ADRIANA CARNEIRO DA SILVA

USO DA CITOMETRIA DE FLUXO PARA QUANTIFICAÇÃO DE *Leishmania* sp. INTRACELULAR

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 M. da Silva

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BIOGRAFIA

Adriana Carneiro da Silva nasceu na cidade de Patos de Minas/MG, no dia 21 de outubro de 1983, filha de Armindo Carneiro da Silva e Otacília Dias Cardoso. Estudou por toda infância na Escola Estadual Carlos Nogueira da Gama, no munícipio de Reduto/MG. No período do ensino médio, estudou na Escola Estadual Maria de Lucca Pinto Coelho, na cidade de Manhuaçu/MG. No ano de 2003, foi aprovada no curso de Farmácia, na Faculdade Federal de Ouro Preto (UFOP), onde se formou em 2008. Após a formatura, veio para Viçosa/MG, onde trabalhou no Laboratório de Análises Clínicas Santa Rita de Cássia, como Responsável Técnica da unidade hospitalar de 2008 a 2015. Em 2010, especializou-se em Análises Clínicas pela UFOP em parceria com a Sociedade Brasileira de Análises Clínicas (SBAC). No ano de 2015, foi aprovada no mestrado do Programa de Pós-Graduação em Biologia Celular e Estrutural da Universidade Federal de Viçosa (UFV), sob orientação do Professor Eduardo de Almeida Marques da Silva, onde desenvolveu pesquisa de avaliação *in vitro* do potencial leishmanicida de derivados triazólicos e de benzofenonas. No ano de 2017, obteve o título de mestre em Biologia Celular e Estrutural e foi aprovada no doutorado no mesmo programa de Pós-Graduação, trabalhando principalmente no desenvolvimento de um novo método para quantificação *in vitro* de *Leishmania* intracelular por citometria de fluxo. Em 2020, especializou-se em Saúde e Estética pela FAPUGA/NEPUGA. Em 2020, fundou a microempresa AC-Estética Avançada e, em 2022, lançou, em parceria com a UNIVIÇOSA, a Pós-graduação em Saúde e Estética.

RESUMO

DA SILVA, Adriana Carneiro, D.Sc., Universidade Federal de Viçosa, março de 2022. **Uso da citometria de fluxo para quantificação de *Leishmania* sp. intracelular.** Orientador: Eduardo de Almeida Marques da Silva.

Investir em novas metodologias de pesquisa que tornem o trabalho na busca por novas drogas mais preciso, ágil e com maior padronização é muito importante para o estudo da leishmaniose. A técnica mais utilizada para avaliação do efeito leishmanicida de uma determinada droga *in vitro*, a contagem de lâminas, apesar de consolidada no meio científico, apresenta inúmeras dificuldades em sua execução, avaliação e obtenção de resultados. Além de trabalhosa, esta técnica demanda tempo, tanto para a preparação do material para análise quanto para a própria contagem. Partindo desta necessidade, nosso grupo de pesquisa propõe uma nova metodologia baseada na marcação nuclear, utilizando iodeto de propídeo e citometria de fluxo para quantificação de carga parasitária de *Leishmania* sp. em macrófagos *in vitro*. Nossos resultados mostram que a fluorescência de amostras infectadas aumenta à medida que aumenta a taxa de infecção. Pelo uso de análise de Correlação de Pearson, foi possível estabelecer um coeficiente de correlação (Pearson r = 0,9473) positivo, linear e diretamente proporcional entre as variáveis fluorescência e taxa de infecção. Assim, foi possível inferir uma equação matemática, por meio de regressão linear, para se obter a estimativa do número de parasitos de uma dada amostra por meio de valores de Unidades de Fluorescência Relativa (RFU). Essa nova metodologia abre espaço para a possibilidade de uso desse recurso metodológico na quantificação *in vitro* de *Leishmania* sp. em macrófagos.

Palavras–chave: Citometria de fluxo. Leishmanioses. Iodeto de Propídeo

ABSTRACT

DA SILVA, Adriana Carneiro, D.Sc. Universidade Federal de Viçosa, March 2022. **Use of flow cytometry to quantify intracellular *Leishmania* sp**. Advisor: Eduardo de Almeida Marques da Silva.

The search for new methodologies that make the work in the search for new drugs more precise, agile, and with greater standardization is very important to the study of leishmaniasis. The technique most used to assess the leishmanicidal effect of a given drug in vitro, the slide count, despite being consolidated in the scientific environment, presents several difficulties in its execution, assessment, and results. In addition to being laborious, this technique takes time, both for the preparation of the material for analysis and for the counting itself. Based on this need, our research group proposes a new methodology based on nuclear labeling, using propidium iodide and flow cytometry to quantify the parasite load of Leishmania sp. in macrophages in vitro. Our results show that the fluorescence of infected samples increases as the infection rate increases. Using Pearson's Correlation analysis, it was possible to establish a correlation coefficient (Pearson r = 0.9473) that was positive, linear, and directly proportional to the variables fluorescence and infection rate. Thus, it is possible to infer a mathematical equation, through linear regression, to obtain an estimate of the number of parasites in each sample through the Relative Fluorescence Units (RFU) values. This new methodology opens space for the possibility of using this methodological resource in the in vitro quantification of *Leishmania* sp. in macrophages.

Keywords: Flow cytometry. Leishmaniasis. Propidium iodide.

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ANEXO 1

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1. **INTRODUÇÃO**

1.1 Epidemiologia das leishmanioses

As doenças tropicais negligenciadas (DTN) são um grupo diversificado de 20 condições patológicas causadas por uma série de microrganismos, incluindo bactérias, vírus, protozoários, toxinas e fungos. Elas causam consequências avassaladoras tanto na saúde como nas condições socioeconômicas para bilhões de pessoas, especialmente em países em desenvolvimento da África, Ásia e América Latina (WHO, 2021). São assim classificadas por serem largamente ignoradas pela indústria farmacêutica e devido ao pouco investimento dos órgãos de fomento à saúde pública em pesquisas referentes ao seu controle (Beaumier et al., 2013).

Segundo a Organização Mundial de Saúde (OMS), a epidemiologia das DTN é complexa e muitas vezes relacionada às condições ambientais. Muitos agentes etiológicos relacionados a essas doenças são transmitidas por vetores, possuem reservatórios animais e estão associados a ciclos de vida complexos. Todos esses fatores tornam seu controle um desafio para a saúde pública (WHO, 2021).

Dentre as DTN, a leishmaniose é classificada como uma das parasitoses negligenciadas mais importantes do mundo, com um total de 0,7-1,0 milhão de novos casos por ano em países endêmicos (WHO, 2021). Os agentes infecciosos das diferentes formas clínicas de leishmaniose são parasitos do gênero *Leishmania* que, dependendo da espécie e da relação parasito/hospedeiro, causam as diferentes formas cutâneas e a forma visceral da doença. Sua transmissão se dá pela picada de flebotomíneos fêmeas infectadas, que se alimentam de sangue para produzir ovos (Kaye; Scott, 2011). A epidemiologia da leishmaniose depende das características do parasito e das espécies de flebotomíneos, das características ecológicas locais dos locais de transmissão, da exposição atual e passada da população humana ao parasito e do comportamento humano. Cerca de 70 espécies animais, incluindo humanos, foram encontradas como hospedeiros reservatórios naturais de *Leishmania* (WHO, 2021).

A forma cutânea de leishmaniose (a forma mais comum) está relacionada, muitas vezes, ao surgimento de cicatrizes desfigurantes em partes expostas do corpo, que atingem de sobremaneira as condições físicas e psíquicas dos pacientes afetados (Costa et al., 1987). Segundo a OMS, aproximadamente 95% dos casos de leishmaniose cutânea (LC) ocorrem nas Américas, bacia do Mediterrâneo, Oriente Médio e Ásia Central. A estimativa é que ocorram cerca de 0,6 a 1 milhão de novos casos ao ano, mundialmente. Quando as mucosas também são afetadas, tem-se o quadro de leishmaniose mucocutânea. Neste quadro, há destruição parcial ou total das mucosas de nariz, boca e garganta. Mais de 90% dos casos de leishmaniose mucocutânea ocorrem na Bolívia, Brasil, Etiópia e Peru (WHO, 2021). A leishmaniose visceral (LV), também conhecida como calazar, por sua vez, está relacionada a quadros graves de espleno e hepatomegalia, leucopenia, petéquias, hemorragias das mucosas e emagrecimento que, na ausência de tratamento adequado, pode levar à morte em 95% dos casos (van Griensven et al., 2019; PAHO, 2021). A maioria dos casos ocorre no Brasil, na África Oriental e na Índia. Acredita-se que cerca de 50.000 a 90.000 novos casos de LV ocorram ao ano mundialmente e ela continua sendo uma das doenças parasitárias com maior potencial de surto e mortalidade. Brasil, China, Etiópia, Eritreia, Índia, Quênia, Somália, Sudão do Sul, Sudão e Iêmen abrangem noventa por cento dos casos notificados de LV à OMS em 2020.

A leishmaniose dérmica pós-calazar (LDPK) apresenta-se como uma sequela da LV, cujos sinais são erupção papular, nodular ou macular que ocorrem principalmente nas áreas da face, troncos e braços. A LDPK ocorre principalmente no subcontinente indiano e na África oriental, onde 5 a 10% dos pacientes que apresentaram quadro de LV desenvolvem a forma dérmica. *Leishmania donovani* é o agente causador estabelecido, mas outros como *Leishmania tropica*, *Leishmania infantum* ou *Leishmania chagasi* podem ocasionalmente levar à doença, especialmente com a coinfecção por HIV. As pápulas aparecem de 6 a 12 meses ou mais anos após a cura da LV, porém podem ocorrer mais cedo. Pacientes com este quadro são considerados uma fonte em potencial para transmissão do parasito (Ghosh et al., 2021).

Vários fatores de risco estão associados à ampla expansão das leishmanioses pelo globo terrestre. A pobreza é um deles. Condições sanitárias e de habitação precárias aumentam os locais de procriação dos flebotomíneos, assim como o acesso aos humanos. Habitações lotadas fornecem uma boa fonte de alimentação (Calderon-Anyosa et al., 2018). A falta de nutrientes, como fontes ricas em proteína, ferro, zinco e vitaminas, é um outro fator que contribui para o desenvolvimento do parasito no organismo do hospedeiro. A desorganização do sistema de defesa do hospedeiro como resultado da desnutrição é responsável por infecções assintomáticas e até doenças graves (Nweze et al., 2020). Isso se agrava com a questão da maior mobilidade da população mundial. No mundo globalizado, a mobilidade humana provoca mudanças na epidemiologia das doenças e na disseminação de diversas infecções pelos continentes. As epidemias de LC e LV estão frequentemente ligadas à migração e ao movimento de pessoas não imunes para áreas com ciclos existentes de transmissão. A exposição ocupacional, bem como o desmatamento generalizado, continuam sendo fatores importantes (Akdur et al., 2017; WHO, 2021). Além disso, a ocorrência de leishmaniose pode ser afetada por mudanças na estrutura de urbanização e pela incursão humana em áreas florestais antes isoladas.

Conter a leishmaniose é uma tarefa difícil e complicada, já que existem inúmeros agentes, reservatórios, vetores, situações epidemiológicas diversas, além de conhecimento ainda insuficiente sobre os vários elementos que compõem a cadeia de transmissão. As técnicas de controle desta parasitose ainda são pouco efetivas e estão baseadas em diagnóstico e tratamento precoce dos casos, eliminação dos reservatórios, diminuição da população de flebotomíneos e programas de educação em saúde.

Existem inúmeros desafios no tocante ao controle das leishmanioses. O diagnóstico precoce e o tratamento imediato reduzem a prevalência da doença, além de prevenirem morbidade, mortalidade e reduzirem a transmissão (WHO, 2021).

1.2 Tratamento das leishmanioses e seus desafios

O tratamento de humanos infectados ainda é a melhor e principal forma de controle das diferentes formas clínicas de leishmaniose (Olías-Molero et al., 2021). Este depende de inúmeros fatores: tipo de doença, patologias concomitantes, espécies parasitárias e localização geográfica. A leishmaniose é uma doença tratável e curável, que requer um sistema imunocompetente.

Dos tratamentos disponíveis para leishmaniose, todos enfrentam diferentes obstáculos: desde a complexidade da natureza parasitária até a negligência das grandes empresas farmacêuticas em desenvolver fármacos adequados devido à baixa renda dos países afetados (Taslimi et al., 2018). Atualmente, apenas o antimônio pentavalente é projetado especificamente para o tratamento da leishmaniose. O tratamento com derivados antimoniais causa uma série de efeitos adversos, como mialgia, náuseas, vômitos, pancreatite, arritmia cardíaca, hepatite, o que leva até mesmo ao término do tratamento antes do tempo estipulado (Sundar; Chakravarty, 2010). Outros medicamentos, como: miltefosina, anfotericina B lipossomal, paromomicina e pentamidina também apresentam problemas de segurança, efeitos colaterais, alto custo, necessidade de administração parenteral, além da taxa crescente de falhas no tratamento, o que os torna inaplicáveis para pacientes em áreas endêmicas (Singh et al., 2016; Hendrickx et al., 2018).

Durante décadas, os antimoniais pentavalentes foram as drogas de escolha para o tratamento das leishmanioses, mas a crescente resistência os tornou obsoletos como terapia de primeira linha nas áreas endêmicas, como no subcontinente indiano, por exemplo (Ponte-Sucre et al., 2017). Miltefosina e anfotericina B lipossomal têm sido empregadas como tratamento eficaz de primeira linha. Terapias combinadas têm sido uma opção bem-sucedida no tratamento das leishmanioses (Bastos et al., 2022), mas mesmo estas não estão imunes à seleção de resistência, o que representa desafios potenciais para a próxima geração de terapias combinadas (Hendrickx et al., 2018). Além dos desafios com a resistência aos medicamentos nas leishmanioses humanas, outra situação desafiadora é o amplo uso destes medicamentos na prática veterinária: fato importante que merece atenção, uma vez que cria uma pressão adicional significativa de seleção de drogas para *Leishmania* sp. O tratamento repetido de cães infectados pode selecionar parasitos resistentes, aumentando a transmissão zoonótica a humanos (Noli et al., 2014).

Apesar de grandes esforços realizados na tentativa de se conter as diferentes formas da leishmaniose, os resultados não são como o esperado e a doença continua a atingir a população de diversos países, principalmente na África e nas Américas do Sul e Central (Olías-Molero et al., 2021).

1.3 O desafio de se desenvolver novas drogas

O desenvolvimento de novas drogas para o tratamento das leishmanioses do Velho e do Novo Mundo é urgentemente necessário, porque a falha na terapia é um problema crescente. A resistência aos medicamentos é um fator fundamental para o fracasso do tratamento, embora outros fatores também contribuam para esse evento, como a epidemia global de HIV/AIDS com seu impacto negativo no sistema imunológico (Ponte-Sucre et al., 2017). Além do mais, os fármacos disponíveis atualmente apresentam eficácia variável com efeitos colaterais marcantes, que levam ao abandono do tratamento e a uma seleção natural dos parasitos, como citado anteriormente. Junto a isso, existe a falta de investimento financeiro por parte das grandes indústrias farmacêuticas para o controle das leishmanioses (Briones et al., 2021). Portanto, há uma necessidade contínua e urgente de desenvolvimento de novas terapias contra a leishmaniose que sejam seguras e eficazes na indução de cura.

Um problema encontrado por pesquisadores na busca por novas drogas são as técnicas empregadas nestes estudos. A técnica mais utilizada para avaliar o efeito leishmanicida de uma determinada droga sobre a forma amastigota é a contagem em lâmina de parasitos intracelulares (Guru et al., 1989; Tandon et al., 1991; Roy et al., 2017). Apesar desta metodologia ser consolidada para este tipo de estudo, existem várias dificuldades operacionais relacionadas à avaliação e à obtenção de resultados. Além de trabalhosa, esta técnica demanda tempo tanto para a preparação do material para análise quanto para a própria contagem. Deve-se ressaltar ainda a questão da padronização da proporção de parasitos por macrófagos durante a infecção, a quantidade de células que deverão ser contadas ao microscópio óptico, o corante ideal, bem como o tempo de exposição ao mesmo. A contagem das lâminas também é um processo que pode agregar muitos erros durante sua execução: o ideal é que apenas um indivíduo faça todas as contagens, a fim de reduzir o viés do observador/contador. Porém, esta é umas das questões mais complicadas para se resolver, já que durante a triagem de drogas o número de lâminas é muito elevado. Isso acaba levando à divisão do trabalho e, junto, à soma de erros intrínsecos de cada contador.

O uso de parasitos geneticamente modificados vem ganhando espaço nos meios de pesquisa na área biológica, o que não é diferente para a área de prospecção de novas drogas para o tratamento das leishmanioses e de outras doenças infecciosas. Estudos recentes usam parasitos que expressam proteínas que emitem fluorescência quando submetidos à luz com determinados comprimentos de onda. Isso permite a detecção do parasito intracelular utilizando microscópios de fluorescência, fluorímetros ou mesmo citômetros de fluxo. Desta forma, é possível determinar a carga parasitária indiretamente por intermédio da intensidade de fluorescência emitida nestas condições (Bastos et al.; 2017). Apesar de ser uma excelente opção para a triagem de drogas leishmanicidas, muitas vezes os laboratórios esbarram em questões tais como a dificuldade de obtenção do parasito, a falta de estrutura e de credenciamento para trabalhar com organismos geneticamente modificados ou questões burocráticas que acabam por impedir o uso de tal técnica.

Diante de todos os empecilhos e dificuldades encontrados nas técnicas usadas para o estudo de novas drogas para o tratamento das leishmanioses, propomos avaliar o potencial de uma nova técnica baseada na marcação nuclear utilizando iodeto de propídeo, com leitura de fluorescência emitida em citômetro de fluxo.

As vantagens desta técnica em comparação com a contagem de lâminas ao microscópio óptico seriam: maior rapidez (contagem de células em um tempo menor - milhares de células/segundo - quando comparada à microscopia), minimização de erros decorrentes de análises feitas por diferentes observadores/contadores e maior sensibilidade. Apesar dos procedimentos para a preparação do material para a citometria serem muito parecidos com aqueles utilizados na microscopia, para a primeira não há procedimento posterior de montagem de lâminas. Além disso, os resultados são obtidos e podem ser analisados logo após serem adquiridos pelo citômetro de forma rápida e dinâmica (Azas et al., 1997; Giorgio et al., 2000).

Com a disseminação cada vez maior das leishmanioses pelo globo, a falta de medidas efetivas para evitar sua transmissão e ausência de medicamentos seguros para seu tratamento, parece interessante investir em técnicas que sejam mais confiáveis, de fácil execução, além de ágeis. Isto permitiria um intercâmbio mais eficiente de informações, além de manter um padrão de comparação, tornando a pesquisa por novas drogas para o combate à leishmaniose mais dinâmica e eficaz. Pensando neste cenário, foi proposta a execução deste trabalho

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2. OBJETIVOS

2.1 Objetivo geral

Desenvolver nova técnica em citômetro de fluxo, para contagem de formas evolutivas de *Leishmania* intracelular em macrófagos.

2.2 Objetivos específicos

1. Estabelecer a correspondência entre as taxas de infecção de macrófagos infectados por *L. chagasi* feitas em lâminas e as obtidas pela nova metodologia;

2. Estabelecer se existe um aumento da Unidade Relativa de Fluorescência (RFU) à medida que aumenta a taxa de infecção dos macrófagos;

3. Estabelecer a correlação entre as metodologias estudadas (contagem de lâminas e leitura de fluorescência emitida em citômetro de fluxo de macrófagos infectados marcados com iodeto de propídeo);

4. Estabelecer uma equação matemática para obtenção de número de parasitos/100 macrófagos utilizando RFU.

3. **RESULTADOS**

Os resultados obtidos durante o período de doutorado originaram dois manuscritos:

a. O primeiro manuscrito (Capítulo 1), intitulado “Development of a new method of quantification of intracellular *Leishmania* sp. using flow cytometry”, mostra a possibilidade de se utilizar a citometria de fluxo para quantificação intracelular de *Leishmania* sp. Este manuscrito foi submetido para publicação no periódico científico *“Cytometry – part A”*

b. O segundo manuscrito (Anexo 1), intitulado “Could combination chemotherapy be more effective than monotherapy in the treatment of visceral leishmaniasis? A systematic review of preclinical evidence”, evidencia o uso da quimioterapia combinada, principalmente aquelas baseadas em drogas clássicas, como sendo um tratamento com interações aditivas e sinérgicas, cujo resultado é um efeito leishmanicida e imunomodulador, associado a redução da carga parasitária, a danos aos órgãos e melhores taxas de cura nos quadros de leishmaniose visceral. Este manuscrito foi publicado no periódico científico “*Parasitology*”.

CAPÍTULO 1

Development of a new method of quantification of intracellular *Leishmania* sp. using flow cytometry

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SUMMARY

The search for new methodologies that make the work in the search for new drugs more precise, agile, and with greater standardization is very important to the study of leishmaniasis. The technique most used to assess the leishmanicidal effect of a given drug *in vitro*, the slide count, despite being consolidated in the scientific environment, presents several difficulties in its execution, assessment, and results. In addition to being laborious, this technique takes time, both for the preparation of the material for analysis and for the counting itself. Based on this need, our research group proposes a new methodology based on nuclear labeling, using propidium iodide and flow cytometry to quantify the parasite load of *Leishmania* sp. in macrophages *in vitro*. Our results show that the fluorescence of infected samples increases as the infection rate increases. Using Pearson's Correlation analysis, it was possible to establish a correlation coefficient (Pearson r = 0.9473) that was strongly positive, linear, and directly proportional to the variables fluorescence and infection rate. Thus, it is possible to infer a mathematical equation, through linear regression, to obtain an estimate of the number of parasites in each sample through the Relative Fluorescence Units (RFU) values. This new methodology opens space for the possibility of using this methodological resource in the *in vitro* quantification of *Leishmania* in macrophages.

Keywords: Flow cytometry. Leishmaniasis. Propidium iodide.

KEY FINDINGS

- Fluorescence of *Leishmania*-infected macrophage samples stained with propidium iodide increases as the rate of infection increases;
- There is a positive, linear, and directly proportional correlation coefficient (Pearson r = 0.9473) between the variables fluorescence and infection rate;
- It was possible to infer a mathematical equation, through linear regression, to obtain an estimate of the number of parasites in each sample through the values of Relative Fluorescence Units (RFU);
- It is possible to use this methodological resource in the *in vitro* quantification of *Leishmania* sp. in macrophages.

INTRODUCTION

The development of new drugs that could be used effectively in the treatment of different clinical forms of leishmaniasis is a relevant action to leishmaniasis research. A problem found in research works in the search for new drugs is the techniques used in these studies. The most used technique to assess the leishmanicidal effect of a given drug on the amastigote form is the slide count of intracellular parasites after treatment (Guru et al., 1989; Tandon et al., 1991; Roy et al., 2017). Despite this methodology being consolidated for this type of study, there are several operational difficulties related to the assessment and obtaining results. In addition to being laborious, this technique requires time both for the preparation of the material for analysis and for the counting itself. It should also be highlighted the issue of standardizing the volume capacity of the plate, the number of cells to be counted under the optical microscope, the ideal dye, as well as the exposure time to it. Counting slides is also a process that adds many errors during its execution: ideally, only one individual should perform all the counts, to reduce observer/counter bias. However, this is one of the most complicated issues to solve, since during some procedures, such as drug screening, for example, the number of slides is very high. These results lead to the division of labor and, together, the sum of intrinsic errors of each counter.

The use of genetically modified parasites has been gaining ground in research in the biological area, which is not different in the area of prospecting new drugs for the treatment of leishmaniasis (Pandey et al., 2020). Recent studies use parasites that express proteins that emit fluorescence when subjected to light with certain wavelengths. This allows the visualization of the intracellular parasite in fluorescence microscopes, fluorimeters, or even in flow cytometers. Thus, it is possible to determine the parasite load indirectly through the intensity of fluorescence emitted under these conditions (Bolhassani et al., 2011; Patel et al., 2014; Bastos et al.; 2017). Despite being an excellent option for the screening of leishmanicidal drugs, laboratories often face issues such as the lack of the genetically modified parasite or the lack of structure and accreditation to work with the same modified ones, or even bureaucratic issues that end up impeding the use of this technique.

Due to all the obstacles and difficulties found in the techniques used to study new drugs for the treatment of leishmaniasis, we propose to assess the potential of a new methodology based on nuclear labeling using propidium iodide, with fluorescence reading emitted in a flow cytometer.

Flow cytometry is currently the most used method to establish the nuclear DNA content of plant and animal cells. The technique, based on the use of single or multiple lasers, provides a multi-parametric analysis of the sample (Givan, 2011; Adan et al., 2015; McKinnon, 2018). Each cell or particle is analyzed by visible light scattering or by fluorescence as the cells flow under the incidence of each laser. Independent of light scattering analysis, fluorescence measurements are obtained by transfection and/or expression of common fluorescent proteins, such as green fluorescent protein, (GFP) or Red Fluorescent Protein (RFP), and by labeling with fluorescent-conjugated antibodies or simply using fluorescent dyes such as propidium iodide (PI) (Sereno et al., 2012).

The advantages of this new methodology compared to the counting of slides under the optical microscope would be 1. Quickness: faster (counting cells in a shorter time - thousands of cells/second) when compared to microscopy; 2. Reliability: minimization of errors resulting from analyzes performed by different observers/counters; and 3. Greater sensitivity. Although the procedures for preparing material for cytometry are very similar to those used in microscopy, they do not require slide mounting procedures. In addition, the results are obtained and can be analyzed immediately after being acquired by the cytometer, quickly and dynamically (Azas et al., 1997; Giorgio et al., 2000).

With the increasing spread of leishmaniasis across the globe, the lack of effective measures to prevent its transmission, and the absence of safe drugs for its treatment, it seems interesting to invest in techniques that are more reliable, easy to perform, and agile. This would allow a more efficient exchange of information, in addition to maintaining a standard of comparison, making, for example, the search for new drugs against leishmaniasis more dynamic.

MATERIAL AND METHODS

Parasites

Promastigote forms of *Leishmania chagasi* strain (MHOM/BR/75/M2682) L. chagasi strain MHOM/BR/1975/ M2682, kindly provided by Dra. Norma Maria de Melo, Department of Parasitology, Federal University of Minas Gerais (UFMG), Belo Horizonte, Minas Gerais, Brazil, were cultured in Dulbecco's Modified Eagle Medium (DMEM; SIGMA-ALDRICH, St. Louis, MO, USA) supplemented with 10% human urine, 1% adenine 1 mmol x L-1, 10% Fetal Bovine Serum (FBS; LGC Biotechnology, Cotia, SP, Brazil), 2 mmol x L^{-1} L-glutamine (GIBCO BRL, Grand Island, N.Y., USA), Cleveland, OH, USA), and 0.25% hemin in 50% triethanolamine (SIGMA-ALDRICH), pH 6.5 at 25°C.

Mammal cells

RAW 264.7 macrophages were maintained in DMEM medium (SIGMA-ALDRICH) supplemented with 10% fetal bovine serum (LGC Biotechnology) and penicillin G 100 IU x mL^{-1} (USB Corporation), pH 7.2 at 37°C/5% CO_2.

Macrophage infection and quantification of amastigotes labeled with propidium iodide by flow cytometry

Suspensions containing $1x10^5$ macrophages/well were incubated at 37°C/5% CO_2 for 4 h in a 24-well plate. After this period, we added different concentrations of stationary phase promastigotes to each well (parasites/macrophage: 0/1; 0.2/1; 1/1; 5/1; 10/1; 15/1; 20/1) and the material was incubated for 2 h at 37°C/5% CO_2. Then, each well was washed 3 times with Phosphate-Buffered Saline (PBS). Each plate was again incubated for 24 h at 37°C/5% CO_2. We used 250 µL of 0.2% EDTA solution in PBS to detach the cells adhered to the plate. After 5 minutes, we added 750 µL of DMEM (SIGMA-ALDRICH) to each well. One microliter of the cell suspension was transferred to conical bottom microtubes and centrifuged at 540 x *g*/5 min/4°C. The supernatant was discarded, and the precipitate was suspended in 100 µL of DMEM

(SIGMA-ALDRICH). This suspension was dropped into 400 µL of 4% formalin solution in the vortex to fix the cells. We added 50 µL of propidium iodide at 0.5 µg x mL-1 to each microtube. We incubated the samples for 30 minutes in the dark and at room temperature. From each sample, we took a fraction to make slides for counting under an optical microscope. The remainder was acquired on a flow cytometer, in a total of 10,000 events for each sample. We used the FACSuite Software® program for data acquisition and analysis.

Quantification of amastigotes by counting slides under an optical microscope

Macrophages were infected as mentioned above and slides were prepared and stained with rapid hematological dye (Panotic, Laborclin PR, BRAZIL), as described by the manufacturer. The numbers of infected cells and intracellular parasites were counted in a total of 100 cells/slide, using an inverted trinocular optical microscope at 60X magnification. We performed the assay in triplicate, in a total of three independent experiments.

Statistical Analyses

The statistical analyzes were made using GraphPad Prism 9 software. Shapiro-Wilk test was used to verify the Gaussian distribution. The results obtained both in the counting of slides and in the analysis of flow cytometry were analyzed using the ANOVA test one-way. Pearson's Correlation test was used to obtain the degree of correlation between the two variables (Relative Fluorescence Unit x infection rate).

RESULTS

The infection rate and Relative Fluorescence Units (RFU) increase as the proportion of parasites available in the medium increases

Table 1 and figure 1 show a gradual increase in the infection rate of RAW 264.7 macrophages as the proportion of parasites supplied during the assay increases.

Table 1 also demonstrates an increase in RFU as the infection rate increases from the same samples that were counted on a slide. This increase in RFU as the infection rate increases can also be observed in figure 2. Also, according to the data presented in figure 2, it is interesting to highlight that from 4.8 parasites/100 macrophages, a difference in RFU between uninfected and infected samples starts to occur. The ANOVA test of variance shows a significant difference between the sequential readings made by flow cytometry and by the slide counting ($p<0.05$).

Table 1: RFU of RAW 264.7 macrophages infected with different proportions of *L. chagasi* and stained with propidium iodide.

Number of parasites/MØ	Parasites/100 cells*	RFU*
0	0,0 ± 0	3229,7 ± 451,9
0,2	1,9 ± 1,31	3315,7 ± 300,3
1	2,0 ± 1,01	4300,3 ± 1307,8
5	4,8 ± 0,91	7043 ± 4,2
10	5,3 ± 1,54	8315 ± 1468
15	6,0 ± 0,75	8466 ± 244,7
20	6,6 ± 0,51	9764,5 ± 328,8

Parasites/100 cells: number of parasites/100 macrophages counted in slides. RFU: the measure of fluorescence obtained by flow cytometry. *Mean ± standard deviation of 3 independent assays. MØ = macrophage; RFU = Relative Fluorescence Unit.

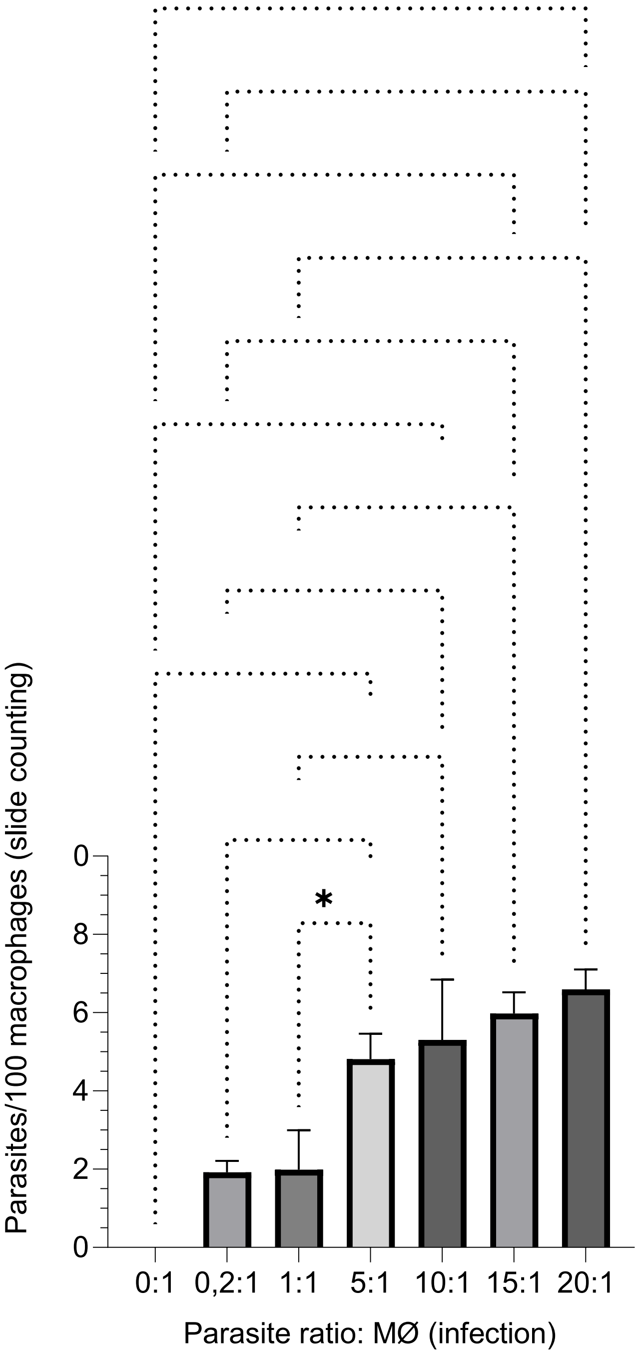

Figure 1: Infection rate of RAW 264.7 macrophages after infection with different proportions of *L. chagasi*. MØ = macrophage. Each bar represents the mean ± standard deviation of 3 independent experiments. Statistically significant differences ($p<0.05$, ANOVA) are indicated by an asterisk.

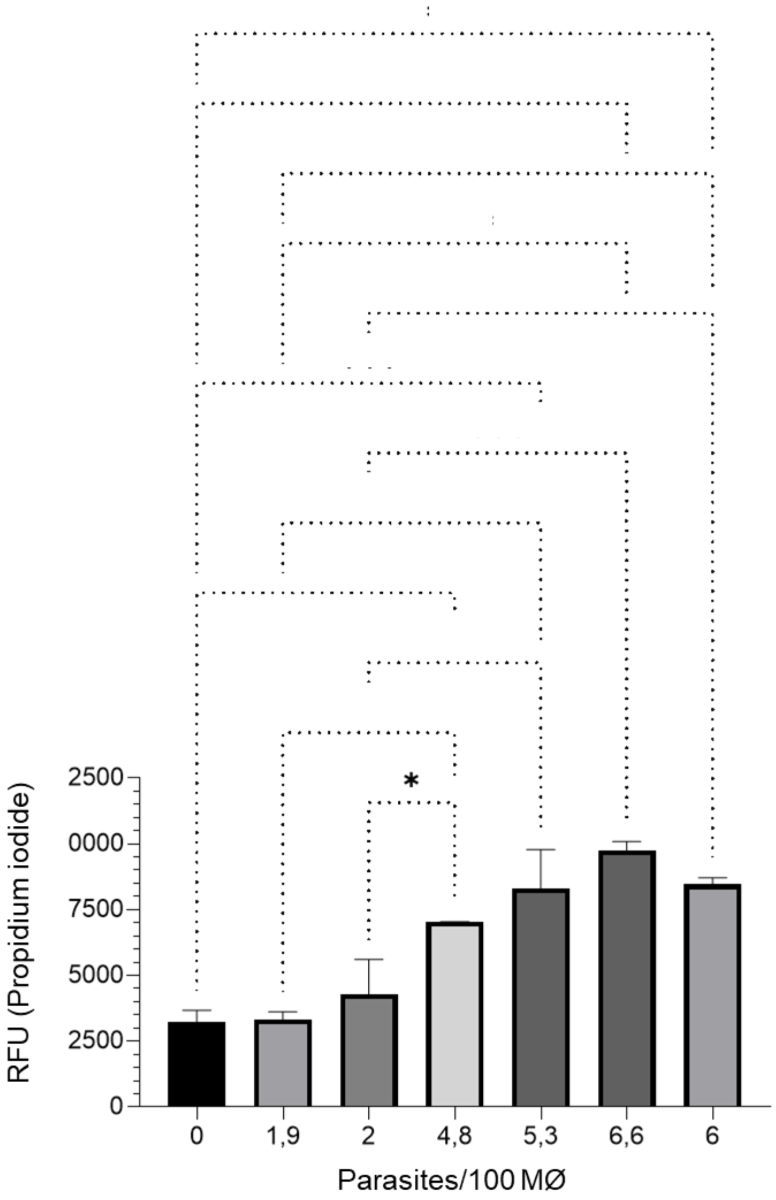

Figure 2: Measurement of RFU for different infection rates of RAW 264.7 macrophages by *L. chagasi*. MØ = macrophage. Each bar represents the mean ± standard deviation of 3 independent experiments. Statistically significant differences ($p<0.05$, ANOVA) are indicated by an asterisk.

When the variables fluorescence and macrophage infection rate are plotted in a scatter plot (figure 3), we observe a positive correlation between the RFUs and the infection rates obtained by the slide counting. We observe that there is a linear correlation, directly proportional between the two variables, a fact confirmed by the Pearson's constant r = 0.9437.

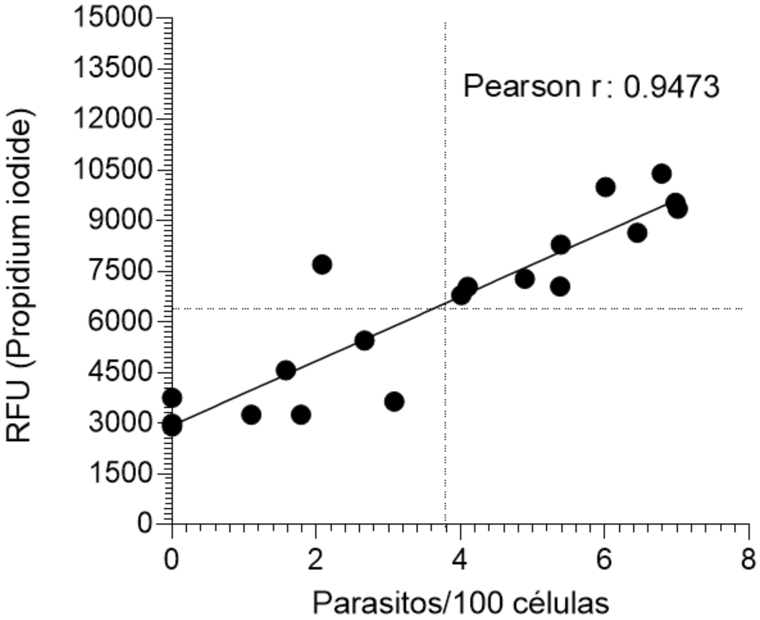

Figure 3: Pearson's correlation between the variables fluorescence and infection rate of RAW 264.7 macrophages by *L. chagasi*. MØ = macrophage. Data were obtained from 3 independent assays. Pearson's r = 0.9473; p<0.0001.

The mathematical equation for estimating the infection rate using fluorescence values

Due to the positive, linear, and directly proportional correlation of the variables fluorescence and infection rate, it is possible to establish a mathematical equation to calculate the estimated rate of infection of macrophages using the fluorescence values (figure 4).

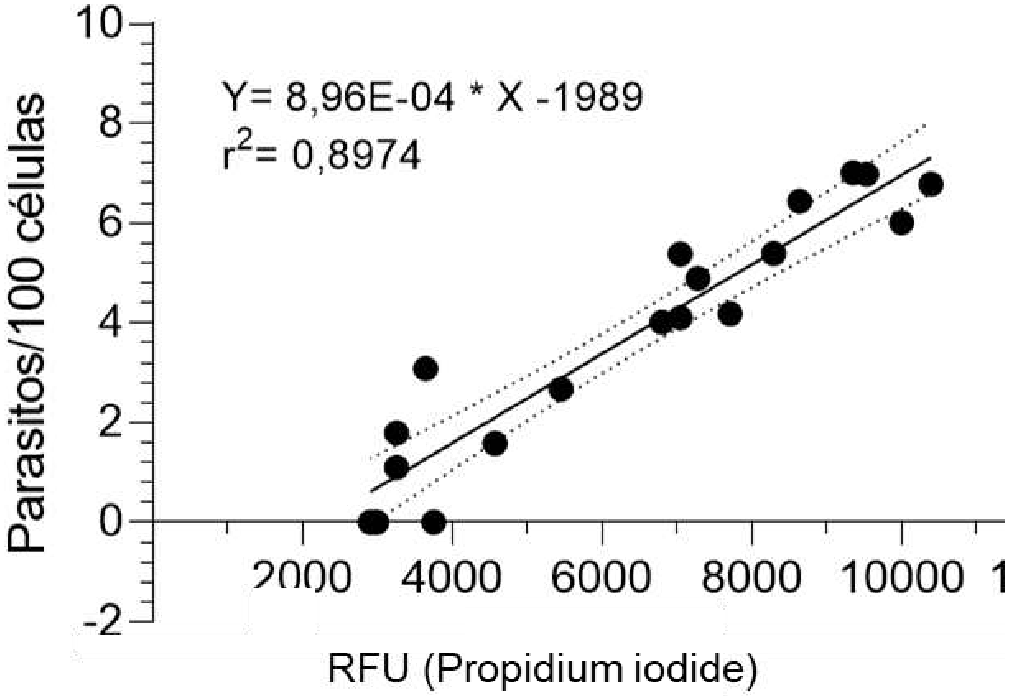

Figure 4: Equation for estimating the infection rate, obtained by linear regression of the fluorescence variables X infection rate. MØ = macrophage.

DISCUSSION

In this work, we purpose a new technique to quantify the *in vitro* parasitism of *Leishmania* infected macrophages. The techniques currently used for this purpose present some problems that mainly make it difficult to standardize and perform tests using large amounts of samples. The most used technique to assess the leishmanicidal effect of a given drug on the amastigote form, for example, is the slide count of intracellular parasites after treatment. Despite this methodology being consolidated for this type of study, there are several operational difficulties related to the assessment and obtaining results. In addition to being laborious, this technique requires time both for the preparation of the material for analysis and for the counting itself.

Slide counting is also a process that adds many errors during its execution: ideally, only one individual should perform all the counts, to reduce observer/counter bias. However, this is one of the most complicated issues to solve, since during some procedures, such as drug screening, for example, the number of slides is very high. This leads to the division of labor and, together, the sum of intrinsic errors of each counter.

Due to all the obstacles and difficulties found, our results show the possibility of using flow cytometry to quantify the parasite load of macrophages infected with *L. chagasi*, using nuclear labeling with propidium iodide. Another possibility for the use of flow cytometry for this purpose would be using genetically modified parasites that express fluorescent proteins, but laboratories do not always have accreditation for this procedure or even have access to this type of microorganism.

In this work, we start from the idea that as more *Leishmania* is available for the same proportion of macrophages, there should be an increase in their infection rate due to the greater supply of parasites, and the presence of intracellular amastigotes adds an extra load of DNA and RNA to the system. When a fraction of the same samples counted on slides were stained with propidium iodide and acquired in a cytometer, the results found showed the same profile for both methodologies: a gradual increase in the number of parasites per 100 macrophages and in fluorescence as the infection rate increases (Table 1 and Figure 2). This is because, in the presence of the fluorescent intercalating agent propidium iodide, not only the DNA/RNA of the fixed macrophages was labeled, but also the DNA/RNA of the parasites present in their parasitophorous vacuoles. In our experiments, we evidenced that it is necessary to reach a minimum value of parasites per 100 macrophages in the assessed sample to generate RFU significantly higher than that presented by uninfected macrophages: there is a significant difference in RFU between the non-infected sample and the sample with an infection rate of 4.8 parasites/100 macrophages, reflecting a significant increase in the DNA/RNA of the parasites in the system, detectable by the technique (Figure 2). Values lower than this (4.8 parasites/100 macrophages in the slide count or 7043 RFU in the flow cytometry) do not present significant differences in this type of comparison. Therefore, it would be necessary, for the use of the methodology in drug screening, to use a proportion of at least 5 parasites per macrophage in the infection procedure for the minimum value of parasites per macrophage to generate an RFU different from that obtained from macrophages not infected by *L. chagasi*. Importantly, this same limitation is observed when the slide counting methodology is used (Figures 1 and 2).

Another fact evidenced during the experiments is that there is a limit for macrophage infection that is reflected in the RFU of infected samples, that is, no matter how much a greater proportion of parasites per macrophage is added, a fluorescence reading limit will not be exceeded. This ratio is 4.8 parasites per 100 macrophages or 7043 RFU. Even if more parasites are added to the system during the infection process, the infection rate or RFU values does not change significantly (Figures 1 and 2). Therefore, the ideal for drug screening, in the example of infection by *L. chagasi* presented in our work, is to use a proportion of five parasites for each macrophage during the execution of the infection assays (Table 1). Curiously, this value coincided with the minimum value of parasites per macrophage used in the infection to generate numbers of parasites per macrophage and RFU different from those obtained from macrophages not infected by *L. chagasi*, as shown in the previous paragraph. This limit for infection can be explained by the fact that the acquisition of promastigote forms of *Leishmania* by macrophages is a process mediated by classical receptors that initiate the process of phagocytosis (Liu; Uzonna, 2012). There is a certain number of receptors per cell. The exacerbated number of parasites may have saturated the system.

Infections made with different proportions of parasites by macrophages were an artifice used by us to obtain a variable number of intracellular parasites per sample. However, when an infection happens to lead to a low rate of infection or a low rate of proliferation of the parasite in the parasitophorous vacuole, it is possible that an appreciable number of parasites that reflect the RFU in a significant way is not always obtained. It is also important to consider that, even though *Leishmania* is in the stationary phase of the growth curve when there is a greater proportion of infective parasites in the culture (metacyclic forms), there is still a proportion of parasites that are not in such a phase. In this case, even if respecting the use of a fixed number of parasites for each infection, the actual number of infective forms may be different in different experiments and, consequently, the infection rate as well. Therefore, the potential for infection of the parasite in culture, at the time of the assay, is a factor to be considered for the validation of the methodology. However, this same reasoning is valid when using slide counting, since the minimum infection values at which reading differences are detected in relation to uninfected macrophages are the same for both techniques.

We determined the Pearson’s correlation coefficient (r) for the data obtained to verify if there is a correlation between the infection rate and RFU variables of the samples (Figure 3). According to the results obtained ($r = 0.9473$; $p<0.001$), we can say that there is a positive and directly proportional linear correlation between the variables fluorescence and infection rate, that is, there is a similarity between the distribution of the scores variables. Therefore, as it has a linear and directly proportional distribution, we can infer a mathematical equation, through linear regression, to obtain an estimate of the number of parasites in each sample through the RFU values (Figure 4).

Therefore, our results demonstrate that, as the infection rate increases, there is a proportional increase in RFU of the analyzed samples. Considering these results, we can admit the possibility of using flow cytometry without the need to use genetically modified organisms for the intracellular quantification of amastigotes in infected macrophages *in vitro*, such as in the screening of leishmanicidal drugs, during the first stages of the procedure, in place of the use of the slide counting technique. In the initial screening, the main objective is to verify whether there is a potential leishmanicidal activity against amastigote forms of a given substance being tested, that is, whether, in relation to the control, there is a reduction in the infection rate of samples treated with a certain substance. This initial screening is one of the most laborious procedures due to the large number of substances commonly tested. The use of flow cytometry in this step would reduce its execution time, in addition to obtaining more accurate results with greater potential for standardization in relation to the methodologies currently used.

CONCLUSION

As the infection rate increases, there is also a directly proportional increase in RFU in macrophage infection samples labeled using propidium iodide. In this way, it is possible to use flow cytometry with samples labeled with propidium iodide for fast, accurate, and easy standardization for the quantification of intracellular parasites, as shown in the experiments of infection of macrophages with *L. chagasi*. Similarly, it is possible to extend the use of this technique in the quantification of other intracellular agents, such as *Plasmodium* sp. and *Trypanosoma cruzi*, without the need to use genetically modified organisms in the tests.

CONFLICT OF INTEREST

The authors declare that they have no conflict of interest.

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4. CONCLUSÕES GERAIS

As leishmanioses fazem parte do quadro de doenças negligenciadas que afetam milhões de pessoas, todos os anos. É um grande problema de saúde em muitas regiões tropicais e subtropicais do mundo. Não existem meios reconhecidos e confiáveis de quimioprofilaxia ou vacinação contra infecções por diferentes formas de leishmaniose. Além disso, a quimioterapia, infelizmente, permanece, em muitos aspectos, insatisfatória. Portanto, há uma necessidade contínua e urgente de novas terapias contra a leishmaniose que sejam seguras e eficazes na indução de uma cura a longo prazo.

Este trabalho visa contribuir com uma nova técnica que tem potencial para ser utilizada nos primeiros passos na busca por novas terapias, com o objetivo de tornar o trabalho mais ágil, confiável e de fácil execução. Isto permitiria um intercâmbio mais eficiente de informações, além de manter um padrão de comparação, tornando a pesquisa na busca de novas drogas no combate a leishmaniose mais dinâmica e eficaz.

ANEXO 1

Could combination chemotherapy be more effective than monotherapy in the treatment of visceral leishmaniasis? A systematic review of preclinical evidence

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SUMMARY

From a systematic review framework, we assessed the preclinical evidence on the effectiveness of drug combinations for visceral leishmaniasis (VL) treatment. Research protocol was based on the PRISMA guideline. Research records were identified from Medline, Scopus and Web of Science. Animal models, infection and treatment protocols, parasitological and immunological outcomes were analyzed. The SYRCLE's toll was used to evaluate the risk of bias in all studies reviewed. Fourteen papers using mice, hamster and dogs were identified. *Leishmania donovani* was frequently used to induce VL, which was treated with 23 drugs in 40 different combinations. Most combinations allowed to reduce the effective dose, cost and time of treatment, in addition to improving the parasitological control of *Leishmania spp*. The benefits achieved from drug combinations were associated with an increased drug's half-life, direct parasitic toxicity, and improved in immune defenses in infected hosts. Selection, performance and detection bias were the main limitations identified. Current evidence indicates that combination chemotherapy, especially those based on classical drugs (miltefosine, amphotericin B antimony-based compounds) and new drugs (CAL-101, PAM3Cys, tufisin and DB766), develops additive or synergistic interactions, which trigger trypanocidal and immunomodulatory effects associated with reduced parasite load, organ damage and better cure rates in VL.

Key words: Antiparasitic chemotherapy. Leishmaniasis. Drug association.

KEY FINDINGS

- The relevance of drug combination to treat visceral leishmaniasis (VL) was analyzed.
- The current evidence and methodological bias were systematically evaluated.
- Drug combination may have additive or synergistic interaction.
- Drug combination improves immunological defenses and parasite control.
- Drug combination may be more effective than monotherapy in treating VL.

INTRODUCTION

Visceral Leishmaniasis (VL) or kala-azar is an infectious disease caused by infection of the parasite protozoans *Leishmania infantum chagasi*, *Leishmania donovani* and *Leishmania infantum* (Freitas *et al.*, 2012). This is a severe neglected disease that is fatal if left untreated in over 95% of cases, closed correlated to poverty, precarious conditions of basic sanitation, and limited access to health services (WHO, 2022). Although VL is endemic in more than 79 countries, an estimated 50 000 to 90 000 new cases of VL occur worldwide annually and a total of 3813 deaths were reported to WHO in 2014–2020 (Ruiz-Postigo *et al.*, 2021). These cases reported by the World Health Organization (WHO) were associated to 10 countries, specifically Brazil, China, Ethiopia, Eritrea, India, Kenya, Somalia, South Sudan, Sudan and Yemen (WHO, 2022). 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 (Ready, 2014).

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 often associated with VL (Serafim *et al.*, 2010; Mwololo *et al.*, 2015; WHO, 2016).

The specific treatment of VL is limited to pentavalent antimonies (sodium stibogluconate and meglumine antimoniate), followed by p paromomycin, oral miltefosine or amphotericin B as a second-choice (Corral *et al.*, 2014; Joice *et al.*, 2017; Alves *et al.*, 2018). 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 in several endemic areas (Corral *et al.*, 2014; Ponte-Sucre *et al.*, 2017). Although 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 VL treatment (Mwololo *et al.*, 2015; Ponte-Sucre *et al.*, 2017). Thus, developing more effective and less toxic treatment protocols for VL is necessary and urgent (Khadem *et al.*, 2017a; Joice *et al.*, 2017).

In the last years, therapy based on drugs association has emerged as an alternative to VL treatment (Mwololo *et al.*, 2015; Joice *et al.*, 2017; Rebello *et al.*, 2019). As combination 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 are proposed (van Griensven *et al.*, 2010). With the increase in therapeutic failures after the administration of antimonial drugs and miltefosine, combination chemotherapy is also relevant 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 chemotherapy alternative for 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).

Drug combination has been successfully used in the treatment of several others infectious diseases, such as tuberculosis, malaria, and leprosy (Nosten and Brasseur, 2002; van Griensven *et al.*, 2010; Ramón-García *et al.*, 2011). However, as the current evidence is fragmented, it is difficult to establish a clear profile of 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, all preclinical models and treatment protocols used, as well as the risk of bias related to the studies that supports the current 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 in the search of parasitological cure for this disease.

MATERIALS AND METHODS

Guiding questions

The main questions to be answered in this systematic review were: Are combinations of antileishmanial drugs effective in the treatment of visceral leishmaniasis? What are the main chemotherapy protocols and primary research outcomes used to determine treatment effectiveness?

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). In our research strategy, an extensive literature search using three comprehensive databases was used: (i) PubMed/Medline, (ii) Scopus, and (iii) Web of Science. We used an advanced research strategy based on search filters optimized according specifically algorithms and syntaxes adopted in each database. The search filters were structured in three complementary levels as follows: (i) Treatment: combination chemotherapy, (ii) Disease: visceral leishmaniasis, and (iii) Research type: preclinical models *in vivo*. A search filter was initially developed for PubMed according to 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 from PubMed, a standardized animal filter was applied (Pereira *et al.*, 2017). The same search filter used to disease and treatment was adapted to Scopus and Web of Science. A search limit for animal models in Scopus, and language limit (English) for Scopus and Web of Science were applied. No chronological restrictions were adopted in the search strategy. The complete search strategies applied in both databases can be consulted in the supplementary material (Table 1). 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 publicati

Table 1. Search strategy used in each database.

PUBMED			
Group	Filter	Date and time	N° studies
#1 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]]	03/24/20	16,432
#2 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]]	03/24/20	704,431
#3 Animal model	Standardized filter 1 (Pereira et al., 2017)	03/24/20	6,752,043
#3 Animal model	Standardized filter 2 (Pereira et al., 2017)	03/24/20	98,646
#4 Combination animal filter	Standardized filter 1 OR Standardized filter 2	03/24/20	6,850,430
#5 Combination	#1 Visceral leishmaniasis AND #2 Drugs AND #4 Combination animal filter	03/24/20 06:37 pm	1,084

Table 1 (*continuation*). Search strategy used in each database.

SCOPUS			
Group	Filter	Date and time	N° studies
#1 Visceral leishmaniasis	**(**title-abs-key (amphotericin b) or title-abs-key (pentavalent antimonial) 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)	03/24/20	156,236
#2 Drugs	(title-abs-key (leishmaniasis, visceral) or title-abs-key (leishmania infantum) or title-abs-key (leishmania infantum chagasi) or title-abs-key (leishmania donovani))	03/24/20	20,583
#1 and #2	Combination #1Visceral leishmaniasis AND #2 Drugs	03/24/20	4,229
#4 Combination	Search limit: Animal model	03/24/20 09:12 pm	397

WEB OF SCIENCE			
Group	Filter	Date and time	N° studies
#1 Visceral leishmaniasis	TS=(Visceral AND leishmaniasis) OR TS=(Leishmania AND infantum) OR TS=(Leishmania AND infantum AND chagasi) OR TS=(Leishmania AND donovani)	03/23/20	18,607
#2 Drugs	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)	03/23/20	56,560
#3 Animal model	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)	03/23/20	4,309,416
#4 Combination	Combination #1Visceral leishmaniasis AND #2 Drugs AND #3 Animal model	03/23/20 09:06 pm	758

Inclusion and exclusion criteria

Only studies investigating effectiveness of drug combination in preclinical models of VL were included in the systematic review. Irrelevant studies were excluded (not related to the subject) after the initial screening, and 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 on *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) absence of monotherapy as control, (vi) non-pharmacological treatments (i.e., plant extracts, cytokines, peptides) and vaccines, (vii) absence of groups treated with drug combination, and (viii) studies published in other language than English. After identifying all 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 two other researchers (RDN and EAMS).

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) Primary research outcomes: parasitism, immunological, biochemical and survival results. For parasitism, data available in figures were digitized and the means was obtained using ImageJ software (Schneider *et al.*, 2012) after calibrating each picture to the nearest 0.01 mm.

Reporting quality as a risk of bias

The risk of bias in animal studies was analyzed from the SYRCLE’S (Systematic Review Center for Laboratory Animal Experimentation) guidelines, which is based on the Cochrane Collaboration risk-of-bias (RoB) tool for randomized trials (Hooijmans *et al.*, 2014). This instrument is 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 levels: 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.

RESULTS

Research records retrieved

Our primary search strategies recovered 2239 articles from PubMed, Scopus and Web of Science, of which 608 were duplicates. After title and abstract screening, 1631 studies were excluded due to inadequate research topic. Of these, 349 studies were based only *in vitro* parasite viability assays, 595 investigated treatments without chemotherapeutic combination, 238 studies evaluated other diseases. In addition, 386 studies corresponded to non-original papers, 5 papers were based on clinical investigations, and 23 studies were not written in English. Considering papers that investigated drug combination for VL treatment, 40 studies were selected for full text evaluation. Thus, 14 relevant studies were identified and included in this systematic review (Fig. 1).

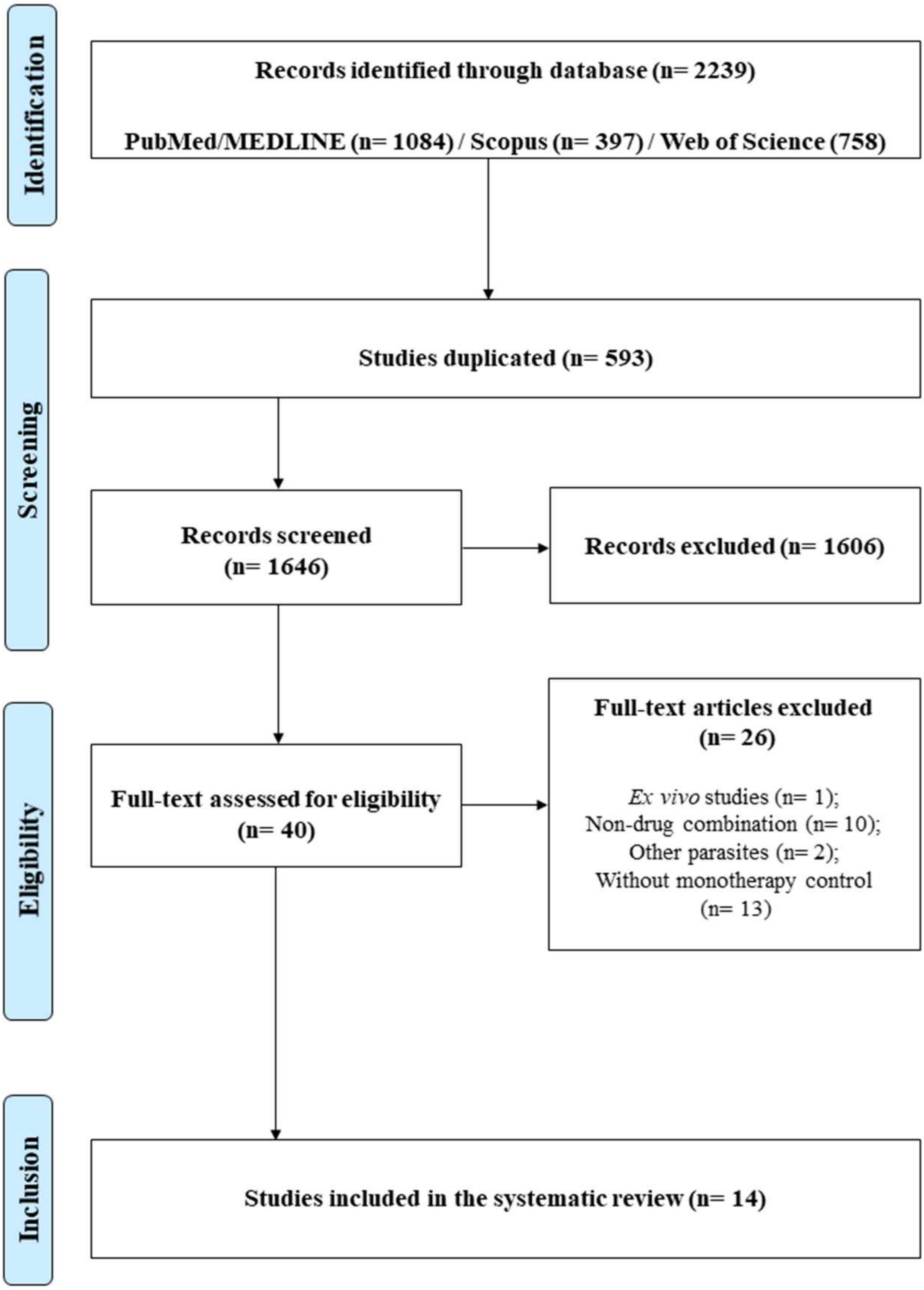

Figure 5: Flow diagram of the systematic review literature search results. Based on PRISMA statement "Preferred Reporting Items for Systematic Reviews and Meta-Analyses". www.prisma-statement.org.

Animal models of visceral leishmaniasis

General characteristics of the animal models used in all studies are showed in supplementary files (Table S1). Most studies were produced in India (35.71%, n = 5), followed by Kenya (14.29%, n = 2). The remain studies (50%) were conducted in the United States of America, Italy, United Kingdom, Spain, Canada, Belgium and Brazil. BALB/c mice were used as animal model in most studies (71.43%, n = 10), followed by hamster (21.43%, n = 3), especially the golden lineage (14.29%, n = 2). Hamster lineage was underreported in one study. Dogs belonging to different breeds were used in only one study.

The report about the sex of the animals used in the studies show that females (35.71% n = 5) and works using males and females together (35.71% n = 5) were the most found. Male animals were adopted in only one study (7.14%, n = 1), while this parameter was underreported in 3 other studies (21.43%). Mice and hamster age ranged from 4 to 8 weeks, and dogs age ranged from 2 to 10 years old (7.14%, n = 1). This variable was neglected in 6 studies (21.43%). Animal’s weight ranged from 18-25 g for mice, and 80-100 g for hamster. Most studies did not report this data (57.14, n = 8).

Visceral leishmaniasis characteristics

Leishmania donovani species was used in 9 studies (64.29%), followed by *L. infantum* (35.71%, n = 5). In studies with dogs, the animals were naturally infected and the *Leishmania* species was not identified. Four different *Leishmania* strains were used. The strains MHOM/KE/82/LRC-L445/NLB065 and MHOM/MA/67/ITMAP263 were used in 3 studies (21.43%) each, while MHOM/IN/89/GE1F8R and MHOM/ET/67/HU3 strains were found in 2 studies (14.29%) each. The remaining studies used 5 different strains. The strain used to infect the animals was not reported in 1 study (6.25%) (Table S2). Intravenous and intraperitoneal parasites inoculation were used in 5 studies (35.71%) each, followed by intracardiac route in 3 studies (21.43%) and only one study reported natural infection (7.14%). The inoculum size ranged from 1×10^6 to 1×10^8 parasites in studies using mice and hamsters (Table S2).

Protocols of combination chemotherapy

Considering all 14 studies, 23 drugs were tested in 40 different combinations (Table 2 and S3). In mice models, 28 combinations (70%) based on 23 different drugs were used. The most used drug (n = 3, 13.64%) was miltefosine (MTF), followed by sodium antimony gluconate (SAG), diminazene (DIM) and DB766 compound (n = 2, 9.09% combinations each). Glycyrrhizic acid (GA), sodium stibogluconate (SSG), CAL-101 (CAL), amphotericin B (AMB), artesunate (ART), diperoxovanadate (PV6), N-palmitoyl-S-(2, 3-bis (palmitoyloxy)-(2RS)-propyl)-Cys-Ser-Lys4.Hydrochloride (Pam3Cys), buthionine sulfoximine (BSO), chloroquine (CHQ), ketoconazole (KET), lopinavir (LPV), posaconazole (POS) and tufisin (TUF) were also used in combination for VL treatment (Tables 2 and S3). For hamster models, 11 (27.5%) combinations based on 7 different drugs were investigated. The combinations were based on trans-aconitic acid (TAA) SSG, pentamidine (PET), allopurinol (ALO), allicin (ALL), AAMB, paromomycin (PAR) and MTF. Paromomycin and meglumine antimoniate (MEG) were combined to treat dogs with VL (Tables 2 and S3).

Intraperitoneal (n = 12, 48%) and oral (n = 6, 24%) routes were mainly used for drug administration. Intramuscular, subcutaneous and intravenous routs were reported in 24% of the studies. Only one study did not report this data. Most 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 studies twice a week (11.11%). Only one study chose to administer the drugs twice a day (5.56%). Most of studies (n = 14, 71.42%) used doses below than those recommended for monotherapy (sub doses), to reduce the risk of toxicity (Table 3).

Main outcomes

The main outcomes were shown in Tables 2, 3 and Figure 2. Preclinical studies demonstrated that combination chemotherapy is effective for VL treatment. In most studies, parasitism was used as a primary outcome to assess the success of antiparasitic chemotherapy. Thus, in the entire dataset with 14 studies, 100% reported a significant reduction in organ parasitism, especially in spleen (n = 11, 44%), liver (n = 11, 44%) and bone marrow (n = 3, 12%). Compared to monotherapy, the improved parasite clearance achieved from drug combination was often associated to the induction of a protective Th1 immunological response, which was mainly evidenced by

IL-12, IL-6, TNFα, IFN-γ, and IgG2 upregulation (Haldar *et al.*, 2009; Shakya *et al.*, 2012a,b; Bhattacharjee *et al.*, 2015). Conversely, combined pharmacological regimens were associated with an attenuated production of Th2 and/or Treg cytokines, such as IL-10, IL-4 or TGFβ upregulation (Haldar *et al.*, 2009; Shakya *et al.*, 2012a; b; Bhattacharjee *et al.*, 2015; Khadem *et al.*, 2017a). In addition, beneficial antiparasitic effects were associated with marked modulation of redox metabolism, especially attributed to upregulation of reactive oxygen (ROS) and nitrogen species (RNS) biosynthesis (Haldar *et al.*, 2009; Shakya *et al.*, 2012a; b; Bhattacharjee *et al.*, 2015), and increase of hepatic and splenic reduced and total glutathione levels (Carter *et al.*, 2003). Improved cellular parameters, such as macrophage phagocytic ability and T cell proliferation were also associated with a better parasitological control in animals treated with combination chemotherapy than monotherapy (Haldar *et al.*, 2009; Shakya *et al.*, 2012a; b; Khadem *et al.*, 2017a). Improved score and clinical signs (i. e. decrease of hematochemical parameters, cutaneous alterations, anemia, reduction in size of spleen and/or lymph nodes and increase in weight), as well as reduction in organs hypertrophy and increase in body weight (Oliva *et al.*, 1998) and survival rates (Mutiso *et al.*, 2011) were also reported as good markers of drug combination effectiveness.

As a critical parameter in drug combination, 8 studies (57.14%) used integrated *in vitro* models to determine the nature of pharmacological interaction from specific drug combinations. From 4 studies (28.57%), seven synergistic interactions were reported: (i) glycyrrhizic acid + sodium antimony gluconate, (ii) diminazene + chloroquine, (iii) DB766 + posaconazole (7 different combinations), (iv) DB766 + ketoconazole, (v) trans-aconitic acid + sodium stibogluconate, (vi) trans-aconitic acid + pentamidine, and (vii) trans-aconitic acid + allopurinol. Additive interaction was reported in 4 studies (28.57 %) to: (i) diperoxovanadate + sodium antimony gluconate, (ii) miltefosine + lopinavir, (iii) allicin + amphotericin B (iv) DD766 + ketoconazole. Antagonism interaction was reported in one study using two different combinations with DD766 + ketoconazole. One study (7.14%) investigating paromomycin + miltefosine reported no pharmacological interaction.

Table 2. Control monotherapy, drug combinations, target organs, parasitism suppression and nature of pharmacological interaction.

	Target organs	Control monotherapy (Dose)	Parasitism suppression (%)	Combination chemotherapy (Dose)	Parasitism suppression (%)	Drug interaction†
Mouse model						
(Carter *et al.*, 2003) ^{1}200016 strain ^{2}200011 strain	^{1}Liver ^{1}Spleen ^{1}Bone-marrow	BSO (34 mg/kg) SSG (74 mg SB^{v}/kg) SSG (282 mg SB^{v}/kg)	36^{L}, 3.5^{S}, 11^{BM} 77^{L}, 5^{S} 25^{BM} 98^{L}, 40^{S}, 72^{BM}	BSO (34 mg/kg) + SSG (74 mg SB^{v}/kg)	99.45^{L}, 85^{S}, 85^{BM}	-
	^{2}Liver ^{2}Spleen ^{2}Bone-marrow	BSO (34 mg/kg) SSG (74 mg SB^{v}/kg) SSG (282 mg SB^{v}/kg)	14^{L}, 6.5^{S}, 19^{BM} 4^{L}, 4^{S}, 10^{BM} 30^{L}, 22^{S}, 6^{BM}	BSO (34 mg/kg) + SSG (74 mg SB^{v}/kg)	94.5^{L}, 0^{S}, 11^{BM}	-
(Haldar *et al.*, 2009) ^{3}S strain ^{4}R strain	^{3}Liver ^{3}Spleen	PV6 (0.5 µmol/30g) SAG (50 mg/kg) SAG (250 mg/kg)	72.7^{L}, 75.8^{S} 47.5^{L}, 48.8^{S} 92.9^{L}, 91.0^{S}	PV6 (0.5 µmol/30g) + SAG (50 mg/kg)	84.2^{L}, 83.4^{S}	Additive
	^{4}Liver ^{4}Spleen	PV6 (0.5 µmol/30g) SAG (50 mg/kg) SAG (250 mg/kg)	49.9^{L}, 53.4^{S} 17.8^{L}, 17.3^{S} 49.2^{L}, 45.8^{S}	PV6 (0.5 µmol/30g) + SAG (50 mg/kg)	77.1^{L}, 79.2^{S}	Additive
(Mutiso *et al.*, 2011)	Spleen	DIM (12.5 mg/kg) ART (12.5 mg/kg) AMB (12.5 mg/kg)	27.78^{S} 33.05^{S} 92.91^{S}	DIM (12.5 mg/kg) + ART (12.5 mg/kg)	80.33^{S}	-
(Shakya *et al.*, 2012a)	Liver	Pam3Cys (100 µg) MTF (2.5 mg/kg) MTF (5 mg/kg) MTF (20 mg/kg)	58.2^{L} 69.9^{L} 48.2^{L} 96.5^{L}	PAM3Cys (100 µg) + MTF (2.5 mg/kg) PAM3Cys (100 µg) + MTF (5 mg/kg)	82.6^{L} 92.5^{L}	-

†Drug interaction evaluated from *in vitro* or *in vivo* parasitological/cytotoxicity tests, ^{L}**:**Liver, ^{BM}**:** Bone-Marrow, ^{LN}**:** Lymph-node, ^{1}**:** 200016 strain $^{2:}$ 200011 strain, ^{3}**:** resistant strain, ^{4}**:** susceptible strain, **BSO**: Buthionine sulfoximine, **SSG**: Sodium stibogluconate, **MTF:** Miltefosne, **DIM:** Diminazene, **ART:** Artesunate, **PV6:** Diperoxovanadate, **Pam3Cys:** N-palmitoyl-S-(2, 3-bis (palmitoyloxy)-(2RS)-propyl)-Cys-Ser-Lys4.Hydrochloride), **AMB:** Amphotericin B. $^{1,2 \text{ and } 3,4}$**:** Studies using 2 different strains

Table 2 (*continuation*). Control monotherapy, drug combinations, target organs, parasitism suppression and nature of pharmacological interaction.

	Target organs	Control monotherapy (Dose)	Parasitism suppression (%)	Combination chemotherapy (Dose)	Parasitism suppression (%)	Drug interaction†
Mouse model						
(Shakya *et al.*, 2012b)	Liver	F-TUF (60 µg)	34^L	F-TUF (60 µg) + MTF (2.5 mg/kg)	66^L	-
		L-TUF (60 µg)	48^L			
		MTF (2.5 mg/kg)	49^L	L-TUF (60 µg) + MTF (2.5 mg/kg)	81^L	
		MTF (5 mg/kg)	72^L			
		MTF (20 mg/kg)	98^L	L-TUF (60 µg) + MTF (5 mg/kg)	93^L	
(Bhattacharjee *et al.*, 2015)	Liver	SAG (250 mg/kg)	9^L, 12^S	GA (50 mg/kg) + SAG (250 mg/kg)	92^L, 93^S	Synergistic
	Spleen	GA (50 mg/kg)	26^L, 31^S			
(Mwololo *et al.*, 2015)	Spleen	DIM (12.5 mg/kg) CHQ (12.5 mg/kg) AMB (1 mg/kg)	29^S 8^S 96^S	DIM (12.5 mg/kg) + CHQ (12.5 mg/kg)	68^S	Synergistic
(Khadem *et al.*, 2017)	Liver Spleen	CAL-101 (0.05 mg) AMB (0.1 mg/kg)	88^L, 84^S 65^L, 69^S	CAL-101(0.05 mg) + AMB (0.1 mg/kg)	$100^{¨L}$, $100^{¨S}$	-
(Joice *et al.*, 2017)	Liver	POS (30 mg/kg)	57^L	DB766 (75 mg/kg) + POS (30 mg/kg)	86^L	Additive
		POS (15 mg/kg)	21^L	DB766 (75 mg/kg) + POS (15 mg/kg)	88^L	Synergistic
		POS (7.5 mg/kg)	6^L	DB766 (75 mg/kg) + POS (7.5 mg/kg)	75^L	Additive

†Drug interaction evaluated from *in vitro* or *in vivo* parasitological/cytotoxicity tests, ^{L}**:** Liver, ^{BM}**:** Bone-Marrow, ^{LN}**:** Lymph-node, **MTF:** Miltefosne, **DIM:** Diminazene, **AMB:** Amphotericin B, **POS:** Posaconazole, **ART:** Artesunate **CHQ:** Chloroquine **CAL-101:** p110δ-specific pharmacological inhibitors **SAG:** sodium antimony gluconate**, GA:** Glycyrrhizic acid**, F-TUF:** Free-Tufisin**, L-TUF:** Lipo-Tufisin, **DB766:** 2,5-bis[2-(2-i-propoxy)-4-(2-pyridylimino) aminophenyl]furan hydrochloride. ¨No parasite was detected.

Table 2 (*continuation*). Control monotherapy, drug combinations, target organs, parasitism suppression and nature of pharmacological interaction.

	Target organs	Control monotherapy (Dose)	Parasitism suppression (%)	Combination chemotherapy (Dose)	Parasitism suppression (%)	Drug interaction†
Mouse model						
(Joice *et al.*, 2017)	Liver			DB766 (38 mg/kg) + POS (30 mg/kg)	83^{L}	Synergistic
				DB766 (38 mg/kg) + POS (15 mg/kg)	80^{L}	Synergistic
				DB766 (38 mg/kg) + POS (7.5 mg/kg)	69^{L}	Synergistic
		DB766 (75 mg/kg)	68^{L}			
		DB766 (38 mg/kg)	40^{L}	DB766 (19 mg/kg) + POS (30 mg/kg)	66^{L}	Synergistic
		DB766 (19 mg/kg)	22^{L}			
				DB766 (19 mg/kg) + POS (15 mg/kg)	67^{L}	Synergistic
		KET (30 mg/kg)	76^{L}			
		KET (15 mg/kg)	59^{L}	DB766 (19 mg/kg) + POS (7.5 mg/kg)	48^{L}	Synergistic
		KET (7.5 mg/kg	41^{L}			
				DB766 (45 mg/kg) + KET (18 mg/kg	92^{L}	Synergistic
		MTF (10 mg/kg)	93^{L}			
				DB766 (30 mg/kg) + KET (12 mg/kg	44^{L}	Antagonism
				DB766 (15 mg/kg) + KET (6 mg/kg	36^{L}	Antagonism
				DB766 (7.5 mg/kg) + KET (3 mg/kg)	33^{L}	Additive

†Drug interaction evaluated from *in vitro* or *in vivo* parasitological/cytotoxicity tests, **^{L}:** Liver, **^{BM}:** Bone-Marrow, **^{LN}:** Lymph-node, **MTF:** Miltefosine **DB766:** 2,5-bis[2-(2-i-propoxy)-4-(2-pyridylimino) aminophenyl] furan hydrochloride, **POS:** Posaconazole, **KET:** Ketoconazole.

Table 2 (*continuation*). Control monotherapy, drug combinations, target organs, parasitism suppression and nature of pharmacological interaction.

	Target organs	Control monotherapy (Dose)	Parasitism suppression (%)	Combination chemotherapy (Dose)	Parasitism suppression (%)	Drug interaction†
Mouse model						
Rebello et al., 2019	Liver Spleen			LPV (493.2 mg/kg) + MTF (7.2 mg/kg)	100^{L}, 100^{S}	Additive
		MTF (15.4 mg/kg)	100^{L}, 100^{S}	LPV (493.2 mg/kg) + MTF (3.85 mg/kg)	70^{L}, 52^{S}	
		MTF (7.2 mg/kg)	100^{L}, 100^{S}	LPV (493.2 mg/kg) + MTF (1.92 mg/kg)	44^{L}, 77^{S}	
		MTF (3.85 mg/kg)	46^{L}, 67^{S}	LPV (246.6 mg/kg) + MTF (7.2 mg/kg)	$100^{\text{¨L}}$, $100^{\text{¨S}}$	
		LPV (246.6 mg/kg)	21^{L}, 0^{S}	LPV (246.6 mg/kg) + MTF (3.85 mg/kg)	71+, 83^{S}	
		LPV (493.2 mg/kg)	40^{L}, 52^{S}	LPV (246.6 mg/kg) + MTF (1.92 mg/kg)	12^{L}, 12^{S}	
Dog model						
(Oliva *et al.*, 1998)	Bone-marrow Lymph node	PAR (3.5 mg/kg) MEG (30 mg Sb/kg)	35^{BM}, 10^{LN} 41^{BM}, 38^{LN}	PAR (3.5 mg/kg) + MEG (20 mg Sb/kg)	36^{BM}, 60^{LN}	

†Drug interaction evaluated from *in vitro* or *in vivo* parasitological/cytotoxicity tests, ^{L}**:** Liver; ^{BM}**:** Bone-Marrow; ^{LN}**:** Lymph-node; ^{S}**:** Spleen **MTF:** miltefosine, **MEG:** meglumine antimoniate, **PAR:** paromomycin, **LPV:** Lopinavir. ¨No parasite was detected.

Table 2 (*continuation*). Control monotherapy, drug combinations, target organs, parasitism suppression and nature of pharmacological interaction.

	Target organs	Control monotherapy (Dose)	Parasitism suppression (%)	Combination chemotherapy (Dose)	Parasitism suppression (%)	Drug interaction†
Hamster model						
(Kar *et al.*, 1993) ^{5}*8-day model* ^{6}*1-month model*	Spleen	SSG (100 mg Sb/kg)	$^{5}(-)^{S}/^{6}72^{S}$	SSG (50 mg Sb/kg) + TAA (200mg)	$^{5}87^{S}/^{6}98^{S}$	Synergistic
		SSG (50 mg Sb/kg)	$^{5}30^{S}/^{6}35^{S}$	SSG (50 mg Sb/kg) + TAA (400mg)	$^{5}98^{S}/^{6}100^{¨S}$	
		PET (8 mg/kg)	$^{5}7^{S}/^{6}20^{S}$	PET (8 mg/kg) + TAA (200mg)	$^{5}90^{S}/^{6}98^{S}$	
		ALO (15 mg/kg)	$^{5}18^{S}/^{6}22^{S}$	PET (8 mg/kg) + TAA (400mg)	$^{5}99^{S}/^{6}100^{¨S}$	
		TAA (200 mg/kg)	$^{5}62^{S}/^{6}73^{S}$	ALO (15 mg/kg) + TAA (200mg)	$^{5}79^{S}/^{6}97^{S}$	
		TAA (400 mg/kg)	$^{5}98^{S}/^{6}99^{S}$	ALO (15 mg/kg) + TAA (400mg)	$^{5}99^{S}/^{6}100^{¨S}$	
				SSG (50 mg Sb/kg) + ALO (15 mg/kg)	$^{5}(-)/^{6}45^{S}$	
				SSG (100 mg Sb/kg) + ALO (15 mg/kg)	$^{5}(-)/^{6}89^{S}$	
(Corral *et al.*, 2014)	Liver Spleen	AMB (5 mg/kg) AMB (1 mg/kg) ALL (5 mg/kg)	90.6^{L}, 94.5^{S} $\sim70^{L}$, $\sim70^{S}$ $(-)^{L}$, $(-)^{S}$	AMB (1 mg/kg) + ALL (5 mg/kg)	100¨, 96.5^{S}	Additive
(Hendrickx *et al.*, 2017)	Liver Spleen Bone-marrow	MTF (10 mg/kg) MTF (20 mg/kg) MTF (40 mg/kg)	18^{L}, 54^{S}, 48^{BM} 80^{L}, 94^{S}, 76^{BM} 95^{L}, 99^{S}, 86^{BM}	MTF (20 mg/kg) + PAR (350 mg/kg)	99^{L}, 99^{S}, 98^{BM}	Indifferent
		PAR (180 mg/kg) PAR (350 mg/kg)	79^{L}, 64^{S},0^{BM} 85^{L}, 74^{S}, 84^{BM}	MTF (10 mg/kg) + PAR (180 mg/kg)	97^{L}, 96^{S}, 88^{BM}	

†Drug interaction evaluated from *in vitro* or *in vivo* parasitological/cytotoxicity tests, ^{L}**:** Liver; ^{BM}**:** Bone-Marrow; ^{LN}**:** Lymph-node; ^{S}**:** Spleen; **AMB:** Amphotericin B, **ALL:** Allicin, **ALO:** allopurinol, **PAR:** paromomycin, **MTF:** Miltefosine, **PET:** Pentamidine, **MEG:** Meglumine Antimoniate, **TAA**, trans-Aconitic acid. ¨No parasite was detected. **(-)** not reported or evaluated. ^{5},^{6}**:** study evaluated the drug combination in different models of infectio

Table 3. Effective drug combinations able to induce an efficient parasite clearance in different animal models of visceral leishmaniasis.

	Control monotherapy (Dose)	**Effective combination chemotherapy (Dose)**	**Sub dose**	**Main outcomes†**
Mice model				
(Carter *et al.*, 2003)	BSO (34 mg/kg) SSG (74 or 282 mg SB^v/kg)	BSO (34 mg/kg) + SSG (74 mg SB^v/kg)	yes	↑ Reduced and total glutathione ↑ drug efficacy in sub doses ↓ Efficacy in clear parasitism in liver and bone-marrow ↓ Efficiently against resistant strain
(Shakya *et al.*, 2012a)	Pam3Cys (100 µg) MTF (2.5, 5 or 20 mg/kg)	PAM3Cys (100 µg) + MTF (5 mg/kg)	yes	↑ NO, ROS, H_2O_2; ↑ drug efficacy in sub doses; ↑ Phagocytic Index ↑ IL-12, TNFα, IFN-γ ↓ IL-6, IL-10
(Shakya *et al.*, 2012b)	F-TUF (60 µg) L-TUF (60 µg) MTF (2.5, 5 or 20 mg/kg)	L-TUF (60 µg) + MTF (5 mg/kg)	yes	↑ TNFα, IL-12 IFN-γ; ↑ NO, ROS; ↑ drug efficacy in sub doses ↑ Phagocytic Index; ↓ IL-10
(Bhattacharjee *et al.*, 2015)	SAG (250 mg/kg) GA (50 mg/kg)	GA (50 mg/kg) + SAG (250 mg/kg)	no	↑ TNFα, IL-12, IFN-γ, ↑ NO, ↓ TGF-β, IL-10, IL-4 (*in vitro* and *in vivo*) ↓ antimony efflux ↑ drug efficacy;
(Khadem *et al.*, 2017)	CAL-101 (0.05 mg) AMB (0.1 mg/kg)	CAL-101(0.05 mg) + AMB (0.1 mg/kg)	no	↓ T regulatory cells ↓ Treatment time ¨ Parasitological cure

¨No parasite was detected. † Main outcomes compared to the group treated with monotherapy. H_2O_2: Hydrogen peroxide; IFN-γ: Interferon gamma, IL: Interleukin, NO: nitric oxide, ROS: Reactive oxygen species, TGF-β: transforming growth factor beta, TNF-α: tumor necrosis factor alpha. Drugs: BSO: Buthionine sulfoximine, SSG: Sodium stibogluconate, PAM3cys: N-palmitoyl-S-(2, 3-bis (palmitoyloxy)-(2RS)-propyl)-Cys-Ser-Lys4.Hydrochloride), F-TUF: Free-Tufisin, L-TUF: Lipo-Tufisin, MTF: Miltefosine, SAG: Sodium antimony gluconate, GA: Glycyrrhizic acid, CAL-101: p110δ-specific pharmacological inhibitors, AMB: Amphotericin B.

Table 3 (*continuation*). Effective drug combinations able to induce an efficient parasite clearance in different animal models of visceral leishmaniasis.

	Control monotherapy (Dose)	Effective combination chemotherapy (Dose)	Sub dose	Main outcomes†
Mice model				
(Joice *et al.*, 2017)	POS (7.5, 15, 30 mg/kg) DB766 (19, 38, 75 mg/kg) KET (7.5, 15, 30 mg/kg) MTF (10 mg/kg)	DB766 (45 mg/kg) + KET (18 mg/kg) DB766 (75 mg/kg) + POS (30 mg/kg)	yes	↑ Liver concentrations and half-lives of DB766 and POS; ↑ drug efficacy
Rebello et al., 2019	MTF (3.85, 7.2, 15.4 mg/kg)	LPV (493.2 mg/kg) + MTF (7.2 mg/kg)	yes	↓Organ weight ↑ drug efficacy in sub doses ¨ Parasitological cure
	LPV (246.6 or 493.2 mg/kg)	LPV (246.6 mg/kg) + MTF (7.2 mg/kg)	yes	
Hamster model				
(Kar *et al.*, 1993)	SSG (50 or 100 mg Sb/kg)	ALO (15 mg/kg) + TAA (400mg)	no	¨ Parasitological cure (1-month model); Inhibited *Leishmania* transformation, multiplication and infectivity; ↑ drug efficacy in sub doses
	PET (8 mg/kg)	SSG (50 mg Sb/kg) + TAA (400mg)	yes	
	ALO (15 mg/kg)	PET (8 mg/kg) + TAA (400mg)	no	
	TAA (200 or 400 mg/kg)	PET (8 mg/kg) + TAA (200mg)	yes	
(Corral *et al.*, 2014)	AMB (1 or 5 mg/kg) ALL (5 mg/kg)	AMB (1 mg/kg) + ALL (5 mg/kg)	yes	↑ drug efficacy in sub doses ¨ Parasitological cure (50% of animals)
(Hendrickx *et al.*, 2017)	MTF (10, 20 or 40 mg/kg) PAR (180 or 350 mg/kg)	MTF (20 mg/kg) + PAR (350 mg/kg)	yes	No cross-resistance *in vivo* or *in vitro* after repeatedly exposed ↑ drug efficacy in sub doses ↓ Effectivity in lower doses and short treatment
		MTF (10 mg/kg) + PAR (180 mg/kg)	yes	

¨ No parasite was detected. † Main outcomes compared to the group treated with monotherapy. Drugs: POS: Posaconazole, DB766: 2,5-bis[2-(2-i-propoxy)-4-(2-pyridylimino) aminophenyl] furan hydrochloride, KET: Ketoconazole, MTF: Miltefosine, LPV: Lopinavir, SSG: Sodium stibogluconate, PET: Pentamidine, ALO: Allopurinol, TAA: *trans*-Aconiticc acid, All: Allicin, AMB: Amphotericin B, PAR: Paromomycin

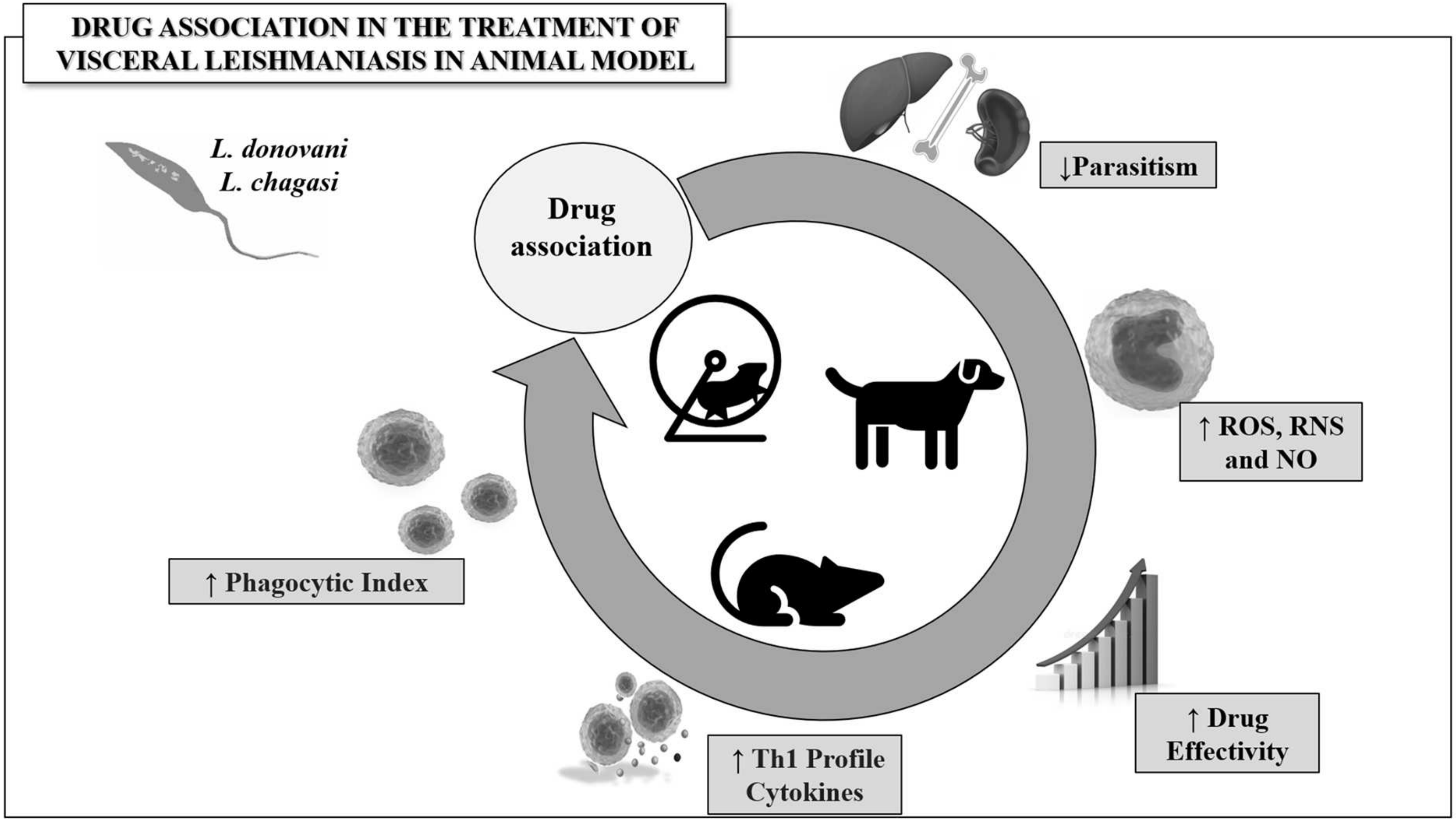

Figure 6: Representative model of the main outcomes obtained from drug combination used in the treatment of visceral leishmaniasis. Drug combination is potentially useful in potentiates chemotherapy effectivity by upregulating biosynthesis of reactive species and protective Th1 cytokines, stimulating proliferation and activity of immune cells, consequently reducing tissue parasitism in *Leishmania spp.*-infected animals.

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 a mean of 43.57 ± 6.79% items reported in SYRCLE's toll was covered from all studies reviewed. The criteria not met were predominantly underreported, indicating an unknown or high risk of bias. The chronological analysis of all studies indicated that the risk of bias exhibited no time-dependent influence, suggesting that methodological limitations have been systematically replicated over the years of research in the area. Eight studies (57.14%) 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), allocation concealment or the criteria about randomization (housing and outcome assessment) of which resulted in high risk of bias. The random sequence generator was performed in only one study (7.14%) (Joice *et al.*, 2017). Therefore, three studies (21.43%) randomization the animals but not reported the method used (Shakya *et al.*, 2012a; b; Corral *et al.*, 2014). The baseline characteristics for animal models were reported in most studies (n = 7, 50%). Interestingly, five studies started the experiment with animals from different sexes (Carter *et al.*, 2003; Mutiso *et al.*, 2011; Shakya *et al.*, 2012a,b; Mwololo *et al.*, 2015). Oliva *et al.*, 1998 started the treatment using dogs from different breeds, weight, age and clinical conditions. Incomplete outcome data were adequately addressed in 11 studies (78.57%). Three studies (21.43%) unreported the parasitism data for some treatment group (Kar *et al.*, 1993; Corral *et al.*, 2014; Khadem *et al.*, 2017a). Other potential sources of bias detected were the absence of information such as strain used, route of drug administration and lineage of animals.

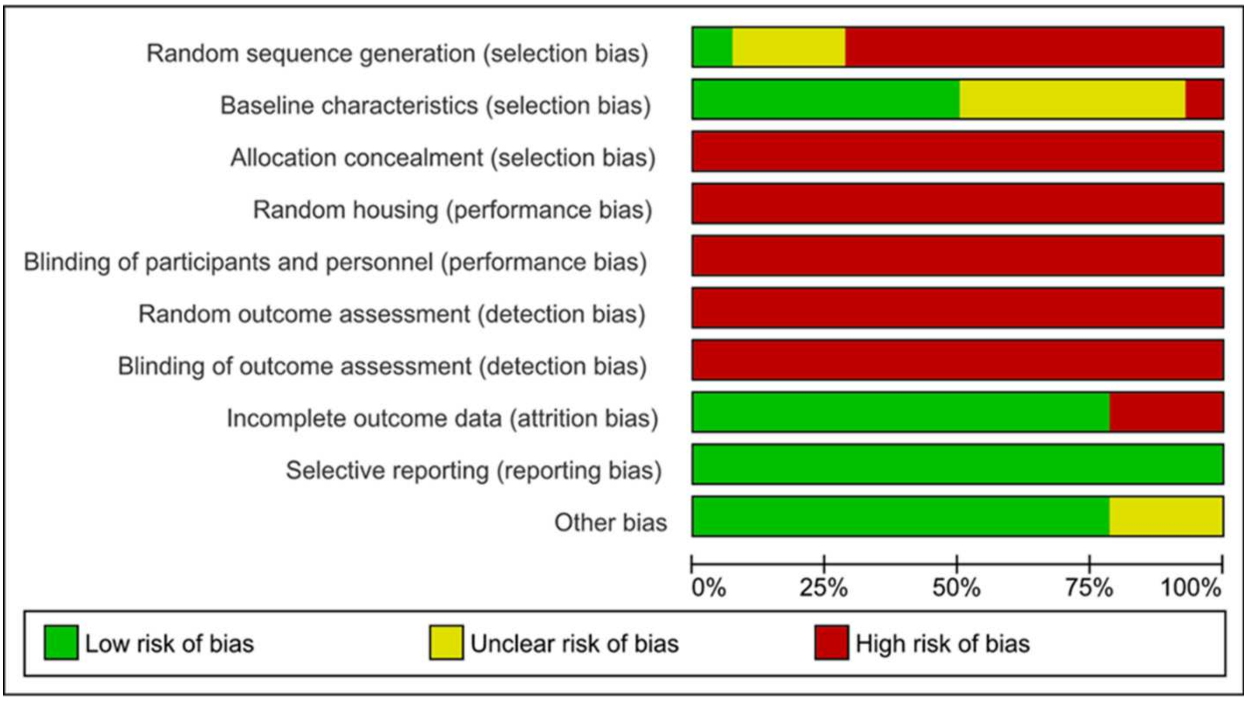

Figure 7: Results for the risk of bias and methodological quality indicators for all studies included in this systematic review that evaluated the effect of drugs combination for treatment of visceral leishmaniasis. The items covered by the Systematic Review Centre for Laboratory Animal Experimentation (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.

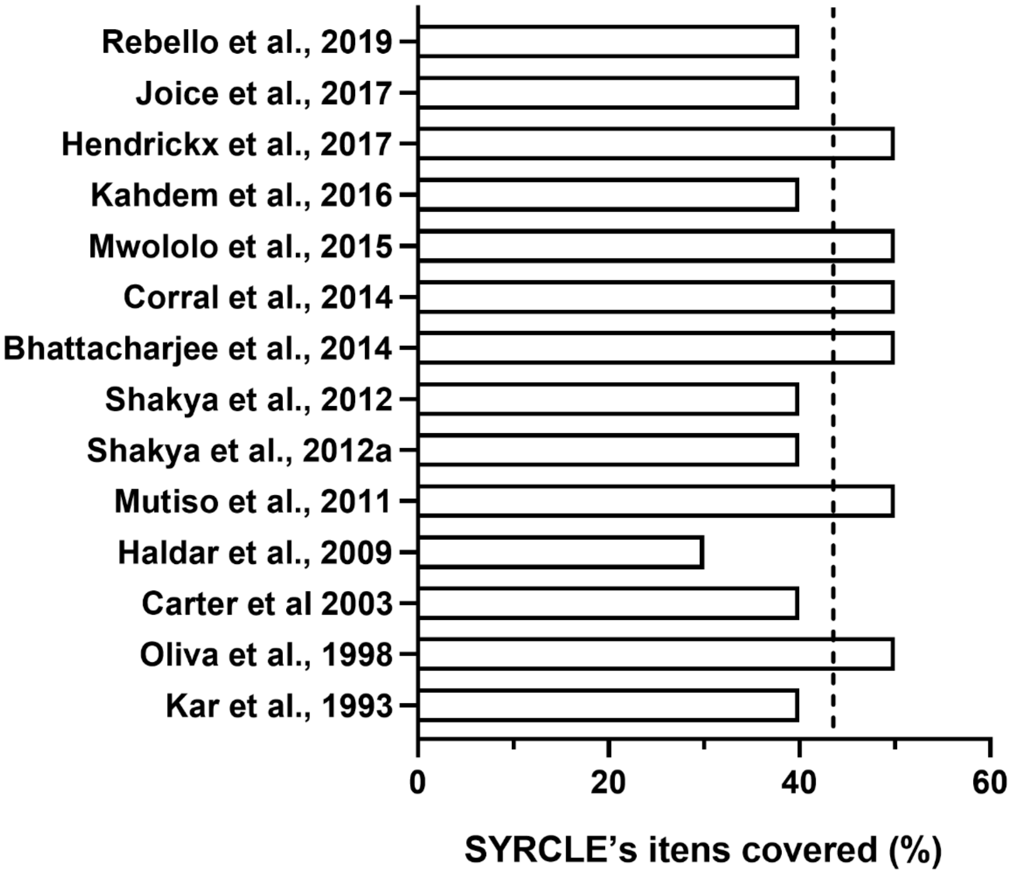

Figure 8: Analysis of the risk of bias in each study included in the systematic review. Based on the SYRCLE's risk of bias tool for animal studies. The dotted line indicates the average score obtained for all studies reviewed.

DISCUSSION

In the present review, our findings are discussed considering two guiding questions: (I) Are combinations of antileishmanial drugs effective in the treatment of VL? and (II) What are the main chemotherapy protocols and primary research outcomes used to determine treatment effectiveness? By answering these questions, we identified that specific protocols based on drugs combination can be used in realistic and rational strategies to potentiate the effectiveness of antiparasitic chemotherapy in different animal models of leishmaniasis compared to monotherapy. Interestingly, beneficial antiparasitic results were specially determined by the combination of complex pharmacological effects, especially stimulation of leukocyte replication and activity (Haldar *et al.*, 2009; Shakya *et al.*, 2012a; b), upregulation of Th1 cytokines, oxygen and nitrogen reactive species (ROS and RNS) production (Haldar *et al.*, 2009; Shakya *et al.*, 2012a; b; Bhattacharjee *et al.*, 2015), attenuation of the parasitic load and increase in the cure rates of infected hosts (Kar *et al.*, 1993; Carter *et al.*, 2003; Haldar *et al.*, 2009; Shakya *et al.*, 2012b; a; Corral *et al.*, 2014; Khadem *et al.*, 2017b; a; Hendrickx *et al.*, 2017; Joice *et al.*, 2017; Rebello *et al.*, 2019).

From our search strategy, we identified that studies investigating combination chemotherapy were mainly concentrated in five countries, such as Bangladesh, Brazil, Ethiopia, India, and Sudan. Considering that VL exhibits a broad geographic distribution in 98 countries (Alvar *et al.*, 2012; WHO, 2016), this finding indicates that drug combination is not yet widely explored for VL, although studies in the field have expanded in the last two decades. However, the countries in which the studies were developed are consistent with endemic areas for leishmaniasis, since VL cases are especially concentrated in Latin America, Africa and the Middle East (Alvar *et al.*, 2012; WHO, 2016; Singh *et al.*, 2016). In this areas, social and environmental conditions such as humidity, temperature, rainfall, poverty, hygiene habits and limited access to health services are favorable to vector spread, making it difficult to control the disease (Oryan and Akbari, 2016). Interestingly, most of the identified studies originated from India, which is the world leader in VL cases (Alvar *et al.*, 2012; WHO, 2016). Especially in this country, the governmental strategies associated to combination chemotherapy has been successfully integrated to clinical VL management (WHO, 2015; Oryan and Akbari, 2016). Despite the high incidence of VL in South America, only one study was identified (Rebello *et al.*, 2019). This finding is still poorly understood, although it is potentially related to limited investments in research and development, reinforcing the neglected characteristic of this tropical disease (Hotez *et al.*, 2008; Lindoso and Lindoso, 2009). A smaller number of studies were also derived from Italy, Belgium, Spain, United Kingdom, Canada and United States of America. In these countries, VL cases have been frequently associated with exotic *Leishmania* species, which are introduced in these areas by humans and dogs infected and that come from endemic countries (Pérez-Ayala *et al.*, 2009).

Despite geographical divergence of the studies reviewed, the animal model choice (i.e. BALB/c mice and golden hamster) reflected a convergent research perspective. The genetic similarity from mice inbred strains (i.e., BALB/c and C57BL/6), its high reproductive efficiency and wide available of analytical tools make these animals attractive models for preclinical research (Loria-Cervera and Andrade-Narvaez, 2014). Although extensively used (Carter *et al.*, 2003; Haldar *et al.*, 2009; Mutiso *et al.*, 2011; Shakya *et al.*, 2012a; b; Mwololo *et al.*, 2015; Khadem *et al.*, 2017a; b; Joice *et al.*, 2017), *Leishmania*-infected BALB/c mice presented limitations associated to disease development. Accordingly, these animals exhibit a natural profile of parasite resistance and, therefore, they do not represent the most adequate model to simulate human disease (Loeuillet *et al.*, 2016; McFarlane *et al.*, 2019). On the other hand, the hamster was reported as the best preclinical model for VL in three reviewed studies (Melby *et al.*, 2001; Loria-Cervera and Andrade-Narvaez, 2014). Characteristically, these animals exhibit immunopathological similarities of human VL, especially the limited ability in controlling parasites replication in target organs (i.e., liver, spleen, and bone marrow) at despite a strong Th1 response. Thus, the hamster infection culminates with the typical development of hepatosplenomegaly and hypergammaglobulinemia (Melby *et al.*, 2001; Loria-Cervera and Andrade-Narvaez, 2014). However, the applicability of hamsters is still limited due the scarcity of molecular tools available to investigate the immunological mechanisms associated with resistance or susceptibility to infection by *Leishmania spp*. (Gupta and Nishi, 2011; Loria-Cervera and Andrade-Narvaez, 2014). This limitation has been also observed in the dog model, which was reported in only study. Dogs are a realistic VL model (Quinnell and Courtenay, 2009), especially considering that these animals are natural reservoirs of *Leishmania spp.*, providing a more accurate preclinical understanding of the disease immunopathology with translational potential (Loria-Cervera and Andrade-

Narvaez, 2014). The limited use of dogs is not surprising, as they are generally associated with high animals cost for acquisition and maintenance, need of large facilities, and more restrictive ethical requirements compared to preclinical studies with rodent models (Loria-Cervera and Andrade-Narvaez, 2014).

In addition to the preclinical model, animals' age and sex are still highlighted as potential factors influencing the evolution of parasitic infections. There are consistent disagreements about the ideal age in animal models of *Leishmania ssp*. infection. While some studies reinforce that younger animal are more susceptible to infection (Müller *et al.*, 2008; Boldizsar *et al.*, 2010; Lockard *et al.*, 2019), others pointed that extreme of ages favor the infection due to the curvilinear profile of activation and effectiveness of the innate and acquired immune responses throughout the host life cycles in animals and humans (Boldizsar *et al.*, 2010; Fuentes *et al.*, 2017). As the animals' age was often underreported, the influence of this parameter on the immune response and on the results of antiparasitic chemotherapy cannot be accurately determined, requiring further investigation. Unlike age, the animals were consistently reported in most studies reviewed, which used female and male animals in a homogeneous proportion. There is evidence that sex hormones can interact with leucocytes such as monocytes/macrophages and lymphocytes, determining idiosyncratic immune patterns in male and female organisms (Snider *et al.*, 2009; Bhatia *et al.*, 2014; Kovats, 2015). Accordingly, differential immunosuppressive effects are potentially attributed to testosterone and progesterone levels, which can differentially down-regulate NFκB cell signal pathway, cytokines production, NK cells and macrophages activity (D'Agostino *et al.*, 1999; Snider *et al.*, 2009; Bhatia *et al.*, 2014). Previous studies also indicated that increased levels of these steroidal hormones were associated with a more intense production of Th2/Treg effectors such as IL-4 and IL-10 (Piccinni, 2000; Snider *et al.*, 2009), which are recognized by increase host susceptibility to VL (Shakya *et al.*, 2012b). From this physiological perspective, previous studies indicated that male hamster infected with are more susceptible to *L. donovani* infection, exhibiting higher parasite load compared female counterparts. Thus, male animals are suggested as a more appropriate preclinical model of leishmaniasis, an aspect also related to lower hormonal variability (Travi *et al.*, 2002; Lockard *et al.*, 2019).

Alongside the characteristics of the animal model, genetic and phenotypic variability of *Leishmania* strains also exerts a marked influence on host-pathogen interaction, time-course of infection and pathological outcomes (Loeuillet *et al.*, 2016; Samarasinghe *et al.*, 2018). From the studies reviewed, VL was especially induced by *L. donovani* MHOM/KE/82/LRC-L445/NLB065 and *L. infantum* MHOM/MA/67/ITMAP263 strains, which proved to be infective and pathogenic. In addition, parasite strains (i.e., *L. donovani* pentavalent antimonial-resistant MHOM/IN/1989/GE1) with recognized pharmacological resistance to classical leishmanicidal drugs (i.e., pentamidine, sodium antimony gluconate, sodium stibogluconate) were used (Haldar *et al.*, 2009; Bhattacharjee *et al.*, 2015). Interestingly, these strains were intentionality used in realistic strategies to identify more efficient chemotherapeutic strategies to overcome drug resistance observed in several cases of VL (Haldar *et al.*, 2009; Bhattacharjee *et al.*, 2015). In preclinical models, the parasite inoculum is additionally relevant and must be carefully delimited. Accordingly, the inoculum size can exert a marked impact on the parasite load, tissue parasitism, immunological sensitization, infection severity and mortality rates of the infected host (Loeuillet *et al.*, 2016). In the studies reviewed, the use of medium (1×10^6) or higher (1×10^8) parasite inoculum was effective in inducing a marked parasitism in target organs (i.e., liver, spleen, and bone marrow), which was consistent with the human infection (Rolão *et al.*, 2004; Oliveira *et al.*, 2012). There is evidence that poorly sized inoculum (i.e., 10^3 to 10^4 parasites) may be inadequate to induced VL, since Th1 immunological effectors can resolve the infection resolution before relevant pathological manifestations develop (Kaur *et al.*, 2008; Oliveira *et al.*, 2012). Conversely, high inoculum ($\cong 10^7$ parasites) can trigger an exacerbated Th2 immune response, which aggravates tissue parasitism and infection severity (Rolão *et al.*, 2004; Kaur *et al.*, 2008). When evaluating the parasite load induced by different inoculation routes, Kaur *et al.*, (2008) concluded that subcutaneous is less efficient than intradermal, intraperitoneal and intracardiac routes to induce liver parasitism. In this sense, intracardiac and intraperitoneal routes were used in 80% of the studies reviewed. These routes were consistent with the development of a more prominent Th2 phenotype, which was suitable to stimulate tissue parasitism and a persistence infection (Mukherjee *et al.*, 2003).

From the 14 studies investigating the *in vivo* models of VL described, 7 studies also evaluated drug combinations *in vitro*. Most studies were consistent in report a potent leishmanicidal effect *in vitro* (Kar *et al.*, 1993; Mutiso *et al.*, 2011; Bhattacharjee *et al.*, 2015; Mwololo *et al.*, 2015; Hendrickx *et al.*, 2017; Joice *et al.*, 2017), and only one study indicated cytotoxicity on host cells (Rebello *et al.*, 2019). According to Huang *et al*., (2019) *in vitro* studies are relevant to investigate potential drug interactions. Thus, the combination of drugs with additive and especially synergistic effects generally results in better therapeutic outcomes compared to monotherapy in preclinical models (Huang *et al.*, 2019). In this perspective, 23 drugs administered in 40 different combinations were identified from all studies reviewed. These combinations were especially based on the following drugs: (i) BSO + SSG (Carter *et al.*, 2003); (ii) PV6 + SAG (Haldar *et al.*, 2009); (iii) DIM + ART (Mutiso *et al.*, 2011); (iv) Pam3Cys + MTF (Shakya *et al.*, 2012a); (v) TUF + MTF (Shakya *et al.*, 2012b); (vi) GA + SAG (Bhattacharjee *et al.*, 2015); (vii) DIM + CHQ (Mwololo *et al.*, 2015); (viii) CAL-101 + AMB (Khadem *et al.*, 2017a); (ix) DB766 + POS or DB766 + KET (Joice *et al.*, 2017); (x) MTF + LPV (Rebello *et al.*, 2019); (xi) PAR + MEG (Oliva *et al.*, 1998); (xii) SSG + TAA, ALO + TAA, PET + TAA and SSG + ALO (Kar *et al.*, 1993); (xii) AMB + ALL (Corral *et al.*, 2014) and (xiv) MTF + PAR (Hendrickx *et al.*, 2017).

Based on the results of parasite control compared to antiparasitic monotherapy, superior therapeutic responses were obtained from the following combination strategies: (i) BSO (34 mg/kg) + SSG (74 mg SBv/kg) compared to SSG (282 mg SBv/kg) (Carter *et al.*, 2003); (ii) PV6 (0.5 µmol/30g) + SAG (50 mg/kg) compared to SAG (250 mg/kg) (Haldar *et al.*, 2009); (iii) Pam3Cys (100 µg) + MTF (5 mg/kg) compared to MTF (20mg/kg) (Shakya *et al.*, 2012a); (iv) L-TUF (60 µg) + MTF (5 mg/kg) compared to MTF (20mg/kg) (Shakya *et al.*, 2012b); (v) GA (50 mg/kg) + SAG (250 mg/kg) compared to SAG (250 mg/kg) (Bhattacharjee *et al.*, 2015); (vi) CAL-101(0.05 mg) + AMB (0.1 mg/kg) compared to AMB (0.1 mg/kg) (Khadem *et al.*, 2017a); (vii) DB766 (45 mg/kg) + KET (18 mg/kg) compared to MTF (10mg/kg) (Joice *et al.*, 2017); (viii) LPV (493.2 mg/kg) + MTF (7.2 mg/kg) and LPV (246.6 mg/kg) + MTF (7.2 mg/kg) compared to MTF (15.4 mg/kg) (Rebello *et al.*, 2019); (ix) PAR (3.5 mg/kg) + MEG (20 mg Sb/kg) compared to MEG (30 mg Sb/kg); (x) SSG (50 mg Sb/kg) + TAA (400mg) or PET (8 mg/kg) + TAA (200mg) or PET (8 mg/kg) + TAA (400mg) and ALO (15 mg/kg) + TAA (400mg) compared to monotherapy using SSG (100 mg Sb/kg), SSG (50 mg Sb/kg), PET (8 mg/kg) or ALO (15 mg/kg) (Kar *et al.*, 1993) (xi) AMB (1 mg/kg) + ALL (5 mg/kg) compared to AMB (5 mg/kg) (Corral *et al.*, 2014) and (xii) MTF (20 mg/kg) + PAR (350 mg/kg) or MTF (10 mg/kg) + PAR (180 mg/kg) compared to MTF (40 mg/kg) (Hendrickx *et al.*, 2017).

Interestingly, combination strategies based on MTF and antimony-based drugs (SSG, SAG and MEG) were more frequently used in all studies analyzed. In general, this is a rational strategy considering a more favorable time and cost of treatment, reducing the dose (use of subdoses) and toxicity of the combined drugs, which can act through complementary ways to overcome the parasite's pharmacological resistance (Olliaro et al., 2005). Despite the drug combination exhibits a theoretical potential to improve the host response to VL treatment, find an effective dosage schedule is still a challenge task, which is not always successful. Thus, worse results than those obtained for AMB or MTF monotherapy were reported in 7 studies (Mutiso *et al.*, 2011; Shakya *et al.*, 2012a; b; Mwololo *et al.*, 2015; Hendrickx *et al.*, 2017; Joice *et al.*, 2017; Rebello *et al.*, 2019). Therapeutic schemes such as PAR (3.5 mg/kg) + MEG (30 mg Sb/kg) for dog model (Oliva *et al.*, 1998), DIM (12.5 mg/kg) + ART (12.5 mg/kg) in mice (Mutiso et al., 2011), and DIM (12.5 mg/kg) + CHQ (12.5 mg/kg) also applied in mice (Mwololo et al., 2015) showed lower or the same effectivity achieved from the monotherapy with the reference drugs MEG, PAR or the experimental drugs used alone, as DIM, ART or CHQ.

Considering the studies investigating the hamster model, 8 different drugs used in 11 therapeutic combinations were identified. Among these combinations, 7 were successful in reducing parasitism and increasing chemotherapy effectiveness when administered in subdoses (Kar *et al.*, 1993; Corral *et al.*, 2014; Hendrickx *et al.*, 2017). In these studies, was reported that drug combination was also effective in inhibiting parasite multiplication, transformation and infectivity (Kar *et al.*, 1993), as well as achieving parasitological cure (Kar *et al.*, 1993; Corral *et al.*, 2014) without cross-resistance *in vivo* or *in vitro* after repeated exposures to combined treatment (Hendrickx *et al.*, 2017). This is a remarkable finding, especially considering that monotherapy strategies currently prescribed to treat VL have been associated to parasite cross-resistance to alternative or second-line leishmanicidal drugs, especially in endemic areas (Rijal *et al.*, 2013). Thus, the combination of drugs becomes relevant as a safe and effective alternative to reduce the dose and toxicity of the treatment, ensuring the desirable antiparasitic efficacy (Sundar *et al.*, 2011; Hendrickx *et al.*, 2017). In this since, MEG and PAR coadministration was effective in reducing parasite load in the liver and bone marrow in naturally infected dogs, although this combination did not induce parasitological cure (Oliva *et al.*, 1998). However, by attenuating disease severity, MEG plus PAR cannot be disregarded as an alternative combined treatment for natural canine leishmaniasis (Oliva *et al.*, 1998). Although this experimental model has been little used, naturally infected dogs of varying breeds are excellent tools to investigate the therapeutic efficacy of drug combinations for VL. Accordingly, the variable genetic and immunological background modulates disease evolution and increases the heterogeneity of the research outcomes, characteristics that bring this model closer to the epidemiological reality associated with the domestic cycle of VL transmission (Quinnell *et al.*, 2003; de Vasconcelos *et al.*, 2019).

In addition to the hamster model, 17 drugs administered in 28 different combinations were identified in VL mice models. In general, most studies obtained better therapeutic effects from drug combinations than monotherapy. Accordingly, the beneficial effects achieved from combined therapy were mainly associated to the improvement of the following parameters: Half-life or drug retention (Bhattacharjee *et al.*, 2015; Joice *et al.*, 2017), sub-doses efficacy (Carter *et al.*, 2003; Bhattacharjee *et al.*, 2015; Khadem *et al.*, 2017a; Joice *et al.*, 2017; Rebello *et al.*, 2019), immunomodulation (Shakya *et al.*, 2012a; b; Bhattacharjee *et al.*, 2015; Khadem *et al.*, 2017a), and activation of oxidative defenses (Carter *et al.*, 2003; Shakya *et al.*, 2012a; b). In addition, parasitological cure was achieved in mice models treated with CAL-101 (0.05 mg) + AMB (0.1 mg/kg) (Khadem *et al.*, 2017a), and LPV (493.2 mg/kg) + MTF (7.2 mg/kg) (Rebello *et al.*, 2019). More than two thirds of the studies reviewed reported low toxicity of the new drug combinations, down-regulation of Th2/Treg cytokines (i.e., IL-4/IL-10) and upregulation of antiparasitic effectors, especially reactive oxygen (ROS) and nitrogen (RNS) species and cytokines Th1 cytokines (i.e., IFN-γ, IL-12 and TNF). Thus, success in VL treatment was especially associated to the development of a more effective Th1 phenotype, which potentiated the ROS and RNS production and the elimination of intracellular amastigotes by macrophages (Kaur *et al.*, 2008; Khadem *et al.*, 2017a). Considering the analyzed pathological outcomes, it becomes evident that the reviewed studies direct drug combinations to stimulate the Th1 phenotype, a rational strategy considering that this phenotype is associated with greater host resistance against *Leishmania spp*. infection (Haldar *et al.*, 2009; Mutiso *et al.*, 2011; Shakya *et al.*, 2012a; b; Bhattacharjee *et al.*, 2015; Khadem *et al.*, 2017a). In fact, the immunomodulatory effects of drug combinations seem to be a convergent mechanism associated with the improvement of leishmanicidal defenses in mice (Musa *et al.*, 2010; Shakya *et al.*, 2012a; b). Thus, current evidence is consistent in demonstrating that curing VL depends simultaneously on the direct ability to kill the parasite and a protective immunological profile, aspects that were simultaneously stimulated in different pharmacological combinations used as AMB + CAL (Khadem *et al.*, 2017a), MTF + TUF (Shakya *et al.*, 2012b), MTF + PAM3Cys (Shakya *et al.*, 2012a) and SAG + GA (Bhattacharjee *et al.*, 2015).

Considering a critical interpretation of the evidence, the objective assessment of methodological quality indicated important elements of bias in the reviewed studies. Even considering the specificities of each research design in the context of the bias analysis, no study fulfilled all methodological criteria, with an average of 43.57% fulfilled criteria. In addition, the studies presented variable methodological score without a temporal influence (year of publication), indicating that elements of bias are systematically replicated in this area of parasitological research, despite methodological advances and the greater availability of more sensitive and specific analytical tools. 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, allocation concealment, and complete description of research results undermine the reproducibility, internal and external validity of the reviewed studies, limiting the reliability research evidence. Methodological bias analysis corroborated the low quality of research reports, showing high or unknown risk of bias for most studies and categories evaluated. In general, the description of baseline characteristics (characterization of models and experimental conditions) represented the criteria best performed by studies with murine models. However, studies using dogs had obvious limitations in describing these baseline characteristics. Considering that naturally infected dogs were used, the difficulty to delimit aspects related to race, sex, weight, age and time of infection are justifiable, representing a methodological limitation inherent to this preclinical model. However, it is imperative that experimental models artificially constructed to simulate human VL overcome the bias factors previously identified from current scientific evidence. In this sense, by mapping the risk of bias in all investigated studies, this review provides objective support to delimit further studies with greater methodological rigor, providing unequivocal evidence on the relevance and effectiveness of drug combinations for VL treatment.

From this systematic review, we identified that despite exhibiting some risk of methodological bias, current evidence indicates that the combination of drugs with different mechanisms of action is potentially relevant to treat VL. In general, combinations based on MTF + CAL-101, MTF + LPV, AMB + ALL, AMB + PAR, TAA + SSG, TAA + ALO and TAA + PET achieved better therapeutic effects compared to monotherapy with the currently prescribed reference leishmanicidal drugs, such as SAG, MEG and SSG. From a mechanistic point of view, the improved therapeutic effects induced by the pharmacological associations were achieved by the combination of direct parasitic toxicity and the upregulation of immunological effectors linked to the Th1 protective phenotype, which increases the host's natural defenses against infection by *Leishmania spp.* By acting in an additive or synergistic way, drug combinations can improve the management of VL, reducing the costs, doses, time and adverse effects associated with the treatment of this infection. Thus, drug combination offers a realistic opportunity to overcome parasitic resistance to leishmanicidal chemotherapy, alleviating infection severity and increasing the parasitological cure rates in *Leishmania spp.*-infected hosts. However, combinations based on SSG + ALO, PAR + MEG, DB766+ POS, DB766 + KET, DIM + CHQ, MTF + Pam3Cys, MTF + TUF, PV6 + SAG and DIM + ART did not show additional therapeutic benefits compared to monotherapy with AMB, MTF. Thus, drugs used in combination strategies must be carefully and rationally delimited, as they can trigger similar or even worse effects than monotherapy, characterizing relative or absolute contraindications to treat VL.

CONFLICT OF INTEREST

The authors declare that they have no conflict of interest.

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SUPPLEMENTARY TABLES

Table S1. 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, PET, ALO, TAA
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	?	BSO, SSG
Haldar et al., 2009	IN	Mice	Balb/c	?	4-6w/?	?	PV6, SAG
Mutiso et al., 2011	KE	Mice	Balb/c	♀/♂	6-8 w/?	30	DIM, AMB, ART
Shakya et al., 2012a	IN	Mice	Balb/c	♀/♂	?/18-20g	?	MTF, Pam3Cys
Shakya et al., 2012b	IN	Mice	Balb/c	♀/♂	?/18-20g	?	MTF, TUF
Bhattacharjee et al., 2014	IN	Mice	Balb/c	?	4-6w/?	20	SAG, GA
Corral et al., 2014	ES	Hamster	?	♀	?/80-90g	35	AMB, ALL
Mwololo et al., 2015	KE	Mice	Balb/c	♀/♂	6-8 w/?	40	AMB, CHQ, DIM
Kahdem et al., 2016	CA	Mice	Balb/c	♀	6-8w/20g	?	AMB, CAL-101
Hendrickx et al., 2017	BE	Hamster	Golden	♀	?/80-100g	45	MTF, PAR
Joice et al., 2017	US	Mice	Balb/c	♀	6-8w/?	?	POS, KET, MTF, DB766
Rebello et al., 2019	BR	Mice	Balb/c	♀	6-8w/?	?	MTF, LPV

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; **SAG:** sodium antimony gluconate, **MEG**: Meglumine Antimoniate; **PAR**: Paromomycin; **MTF**: Miltefosine; **AMB**: Amphotericin B; **MET**: Metronidazole; **KET**: Ketoconazole**, TUF:** Tufinsin, **GA:** Glycyrrhizic acid, **ALL:** Allicin, **DIM:** Diminazene, **CAL-101:** p110δ-specific pharmacological inhibitors, **PAR:** Paromomycin, **LPV:** Lopinavir, **DB766:** 2,5-bis[2-(2-i-propoxy)-4-(2-pyridylimino) aminophenyl]furan hydrochloride, **Pam3Cys:** N-palmitoyl-S-(2, 3-bis (palmitoyloxy)-(2RS)-propyl)-Cys-Ser-Lys4.Hydrochloride), **ART:** Artesunate **CHQ:** Chloroquine, **PET:** Pentamidine, **ALO:** Allopurinol, **TAA:** *trans*-Aconiticc acid, **PV6:** Diperoxovanadate.

Table S2. 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	*L. infantum*	?	?	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., 2012b	*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
Mwololo et al., 2015	*L. donovani*	MHOM/KE/82/LRC-L445/NLB-065	$1x10^6$	intraperitoneal
Kahdem et al., 2016	*L. donovani*	MHOM/ET/67/HU3	$5x10^7$	intravenous
Hendrickx et al., 2017	*L. infantum*	MHOM/MA/67/ITMAP263	$2x10^7$	intracardiac
Joice et al., 2017	*L. donovani*	MHOM/ET/67:LV82	$5x10^7$	intravenous
Rebello, 2019	*L. infantum*	MHOM/MA/67/ITMAP-263	$1x10^8$	intraperitoneal

Table S3. 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)	daily (PAR); bis in d. (MEG)	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., 2012b	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

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 S3 (*Continuation*). General characteristic of the treatment protocols used in all studies included in the systematic review.

Author	Combination chemotherapy	Dose	Frequency	Route
Corral et al., 2014	Allicin (ALI) + Amphotericin B (AMB)	ALI (5 mg/kg) AMB (1, 5 mg/kg)	daily	ip/ip
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; **po**: per oral route; **sc**: subcutaneous; **im**: intramuscular. **alt. d.**: every alternate day; **bis in 7d**: twice in week; **bis in d.**: twice in day