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Caroline Sobotyk de Oliveira

AVALIAÇÃO DE MINERAIS E ELEMENTOS TRAÇO NA LEISHMANIOSE CUTÂNEA E SUA CORRELAÇÃO COM A PATOGÊNESE DA DOENÇA

Santa Maria, RS

2019

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Tese apresentada ao Curso de Pós-graduação em Medicina Veterinária, da Universidade Federal de Santa Maria (UFSM, RS), como requisito parcial para obtenção do título de **Doutora em Sanidade e Reprodução Animal.**

Orientador: Prof. Dr. Luis Antonio Sangioni

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A diferença entre o cientista e o mítico é que o cientista tem um modo sereno de examinar fatos, enquanto o misticismo tanto poderá ser orientado pelos bons profetas, como também pelos maus, pois o misticismo não raciocina e precisamos de lucidez.

(Bertha Lutz, 1936)

RESUMO

AVALIAÇÃO DE MINERAIS E ELEMENTOS TRAÇO NA LEISHMANIOSE CUTÂNEA E SUA CORRELAÇÃO COM A PATOGÊNESE DA DOENÇA

AUTORA: CAROLINE SOBOTYK DE OLIVEIRA
ORIENTADOR: LUIS ANTONIO SANGIONI

A leishmaniose é uma doença ocasionada por parasitos do gênero *Leishmania* e pode se manifestar através de diversas formas clínicas com lesões cutâneas localizadas, disseminadas ou até a forma visceral. O resultado clínico de uma infecção por *Leishmania* spp. é variável e depende de diversos fatores, tanto relacionados ao parasito, quanto ao hospedeiro. A imunidade ou susceptibilidade às doenças infecto-parasitárias estão relacionadas com o estado nutricional do paciente. Nos últimos anos algumas pesquisas têm relacionado o estado nutricional e a presença ou ausência de minerais e elementos traço com a gravidade da doença, tanto para a leishmaniose tegumentar, quanto para a visceral. Sendo assim, este estudo teve como objetivo identificar e quantificar alterações de macro e microelementos durante o curso de infecção experimental por *Leishmania (Leishmania) amazonensis* e avaliar sua influência na patogenicidade da doença, correlacionando-os com a carga parasitária, análise histopatológica e parâmetros de estresse oxidativo. Para isto, camundongos BALB/c foram inoculados com as formas promastigotas de *L. (L.) amazonensis* em fase estacionária de crescimento. Os animais foram divididos em diferentes grupos, simulando quadros de infecções em hospedeiros imunocompetentes e imunocomprometidos. Amostras de sangue, baço, fígado e rim foram coletadas e submetidas à análise de espectrometria de Emissão Óptica por Plasma Acoplado Indutivamente, realizado testes histopatologicos, bem como da atividade da butirilcolinesterase, mieloperoxidase, substâncias reativas ao ácido tiobarbitúrico, superóxido dismutase, tióis totais e glutationa peroxidase. Foi possível observar a doença sistêmica com lesões histopatológicas em diferentes órgãos dos animais infectados, além de diminuição nos níveis de zinco e manganês no baço de animais infectados com *L. (L.) amazonensis* e imunossuprimidos pela depleção de células T $CD4^+$ quando comparado ao grupo controle ($P<0,05$). No entanto não foi observado alteração nos parâmetros de estresse oxidativo evidenciando a resistência dos protozoários de *L. (L.) amazonensis* frente à resposta antioxidante de hospedeiros imunocompetentes e imunossuprimidos, possibilitando assim a persistência da infecção.

Palavras-chave: *Leishmania amazonensis*. Leishmaniose tegumentar. Macronutrientes. Micronutrientes. Estresse oxidativo.

ABSTRACT

ASSESSMENT OF MINERALS AND TRACE ELEMENTS IN CUTANEOUS LEISHMANIOSIS AND CORRELATION WITH PATHOGENESIS OF THE DISEASE

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Leishmaniasis is a disease caused by parasites of the genus *Leishmania* and can manifest several clinical forms like localized, disseminated skin lesions or visceral form. The clinical result of a *Leishmania* spp. infection is variable and depends on several factors, both related to the parasite and to the host. Immunity or susceptibility to infectious-parasitic diseases is related to the nutritional status of the patient. In recent years, some research has correlated nutritional status and the presence or absence of minerals and trace elements with the severity of the disease, both for tegumentary and visceral leishmaniasis. This study aimed to identify and quantify changes in macro and microelements during the course of experimental infection by *Leishmania* (*Leishmania*) *amazonensis* and to evaluate its influence on the pathogenicity of the disease, correlating them with the parasite load, histopathological analysis and parameters oxidative stress. For this, BALB/c mice were inoculated with the promastigote forms of *L.* (*L.*) *amazonensis* in stationary phase of growth. The animals were divided into different groups and submitted to different clinical situations, simulating immunocompetent and immunocompromised hosts. Samples of blood, spleen, liver and kidney were collected and subjected to Inductively Coupled Plasma Optical Emission Spectroscopy analysis, histopathology, butyrylcholinesterase activity, myeloperoxidase, thiobarbituric acid reactive substances, superoxide dismutase, total thiols and glutathione peroxidase. It was possible to observe visceralization of the disease with histopathological lesions in several organs of the infected animals, as well as a decrease in the levels of zinc and manganese in the spleen of *L.* (*L.*) *amazonensis* infected animals and immunosuppressed by $CD4^+$ T cell depletion when compared to the control group ($P < 0.05$). However, no alteration in the oxidative stress parameters was observed evidencing the resistance of *L.* (*L.*) *amazonensis* protozoans to the antioxidant response of immunocompetent and immunosuppressed hosts.

Key-words: *Leishmania amazonensis*. Cutaneous leishmaniasis. Macronutrients. Micronutrients. Oxidative stress.

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LISTA DE ABREVIATURAS E SIGLAS

º	- Grau
%	- Por cento
µA	- Microamper
µg	- Micrograma
µL	- Microlitro
µmol	- Micromolar
AAP	- 4-aminoantipirina
ANOVA	- Análise de Variância
B.O.D.	- Demanda Biológica de Oxigênio
BChE	- Butirilcolinesterase
C	- Centígrado
Ca	- Cálcio
CAM	- Concentração Alveolar Mínima
Cm	- Centímetros
CCD	- Dispositivo de Carga Acoplada
CEUA	- Comissão de Ética no Uso de Animais
Co.	- Companhia
CONCEA	- Conselho Naconal de Controle de Experimentação Animal
$CuSO_4$	- Sulfato de Cobre
d.C.	- Depois de Cristo
DNA	- Ácido desoxirribonucleico
DTNB	- 5,5'-ditio-bis (ácido 2-nitrobenzóico)
DTPA	- Ácido dietilenotriaminopentacético
DTT	- Ditiotreitol
EDTA	- Ácido etilenodiamino tetra-acético
EDXRF	- Fluorescência de raios-X por dispersão de energia
ELISA	- Ensaio de Imunoabsorção Enzimática
ERRO	- Espécie Reativa do Oxigênio
GPx	- Glutationa Peroxidase
GSH	- Glutationa
GSH-Px	- Glutationa Peroxidase
H_2O_2	- Peróxido de Hidrogênio
H_2SO_4	- Ácido Sulfúrico
HCl	- Ácido Clorídrico
HNO_3	- Ácido Nítrico
HE	- Hematoxilina e Eosina
HEPES	- Ácido 2-[4-(2-hidroxietil)-piperazina-1-il]-etanossulfônico
ICP-OES	- Espectrometria de Emissão Óptica por Plasma Acoplado Indutivamente
Ip	- Intraperitoneal
Kg	- Quilo
kV	- Quilovolt
L	- Litro
LC	- Leishmaniose Cutânea
LM	- Leishmaniose Muco-cutânea
LTA	- Leishmaniose Tegumentar Americana
LV	- Leishmaniose Visceral
M	- Molar
MDA	- Malondialdeído

Mg	- Magnésio
Mg	- Miligrama
min	- Minutes
mL	- Mililitro
Mm	- Milímetro
mM	- Minimolar
Mol	- Massa molecular
MPO	- Mieloperoxidase
N	- Número
Na_2CO_3	- Carbonato de sódio
NADP	- Fosfato de Dinucleotídeo de Adenina e Nicotinamida
Nm	- Nanômetro
NPSH	- Tióis não proteicos
PBS	- Tampão fosfato-salino
Ph	- Unidade de medida para especificar acidez ou basicidade
Rh	- Ródio
Rpm	- Rotações por minuto
RIFI	- Reação Imunofluorescência Indireta
SDS	- Dodecilsulfato de Sódio
SFB	- Soro Fetal Bovino
SOD	- Superóxido Dismutase
TBA	- Ácido Tiobarbitúrico
TBARS	- Substâncias Reativas ao Ácido Tiobarbitúrico
TCA	- Ácido Tricloroacético
Tris	- Trisaminometano
T-SHs	- Tióis totais
U	- Unidade
UFCSPA	- Universidade Federal de Ciências da Saúde de Porto Alegre
UFSM	- Universidade Federal de Santa Maria
UV-VIS	- Ultravioleta-visível
vs.	- Versos
v/v	- Concentração/Volume
W	- Watts
w/v	- Massa/Volume
WHO	- Organização Mundial da Saúde
X	- Vezes
x g	- Força Gravitacional
Zn	- Zinco

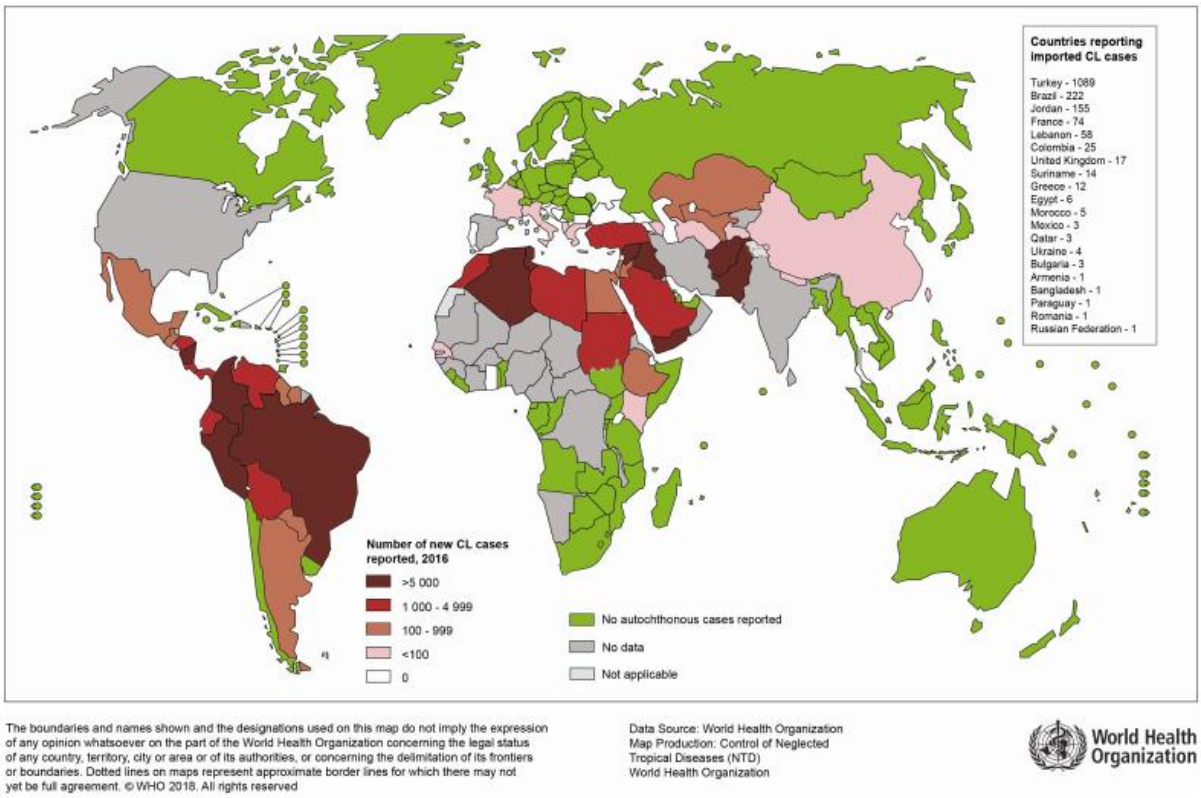
SUMÁRIO

1 APRESENTAÇÃO

A Leishmaniose é uma doença zoonótica, de transmissão vetorial causado por diversas espécies de protozoários do gênero *Leishmania*. A enfermidade tem distribuição mundial e é considerada um grave problema de saúde pública, com uma incidência de 2 milhões casos por ano. Estima-se que 350 milhões de pessoas estão sob o risco de contrair a doença. Atualmente a Leishmaniose encontra-se distribuída em 88 países, estimando-se uma prevalência de 14 milhões de casos e 59 mil óbitos (WHO, 2010). A doença esta classificada no grupo de doenças tropicais negligenciadas tendo alta prevalência em áreas de vulnerabilidade social e em condições de pobreza contribuindo, assim, com o quadro de desigualdade social e econômica do país. Além disso, o reduzido potencial de retorno lucrativo para a indústria farmacêutica gera baixo interesse no desenvolvimento de pesquisas, novas drogas e vacinas para o controle da doença (COURA e CASTRO, 2002; OLIVEIRA, B. et al., 2016; RIBEIRO et al., 2018).

As leishmanioses apresentam-se de duas formas: leishmaniose cutânea (LC) ou leishmaniose visceral (LV). A patogenicidade e a apresentação clínica da doença variam conforme a espécie envolvida, a carga parasitária e o estado imunológico do hospedeiro (SCOTT e NOVAIS, 2016). Diferentemente da LV, a LC não é uma doença letal. No entanto a sua importância reside, além da alta incidência e ampla distribuição, no quadro clínico gerado, pois é altamente traumática resultando em grande impacto tanto no campo sanitário como no social (VAN DER AUWERA e DUJARDIN, 2015). Na útima década, foram relatados aproximadamente 1,5 milhão de casos por ano, compreendendo as duas principais formas clínicas da doença: forma clínica cutânea, encontrada principalmente em regiões da América do Sul e Oriente Médio, e forma clínica muco-cutâneas, a qual se concentra quase que na sua totalidade (90% dos casos) no Brasil, Peru e Bolívia (Figura 1) (ELMAHALLAWY et al., 2014; POURMOHAMMADI et al., 2010).

Figura 1 - Situação mundial da endemicidade da leishmaniose cutânea em 2016.



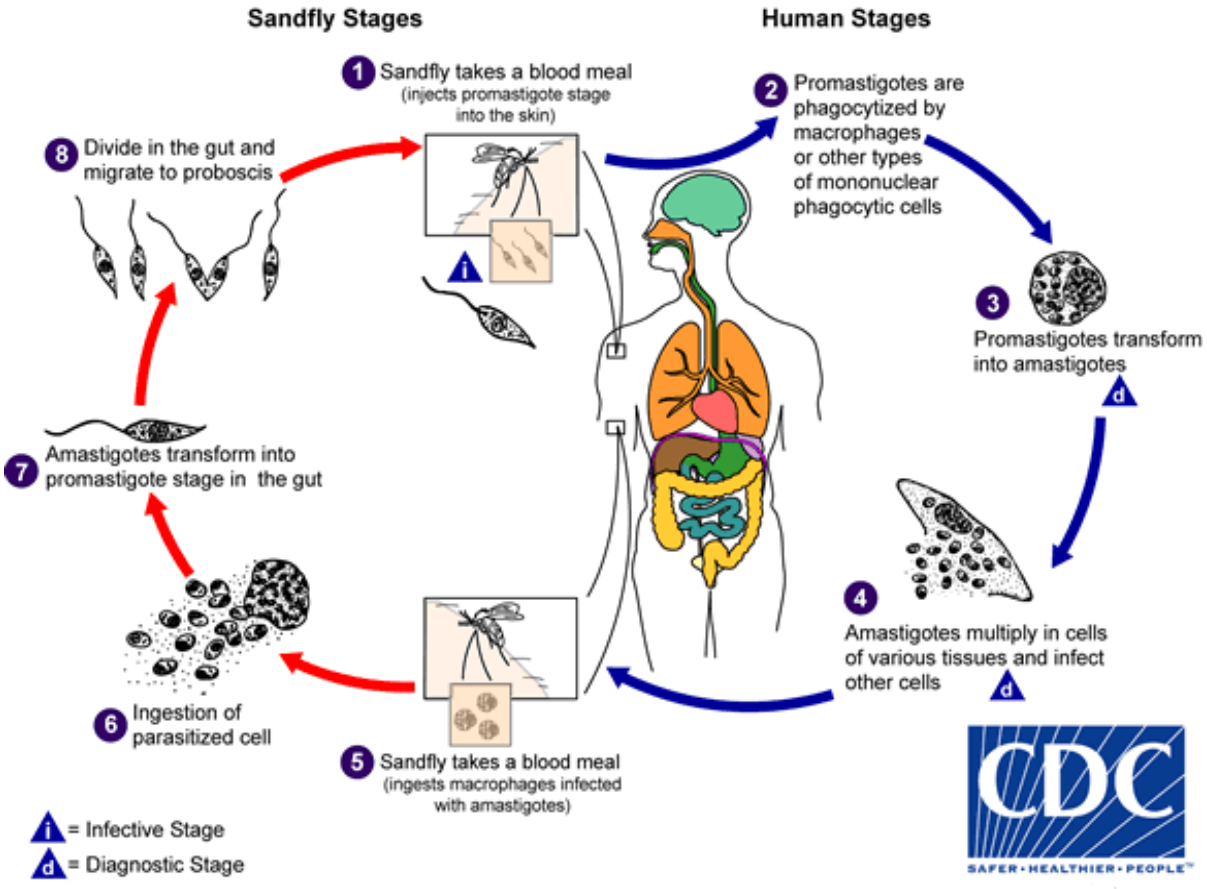
Fonte: (WHO, 2016)

A enfermidade apresenta algumas variações epidemiológicas, conforme a espécie do agente etiológico envolvido, a região geográfica e as espécies de hospedeiros acometidos (SCHIMMING e PINTO E SILVA, 2012). Até o momento, sete espécies de *Leishmania*, pertencentes aos subgêneros *Leishmania* e *Viannia*, foram identificadas no Brasil como causadoras de LC: *Leishmania (Viannia) braziliensis, Leishmania (Viannia) guyanensis, Leishmania (Leishmania) amazonensis, Leishmania (Viannia) naiffi, Leishmania (Viannia) lainsoni, Leishmania (Viannia) shawi* e *Leishmania (Viannia) lindenbergi* (BRASIL, 2014; GUERRA et al., 2015). Na área urbana o cão (*canis familiaris*) é o principal reservatório do protozoário. No ambiente selvagem, a raposa (*Dusicyon ventulus* e *Cerdocyon thous*) e os marsupiais (*Didelphis albiventris*) atuam como reservatórios (CORREDOR et al., 1989). Estes animais favorecem a infecção dos vetores, por apresentarem elevada carga parasitária de formas amastigotas na pele, tornando-se altamentente eficiente na manutenção e circulação do parasito em áreas endêmicas (MORENO e ALVAR, 2002).

Os agentes etiológicos da leishmaniose são protozoários tripanosomatídeos do gênero *Leishmania*, parasito intracelular obrigatório das células do sistema fagocítico mononuclear.

Possuem ciclo biológico heteroxênico, ou seja, necessitam de um hospedeiro invertebrado, representado pelos flebotomídeos, os quais abrigam nas células intestinais a forma flagelada ou promastigota; e um vertebrado, representado por animais silvestres, domésticos, sinantrópicos, além do homem, os quais albergam nos seus tecidos a forma aflagelada ou amastigota (Figure 2) (ANTOINE, 1995; SCHLEIN, 1993).

Figura 2 – Esquematização do ciclo biológico do protozoário *Leishmania* spp.



Fonte: (CDC, 2018)

Os animais e o homem se infectam durante o repasto sanguíneo de fêmeas de insetos pertencentes à ordem Diptera, família Psychodidae, também denominados de flebótomos (LEWIS, 1974). Existem inúmeras espécies de flebotomídeos capazes de transmitir Leishmaniose, variando conforme a região geográfica e suas características climáticas. No Oriente os transmissores da leishmaniose são os insetos pertencentes ao gênero *Phlebotomus*, contudo, no Ocidente são os mosquitos do gênero *Lutzomya* (GARDINER et al., 1988). No Brasil, as principais espécies envolvidas na transmissão da LC são *Lutzomya whitmani*,

Lutzomya intermedia, *Lutzomya umbratilis*, *Lutzomya wellcomei*, *Lutzomya flaviscutellata* e *Lutzomya migonei* (BRASIL, 2014).

As técnicas de diagnóstico imunólogico recomendadas atualmente pelo Ministério da Saúde são o Teste Imunocromtográfico Rápido (TR), a Reação Imunofluorescência Indireta (RIFI) e o Ensaio de Imunoabsorção Enzimática (ELISA) (BRASIL, 2016). A RIFI ainda é o teste de eleição por reunir uma série de vantagens, como fácil execução, rapidez, baixo custo e, sensibilidade e especificidade adequadas quando comparada a outras técnicas (ALVES e BEVILACQUA, 2004). O TR e o teste ELISA são indicados como triagem e avaliação da prevalência da leishmaniose na população canina (BRASIL 2016). Contudo, a sensibilidade e a especificidade do ELISA dependem do tipo de antígeno e do protocolo empregado (REITHINGER et al., 2002). Pelos exames parasitológicos, as formas amastigotas do parasito podem ser observadas em esfregaços de linfonodo, medula óssea, aspirado esplênico, biópsia hepática e esfregaços sanguíneos corados com Giemsa, Wright e Panótico (BRASIL, 2003). Além disso, existem, atualmente, as técnicas imunoistoquímicas que oferecem a vantagem de aumentar a sensibilidade e a especificidade das técnicas parasitológicas por apresentarem facilidades na execução e alto grau de contraste entre os parasitas e as células hospedeiras (FERRER et al., 1988; TAFURI et al., 2001). A apresentação clínica da LC depende de diversos fatores como a espécie do parasito, o vetor e as características imunológicas e genéticas do hospedeiro (OLIVEIRA, B. et al., 2016). A LC produz um amplo espectro de situações clínicas, que se apresentam clinicamente na forma assintomática até a presença de danos cutâneos severos ao hospedeiro, variando de lesões cutâneas localizadas até a destruição da mucosa nasal e oral (OLIVEIRA et al., 2016). Classicamente, a doença se manifesta sob duas formas clínicas: leishmaniose cutânea e leishmaniose mucosa (ou mucocutânea). A forma cutânea caracteriza-se por apresentar lesões indolores, com formato arredondado ou ovalado, com bordas bem delimitadas e elevadas, apresentando base eritematosa, infiltrada e de consistência firme, fundo avermelhado e com granulações grosseiras. A forma mucosa caracteriza-se pela presença de lesões destrutivas localizadas na mucosa, ou cartilagens, em geral nas vias aéreas superiores (BRASIL, 2014).

No cão, existe uma grande variedade de sinais clínicos associados à leishmaniose tegumentar. A evolução clínica da LC manifesta-se normalmente de forma crônica, sem comprometer o estado geral do animal (MARCO et al., 2001). Normalmente o quadro inicia com lesão de pele primária, não ulcerante, podendo, inclusive, não ser detectada. Posteriormente podem surgir áreas de alopecia e/ou hipotricose associadas a uma dermatite esfoliativa aprurítica, nódulos, erosões, crostas e úlceras (WILLEMSE, 1994). Nos casos de

ulcerações, as lesões cutâneas localizam-se principalmente nas orelhas, focinho, face, coxins e escroto e podem progredir em número e extensão, ou evoluir para cura clínica espontânea com reativações posteriores, ou ainda acometer tardiamente a mucosa nasal (MADEIRA et al., 2003; SERRA et al., 2003).

No aspecto imunopatológico, a doença nos cães sintomáticos desenvolve um modelo imune caracterizado pela elevada atividade das células B e pela ausência da imunidade celular (SLAPPENDEL, 1988). No entanto, as alterações patológicas apresentam intensidades de diferentes graus, variando de acordo com a espécie envolvida na doença e da resposta imune do hospedeiro frente à infecção (SCOTT e NOVAIS, 2016). Além disso, estudos demonstraram que a cronicidade do quadro patológico em infecções parasitárias esta relacionado com a geração de radicais livres de oxigênio altamente reativos (BISWAS et al., 1997; EREL et al., 1998). Atualmente, estudos tem relacionado a patogênese da leishmaniose com alterações no equilíbrio dinâmico entre moléculas oxidantes e antioxidantes, denominadas como estresse oxidativo (BILDIK et al., 2004; BRITTI et al., 2008).

A produção de radicais livres constitui um processo contínuo e fisiológico. Durante os processos metabólicos, esses radicais atuam como mediadores para a transferência de elétrons nas várias reações bioquímicas. Sua produção, em quantidades adequadas é essencial para o desenvolvimento de diferentes atividades celulares importantes, porém a produção excessiva pode levar a dano oxidativo (FERREIRA e MATSUBARA, 1997). Mecanismos de defesa antioxidante são os responsáveis pelo controle da atividade dos radicais livres durante os processos metabólicos e atuam limitando os níveis intracelulares de tais moléculas reativas, controlando assim a ocorrência de danos celulares (BIANCHI e ANTUNES, 1999).

A ação dos agentes oxidantes nas moléculas biológicas pode ocorrer por meio da abstração de uma molécula de hidrogênio ou de um elétron, ou pela adição de oxigênio. As moléculas oxidantes geralmente são classificadas como espécie reativa do oxigênio (ERO) e representam a classe mais importante das espécies de radicais livres (VALKO et al., 2007). ERO são encontradas em baixas concentrações nas células e tecidos biológicos, no entanto em quantidades excessivas são prejudiciais às células (DRÖGE, 2002). Como sistema de defesa frente a esse dano, as células atuam como detoxificadora desses agentes oxidantes, ou através da reparação de lesões. Superóxido dismutase (SOD) e glutationa peroxidase (GPx) são os principais componentes enzimáticos responsáveis por inativar relevantes quantidades de oxidantes, auxiliando assim na defesa do organismo contra o desequilíbrio da homeostase provocado por agentes oxidantes (NAITO et al., 2010). Além disso, diversos minerais como Cálcio (Ca), Manganésio (Mg) e Zinco (Zn) apresentam funções importantes no reequilíbrio

da homeostase, pois funcionam como catalisadores da atividade enzimática (KIRSCHVINK et al., 2008; KLOTZ et al., 2003).

O resultado clínico de uma infecção por *Leishmania* é variável e depende de fatores do parasito e do hospedeiro, podendo uma mesma cepa causar diferentes manifestações clínicas em hospedeiros distintos. A resposta imunológica do indivíduo é um fator determinante no resultado da infecção por *Leishmaniose,* sendo que a especificidade e eficiência contra o patógeno dependem de inúmeras variáveis (ROGERS et al., 2002). A imunidade ou a susceptibilidade às doenças infecto-parasitárias está diretamente relacionada com o estado nutricional do paciente (OLIVEIRA, J. et al., 2010). Diversos estudos mencionam que a deficiência de nutrientes afeta particularmente a função fagocítica, produção de anticorpos, citocinas, afinidade do anticorpo para com o antígeno e o sistema complemento o que aumentaria risco de manter a infecção e ocasionar a morte (FOCK et al., 2007).

Um mineral pode ser definido como uma substância inorgânica homogênea (JOBLING, 2001). Todas as formas de vida exigem minerais para manter seu processo biológico regular e em adequado funcionamento. Assim, 29 dos 90 elementos químicos encontrados na natureza, são essenciais para a vida animal (McDOWELL, 2003). Estes são classificados em dois grupos dependendo da quantidade exigida na dieta e armazenagem no tecido do animal: os macrominerais que são exigidos em maior quantidade e os microminerais, que são exigidos em menor proporção (SAVOLAIDEN e GATLIN III, 2010). Os macrominerais servem como componentes para estruturas de tecidos, rotas metabólicas e possuem papéis importantes na osmorregulação e no balanço ácido-básico (JOBLING, 2001). Os microminerais, ou também denominados de elementos traço, são importantes estruturas de hormônios e enzimas, servindo como cofatores e ativadores de uma variedade de enzimas, bem como participam de uma variedade de processos bioquímicos (NATIONAL RESEARCH COUNCIL, 2011).

A perda indireta de nutrientes essenciais do corpo, causada por metabolismo ou por consumo acelerado, tem sido pesquisado durante o curso de doenças infecciosas (CHAUDHURI et al., 2008). Valores alterados de macro e microminerais e sua influência na patogênese da leishmaniose foram relatados predominantemente em infecções causadas por *L. infantum*, principal espécie envolvida na LV (HEIDARPOUR et al., 2012; MALAFAIA et al., 2009; PASA et al., 2003). A deficiência de zinco tem sido descrita como a responsável pelo aumento dos danos oxidativos em proteínas, lípidos e DNA em tecidos de ratos (OTEIZA et al., 1995).

A apresentação da tese foi elaborada na forma de artigo científico. Desta forma, este trabalho foi dividido em 3 capítulos: Clinical and Pathological Aspects of Canine Cutaneous Leishmaniasis: A Meta-Analysis; Essencial elements levels in BALB/c mice experimentally infected with *Leishmania (Leishmania) amazonensis;* Oxidative and anti-oxidative markers in immunocompetent and immunodepressed mice infected with *Leishmania* (*Leishmania*) *amazonensis*.

2 ARTIGO I

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Clinical and pathological aspects of canine cutaneous leishmaniasis: A meta-analysis

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ABSTRACT: In the present study, we described the most prevalent clinical symptoms, the most affected organs, and the macro and microscopic lesions associated with cutaneous leishmaniasis. Two independent researchers performed an extensive systematic review of the literature in four stages (identification, screening, eligibility, and inclusion) to identify studies published between January 2002 to November 2018 from the following electronic databases: Web of Science, PubMed, SciELO, Science Direct, and Google Scholar. Meta-analysis was conducted in "Metaprop" package of R 3.4.2 software. The electronic search yielded 3,896 results, out of which 155 were full-text articles. Data extracted from 16 articles were included in the meta-analysis, representing a total of 430 leishmaniasis cases. Only 43% of all animals were identified to exhibit the clinical and cutaneous changes characteristic of leishmaniasis based on the observation that skin lesions were the most prevalent clinical sign and were present in 86% of all cases. Other less prevalent symptoms included weight loss, cachexia, apathy and lymph node enlargement. Histopathological analysis showed that the skin was the

most affected organ, affecting 64% of cases, followed by lymph nodes (12%), spleen (8%) and liver (7%). Therefore, our current findings demonstrated that cutaneous leishmaniasis could lead to visceral disease. Notably, our findings indicated no clinical manifestation patterns in cutaneous leishmaniasis, since the same host species may present different clinical conditions.

Keywords: Leishmania; Dog; Clinical condition; Pathology; Meta-analysis.

1. Introduction

Leishmaniasis is a vector-borne disease caused by protozoa belonging to the order Kinetoplastida, family Trypanosomatidae and genus *Leishmania*, which are transmitted by sand flies of the order Diptera, family Psychodidae, and genera *Lutzomyia* (New World) and *Phlebotomus* (Old World) (Torres-Guerrero et al., 2017). Leishmaniasis is considered a neglected infectious disease that has a high prevalence in developing countries, which could be attributed to poor health care systems, low economic and ecological status, high cost of investment for research and new drug formulations, and lack of interest of pharmaceutical industries in the disease (Coura and Castro, 2002; Oliveira et al., 2016; Ribeiro et al., 2018). Leishmaniasis is usually classified into three categories, namely, visceral, mucocutaneous, and cutaneous or tegumentary, with the latter being the most prevalent (Desjeux, 2004; WHO, 2010; Hashiguchi et al., 2018). Cutaneous leishmaniasis (CL) is prevalent in more than 90 countries and is particularly highly prevalent in the Middle East, Africa, Latin America, and especially in Brazil (WHO, 2010; Kevric et al., 2015).

Approximately 15 species are involved in cutaneous leishmaniasis, including *Leishmania* (*Viannia*) *braziliensis*, *Leishmania* (*Leishmania*) *major*, *Leishmania* (*Viannia*) *guyanensis*, and *Leishmania* (*Leishmania) amazonensis* (Grimaldi and Tesh, 1993; Marzochi and Marzochi, 1994; Auwera and Dujardin, 2015). The transmission cycles of *Leishmania* species are diverse and involve different reservoirs and sandfly species, which predominantly occur in the wild and in peridomiciliary areas (Goto and Lindoso, 2012).

Domestic and wild mammals, as well as humans, are considered hosts of the disease-causing protozoa, although not all are considered reservoirs, i.e. they can potentially infect vectors and/or new hosts (WHO, 2010; Calzada et al., 2015). Canine leishmaniasis stands out among the parasitic diseases due to prominent clinical features and high transmissibility and zoonotic potential (Gavgani et al., 2002). Dogs with cutaneous leishmaniasis exhibit diverse clinical signs, which vary depending on the species involved in the infection, as well as

genetic and immunological factors affecting the host (Ribeiro et al., 2018). The clinical progression of CL is usually chronic and does not compromise the general health of the animal (Marco et al., 2001). In addition, infected dogs can potentially develop the asymptomatic form of the disease, thereby increasing the risk of transmitting the parasite to vectors and humans (Coura-Vital et al., 2013; Travi et al., 2018). Therefore, the present work aimed to identify the most prevalent clinical signs and affected organs and the macro and microscopic lesions associated with cutaneous leishmaniasis.

2. Methods

2.1 Search strategy

To obtain the data to be included in the analysis, we performed an extensive systematic review of the literature to identify the studies that have assessed the clinical and pathological aspects of canine CL and were published between January 2002 and November 2018. The review was conducted in four stages, namely, identification, screening, eligibility, and inclusion, as recommended in the Preferred Reporting Items for Systematic Reviews and Meta-Analyses-PRISMA (Moher et al., 2009). The following online databases were queried: Web of Science, PubMed, SciELO, Science Direct, and Google Scholar. The keywords searched for in the articles included "cutaneous leishmaniasis" or *Leishmania*, pathology or histopathology and dog or canine. All search strategies were conducted independently by two researchers between January and November 2018.

2.2 Eligibility criteria

Studies on experimental or natural *Leishmania* infections in dogs, including research articles, case reports, and short communications, were included in the study. Studies describe the general clinical conditions, the *Leishmania* species involved, the macroscopic and histopathological lesions, and the occurrence of any outcome related to the acquisition of infection by *Leishmania* spp., regardless of the diagnostic assay or of the presence or absence of symptoms in the subject. No restrictions for age, sex, or country were imposed.

We excluded review studies, meta analytic studies, dissertations, theses, and papers published in scientific conferences or articles that were purely descriptive with no possibility of obtaining accurate data from the factors being investigated. With respect to the variables, we excluded studies that satisfied the following criteria: a) the study investigated animal species other than canine species; b) the study lacked description and quantification of the

lesions; c) the study performed drug or vaccine testing; and d) the study investigated visceral leishmaniasis infections. However, we retained studies that presented more data as environmental characteristics and biochemical analyses as long as the eligibility criteria were satisfied. In addition, we excluded studies and variables in cases where the text was impossible to understand or when we observed inconsistencies in the quantitative data presented or flaws that could invalidate the study findings.

2.3 Information extraction

All titles and abstracts of identified articles were analyzed, and studies that were considered irrelevant were excluded. The full text was analyzed in cases where the information provided was not sufficient for the decision or when studies were considered relevant by at least one of the researchers. After exclusion of ineligible articles, the full texts of the remaining articles were again considered based on the inclusion criteria. The reference lists of the included studies were checked to screen for additional studies. At this stage, the collected articles were included as part of the present meta-analysis.

2.4 Statistical analysis

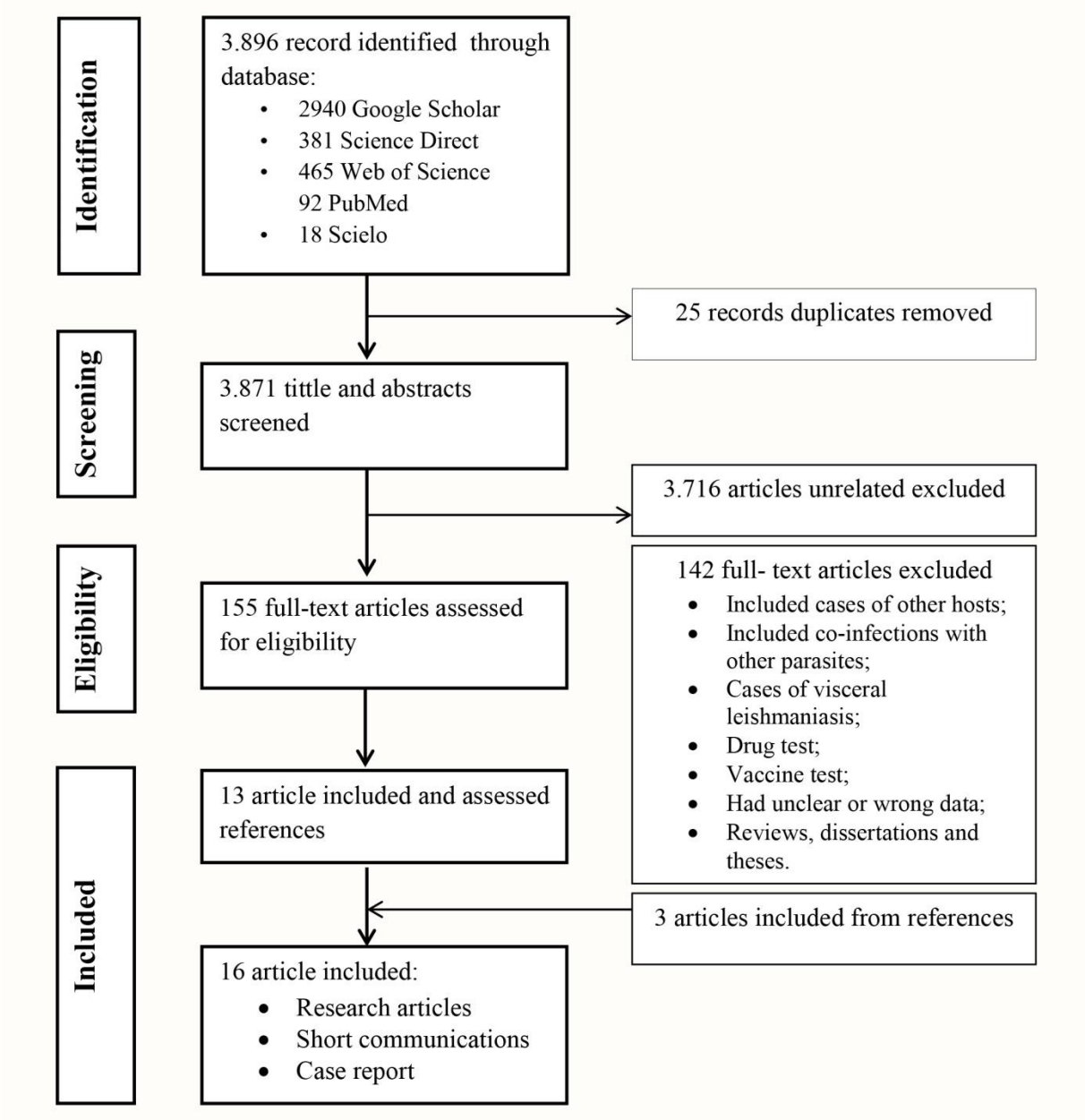
First logit transformation of proportion data. The data of the prevalent clinical signs, the most affected organs and the macro and microscopic lesions involved in cutaneous leishmaniasis isolates were obtained by meta-analysis technique under random effects model. Graphical representation of meta-analysis was done using forest plots. Meta-analysis was conducted in “Metaprop” package of R 3.4.2 software.

3. Results

3.1 Analysis workflow

The electronic search yielded 3,896 results, out of which 155 were further analyzed based on their full text. Data from 16 articles were included in the meta-analysis. Figure 1 illustrates the workflow of the search strategy and study selection and corresponding reasons for exclusion for some articles. Articles were excluded based on the following criteria: (I) the study reported cases of other hosts; (II) the study reported cases of visceral leishmaniasis; (III) the study conducted drug or vaccine test; (IV) the study presented ambiguous or wrong data; (V) the study is a review article, dissertation, or thesis. Data extracted by a third author as a validation procedure were consistent with the data extracted by the two primary authors.

Fig. 1. Flow diagram describing the study design process through the different phases of the systematic review. It maps the number of records that are identified, included and excluded, and the reasons for deletions



In terms of study type, ten were research articles, five were short communications, and one was a case report. Nine studies were conducted in Brazil, two in Iran, two in Israel, one in Colombia, one in Mexico, and one in the United States. In all studies, the target population consisted only of domestic dogs.

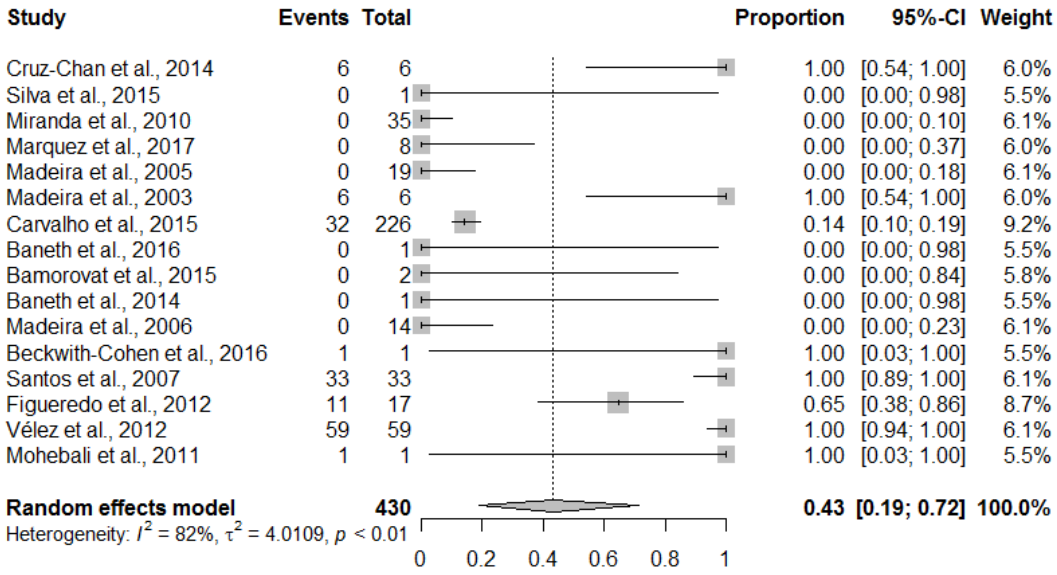
3.2 Study characteristics

A total of 430 leishmaniasis cases were included in the study. Of these, 97.2% (418/430) described *Leishmania braziliensis* infection, out of which 14.1% (59/418) reported co-infection with *Leishmania panamensis*. Only seven cases (1.6%, 7/430), four cases (0.9%; 4/430), and one case (0.2%; 1/430) were associated with *Leishmania mexicana*, *Leishmania tropica* , and *Leishmania major,* respectively.

3.3 Clinical findings

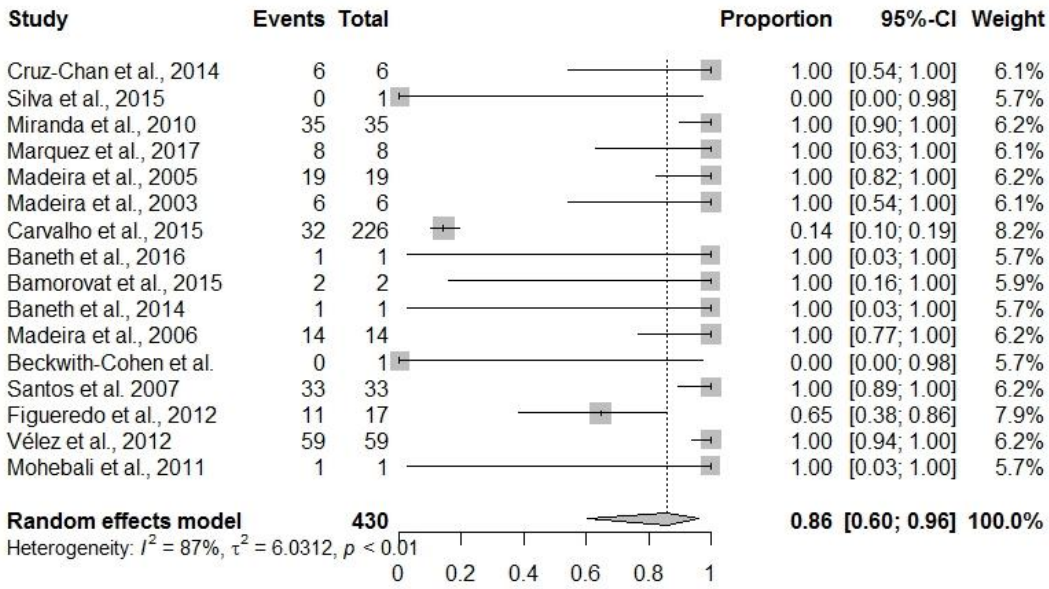
Only 43% of all animals included in the current analysis were observed to exhibit clinical and cutaneous changes characteristic of leishmaniasis (Fig. 2). The remaining cases were considered asymptomatic or showed non-specific signs.

Fig. 2. Forest plot of frequency of symptomatic dogs with clinical and cutaneous changes characteristic of leishmaniasis.



As shown in Fig. 3, the most prevalent clinical sign was skin lesions, which were present in 86% of the cases. Other reported signs included desquamation, alopecia, and hyperkeratosis. Weight loss, cachexia, apathy, and lymph node enlargement were additionally observed in some of the cases.

Fig. 3. Forest plot of frequency of the skin lesion as a clinical sign in symptomatic dogs infected with cutaneous leishmaniasis.



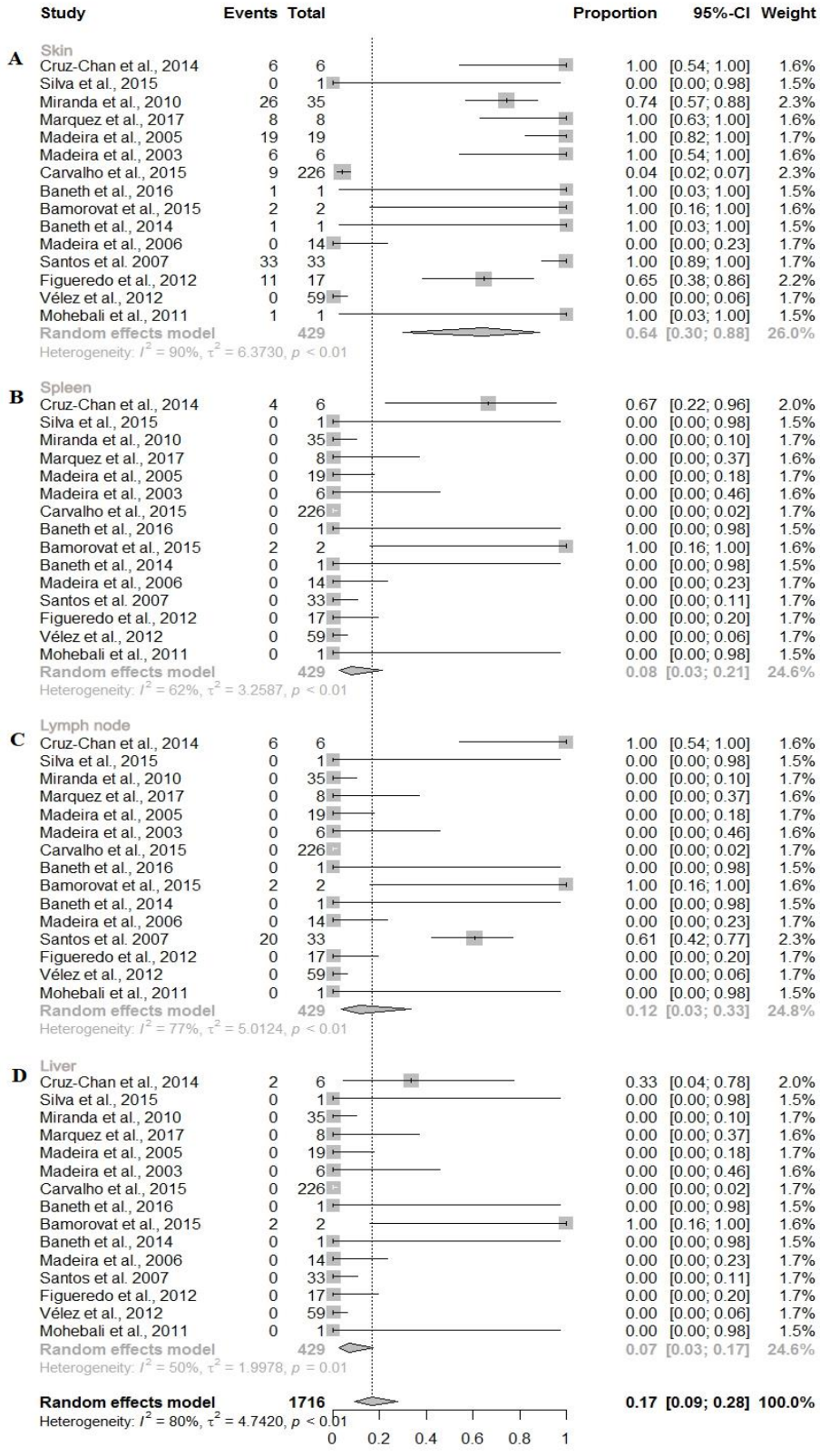
3.4 Pathological findings

Not all selected studies performed histopathological diagnosis; however, we extracted available histopathological data based on microscopic analysis and hematoxylin and eosin (HE) staining. Results obtained from the studies with histopathological findings showed macroscopic and microscopic lesions in the skin, spleen, lymph nodes, and liver.

As shown in Fig. 4, the skin is the organ with the highest occurrence of macro and microscopic lesions (64%) in CL. The most frequently reported macroscopic lesions were ulcers, followed by papules and erythema. Regarding the microscopic lesions, higher frequencies of inflammatory, granulomatous, and piogranulomatous dermal infiltrates were observed along with the presence of plasma cells, lymphocytes, macrophages, and/or neutrophils. Loss of epidermal integrity was observed in some cases.

Macroscopic and microscopic lesions in the lymph nodes were detected in 12% of cases presenting localized or generalized lymphadenitis, vasculitis, disseminated atrophy, interstitial edema, enlarged cortical layer, presence of necrotic fibrinoid zones, and, less frequently, reactive lymphoid follicles and histiocytosis. Microscopic lesions in the spleen and liver were observed in only 8% and 7% of the cases, respectively. In the spleen, the presence of splenomegaly, red pulp hyperplasia, and proliferation of plasma cells, histiocytes, and lymphocytes were detected. Hydropic and severe lipid degeneration and multiple focus of oncotic necrosis were observed.

Fig. 4. Forest plot of frequency of macro and microscopic lesions in the skin, spleen, lymph node and liver in dogs infected with cutaneous leishmaniasis. A: Frequency of macro and microscopic lesions on the skin. B: Frequency of macro and microscopic lesions in the spleen. C: Frequency of macro and microscopic lesions in lymph nodes. D: Frequency of macro and microscopic lesions in the liver.



4. Discussion

Cutaneous leishmaniasis is influenced by several socio-ecological factors that interact and maintain its transmission dynamics (Chaves et al., 2008). *Leishmania* parasites assume various ecological niches and can infect a wide range of hosts and vectors (Alvar et al., 2012; Auwera and Dujardin, 2015). Brazil is the main locality of occurrence of cutaneous leishmaniasis investigated in the present study. The Brazilian territory covers a wide variety of biomes with the highest biodiversities on the planet. The rich diversity of vectors and host species coupled with disturbances or permanent changes in the ecosystem potentially contributes to the considerable increase in the urbanization of the disease transmission cycle (Alexander et al., 2001). Similarly, various factors, such as rapid urbanization and accelerated environmental and climatic changes, are associated with the occurrence of leishmaniasis in other countries, such as Colombia, Israel, and the United States (Handler et al., 2015).

Most cases were considered asymptomatic or presented clinical signs that were non-specific to leishmaniasis. These observations illustrate the high rate of underreporting for CL and low reliability of epidemiological data because of difficulties in obtaining accurate diagnoses (Mitropoulos et al., 2010). Furthermore, diagnostic decisions are complicated by the similarity of the clinical signs of the cases to those found in other diseases, such as bacterial and fungal ulcers, leprosy, sarcoidosis, and lupus vulgaris (Tirelli et al., 2017; Bahrami et al., 2018).

Leishmaniasis is associated with a wide range of clinical manifestations that are influenced by multiple factors, such as the infecting parasite species or strain, interactions between the parasite and host genetics, concomitant infections, and the immunological status of the host (Scott and Novais, 2016). The susceptibility and/or resistance of the host to *Leishmania* infection are dependent on the genetic mechanisms involved in disease pathogenesis. The ability of the host macrophages to support or inhibit the multiplication of the parasite and to control the magnitude, quality, and reaction of the immune system against the parasite antigen are crucial to the disease status (Cardoso et al., 2010). Leishmaniasis resistance in dogs is directly associated with the Th1 immune response, which is mediated by the production of the key Th1 cytokine interferon (IFN)-g. Current results indicated that lymph node involvement in cutaneous leishmaniasis is rare; however, some authors reported differences between asymptomatic and symptomatic dogs. Dogs positive for leishmaniasis with no apparent clinical signs have higher expression of genes encoding IFN-g and tumor necrosis factor (TNF)-a and showed lower parasite load in the lymph nodes. By contrast,

symptomatic dogs have high parasite loads and higher expression of genes encoding the immunosuppressive cytokines IL-10 and transforming growth factor (TGF)-b (Day, 2011).

Certain parasitic species that are responsible for the development of cutaneous leishmaniasis could also cause visceral disease. In experimental conditions, the parasite transmission results in massive infection of lymphoid organs, accompanied by hyperplasia, extensive tissue damage, and the occurrence of a large spectrum of lesions in the liver, bone marrow, bones, and brain (Torrentera et al., 2002; Cardoso et al., 2010). The clinical presentations of symptomatic dogs infected with visceral leishmaniasis are well known. In addition to the presence of dermal inflammatory infiltrates and high parasite loads at the skin tissues and lymph nodes, the spleen is observed to contain mononuclear cellular infiltrate in red pulp and the substitution of lymphocytes by macrophages in the white pulp, accompanied by strong accumulation of IL-10 in those animals with high parasite loads (Lage et al., 2007; Reis et al., 2010). However, little is known about clinical progression of CL, particularly the immunological mechanisms involved in healing or disease progression.

5. Conclusion

The present study showed that mostly dogs were considered asymptomatic or has non-specific signs, confirming the view that the CL presents high prevalence of underreporting and low reliability of epidemiological data. With respect to pathological findings, the skin was the most frequently affected organ system in canine cutaneous leishmaniasis. As a result, our findings demonstrated that dermotropic species leads to the development of visceral disease with involvement of the lymph nodes, spleen and liver.

6. Acknowledgments

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3 ARTIGO II

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Essencial elements levels in BALB/c mice experimentally infected with *Leishmania (Leishmania) amazonensis*

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ABSTRACT: This study used an experimental model to investigate the alterations of trace metals in *Leishmania (Leishmania) amazonensis* infection and the effect of CD4+ T cells depletion on these parameters. BALB/c mice were subjected to four treatments: 1- control (not infected); 2- treated with anti-CD4 antibody; 3- infected with *L.* (*L.*) *amazonensis*; and 4- treated with anti-CD4 antibody and infected with *L. amazonensis*. Inductively coupled plasma optical emission spectroscopy was used to estimate Ca, Cu, Fe, Mg, Mn, and Zn levels in the tissues of the footpad (inoculation site), liver, spleen, and kidneys. A quantitative determination of the elements showed that there was a higher concentration of Zn in animals treated with anti-CD4 antibody (group 2), while Zn and Mn

levels decreased in mice treated with anti-CD4 antibody and infected (group 4) compared to the control (group 1) ($P < 0.05$). The results showed that important alterations in microelement levels occur when BALB/c mice are experimentally infected with *L.* (*L.*) *amazonensis*.

Keywords: Leishmania; Cutaneous leishmaniasis; metal; micro elements; pathogenesis.

1. Introduction

Cutaneous leishmaniasis (CL) is widely distributed, with approximately 0.7 to 1.2 million cases each year. Brazil is among the 10 countries with the highest number of cases of CL, along with Afghanistan, Algeria, Colombia, Iran, Syria, Ethiopia, North Sudan, Costa Rica, and Peru, which together account for 70 to 75% of the incidence estimate of the disease (Alvar et al., 2012). In Brazil, CL is associated with *Leishmania* species within the subgenera *Viannia* and *Leishmania* and the transmission occurs by a variety of species of sand flies resulting in several cycles of transmission in different geographic regions (Costa et al., 2007).

Sand flies from the genera *Phlebtomus* or *Lutzomyia* are the principal vector involved in the transmission of the disease for humans, domestic and wild animals (Chaves et al., 2007). Mammal hosts are infected by *Leishmania* promastigotes inoculated in the skin during the blood meal of infected female of sand fly. In the host, the promastigotes invade macrophages, infect other mononuclear phagocytic cells, and replicate as intracellular amastigotes (Gharbi et al., 2015).

Leishmania (Leishmania) amazonensis has received public health relevance since it has been associated with cutaneous, diffuse cutaneous and mucocutaneous clinical forms of the disease. This species is distributed in several regions of Brazil, mainly in the Amazon basin, in areas of primary and secondary forests, as well as other countries of the American continent such as Bolivia, Colombia, French Guiana, and Paraguay. The main wild host is the rodent *Proechimys* sp., but there are reports in *Oryzomys* sp., *Neacomys* sp., *Nectomys* sp., *Dasyprocta* sp., the marsupials *Metachirus* sp., *Philander* sp., *Didelphis* sp., *Marmosa* sp., and the fox of the species *Cerdocyon thous* (Basano and Camargo, 2004).

The pathogenicity and virulence of *Leishmania* spp. depend on several factors such as the genetic and immunological factors of the host and parasite specie, as well as the interaction between their individual genotypes (Ribeiro et al., 2018). Studies involving the determination of the cellular components of the innate and adaptive immune system during *L. (L.) amazonensis* infection make it clear that these parasites can evade from some protective

mechanisms (Pereira and Alves, 2008). Many immunological functions depend directly on minerals, such as zinc and copper, for their efficient functioning of immunocompetent cells (Mastousek et al., 1993). In addition, they are involved in diverse cellular actions such as cell membrane stability, apoptosis, host metabolism and enzymatic activities (Chvapril, 1973; Panemangalore and Bebe, 1996; Sprietsma, 1997; Kocyigit et al., 1998).

The objectives of this study were to investigate the changes in macro and micro elements in BALB/c experimentally infected with *L.* (*L.*) *amazonensis* and associate with the clinical outcome, parasite load and histopathological lesions, and to determine the effect of depletion of CD4 + T cells in these parameters.

2. Material and Methods

2.1 Parasites

L. (L.) amazonensis promastigotes (IFLA/BR/67/PH8) were cultured *in vitro* with M199 medium supplemented with 10% (v/v) fetal bovine serum (FBS), 2% (v/v) human urine, 0.1 mM adenine, 7.7 mM hemin, 0.1% (v/v) biotin, 40 mM 4-(2-hydroxyethyl) piperazine-1-ethanesulfonic acid sodium salt (HEPES, pH 7.4), 50 unit/mL penicillin, and 50 μg/mL streptomycin (Sigma-Aldrich®). Cultures were incubated at 26 °C with cell densities ranging between 5×10^5 and 3×10^7 parasites/mL (Romão et al., 2006). The virulence of the strain was maintained by repeated passages in BALB/c mice.

2.2 Experimental Design

A total of 28 six-week-old BALB/c mice were divided into four groups (n = seven mice/group): 1- control (no infected animals); 2- treated with anti-CD4 antibody 3- infected with *L. (L.) amazonensis*; 4- treated with anti-CD4 antibody and infected with *L. (L.) amazonensis.* All the animals were purchased and maintained in the animal house of the Universidade Federal de Ciências da Saúde de Porto Alegre (Rio Grande do Sul, Brazil) for a period of six months and provided with drinking water and a balanced commercial food *ad libitum*, following the principles set down by the Ethics Committee on animal experimentation.

2.3 Immunosuppression

The immunossupression was perfomed using the antibody anti-CD4 purified from the hybridome GK 1.5 (ATCC TIB 207). Each mouse received three peritoneal injections. Groups

2 and 4 were anesthetized by isoflurane inhalation and treated intraperitoneally with three doses 50 μg of anti-CD4 antibody, which were applied on days one, five, and eight of the experiment. The efficiency of immunossupression was evaluated by the quantification of peripheral CD4+ T cells using flow cytometry.

2.4 Experimental infection and monitoring of cutaneous lesion

BALB/c mice were anesthetized by isoflurane inhalation and subcutaneously inoculated in the left footpad with 2×10^6 stationary-phase promastigotes of *L. (L.) amazonensis* in 50 μL of phosphate buffered saline (PBS). In immunosuppressed mice the infection were made one day after depletion of CD4+ T cells.

The kinetics of the cutaneous lesion was evaluated weekly. Footpad thickness was measured using a caliper with an accuracy of 0.01 mm and was expressed as the difference between the infected footpad and the uninfected contralateral footpad.

2.5 Collection of tissue samples

After 24 weeks postinfection, the animals were euthanized with an intra-peritoneal injection of lidocaine (10 mg/kg) and a lethal dose of sodium thiopental (150 mg/kg). Later, the tissue samples from the footpad (inoculation site), liver, spleen, and kidneys were collected and stored at –80°C for further analysis.

2.6 Parasite quantification

Parasite burdens were evaluated in inoculation site (footpad) by microtiter culture technique according to Buffet et al. (1995). The samples were harvested and their total weights were determined. The tissue was macerated and the was prepared by grinding the tissue in 1 mL of M199 medium supplemented with 30% (v/v) heat-inactivated bovine fetal serum (FBS), 2% (v/v) human urine, 0.1 mM adenine, 7.7 mM hemin, 0.1% (v/v), 40 mM 4-(2-hydroxyethyl) piperazine-1-ethanesulfonic acid sodium salt (HEPES, pH 7.4), 50 unit/mL penicillin, and 50 μg/mL streptomycin (Sigma-Aldrich®). This suspension was further diluted to reach a final concentration of 1 mg/mL. The cell suspension was used for the determination of the parasite burden and the cellular mass. Four-fold serial dilutions of the homogenized tissue suspensions were plated in a 96-well culture plate and incubated at 26°C for 10 days. The wells were examined for viable promastigotes. The number of parasites per gram (parasite burden) in the footpad was calculated as follows: (geometric mean of the four titers / fragment weight) x400.

2.8 Inductively Coupled Plasma Optical Emission Spectroscopy (ICP-OES)

The quantitative of the elements was determined through ICP-OES (Optima 7000 DV, Perkin Elmer Co. USA) using *in natura* samples of the infected footpad, spleen, liver and kidneys. were used to perform the. For digestion step, 2 mL of 65% nitric acid (4.2 mol/L HNO_3) (ACS reagent grade, Merck, Rio de Janeiro, Brazil) was added each sample. Inside a fume hood, the solution was transferred to a 25 mL beaker and digested with 2.0 mL of 30% hydrogen peroxide (ACS reagent grade, Merck, Rio de Janeiro, Brazil) on a hot plate at 54 °C (Quimis, model Q313A, Sao Paulo, Brazil). After the digestion, the beakers were cooled and the digests were transferred to volumetric flasks. It was prepared diluted solutions for each sample using HNO_3 5% solution (Suprapur® Merck, Darmstadt, Germany). A reagent blank was prepared under the same conditions in order to correct possible error in test results that comes from the reagents themselves. To prepare the solutions were used high-purity deionized water obtained from Milli-Q® purification system (Millipore, Belford, MA, USA). All containers used were cleaned by soaking in 1.50 mol/L HNO_3 during 24 h, rinsing five times with high-purity water, and dried and stored in a class 100 laminar flow hood (Hexiclean, model Clean-5).

Levels of Ca, Cu, Fe, Mg, Mn, and Zn were determined using ICP-OES (Optima 7000 DV, Perkin Elmer Co. USA) calibrated with analytical solutions prepared by suitable dilution of stock solution (ICP phosphorous and 21 multi-element standard solution Inorganic Ventures, Christiansburg, USA) in HNO_3 5% solution (Suprapur® Merck, Darmstadt, Germany). The measurement conditions for analyses are demonstrated in Tables 1 and 2.

Table 1

Instruments and analytical parameters for ICP-OES

Parameters	Conditions
Radio Frequency generator	40 MHz
Plasma torch standard	1 slit
Injector standard	2 mm
Detector	CCD (charge-coupled device)
Optic system	Echelle
Position	Axial
Power	1300 W
Plasma gas flow	15 L/min
Auxiliary gas flow	0.3 L/min
Nebulizer gas flow (Teflon Mira Mist)	0.6 L/min
Operational principle	Spray chamber cyclonic, baffled 12-mm-axial torch connection, 4 mm drain
Sample flow rate	1.5 mL/min

Table 2

Wavelengths used for elemental analysis

Element	Wavelength (nm)
Calcium	317.933
Copper	324.754
Iron	259.940
Magnesium	279.079
Manganese	257.610
Zinc	213.856

2.7 Histopathological study

Three animals from each experimental group were used for the histopathological analysis of the infected footpad, inguinal lymph node, spleen, liver and kidneys. The organs were removed and fixed in 10% buffered formalin for subsequent embedment in paraffin. Sections (5 μm) were cut on a microtome (Zeiss Hyrax M25) and stained with Hematoxylin-Eosin (HE) (Kluver and Barrera, 1953). The analysis involved the determination of the nature of the inflammatory infiltrate and the presence of parasite forms.

2.9 Statistical analysis

The analyses were performed using GraphPad Prisma 5.0, GraphPad Software, Inc., La Jolla, CA, USA. All analyses were carried out randomly, and animals were categorized following the experimental groups (control, immunosuppressed, infected, and immunosuppressed and infected). The results obtained were compared among groups using one-way ANOVA and Turkey post hoc test. The analysis was followed by residual analyses to check for the error distribution and suitability of the normal model. Differences were considered significant when $P \leq 0.05$.

2.10 Ethical Approval

All experimental procedure involving animals were performed in accordance with National Institute of Health Guide for Care and use of animal and with approval of our institutional ethics committee, which also reviewed and approved this work (CEUA, Universidade Federal de Ciências da Saúde de Porto Alegre, number.505/17).

3. Results

3.1 Clinical course of skin lesions

First, the efficiency of CD4 depletion was evaluated using peripheral blood sample taken from animals treated with anti-CD4. As shown in Fig. 1, after the depletion scheme the circulating CD4+ T cells reduced 70%. The lesion development kinetics over 24 weeks revealed a chronic and progressive evolution. Although the crucial role of CD4+ T cells for the control of *Leishmania paraasite*, in our model, the depletion of CD4+ T cells right before the infection did not change the course of *L. amazonensis* infection. All infected animals presented discrete lesions with footpad swelling, which did not ulcerate or heal over the period. No animal presented spontaneous resolution of the lesions. Groups 3 and 4 had similar lesion sizes at all points of the kinetic curve and there were no statistical differences between the groups (Fig. 2).

Fig. 1. Histogram with arithmetic means of percentage values of CD4+ T cells depletion determined by flow cytometry in blood samples from mice inoculated with antibody anti-CD4 purified from the hybridome GK 1.5 (ATCC TIB 207). '1' refers to untreated mice (control); '2' refers to mice treated with anti-CD4 antibody; '4' refers to mice treated with anti-CD4 antibody and infected with *L. (L.) amazonensis.* Significant differences between sample means are indicated: **, $P < 0.01$.

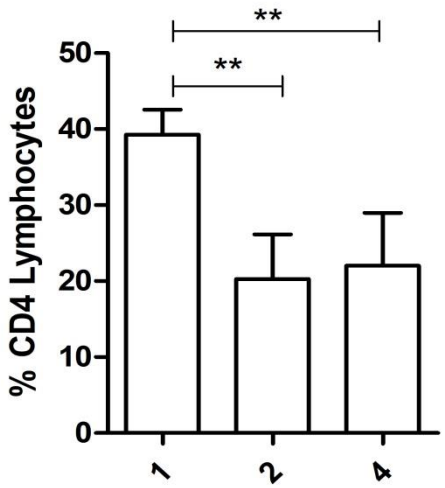
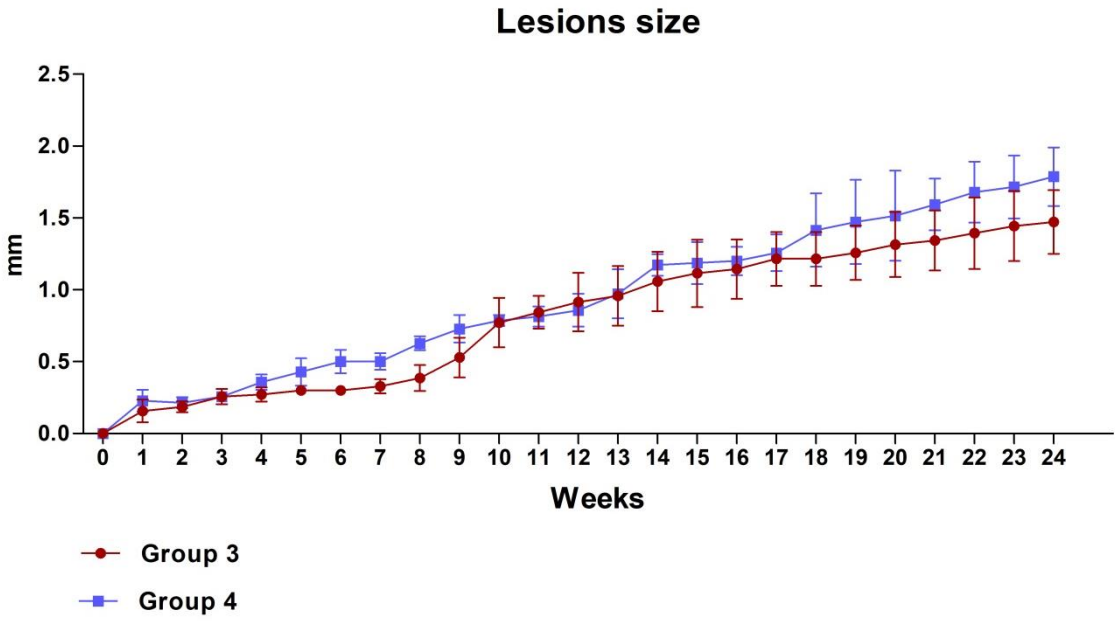


Fig. 2. Evaluation of BALB/c mice skin lesions (footpad) after infection by *L. (L.) amazonensis* over 24 weeks. 'Group 3' represents the group of animals infected with *Leishmania (L.) amazonensi*. 'Group 4' represents the group of animals infected with *L. (L.) amazonensis* and treated with anti-CD4 antibody.



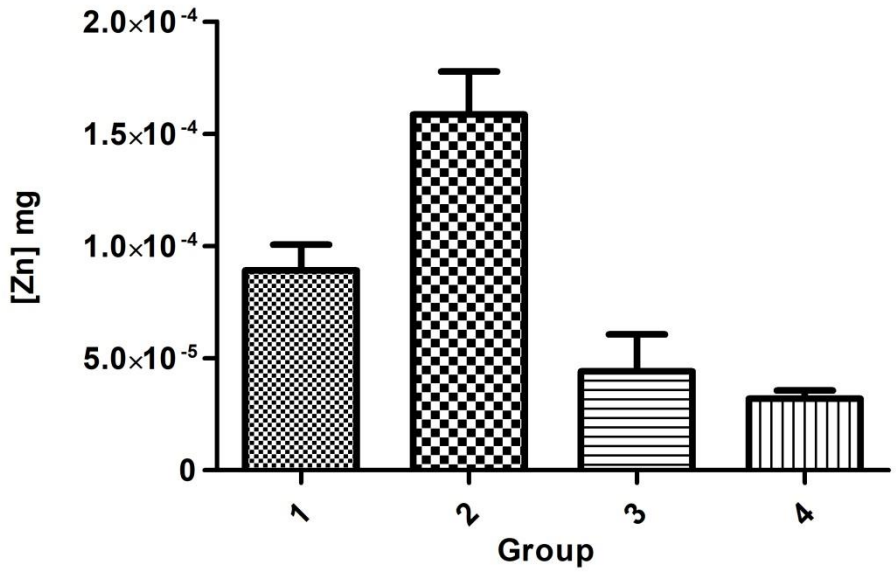
3.2 Parasite quantification

Animals infected with *L.* (*L.*) *amazonensis* (group 3) had a median of 3.6×10^5 parasites/g, and group 4 (infected with *L.* (*L.*) *amazonensis* after CD4 T cells depletion) had a median of 3.9×10^5 parasites/g. There were no significant differences between the median values of the two groups ($P < 0.05$).

3.3 Levels of minerals and trace elements

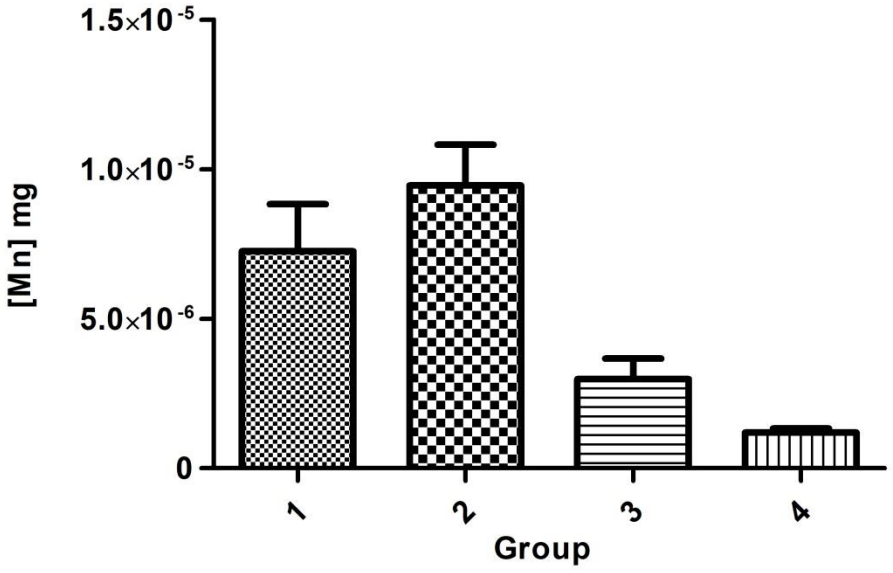
The Zn concentration in the spleen tissue was higher in mice with $CD4^+$ T cells depletion (group 2) compared to control group (group 1). However, Zn levels were significantly lower in the spleen tissues of animals that were treated with CD4 antibody and infected with *L.* (*L.*) *amazonensis* (group 4) compared to control (group 1) ($P < 0.05$; Fig. 3). Animals infected with *L.* (*L.*) *amazonensis* (group 3) were not significantly different than the control.

Fig. 3. Zinc concentrations i in tissue of the spleen in BALB/c mice treated with anti-CD4 antibody (group 2), infected mice with *L.* (*L.*) *amazonensis* (group 3) and mice treated with anti-CD4 antibody and infected with *L. (L.) amazonensis* (group 4) compared to uninfected control mice (group 1). Significant differences ($P < 0.05$).



Mn levels were not significantly different in group 2 compared to the control (group 1), but group 4 animals had significantly lower Mn levels in the spleen than the control and group 2 animals ($P < 0.05$; Fig. 4).

Fig. 4. Manganese concentrations in tissue of the spleen in BALB/c mice treated with anti-CD4 antibody (group 2), infected mice with *L.* (*L.*) *amazonensis* (group 3) and mice treated with anti-CD4 antibody and infected with *L. (L.) amazonensis* (group 4) compared to uninfected control mice (group 1). Significant differences ($P < 0.05$).



No significant differences in levels of Cu, Ca, Fe and Mg were observed in all groups. No significant differences in levels of macro and microelements were observed in liver and kidney tissue.

3.4 Histopathological aspects

We observed presence of *L.* (*L.*) *amazonensis* amastigotes in inguinal lymph node, spleen and liver samples in the infected groups, but no significant differences observed between infected mice with *L.* (*L.*) *amazonensis* (group 3) and treated with anti-CD4 antibody and infected with *L. (L.) amazonensis.* (group 4) ($P > 0,05$). In the infected groups, were observed tissue damage in (i) liver: lymphoplasmocytic and granulomatous periportal hepatitis; (ii) spleen: with pulp hyperplasia; (iii) inguinal lymph node: chronic granulomatous lymphadenitis. No histological alterations were observed in kidneys.

4. Discussion

The mineral elements are essential components of enzyme systems and have intense effects in maturation, activation and function of various components of the immune system for both human and animals (Failla, 2003; Soetan et al., 2010). Alterations in trace mineral levels lead to non-specific inflammatory reaction and have been described in leishmaniasis varying according to host susceptibility (Faryadi and Mohebali, 2003; Nieto et al., 2003; Pasa et al., 2003; Weyenbergh et al., 2004). The trace elements deficiency decreases the possibility of control many infections, and increases the susceptibility to several diseases (Yatoo et al., 2013).

Zinc is the second most abundant trace element in serum and it is classified as micro-elements together with magnesium, iron, copper, cobalt, potassium, iodine, manganese, molybdenum, fluoride, chromium, selenium, and sulfur (Soetan et al., 2010; Wang et al., 2013; Yatoo et al., 2013). These micronutrients demand less than 100 mg/dl and require a daily nutritional support. Furthermore, these mineral elements are involved in several cellular processes such as the catalytic, structural and regulatory processes of keratinization, macronutrient metabolism and cell replication, with directly influence on several systems, as the immune system (Soetan et al., 2010; Shazia et al., 2012). We observe the occurrence of the higher levels of Zn in spleen of mice of the group immunosuppressed. In lymphoid organ tissues are common the necessity of higher Zn levels due to intense proliferation of the cells, mainly immuny system cells (Salgueiro et al., 2000). Studies with Zn supplementation have been demonstrated to significant increase of the effective immune response in face of diverse pathologies (Castillo-Duran et al. 1987; Dardenne et al., 1993; Mocchegiani et al., 1995). In this study, tissue damages and lower levels of Zn and Mn were found in spleen of all animals of the group immunosuppressed and infected with *L.* (*L.*) *amazonensis* (group 4) when compared to control (group 1).

It was verified, in this study, the ability of *L.* (*L.*) *amazonensis* to migrate to other organs. Studies involving *L.* (*L.*) *amazonensis* experimental infection demonstrated extensive tissue destruction in footpad and rapid dissemination to the spleen with high levels of circulating anti-*Leishmania* antibodies (Pereira and Alves, 2008). In addition, researches shown that Zn acts in the activation of immune system cells and maintains the appropriate balance between innate and acquired immunity and its concentration declines during the acute phase response (Beck et al., 1997; Shankar and Prasad, 1998; Scott and Koski, 2000; Yatoo et al., 2013). The decrease Zn level has been implicated as a factor for the inability of the host to eliminate the protozoan and occurrence of the inflammation reaction with deficient production

of several cytokines and enzymes (Warner and Lawrence, 1988; Rofe et al., 1996; Amini et al., 2009).

Both Zn and Mn influence several immunological and enzymatic systems. The innate system uses Mn available in organism human and domestic animals to prevent and control the growth of several pathogens (Hood and Skaar, 2012). However, there is a competition between the host cells and the microorganism for these essential nutrients, which makes this factor extremely important in health and disease progression (Brophy and Nolan, 2015). The Mn is responsible for activation of multiple enzymes as the glycosyl transferase enzymes used in the synthesis of mucopolysaccharides and glycoproteins (Soetan et al., 2010). In addition, Mn ions act as co-factor of different enzymes as hydrolase, decarboxylase and transferase (Murray et al., 2009).

The development and intensity of clinical signs in leishmaniasis are associated with the host immune response (Reis et al., 2010). The adaptive response is activated after macrophages and dendritic cells (DCs) to present *Leishmania* spp. antigens to T cells (Pereira and Alves, 2008). The imbalance between innate and adaptive response, and inadequate T-cell response leads to the occurrence of the clinical signs of the disease and affecting a number of organ systems like skin, bone marrow, spleen, kidneys, liver and lymph nodes (Reis et al., 2006; Prianti, et al., 2007; Reis et al., 2009).

It is essential to understand that the concentration of micro and macro elements change under distinct situations of the infections and/or inflammations. The levels alterations of the trace elements reflect changes in cellular uptake mechanisms and cation binding of plasma proteins (Andrieu, 2008; Yatoo et al., 2013). Thus, the excess or the deficiency of trace elements damage the functioning of the immune system cells and, consequently, increase the probability of occurrence of infectious diseases, which can cause death of the infected host. Therefore, the equilibrium of redox-active metals is essential to the host because the excess can induce free-radical–mediated injury, and infectious microorganisms also may use it for their survival and replication (Failla, 2003).

5. Conclusion

The Zn and Mn spleen concentrations are altered in experimentally situations of CD4+ depletion and infection by *L. (L.) amazonensis*. Zn levels are increase in mice treated with anti-CD4+ antibody, however when these animals were also infected with *L. (L.) amazonensis* the Zn concentration became significatly lower. Mn levels decrease in the spleen of the immunosuppressed and infected animals. This study evidenced important alterations in the

level of microelements, Zn and Mn, that occur in *L.* (*L.*) *amazonensis* experimental infection. Future studies could be performed to mitigate the effects of mineral imbalance in the response and treatment of cutaneous leishmaniasis.

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4 ARTIGO III

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Oxidative and antioxidative markers in BALB/c mice infected with *Leishmania* (*Leishmania*) *amazonensis*

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ABSTRACT: *Leishmania* (*Leishmania*) *amazonensis* is one of the etiological agents of cutaneous leishmaniasis that has been the target of several studies in order to better understand the pathogenic and immunological mechanisms of the disease. This study aimed to investigate the alterations in oxidative and antioxidative status of BALB/c mice experimentally infected with *L.* (*L.*) *amazonensis* and and the effect of CD4+ T cells depletion on these parameters. BALB/c mice were divided into four groups: 1- control (uninfected animals); 2- treated with anti-CD4 antibody; 3- *L.* (*L.*) *amazonensis*-infected; 4- treated with anti-CD4 antibody and *L.* (*L.*) *amazonensis*-infected. 24 weeks after infection, blood samples and fragments of liver, spleen, and kidneys were collected for the following tests: butyrylcholinesterase activity, myeloperoxidase activity, thiobarbituric acid reactive substances, superoxide dismutase assay, total thiols, and glutathione evaluation. We did not

observe differences in the oxidative/antioxidant or protective markers between animals inoculated with *L.* (*L.*) *amazonensis* and those that were previously treated with anti-CD4 monoclonal antibody, suggesting a balance between oxidative-antioxidative systems.

Key-words: Leishmaniasis; Cutaneous leishmaniasis; Oxidative damage; Antioxidantive defenses; $CD4^+$ T cells.

1. Introduction

Leishmaniasis is a chronic zoonotic disease caused by several species of protozoans of the order Kinetoplastida, family Trypanosomatidae, and genus *Leishmania* affecting humans and wild and domestic animals (Bañuls et al. 2007). Although not lethal, cutaneous leishmaniasis (CL) is the main and most frequent clinical form of the disease, and it is usually traumatic, being associated with social stigmatization (Reithinger et al. 2007). *Leishmania* (*Leishmania*) *amazonensis* is one of the etiological agents of CL (Oliveira et al. 2016). The scientific interest in this species has increased in the last years in order to clarify the immunopathogenic mechanisms related to this agent (Silveira et al. 2009).

The parasite is transmitted to the vertebrate host through the bite of a female sandfly during the blood meal (Miró et al. 2017). In the host, the parasites are phagocytized by macrophages and transformed into amastigotes (Hepburn 2003). Therefore, the pathogenesis of the disease is directly related to the process of infection, survival, and multiplication of the parasite within the cells of the phagocytic mononuclear system (Murray and Cartelli 1983).

Free radical molecules assist the immune system in protecting the body against inflammatory and infectious processes (Maeda and Akaike 1998). During the infection, cells from the mononuclear phagocytic system generate highly toxic molecules to fight the disease (Mauel et al. 1991). Reactive oxygen species (ROS) and nitrogen reactive species (RNS) such as superoxide radicals (O_2^-), hydrogen peroxide (H_2O_2), hydroxyl radicals (OH^-), and nitric oxide (NO) are generated (Mauel et al., 1991; Assreuy et al. 1994; Biswas et al. 1997). The aim of this study was to investigate the alterations in oxidative and antioxidative markers in BALB/c mice depleted of CD4+ T cells in relation to naïve animals without CD4+ T cells depletion.

2. Material and Methods

2.1 Parasites

L. (L.) amazonensis promastigotes (IFLA/BR/67/PH8) were cultured *in vitro* with M199 medium supplemented with 10% (v/v) fetal bovine serum (FBS), 2% (v/v) human urine, 0.1 mM adenine, 7.7 mM hemin, 0.1% (v/v) biotin, 40 mM 4-(2-hydroxyethyl) piperazine-1-ethanesulfonic acid sodium salt (HEPES, pH 7.4), 50 unit/mL penicillin, and 50 μg/mL streptomycin (Sigma-Aldrich®). All protozoan cultures were maintained in Bio-Oxygen Demand (BOD) incubator at 26°C, and the densities reaching of 5×10^5 to 3×10^7 parasites/mL (Romão et al. 2006). The virulence of the isolate was maintained by frequent passages in BALB/c mice.

2.2 Experimental Design

Twenty-eight 6-week-old BALB/c mice were equally divided into four groups: 1- control (uninfected animals, n = 7); 2- immunosuppressed at time of infection (by depletion of $CD4^+$ T cells, n = 7); 3- *L. (L.) amazonensis*-infected (n = 7); 4- immunosuppressed and *L. (L.) amazonensis*-infected (n = 7).

All the animals were purchased and maintained in the animal facility at Universidade Federal de Ciências da Saúde de Porto Alegre (Rio Grande do Sul, Brazil) for a period of 6 months and provided with drinking water and a balanced commercial food *ad libitum.*

2.3 Immunosuppression

Mice in the groups 2 and 4 were immunosuppressed by the depletion of $CD4^+$ T cells using the anti-CD4 monoclonal antibody derived from GK 1.5 cell line. Before the treatment, mice were anesthetized and injected intraperitoneally with three doses (50 μg) of anti-CD4 monoclonal antibody at 1, 5, and 8 day. $CD4^+$ T cell depletion was confirmed by immunophenotyping for these specific cells using flow cytometry on peripheral blood samples of animals from groups 2 and 4.

2.4 Experimental infection and monitoring of cutaneous lesion

Before the experiment, BALB/c mice of groups 3 and 4 were anesthetized by isoflurane inhalation. Subsequently, they were inoculated subcutaneously (sc) in their left hind footpad with 2×10^6 stationary-phase promastigotes of *L. (L.) amazonensis* in 50 μL PBS one day after the end of the protocol of CD4+ T cells depletion (day 9). The cutaneous

lesions were evaluated weekly, using a caliper with an accuracy of 0.01 mm (Worker®) for measuring the footpad thickness, and the values were expressed as the difference between the uninfected contralateral footpad.

2.5 Collection and preparation of plasma, serum and tissue samples

The animals were anesthetized with a single intraperitoneal injection containing thiopental (50 mg/kg) + lidocaine (10 mg/kg), and the peritoneal cavity was exposed for collection of caudal vena cava blood. The blood samples were obtained by cardiac puncture and distributed into microtubes without anticoagulants and tubes containing ethylenediaminetetraacetic acid (EDTA). The animals were euthanized by a lethal dose of sodium thiopental (150 mg/kg), and tissues samples of the liver, spleen, and kidneys were collected. The plasma and serum were separated by centrifugation at 3,500 rpm for 5 min. Plasma samples were used for determination of the myeloperoxidase (MPO) activity, while butyrylcholinesterase (BChE) activity and the content of total thiols (T-SHs) were determined in serum samples. All collected tissues were placed on ice (0°C to 4°C) and homogenized in 50 mM Tris-HCl, pH 7.4 (1/10, w/v). The solutions were centrifuged at 2,000 rpm for 10 min, and the supernatant was used for determination of thiobarbituric acid reactive substances (TBARS), superoxide dismutase (SOD), T-SHs, and glutathione reduced levels (GSH). All samples were identified and kept at −80°C, protected from light, until the analyses were carried out.

2.6 Protozoan quantification

The parasite load was evaluated in inoculation site (footpad) using microtiter culture technique according to Buffet et al. (1995). Each collected hind left footpad was weighed and macerated by grinding in 1 mL of M199 medium supplemented with 30% FBS, 2% (v/v) human urine, 0.1 mM adenine, 7.7 mM hemin, 0.1% (v/v), 40 mM HEPES, pH 7.4, 50 unit/mL penicillin, and 50 μg/mL streptomycin. This suspension was further diluted to reach a final concentration of 1 mg/mL. The cell suspension was used for the determination of the parasite burden and cellular mass. Four-fold serial dilutions of the homogenized tissue suspensions were plated in a 96-well culture plate and incubated at 26°C for 10 days. The wells were examined for visualizing the viable promastigotes using an inverted optical microscope. The number of protozoa per gram (protozoan load) in the footpad was calculated as follows: (geometric mean of the four titers / fragment weight) × 400 (Buffet et al. 1995).

2.7 Butyrylcholinesterase (BuChE) activity

Serum BuChE activity was determined by the method of Ellman et al. (1961). 0.1 mol potassium phosphate buffer system, with pH 7.4, 0.30 mmol 5,5'-dithiobis-2-nitrobenzoate (DTNB) and 50 µL of each serum sample was incubated for 2 min at 30 °C and the reaction started by adding the butyrylcholine substrate at the concentration of 1 mmol. The reading was performed by the spectrophotometry method from 2 min to 412 nm. The enzymatic activity was expressed in µmol of BcSCh/h/mg of protein.

2.8 Myeloperoxidase (MPO) activity

MPO was determined in plasma samples by the method described by Metcalf et al. (1986) with slight modification. The MPO activity was analyzed spectrophotometrically by a modified peroxidase-coupled assay system involving phenol, 4-aminoantipyrine (AAP) and H_2O_2. Briefly, 390 µL of solution with 2.5 mM AAP and 20 mM phenol were put in each tube containing each plasma sample, followed by the addition of 450 µL of 1.7 mM H_2O_2. In the presence of H_2O_2 as oxidizing agent, MPO catalyzes the oxidative coupling of phenol and AAP yielding a colored product, quinoneimine, with a maximum absorbance at 500 nm. The results were expressed in micromolar of quinoneimine produced at 30 min.

2.9 Determination of total thiols (T-SHs) and reduced glutathione (GSH)

T-SHs were assayed spectrophotometrically by the method of Boyne and Ellman (1972) with minor modification. An aliquot of 200 µL of serum or 100 µL homogenate from liver, spleen or kidneys in a final volume of 900 µL of solution was tested. The reaction product was measured at 412 nm after the addition of 10 mM 5-5-dithio-bis (2-nitrobenzoic acid) (DTNB) (0.05 mL). A standard curve using cysteine was added to calculate the content of T-SHs in samples, and it was expressed as µmol SH/mL of protein.

The GSH was measured spectrophotometrically with Ellman's reagent (Ellman, 1959). An aliquot of 200 µL of homogenate of each tissue supernatant (liver, spleen or kidneys) were mixed (1:1) with 10% trichloroacetic acid (TCA) and centrifuged at 4000 x g for 10 min. Later, the protein pellet was discarded and the free non-protein sulfhydryl (NPSH) groups were determined in the supernatant. Samples were added in a system containing 1M potassium phosphate buffer, pH 7.4, and 5.5 mM-10 dithiobis (2-nitrobenzoic acid) (DTNB). The reaction product was measured at 412 nm after the addition of 10 mM 5-5-dithio-bis (2-nitrobenzoic acid) (DTNB) (0.05 ml). A standard curve using cysteine was added to calculate the content these thiol groups in samples, and was expressed as nmol GSH/mL.

2.10 Thiobarbituric acid-reactive substance (TBARS) measurement

Malondialdehyde (MDA) levels were determined in liver, spleen and kidneys homogenates samples. In this assay was used the method described by Ohkawa et al., (1979) with modifications. Briefly, the reaction mixture, containing 200 µL of each homogenate sample or standard (0.03 mM MDA), 200 µL of 8.1% sodium dodecyl sulfate (SDS), 500 µL of 0.8% thiobarbituric acid (TBA), and 500 µL of acetic acid solution (2.5 M HCl, pH 3.4) was heated at 95 °C for 120 min. The absorbance was measured at 532 nm. Tissue TBARS levels were expressed as nmol MDA/mg of protein.

2.11 Superoxide dismutase (SOD) assay in tissues

SOD activity was performed according to Mccord and Fridovich (1969) with modifications. The assay was performed in a total volume of 1 mL containing 50 mmol of glycine buffer (pH 10), 60 mmol epinephrine, and each supernatant sample from liver, spleen or kidneys tissues previously homogenized in phosphate buffer and centrifuged. Epinephrine was added and the formation of adrenocrous was recorded at 480 nm with an ultraviolet-visible (UV-VIS) spectrophotometer for 4 min. One unit of SOD activity was equivalent to the amount of enzyme required to inhibit oxidation in 50% epinephrine under the experimental conditions. The results were presented in SOD IU/mg of protein.

2.12 Statistical analysis

The analyses were performed using GraphPad Prism 5.0, GraphPad Software, Inc., La Jolla, CA, USA. All analyses were carried out randomly, and mice were categorized into different groups: group 1 (control, uninfected), group 2 (immunosuppressed), group 3 (*L.* (*L.*) *amazonensis*-infected), and group 4 (immunosuppressed and *L.* (*L.*) *amazonensis*-infected). The results obtained were compared among groups using one-way ANOVA and Turkey post-hoc test. The analysis was followed by residual analyses to check for the error distribution and suitability of the normal model. Differences were considered significant when $P \leq 0.05$.

2.13 Ethical Approval

All experimental procedure with the mice were accomplished in accordance with National Institute of Health Guide for Care and use of animal and with approval of the institutional ethics committee (CEUA) at Universidade Federal de Ciências da Saúde de Porto Alegre, number 505/17.

3. Results

3.1 Protozoal quantification

Although the crucial role of CD4+ T cells for the control of *Leishmaniasis*, in our model, the depletion of CD4+ T cells right before the infection did not change the course of *L.* (*L.*) *amazonensis* infection. The number of parasites recovered from the lesions of animals infected with *L.* (*L.*) *amazonensis* (group 3 - 3.6×10^5 protozoa/g of footpad) was not significantly different from that recovered from animals that were the treatment with anti-CD4 antibody before the infection (3.9×10^5 protozoa/g).

3.2 Oxidantive and antioxidantive markers

The mean of BChE, MPO, TBARS, SOD, T-SHs, and GSH did not reach a statistical significance in group 3 (*Leishmania (L.) amazonensis*-infected) compared to animals of group 4 (depleted of CD4+T cells and infected with *Leishmania (L.) amazonensis*) ($P < 0.05$) (Table 1 and 2).

Table 1

Oxidative and antioxidative parameters in plasma and serum of BALB/c miceinfected and not infected with *Leishmania (Leishamnia) amazonensis.*

Parameters	Group 1[a]	Group 2[b]	Group 3[c]	Group 4[d]
BChE µmol/h/mg	1,20 ± 0,23	2,28 ± 0,45	2,14 ± 0,13	1,46 ± 0,20
MPO µmol/30min	42,6 ± 0,74	40,93 ± 0,32	40,56 ± 4,94	37,65 ± 3,83
T-SHs µmol/mL	982,84 ± 277,19	1112,65 ± 153,71	1172,21 ± 656,73	1476,03 ± 149,95

Values are shown as mean ± standard deviation
[a]Group 1: control
[b]Group 2: mice submitted to depletion of $CD4^+$ T cells
[c]Group 3: mice infected with *Leishamnia (Leishamnia) amazonensis*
[d]Group 4: mice with depletion of of $CD4^+$ T cells and infected with *Leishmania (Leishmania) amazonensis*

Table 2

Comparison of oxidative and antioxidative parameters in liver in BALB/c miceinfected and not infected with *Leishmania (Leishmania) amazonensis.*

Parameters	Sample	Group 1[a]	Group 2[b]	Group 3[c]	Group 4[d]
T-SHs µmol/g	Liver	1558,93 ± 63,97	1428,08 ± 74,09	1560,44 ± 296,22	1635,81 ± 111,76
GSH nmol/g	Liver	3,58 ± 0,73	6,61 ± 1,49	5,99 ± 1,61	6,60 ± 2,22
TBARS nmol/mg	Liver	5,81 ± 0,48	5,96 ± 0,60	7,13 ± 1,76	5,76 ± 1,00
SOD IU/mg	Liver	4,26 ± 1,19	6,23 ± 1,18	3,41 ± 0,49	4,67 ± 0,66

Values are shown as mean ± standard deviation.
[a]Group 1: control
[b]Group 2: mice submitted to depletion of $CD4^+$ T cells
[c]Group 3: mice infected with *Leishmania (Leishmania) amazonensis*
[d]Group 4: mice with depletion of $CD4^+$ T cells and infected with *Leishmania (Leishmania) amazonensis*

Table 3

Comparison of oxidative and antioxidative parameters in spleen in BALB/c miceinfected and not infected with *Leishmania (Leishmania) amazonensis.*

Parameters	Sample	Group 1[a]	Group 2[b]	Group 3[c]	Group 4[d]
T-SHs µmol/g	Spleen	228,13 ± 38,72	240,85 ± 40,30	273,38 ± 38,03	294,51 ± 50,57
GSH nmol/g	Spleen	4,31 ± 0,34	3,79 ± 0,18	4,40 ± 1,78	3,42 ± 6,85
TBARS nmol/mg	Spleen	38,68 ± 5,58	40,46 ± 2,67	33,41 ± 2,09	35,50 ± 5,09
SOD IU/mg	Spleen	16,66 ± 1,31	18,46 ± 5,98	16,39 ± 7,38	16,05 ± 8,16

Values are shown as mean ± standard deviation.
[a]Group 1: control
[b]Group 2: mice submitted to depletion of $CD4^+$ T cells
[c]Group 3: mice infected with *Leishmania (Leishmania) amazonensis*
[d]Group 4: mice with depletion of $CD4^+$ T cells and infected with *Leishmania (Leishmania) amazonensis*

Table 4

Comparison of oxidative and antioxidative parameters in kidneys in BALB/c miceinfected and not infected with *Leishmania (Leishmania) amazonensis*.

Parameters	Sample	Group 1[a]	Group 2[b]	Group 3[c]	Group 4[d]
T-SHs µmol/g	Kidneys	548,33 ± 65,96	577,34 ± 95,98	599,93 ± 88,77	515,25 ± 112,30
GSH nmol/g	Kidneys	4,73 ± 0,97	4,90 ± 1,66	2,69 ± 1,60	4,28 ± 1,62
TBARS nmol/mg	Kidneys	58,89 ± 10,91	59,82 ± 7,39	49,52 ± 6,81	30,71 ± 5,03
SOD IU/mg	Kidneys	21,44 ± 7,27	19,57 ± 10,55	22,03 ± 1,38	13,08 ± 4,00

Values are shown as mean ± standard deviation.
[a]Group 1: control
[b]Group 2: mice submitted to depletion of $CD4^+$ T cells
[c]Group 3: mice infected with *Leishmania (Leishmania) amazonensis*
[d]Group 4: mice with depletion of $CD4^+$ T cells and infected with *Leishmania (Leishmania) amazonensis*

4. Discussion

Several infectious diseases induce a pro-inflammatory response, resulting in the production of toxic molecules such as ROS and RNS, leading to oxidative stress and injury to various biomolecules and cells (Scott and Novais 2016). In some instances, these alterations can contribute to the survival and multiplication of several microorganisms (Sorci and Faivre 2008; Asmaa et al. 2017). *Leishmania* spp. possesses several protective mechanisms to subvert the immune system, allowing them to deal with different levels of oxidative stress during the innate and adaptive immune responses, survive and maintain the infection (McConville and Naderer 2011). The effect of the host defense mechanisms against pathogens is related to the production of ROS and/or RNS by immune cells, targeting structures such as proteins, lipids and DNA (Valko et al. 2006; Wen et al. 2004; Zacks et al. 2005). In this context, the determination of the oxidative status of the host through the analysis of several oxidative and antioxidative markers such as MPO, BChE, TBARS, SOD, T-SHs, and GSH is crucial to understand aspects related to both resistance and pathogenesis (Singh et al. 2014; Tonin et al. 2016).

In our study, we evaluated the oxidative status of susceptible BALB/c mice infected with *L. amazonensis* and BALB/c mice that were pretreated with anti-CD4 antibody in order to cause a more severe infection. However, we did not observe difference in the progression of infection between the groups. It could be due to the augment in the number of CD4+ T cells following the course of infection. Moreover both groups present similar levels of oxidant and protective antioxidant defense markers, indicating that in BALB/c mice *L.* (*L.*) *amazonensis* induce a balance between oxidative-antioxidative mediators favoring the parasite persistence. Silva et al. (2017) demonstrated the presence of an efficient system in *L.* (*L.*) *amazonensi*s protozoa, which leads to the efective adaptation of the parasite to the continuous ROS attack. In contrast, Gaparotto et al. (2017) observed significant alterations in the redox status only in liver samples from experimentally infected mice with *L.* (*L.*) *amazonensi*s. This large variability of results in relation to the oxidative and inflammatory parameters in leishmania infections can occur due to differences in infection time, host and parasite susceptibility and species involved in the infection (Tonin et al., 2016; Magalhães et al., 2018).

Additionally, *Leishmania* spp. has mechanisms capable of evading the host immune system and rebalancing free radical levels (Gupta et al. 2013). In trypanosomes and leishmanias, trypanothione ($T(SH)_2$) plays a central role in parasite protection against mammalian host defence systems by recycling trypanothione disulphide by the enzyme

trypanothione reductase. Trypanothione protects Leishmania to the toxic effect of nitric oxide (Tovar et al. 1998; Mukherjee et al. 2009). Moreover, amastigote forms of the parasite also produce a low-molecular-weight thiol, known as ovothiol, which works as a direct oxygen neutralizer (Steenkamp 2002). In addition, recent studies on genes resistant to oxidative stress demonstrated through genome-wide analysis that the trypanosomatid enzyme pteridine reductase 1 (PTR1) could also have a key role in protozoan oxidative defenses (Nare et al. 2009).

In leishmaniasis caused by *L.* (*L.*) *amazonensis*, the protective immune response is mediated by the development of a Th1 response that leads to the activation of neutrophils, macrophages and dendritic cells and consequent induction of nitric oxide and reactive oxygen species, important mediators that kills intracellular parasites. On the other hand, the IL-4 and Th2 response are correlated with the susceptibility to *L.* (*L.*) *amazonensis* (Guimarães et al., 2006). Different studies with mice models demonstrated that the activity and migration of T cells is directly connected with regulation and performance of the host immune response (Yurchenko et al., 2006). In experimental infections, the host immune system developed a combination of Th1 and Th2 response to *L. (L.) amazonensis* infection, producing IL-4 and IFNγ (Ji et al., 2002). However, the protozoan can control the response of $CD4^+$ T cells mediated by IL-10 persisting even in immunocompetent hosts (Gupta et al., 2013). It should be noted that further to lymphocytes, a variety of cytokines and chemokines perform an important role in immunity in the fight against *Leishmania* spp. infections (Gupta et al., 2011; Gupta et al., 2013). This proteins and receptors are mediators of the immune system and expressed in inflammatory situations, working directly on parasite infections through activation and differentiation of the immune cells that may be contribute to fight or persistence to *Leishmania* protozoa (Kopf et al., 1996; Mattner et al., 1996).

Efficient evasion mechanism against reactive oxygen species produced at each stage of *Leishmania* spp. infection is fundamental to be able to infection and persistence to protozoa in host cell. The development of this mechanism has been the subject of several studies in order to better understand the pathogenesis of the disease, however there is still much to investigate.

5. Conclusion

We did not find significant differences in oxidative and antioxidative markers between the groups, suggesting that *L. (L.) amazonensis* could modulate the host's anti-oxidant response in experimental model, allowing the persistence of this agent in the infected tissues.

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5 DISCUSSÃO

Relatos da ocorrência da leishmaniose cutânea, incluindo descrições em humanos, são encontrados na literatura desde I d.C. permanecendo como um dos principais problemas de saúde pública mundial até os dias atuais (BASANO e CAMARGO, 2004; CAMARGO e BARCINSKI, 2003; HEPBURN, 2003). No presente trabalho, foi observado através de uma pesquisa meta-analítica que o Brasil é o país com o maior número de estudos publicados sobre a LC. Apesar da alta ocorrência da doença e da grande variabilidade de espécies, a primeira confirmação da doença no Brasil ocorreu somente em 1909 e até a década de 70 acreditava-se que a LC era causada exclusivamente pela espécie *L.* (*V.*) *braziliensis*, demonstrando assim o retardamento do país no início do desenvolvimento de políticas públicas para o combate da leishmaniose (BASSANO e CAMARGO, 2004). Embora a leishmaniose apresente epidemiologia e ecologia complexas, a doença permanece classificada integrante do quadro de doenças negligenciadas obtendo baixa relevância nas discussões sobre doenças tropicais (HOTEZ et al., 2004; HOTEZ et al. 2006). A ocorrência de dificuldades no gerenciamento de casos de leishmaniose, além de escassez de dados atualizados de incidências ocorre como consequência da subestimação da doença (ALVAR et al., 2012).

Em um trabalhado realizado em parceria com a Organização Mundial da Saúde foi observado que a incidência mundial de LV é de 0,2 a 0,4 milhão de casos a cada ano, enquanto a LC esta mais amplamente distribuída com incidência global de 0,7 a 1,2 milhão com mais de 70% dos casos concentrados no Afeganistão, Argélia, Colômbia, Brasil, Irã, Síria, Etiópia, Sudão do Norte, Costa Rica e Peru (ALVAR et al., 2012). Apesar dos números alarmantes de incidência é importante destacar que a obtenção de dados na leishmaniose para estudos meta-analíticos é considerada complexa, pois, como as demais doenças tropicais negligenciadas, a incidência ocorre, principalmente, de maneira focal e em locais remotos gerando dados subestimados da doença, o que dificulta a obtenção e estimativa de fontes de dados confiáveis (MATHERS et al., 2007; WHO, 2010).

O quadro clínico resultante da infecção é muito variável dificultando no diagnóstico precoce e preciso como também no tratamento eficaz e no controle da doença (BASANO e CAMARGO, 2004). O desenvolvimento e agravamento da infecção dependem de diversos indicadores ainda pouco compreendidos, como a espécie envolvida, fatores genéticos e imunológicos tanto do hospedeiro como do parasita (HEPBURN, 2003). Dentre a grande variedade de hospedeiros da doença, os cães apresentam um papel fundamental na saúde

pública humana e animal (SHAW et al., 2003; SOLANO-GALLEGO e BANETH, 2008). Os resultados obtidos no trabalho meta-analítico desenvolvido demonstraram a alta incidência de cães positivos para a LC, porém sem a presença de sinais clínicos característicos para a doença dificultando o diagnóstico, a notificação e, consequentemente, o controle da infecção. Dentre as espécies domésticas, os cães apresentam relevante importância nos estudos epidemiológicos da doença nas áreas urbanas. Diversos trabalhos têm relacionado a incidência da leishmaniose canina com a ocorrência de novos casos em seres humanos, bem como o aumento da população canina com o aumento dos riscos da leishmaniose infantil (ACEDO SÁNCHEZ et al., 1996; SOLANO-GALLEGO e BANETH, 2008).

A patogênese das infecções causadas por espécies dermotrópicas geralmente se limitam ao sítio de inoculação, podendo atingir áreas satélites tanto dérmicas como linfáticas (HEPBURN, 2003; PEREIRA e ALVES, 2008). As lesões normalmente são de caráter cutâneo, ou muco-cutâneo, no entanto essa diferenciação fenotípica não é absoluta. A ocorrência da forma mucocutânea destrutiva, conhecida como espundia, é descrita em hospedeiros infectados pela espécie *L. (L.*) *braziliensis*, a qual tem a capacidade de se alojar na região orofaríngea dos mamíferos infectados e ali permanecer na sua forma inativa por longos períodos, até obter condições ideais para a sua reativação (HEPBURN, 2003; LAINSON, 2010). Assim como observado no presente trbalho, diferentes autores relatam que infecções causadas por espécies envolvidas na LC podem levar a ocorrência da forma visceral, atingindo diferentes órgãos como fígado, medula e cérebro (ARA et al., 1998; CARDOSO et al., 2010; MAGILL et al., 1993; TORRENTERA et al., 2002).

Em condições naturais, normalmente a leishmaniose cutânea permanece subclínica em seus hospedeiros, ou ainda auto-resolutivas (VALE e FURTADO, 2005). No entanto, após o período de incubação, que varia de 1 a 12 semanas, lesões na forma de pápula podem desenvolver, podendo inclusive evoluir para a forma de úlcera (HEPBURN, 2003). Em condições experimentais, como observado no presente trabalho, normalmente é possível observar edema no sítio de infecção, podendo evoluir para uma lesão na forma de úlcera, a qual normalmente se manifesta de forma indolor, com feridas de margem elevada, endurecida e uma base necrótica que é frequentemente coberta por uma crosta aderente de exsudado seco. Em humanos, a maioria dos casos descrevem a ocorrência de 1 ou 2 lesões, geralmente em locais expostos, variando em tamanho de 0,5 a 3 cm de diâmetro (HEPBURN et al., 1993).

É importante destacar os dados obtidos no estudo meta-analítico que evidenciou a grande inconstância clínica da LC. O quadro clínico na leishmaniose esta diretamente relacionado a espécie protozoária envolvida, tipo do vetor, espécie hospedeira acometida,

assim como suas características imunológicas e genéticas (GRIMALDI e TESH, 1993; MARZOCHI, 1992). Assim, é fundamental a identificação da espécie infectante para determinar o tratamento adequado e um controle eficiente da doença, bem como ressaltar as pesquisas científicas, estudo clínicos e epidemiológicos específicos para cada espécie envolvida na leishmaniose. As espécies mais relevantes envolvidas na LC são divididas em 5 complexos: *L. donovani, L. tropica, L. braziliensis, L. mexicana* and *L. guyanensis* (VAN DER AUWERA et al., 2014; VAN DER AUWERA e DUJARDIN, 2015). A espécie *L.* (*L.*) *amazonensis* esta classificada dentro do complexo *L. mexicana*, juntamente com *L.* (*L.*) mexicana. O subgênero *Leishmania* ainda permanece pouco explorado quando comparado às demais espécies pertencentes ao subgênero *Viannia*, principalmente na área de variabilidade genética e geográfica, caracterizando assim a relevância dos dados obtidos no presente trabalho (VAN DER AUWERA e DUJARDIN, 2015).

A infecção de humanos e mamíferos domésticos por *L.* (*L.*) *amazonensis* é considerada acidental, pois seus principais hospedeiros são roedores silvestres, principalmente espécies pertencentes ao gênero *Proechymis* (PEREIRA e ALVES, 2008). Diferentemente do que se é observado em humanos, os roedores da espécie *Proechymis* spp. desenvolvem o quadro clínico assintomático durante todo o período de infecção (FORTÉA et al., 2007). Apesar de o ciclo ser prevalentemente silvestre, pesquisadores demonstraram que *L.* (*L.*) *amazonensis* tem aumentando sua distribuição geográfica no Brasil, com quadros clínicos atípicos em novas áreas de transmissão (AZEREDO-COUTINHO et al., 2007).

Para melhor compreensão do ciclo patogênico da leishmaniose e suas relações imunológicas entre hospedeiro e parasita, diversos trabalhos tem utilizado espécies da subfamília Murinae como modelo experimental (OLIVEIRA, J. et al., 2010; PEREIRA e ALVES, 2008; PRIANTI et al., 2007). Como evidenciado no presente estudo, camundongos BALB/c demonstraram ser um adequado modelo animal para infecções experimentais de *L.* (*L.*) *amazonensis.* Em um estudo realizado por Almeida et al. (1996), camundongos BALB/c foram infectados com cepas isoladas da pele, mucosa e vísceras de humanos infectados por *L.* (*L.*) *amazonensis.* As cepas isoladas de lesões cutâneas e mucocutâneas apresentaram lesões ulcerativas, alta carga parasitária e extensa destruição tecidual, metástase cutânea, rápida disseminação dos parasitas ao baço e altos níveis de anticorpos circulantes. Contudo, nos camundongos infectados com as cepas dos casos viscerais foram observados pequenas lesões na pata, baixa carga parasitária, migração tardia para o baço e baixos níveis de imunoglobulina anti-*Leishmania* IgG quando comparado com as cepas cutâneas e mucocutâneas.

A resposta imune de camundongos susceptíveis é considerada semelhante aos humanos, sendo essa a espécie utilizada para mimetizar o comportamento de células humanas do sistema imune frente a infecção por LV (AHMED et al. 2003). A resistência ou suscetibilidade do hospedeiro à infecção por espécies do gênero *Leishmania* estão diretamente relacionadas ao desenvolvimento de respostas por células T $CD4^+$ do tipo Th1 ou Th2 (PEREIRA e ALVES, 2008). Pesquisadores demonstraram que camundongos resistentes desenvolvem uma precoce resposta por Th1, com produção de IFN-γ, ativação de macrófagos, prevenido assim o crescimento do protozoário e, consequentemente, controle da infecção e a cura espontânea (LEHMANN et al., 2000; MATTHEWS et al., 2000). Em animais susceptíveis a resposta Th2 é mais prevalente levando ao desenvolvimento e a persistência de lesões não cicatrizantes e, muitas vezes, ulceradas (HIMMELRICH et al. 2000; LASKAY et al., 1995;).

A infecção experimental de *Leishmania* spp. pode gerar diferentes manifestações clínicas, as quais, assim como na infecção natural, dependem da imunidade do hospedeiro, virulência da cepa, carga parasitária e via de inoculação (TITUS e RIBEIRO, 1988). No presente trabalho, foi observada a ocorrência da forma visceral na infecção por *L.* (*L.*) *amazonensis* em camundongos BALB/c, com alterações nos linfonodos inguinais, fígado e principalmente baço. Em modelos experimentais foi demonstrado que a infecção hepática normalmente é autolimitante, sendo controlada após aproximadamente 1 mês, com prevalência de uma resposta inflamatória granulomatosa e participação de células TCD4+ e TCD8+, além de diversas citocinas. (ENGWERDA et al., 2004). No baço, após a fase aguda da doença, ocorre esplenomegalia, perda da microarquitetura e proliferação de amastigotas (ENGWERDA et al., 2004; MALLA e MAHAJAN, 2006).

Diversos fatores influenciam na resposta imune do hospedeiro frente a infecções por microrganismos. Os elementos traços estão diretamente envolvidos em processos metabólicos críticos para a diferenciação e replicação celular, influenciando no sistema imunológico do hospedeiro (SCUDERI, 1990). Alterações nos níveis de macro e micro-elementos podem contribuir no controle de infecções causadas por protozoários do gênero *Leishmania* no hospedeiro sendo essenciais para diferentes atividades celulares, como estabilidade da membrana celular, apoptose, equilíbrio metabólico e atividades enzimáticas, contribuindo assim nas inúmeras estratégias de defesa de organismos (CHVAPRIL 1973; COUSINS, 1985; KOCYIGIT et al., 1998; PANEMANGALORE e BEBE, 1996; SPRIETSMA, 1997).

Dados obtidos com esta pesquisa demonstraram que hospedeiros imunodeprimidos através da depleção de CD4+ apresentam alteração de níveis de zinco e manganês no baço.

Este resultado corrobora com outros autores e pode ser responsável pela incapacidade destes hospedeiros em eliminar o parasita (AMINI et al., 2009). A dificuldade do sistema imune em controlar a proliferação destes parasitas no baço pode ser atribuída à reação inflamatória resultante da produção de diversas citocinas e enzimas (AMINI et al., 2009; ROFE et al., 1966; WARNER e LAWRENCE, 1988). O Zn é um elemento essencial para diferentes organismos e sua relação com o sistema imune é complexa. Este micro-elemento influencia diretamente no funcionamento dos monócitos, células NK, células B e T e suas citocinas, além de manter o equilíbrio entre as respostas Th1 e Th2 e adequada produção de células T citotóxicas (BECK et al., 1997; SHANKAR e PRASAD, 1998). Assim, a deficiência de Zn esta diretamente ligada a inúmeras doenças, especialmente as doenças do sistema imunológico (BONAVENTURA et al., 2015; PRASAD, 1995).

Diversas funções imunes reduzem com a deficiência de Zn, mas a suplementação oral deste micro-elemento permite a reestabilização dessas atividades (WELLINGHAUSEN et al., 1997). Além disso, trabalhos desenvolvidos ao longo dos anos tem utilizado o Zn como adjuvante em vacinas experimentalmente desenvolvidas para inúmeras doenças tanto em humanos como em animais (BONOMINI et al., 1993; CAKMAN et al., 1996; FRAKER et al., 1986; LIGHART et al., 1984; SANDSTEAD et al., 1982). A utilização do Zn como tratamento experimental no combate às infecções por *Leishmania* também tem sido investigada nos últimos anos. O sulfato de zinco tem sido avaliado em pesquisas *in vitro* e *in vivo*, apresentando resultado semelhante às drogas sistêmicas comumente utilizadas no tratamento de seres humanos com LC (BAFGHI et al., 2014; YAZDANPANAH et al., 2011).

A resistência hepática à infecção pelo protozoário pode estar correlacionada com a produção de intermediários reativos de oxigênio e nitrogênio durante o estágio inicial da leishmaniose visceral (aproximadamente até 14 dias pi), contribuindo significativamente no controle da multiplicação do parasito (KAYE et al., 2004). Após o período inicial da infecção é possível observar declínio do número de amastigotas no tecido hepático e alta expressão do gene que regula a síntese do oxido nítrico (NO_2), o qual leva a ativação de macrófagos dependentes de células T, ativação de diversas citocinas e controle da infecção no tecido (KAYE et al., 2004; MURRAY e NATHAN, 1999).

Durante a infecção por protozoários do gênero *Leishmania* o sistema imune do hospedeiro utiliza de diversos mecanismos oxidativos, como espécies reativas de oxigênio (ROS), com o intuito de controlar e eliminar o parasito, tanto na fase inicial como tardia da doença (THANNICKAL e FANBURG, 2000; WINK et al., 2011). A evasão eficiente do protozoário frente às moléculas tóxicas produzidas pelo hospedeiro em cada estágio da

infecção é importante para que a *Leishmania* seja capaz de iniciar e manter a infecção da célula hospedeira (GANTT et al., 2001). Dois importantes oxidantes produzidos por macrófagos foram identificados como críticos no controle da infecção por *Leishmania*: o íon superóxido (O_2^-) e radicais hidroxila (OH) gerado a partir do peróxido de hidrogénio (H_2O_2) (CHANNON et al., 1984; MILLER et al., 2000; ZARLEY et sl., 1991).

No entanto, diversas pesquisas têm demonstraram a capacidade de resistência dos protozoários do gênero *Leishmania* frente à atuação de EROs, corroborando com os dados observados no presente estudo (ALCOLEA et al., 2016; MILLER et al., 2000; SOUSA-FRANCO et al., 2006). Normalmente a liberação dessas moléculas tem relevância apenas na fase inicial da infecção e por um curto período de tempo (MURRAY e NATHAN, 1999). Paltrinieri et al. (2010), observaram que *L. donovani* é capaz de inibir a produção de ROS em macrófagos caninos e seu aumento ocorre devido ao processo inflamatório decorrente da infecção, ao invés da presença propriamente dita do parasito

Nossos resultados demonstraram a capacidade que a espécie *L.* (*L.*) *amazonensis* tem em resistir e controlar os processos biológicos oxidativos produzidos por células do sistema imunológico do hospedeiro. Os protozoários do gênero *Leishmania* utilizam diversos mecanismos para sobreviver a agentes oxidantes, como enzimas antioxidantes, glicolipídeos de superfície, além de aumentar a expressão de proteínas de choque térmico em resposta a temperatura elevada ou outro estresse ambiental (HIGHTOWER, 1991; ISMAIL et al., 1994; POMPELLA, 1997). Assim, protozoários do gênero *Leishmania* têm a capacidade de adquirir resistência aos efeitos tóxicos de superóxido ou peróxido de hidrogênio após a exposição a condições ambientais desfavoráveis à sua sobrevivência geradas pelo sistema imune dos seus hospedeiros mamíferos (MILLER et al., 2000; WILSON et al. 1994).

6 CONCLUSÃO

O resultado clínico de uma infecção por *Leishmania* é variável e depende de fatores do parasito e do hospedeiro, podendo uma mesma espécie causar diferentes manifestações clínicas em um mesmo hospedeiro. Essa grande variabilidade de quadros clínicos na leishmaniose tem sido um dos principais desafios para a obtenção de um diagnóstico precoce, dificultando o controle desta importante zoonose. Os resultados obtidos neste trabalho, além de evidenciar a grande população de hospedeiros assintomáticos, demonstram a significativa ocorrência da forma clínica visceral da doença mesmo em casos envolvendo apenas espécies comumente classificadas como causadoras de lesões dermotrópicas. Dentre as espécies consideradas dermotrópicas, a *L. (L.) amazonensis* tem apresentado relevância epidemiológica e médica no país devido sua expansão geográfica e aumento do número de casos nos últimos anos. Como observado nas demais espécies, *L. (L.) amazonensis* demonstrou experimentalmente o potencial de migração para outros órgãos além do sítio de infecção e seu impacto patológico nos linfonodos, fígado e baço do hospedeiro. O comprometimento do baço foi significativamente relevante em camundongos com depleção de CD4+, levando a uma expressiva deficiência nos níveis de concentração de zinco e manganês, micro-elementos extremamente importantes na produção e desempenho de diversas células e citocinas do sistema imune. Além disso, foi evidenciada a capacidade do parasita em resistir e controlar os processos biológicos oxidativos produzidos por células do sistema imunológico e assim persistir no organismo do hospedeiro. Assim é almejado que os dados obtidos no presente trabalho sirvam como impulso no desenvolvimento de um tratamento eficaz e estratégias de prevenção e combate à leishmaniose cutânea.

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