	alt. d.	ip/im
Corral et al., 2014	Allicin (ALI) + Amphotericin B (AMB)	ALI (5 mg/kg) AMB (1, 5 mg/kg)	Daily	ip/ip

SSG: Sodium Stibogluconate; **ATO**: Atovaquone; **PAR**: Paromomycin; **MEG**: Meglumine Antimoniate; **EFC**: Enrofloxacin; **KET**: Ketoconazole**; MET**: Metronidazole; **DIM**: Diminazene; **ART**: Artesunate; **MTF**: Miltefosine; **LPV**: Lopinavir. **SbV**: pentavalent antimony; **ip**: intraperitoneal; **p.o.**: per oral route; **sc**: subcutaneous; **im**: intramuscular. **alt. d.**: every alternate day; **bis in 7d**: twice in week; **bis in d.**: twice in day.

Table S4. General characteristic of the treatment protocols used in all studies included in the systematic review.

Author	Combination chemotherapy	Dose	Frequency	Route
Mwololo et al., 2015	Diminazene (DIM) + Chloroquine (CHQ)	DIM (12. mg/kg) CHQ (12. mg/kg)	Daily	ip/ip
Kahdem et al., 2016	CAL-101 (CAL) + Amphotericin B (AMB)	CAL (0.05 mg/animal) AMB (0.1 mg/kg)	Daily	ip/ip
Hendrickx et al., 2017	Paromomycin (PAR) + Miltefosine (MTF)	MTF (10 or 20 mg/Kg); PAR (180 or 350 mg/Kg)	Daily	p.o./ip.
Joice et al., 2017	DB766 + Posaconazole (POS) or Ketoconazole (KET)	POS and KET (7.5, 15, 30 mg/kg); DB766 (19, 38, 75 mg/kg)	Daily	p.o.
Rebello 2019	Miltefosine (MTF) + Lopinavir (LPV)	MTF (1.92; 3.85; 7.7 mg/Kg); LPV (246.6; 493.2 mg/Kg)	bis in 7d	p.o.

SSG: Sodium Stibogluconate; **ATO**: Atovaquone; **PAR**: Paromomycin; **MEG**: Meglumine Antimoniate; **EFC**: Enrofloxacin; **KET**: Ketoconazole**; MET**: Metronidazole; **DIM**: Diminazene; **ART**: Artesunate; **MTF**: Miltefosine; **LPV**: Lopinavir. **SbV**: pentavalent antimony; **ip**: intraperitoneal; **p.o.**: per oral route; **sc**: subcutaneous; **im**: intramuscular. **alt. d.**: every alternate day; **bis in 7d**: twice in week; **bis in d.**: twice in day.

Table S5: Pharmacological classes of drugs used in this systematic review.

Classes	Drugs
Pentavalent antimonial	Pentostan; Sodium stibogluconate; Meglumine antimoniate
Antifungals	Metronidazole; Amphotericin B; Fluconazole; Ketoconazole; Posaconazole
Antibiotic	Pentamidine; Aminosidine sulphate / paramomycin; Spiramycin
Anticancer	Buthionine sulfoximine; Miltefosine
Uricosuric	Allopurinol
Anthelmintic	Levamisole
Antiprotozoals	Atovaquone; Diminazene; Artesunate; Diminazene diaceturate; Chloroquine
Antiretroviral	Nelfinavir
Others	Bovine thymic extract; Trans aconitic acid; Picroliv; 89-215(hexapeptide Val-Glu-Pro-Ile-Gly-Tyr); N. ac-norMur-MeVal-DisoGln (86/448); N.AcMur-Acc-DisoGln (89/729); Stearyllamine Cationic lipossome; Peroxovanadates; Stearylamine bearing phosphatidylcholine; CpG-ODN-2006; Allicin; Triterpenoid glycyrrhizic; p110d-specific pharmacological innibitors; Chemotype 101R; Pyrazolepyridine derivate; DB766; Pam3Cys; p-Tuftsin