Universidade Federal do Maranhão
Centro de Ciências Biológicas e da Saúde
Programa de Pós-Graduação em Ciências da Saúde

Doutorado

PATRÍCIA VALÉRIA CASTELO BRANCO ARAÚJO

MILTEFOSINA (HEXADECILFOSFOCOLINA) INDUZ DANOS GENÔMICOS MODULADOS PELO ANTIOXIDANTE ÁCIDO ASCÓRBICO

São Luís
2019

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Tese de Doutorado apresentado ao Programa de Pós-Graduação em Ciências da Saúde da Universidade Federal do Maranhão como parte dos requisitos para obtenção do título de *Doutora em Ciências da Saúde*.

Orientadora: Profª. Drª. Silma Regina Ferreira Pereira

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aos meus filhos

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A Deus,

- sempre, por tudo.

Aos meus pais, Ilva e Francisco,

- pelo apoio.

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- pelo incentivo.

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"...bom mesmo é ir à luta com determinação,

viver a vida com paixão,

perder com classe e vencer com ousadia,

pois o triunfo pertence a quem se atreve;

e a vida é muito para ser insignificante."

Charles Chaplin

RESUMO

As leishmanioses compreendem um grupo de doenças infecto parasitárias negligenciadas, crônicas e não contagiosas, ocasionadas por protozoários do gênero *Leishmania*. A leishmaniose visceral tem atualmente ocorrência descrita em 102 países. O Brasil se destaca com altas taxas de casos dessa doença, sendo registrado, na última década (2008 a 2017), 37.639 casos e 2.735 óbitos. Devido ao aumento de casos refratários aos antileishmaniais de primeira escolha, a miltefosina foi introduzido como tratamento oral em países como Índia, Bangladesh e Nepal, desde 2002. Entretanto, muito pouco é conhecido sobre a ação da droga, inclusive sua interação com o material genético. Assim, neste estudo foi avaliado o efeito genotóxico e mutagênico desse fármaco, e os mecanismos celulares e moleculares envolvidos na sua ação antileishmanial. Para tanto, foram realizados ensaios *in vitro* e *in vivo* para avaliar a capacidade da miltefosina em induzir instabilididade genômica, de aumentar a frequência de micronúcleos e de causar morte celular. Além disso, foi também determinada a carga parasitária nos animais infectados e tratados com o antileishmanial, bem como foi avaliado o efeito antigenotóxico do ácido ascórbico, reconhecido antioxidante, nos animais tratados com miltefosina, sob diferentes condições de tratamento (dose única, doses repetidas, pré tratamento, pós tratamento e tratamento concomitante). Adicionalmente, foi determinada a atividade das enzimas antioxidantes SOD, CAT e GPx e a expressão de genes relacionados ao sistema de reparo do DNA (*OGG1*, *MUTYH* e *NUDT1*) a fim de identificar possíveis mecanismos moleculares relacionados à ação da miltefosina. Demonstramos inicialmente que a miltefosina causa lesões genômicas por oxidação de bases púricas e pirimídicas do DNA, bem como é indutor de apoptose e necrose em células de mamíferos. Posteriormente, verificamos que esse dano pode ser modulado pelo ácido ascórbico e que o antioxidante não interfere na eficácia antileishmanial da droga. Por fim, verificamos que, em resposta às injúrias oxidativas, a célula responde aumentando a atividade das enzimas SOD e a expressão do gene de reparo *OGG1*.

Palavras-chave: Genotoxicicidade; Estresse oxidativo; Antioxidante; Enzimas antioxidantes; Expressão gênica; *Leishmania*.

ABSTRACT

Leishmaniasis is a group of neglected non-contagious, parasitic and chronic infectious diseases caused by protozoa of the genus *Leishmania*. Visceral leishmaniasis is currently reported in 102 countries. Brazil has high rates of disease cases, registering only in a decade (2008 to 2017), 37,639 cases and 2,735 deaths. Due to increased relapses to first-choice antileishmanial drugs, since 2002, miltefosina has been introduced as oral treatment in countries such as India, Bangladesh and Nepal. However, little is known about the action of the drug and its interaction with the genetic material. Thus, in this study the genotoxic and mutagenic effect of this drug was evaluated, as well as the cellular and molecular mechanisms involved in its antileishmanial action. For this, *in vitro* and *in vivo* assays were performed to evaluate the ability of miltefosina to induce genomic instability, to increase the frequency of micronuclei and to cause cell death. Furthemore, the parasite load was also determined in infected animals and treated with antileishmanial, as well as was evaluated the antigenotoxic effect of ascorbic acid, recognized antioxidant, in animals treated with miltefosina under different treatment conditions (single and repeated doses, simultaneous, pre and post treatment). In addition, the activity of the antioxidant enzymes SOD, CAT and GPx and the expression of genes related to the DNA repair system (*OGG1, MUTYH, NUDT1*) were determined in order to identify possible molecular mechanisms related to the action of miltefosina. We initially demonstrated that miltefosina causes genomic lesions by oxidation of purine and pyrimidine bases of DNA, as well as inducing apoptosis and necrosis in mammalian cells. Subsequently, we verified that this damage can be modulated by ascorbic acid and that the antioxidant does not interfere with the antileishmanial efficacy of the drug. Finally, we verified that, in response to oxidative stress, the cell responds by increasing the activity of the SOD enzymes and the expression of the *OGG1* repair gene.

Key-words: Genotoxicity; Oxidative stress; Antioxidant; Antioxidant enzymes; Gene expression.

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LISTA DE ABREVIATURAS E ESTRUTURA QUÍMICA

APX	Ascorbato peroxidase
BER	Sistema de Excisão de Bases
CAT	Catalase
DNA	Ácido desoxirribonucleico
ENDO III	Endonuclease III
FPG	Formamidopirimidina DNA- glicosilase
GPx	Glutationa peroxidase
LC	Leishmaniose cutânea
LT	Leishmaniose tegumentar
LV	Leishmaniose visceral
MN	Micronúcleo
Mrna	RNA mensageiro
MUTYH	Muty DNA glicosilase
NCE	Eritrócito normocromático
OGG1	8-oxoguanina DNA glicosilase 1
OMS	Organização Mundial de Saúde
NUDT1	diphosphate linked moiety X-type motif 1
PCE	Eritrócito policromático
LDPC	Leishmaniose dérmica pós calazar
PCEMN	Eritrócito policromático micronucleado
RNA	Ácido ribonucleico
RNS	Espécies reativas de nitrogênio
ROS	Espécies reativas de oxigênio
SOD	Superóxido dismutase
SINAN	Sistema de Informação de Agravos de Notificação
SUS	Sistema Único de Saúde
WHO	Worl Health Organization
pH	Potencial hidrogeniônico

Estrutura química

H_2O_2	Peróxido de hidrogênio
O_2	oxigênio
O_2^-	Ânion superóxido
OH-	Radical hidroxila
8-oxoG	8-oxoguanina
8-OH-dGTP	8-oxo-7,8-dihidro-2'-desoxiguanosina 5'-trifosfato
8-oxo-dGMP	8-oxo-7,8-dihidro-2'-desoxiguanosina 5'-monofosfato

SUMÁRIO

1 INTRODUÇÃO

As leishmanioses compreendem um grupo de doenças infecto parasitárias, negligenciadas, crônicas, não contagiosas, ocasionadas por protozoários do gênero *Leishmania*. Tratam-se de patologias com distintas manifestações clínicas, sendo reconhecidas quatro formas principais: leishmaniose cutânea (LC), leishmaniose mucocutânea (LMC), leishmaniose visceral (LV) e leishmaniose dérmica pós calazar (LDPC) (WHO, 2016).

A LV possui ampla distribuição, com ocorrência descrita em 102 países, sendo que 94% dos casos ocorreram em países como: Brasil, Etiópia, Índia, Quênia, Somália, Sudão do Sul e Sudão (WHO, 2019). Estima-se que surjam cerca de 500 mil novos casos por ano da doença, com incidência de 20 a 30 mil mortes (WHO, 2016). No Brasil, a LV atinge em média 3.763 pessoas por ano e o estado do Maranhão representa 15% desses casos (BRASIL, 2019).

O tratamento para leishmaniose divide-se em duas linhas farmacológicas: os antimoniais e os não antimoniais. Os antimoniais pentavalentes continuam sendo bastante utilizados, embora estudos tenham relatado sua alta toxicidade (DEMICHELI et al., 2002; KATO et al., 2014) bem como sua capacidade de induzir danos genotóxicos (LIMA et al., 2010; MOREIRA et al., 2017; de JESUS et al., 2018). Além disso, foi demonstrado vários mecanismos de resistência do parasita ao antimônio, como deleção ou expressão reduzida do gene responsável pelo influxo de droga na célula (*AQP1*), metabolização diminuída de SbV e aumento dos níveis de tripanotiona (PONTE-SUCRE et al., 2017). Dentre os antileishmaniais não antimoniais, destacam-se a anfotericina B, a pentamidina e a miltefosina.

Diante da crescente resistência ao tratamento com antimoniais, desde 2002 países como Índia, Alemanha, Bangladesh e Nepal começaram a utilizar a miltefosina para tratamento antileishmanial (SUNDAR; OLLIARO, 2007; SINGH et al., 2016). Seu uso foi considerado vantajoso por apresentar menor toxicidade e menos efeitos adversos que os antimônios pentavalentes, além de ser administrado por via oral (PRASAD et al., 2004).

Embora nos últimos anos o efeito antileishmanial da miltefosina tenha sido extensivamente estudado, sobretudo como alternativa farmacológica nos casos de resistência medicamentosa, permanecem pouco esclarecidos os mecanismos pelos quais ele exerce seu efeito. Considerando que estudos prévios sugeriram que um dos possíveis mecanismos de ação da droga seria a produção de espécies reativas de oxigênio (ROS) (VINCENT et al., 2014; CARNIELLI et al., 2014), neste trabalho é avaliado, pela primeira vez, se o estresse oxidativo causado pela miltefosina poderia induzir danos genéticos em células de mamíferos *in vitro* e *in vivo*. Essa hipótese também foi testada avaliando-se a capacidade do antioxidante ácido ascórbico em reverter o efeito genotóxico da droga.

2 REFERENCIAL TEÓRICO

2.1 Leishmanioses

A transmissão das leishmanioses ocorre por meio da picada de fêmeas de flebotomíneos pertencentes ao gênero *Phlebotomus*, encontradas no Velho Mundo, e as do gênero *Lutzomyia*, no Novo Mundo, que transmitem o parasita para os animais domésticos/silvestres ou humanos, através do repasto sanguíneo, principalmente em regiões tropicais e subtropicais. O parasita pertence à família Trypanosomatidae, subfamília Phlebotominae, ordem Kinetoplastida e gênero *Leishmania* (BRASIL, 2014). Nas Américas, *Leishmania (Leishmania) infantum* é o agente de VL (BRASIL, 2016). A LV é considerada um grave problema de saúde pública devido a sua magnitude, expansão geográfica e controle complexo. É uma doença relacionada à urbanização e atinge principalmente populações de baixa renda (BRASIL, 2011).

Dentre as quatro principais formas de manifestação da leishmaniose, a LV, conhecida popularmente como calazar, é considerada fatal para o homem se não tratada adequadamente (WHO, 2016). Atualmente essa doença tem ocorrência descrita em 102 países, sendo que, no ano de 2017, 94% dos casos somente em sete países: Brasil, Etiópia, Índia, Quênia, Somália, Sudão do Sul e Sudão (WHO, 2019) (Figura 1).

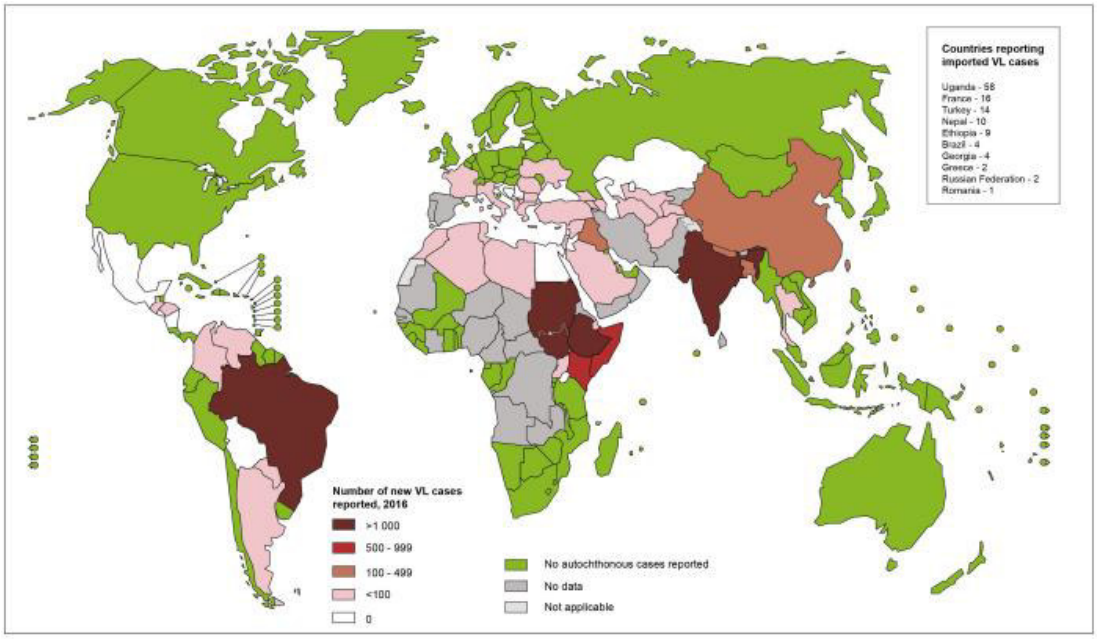


Figura 1. Distribuição de leishmaniose visceral no mundo.

FONTE: https://www.who.int/leishmaniasis/burden/en/ (Acesso em 18/02/2019)

Na América Latina, a doença já foi descrita em pelo menos 12 países (WHO, 2019). No Brasil foram registrados de 2008 a 2017, 37.639 casos de LV, com ocorrência de 2.735 óbitos (BRASIL, 2019) (Figura 2).

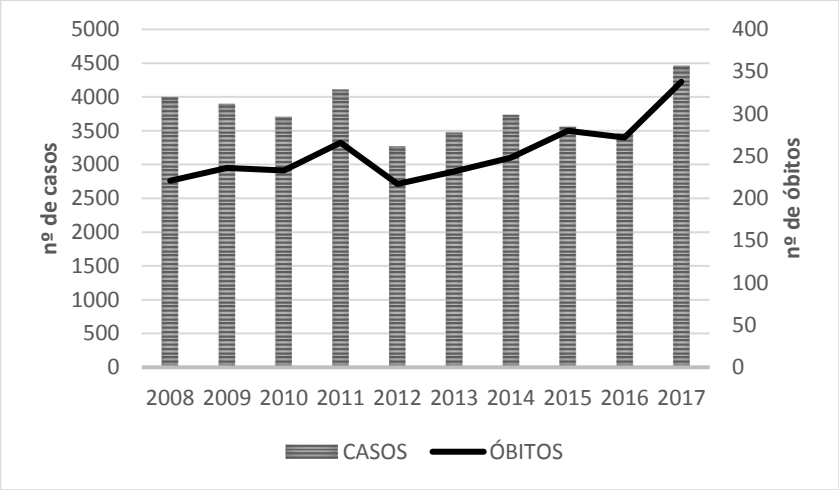


Figura 2. Ocorrência de casos e óbitos de leishmaniose visceral no Brasil, entre o período de 2008 a 2017.

FONTE: Dados colhidos do portal SINAN, atualizado até 04/02/2019 (http://portalsinan.saude.gov.br)

O estado do Maranhão abrange cerca de 15% do total de casos de LV no Brasil. Somente entre os anos de 2008 e 2017, foram registrados 5.705 casos de LV no estado, com 366 mortes nesse mesmo período (BRASIL, 2019) (Figura 3).

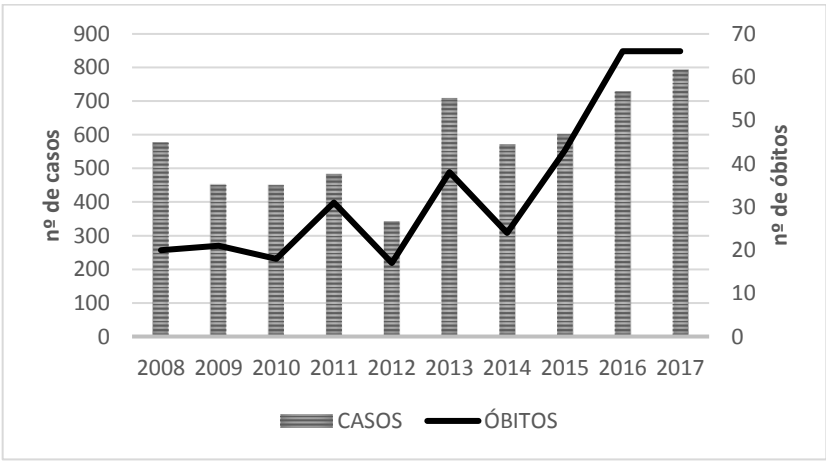


Figura 3. Ocorrência de casos e óbitos de leishmaniose visceral no estado do Maranhão, Brasil, entre o período de 2008 a 2017.

FONTE: Dados colhidos do portal SINAN, atualizado até 04/02/2019 (http://portalsinan.saude.gov.br)

2.2 Tratamento

O tratamento para leishmaniose consiste em duas linhas: os antimoniais e os não antimoniais.

2.2.1 Antimoniais

Dentre os compostos antimoniais, destacam-se os complexos de antimônio trivalentes tais como o tartarato antimonial de potássio (Tartarato emético), antimoniato de bis-catecol-3,5-dissulfonato sódico e tioglicolato de sódio e antimônio, bem como antimoniais pentavalentes, como o antimoniato de *N*-metilglucamina, gluconato de antimônio V sódico (estibogluconato sódico) e ureia estibamina (Estibamine) (RATH et al., 2003). Entretanto, devido a cardiotoxicidade e intolerância gastrintestinal dos antimoniais trivalentes (SbIII), são os antimoniais pentavalentes (SbV) que apresentam maior uso terapêutico. Atualmente existem no mercado duas formulações de SbV: Estibogluconato de sódio (Pentostan®) e o antimoniato de N-metil-glucamina (Glucantime®), não parecendo existir diferenças quanto a eficácia terapêutica destas formulações (BRASIL, 2014).

Desde a década de 40 os antimoniais pentavalentes são utilizados no tratamento das leishmanioses. Atualmente a única formulação disponível é o antimoniato de N-metil-glucamina, cujo mecanismo de ação não está totalmente elucidado. Porém, sabe-se que esta droga atua nas formas amastigotas do parasita, inibindo a atividade glicolítica e a via oxidativa de ácidos graxos dos parasitas (BRASIL, 2014), além de causar oxidação de bases nitrogenadas do DNA (MOREIRA et al., 2017; de JESUS et al., 2018). A farmacocinética dos antimoniais pentavalentes administrados por via intramuscular pode ser dividida em três fases: i) *fase de absorção*, com meia vida de 0,85h; ii) *fase de eliminação rápida,* com meia vida de 2,02h; e, iii) *fase de eliminação mais lenta*, com meia vida de cerca de 76 h. Por outro lado, quando administrado intravenosamente, cerca de 80% do antimonial pentavalente é eliminado em até 8h (GIL et al., 2007).

Devido a essa característica, são necessárias doses elevadas deste fármaco para garantir a eficácia do tratamento, o que pode acarretar diversos efeitos colateriais, tais como dores articulares, dores musculares, náusea e vômito, cefaleia, febre, anorexia, alterações nos testes de função hepática, níveis de lipase e amilase, leucopenia. Além disso, os antimônios são contra-indicados em caso de insuficiência renal, insuficiência hepática, insuficiência cardíaca, gravidez, idade maior de 50 anos e hipersensibilidade aos componentes da formulação (BRASIL, 2011; BRASIL, 2014). Contudo, em razão de sua toxicidade, a OMS preconiza que

a dose desse antimonial não ultrapasse 20 mg SbV/Kg/dia, pela via intramuscular ou intravenosa por, no mínimo, 20 e no máximo, 40 dias (BRASIL, 2014).

2.2.2 Não antimoniais

As drogas não antimoniais mais comumente utilizadas no tratamento de leishmanioses são a anfotericina B, a pentamidina e a miltefosina.

A anfotericina B é um antibiótico macrocíclico, poliênico, com atividade antifúngica e antileishmanial, produzido pela bactéria filamentosa *Streptomyces nodosus*. Atualmente, é o medicamento não animonial mais utilizado no tratamento de LV no Brasil e é considerado a droga mais potente disponível comercialmente, atuando nas formas promastigotas e amastigotas de leishmania. Seu mecanismo de ação se dá através da ligação preferencial com ésteres (ergosterol ou episterol) presentes na membrana plasmática da leishmania (BRASIL, 2014). A escolha entre o antimônio e a anfotericina B leva em consideração a faixa etária, presença de gravidez e comorbidades. Atualmente, existem duas apresentações de anfotericina B: o desoxicolato de anfotericina B e a anfotericina B lipossomal, com eficácias comparáveis, sendo que esta última apresenta menor toxicidade (BRASIL, 2011).

No Brasil, a anfotericina B é considerada como droga de primeira escolha no tratamento de gestantes, e de segunda escolha, quando não é obtida resposta ao tratamento com o antimonial pentavalente ou na impossibilidade de seu uso. Nos casos de resposta insatisfatória aos antimoniais, a anfotericina B deve ser utilizada na dose de 1mg/kg/dia, em dias alternados (máximo de 3g de dose total). Doses acima das recomendadas podem ser usadas em casos especiais. Em crianças, a anfotericina B deve ser utilizada na dose total de 15 a 25 mg/kg de peso, administrada em dias alternados (BRASIL, 2014).

Embora requeira administração parenteral, anfotericina B lipossomal é uma opção terapêutica para pacientes que apresentem insuficiência renal, refratariedade ou toxicidade ao uso de antimoniais, idade superior a 50 anos e transplantados renais, cardíacos e hepáticos (BRASIL, 2011). Seus efeitos colaterais são todos dose-dependentes, sendo altamente tóxico para as células do endotélio vascular. Se administrado rápido (menos de 1 hora), a anfotericina B pode causar cefaleia, febre, calafrios, astenia, dores musculares e articulares, vômitos, hipotensão e alterações cardiovasculares, com parada cardíaca. Alterações pulmonares e complicações renais também são relatados (BRASIL, 2014).

A pentamidina é um derivado sintético da amidina e pertence ao grupo das diamidinas aromáticas. Usado principalmente na Europa e na África, este antileishmanial possui duas formulações comerciais: isotionato e mesilato. A eficácia da pentamidina é inferior à dos antimoniais pentavalentes e anfotericina B, porém seus paraefeitos são maiores. A dose recomendada é de 4mg/kg/dia em dias alternados no total de 15 doses, não devendo ultrapassar a 2g como dose total. Seus efeitos colaterais mais comumente encontrados são anorexia, astenia, náusea, dor abdominal, taquicardia e outras arritmias, insuficiência renal em 25% dos pacientes, geralmente reversível, e pancreatite que pode levar ao aparecimento de *Diabetes mellitus*, em 10 a 15% dos casos (BRASIL, 2014).

2.3 Miltefosina

A miltefosina pertence à classe dos tensoativos zwitteriônicos e é estruturalmente semelhante aos fosfolipídios presentes na membrana biológica (CASTRO et al., 2001) (Figura 4). Tem a capacidade de interagir com a membrana das células dos protozoários, atingindo rapidamente outras membranas subcelulares, sendo capaz de afetar o metabolismo em diferentes níveis (HUANG et al., 2005; MARCO et al., 2009). Desse modo, a membrana celular é considerada o sítio de ação primária desse fármaco, o qual é inserido na membrana fosfolipídica pela miscibilidade e pela interação com esteróis da membrana (RAKOTOMANGA et al., 2004). Seu transporte para dentro da célula ocorre através da maquinaria de transporte e do complexo proteico, localizado na membrana plasmática do parasita (FDA, 2013).

Desenvolvido inicialmente para ser uma droga antitumoral (CROFT; ENGEL, 2006), a descoberta da miltefosina como agente antileishmanial (Impavido®) foi considerado um grande avanço para o tratamento da leishmaniose, por ser uma droga oral e efetiva, inclusive em casos de infecções resistentes ao antimônio pentavalente (SUNDAR et al., 2002). A importância de uma droga de uso oral e efetiva contra a leishmaniose se destaca pelo fato dessa doença ser mais prevalente em regiões mais pobres, onde a adesão ao tratamento é comprometida devido a questões econômicas e de infra-estrutura básica, como dificuldades de deslocamento dos pacientes ao local do tratamento (COSTA-FILHO et al., 2008).

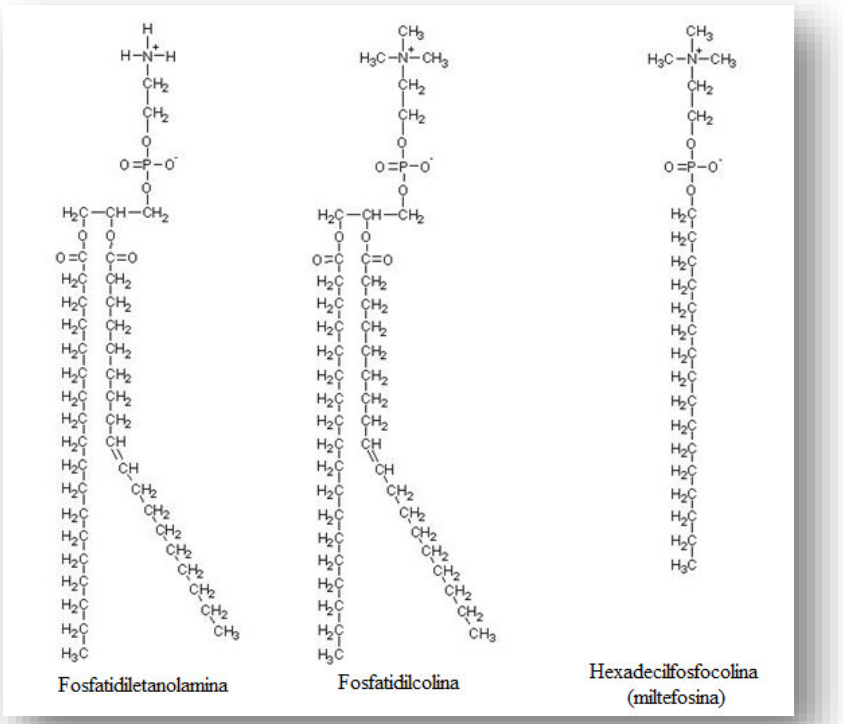


Figura 4. Semelhança entre a estrutura química dos principais fosfolipídios de membrana e a molécula da miltefosina (hexadecilfosfocolina).

FONTE: O autor

A miltefosina é bem absorvida por via oral sendo rapidamente distribuído por todo o organismo, acumulando-se em órgãos como fígado, baço, rins, pulmão e glândulas adrenais, e é lentamente metabolizado por fosfolipases hepáticas, apresentando excreção urinária mínima (MORE et al., 2003; AGRAWAL; SINGH, 2006). Requer tratamento prolongado (28 dias) e tem uma meia-vida longa (cerca de 150 horas) (PÉREZ-VICTORIA et al. 2006b).

Na Índia, devido à ocorrência de recidiva e resistência contra o tratamento tradicional utilizando antimoniais pentavalentes, passou-se a utilizar outras drogas com ação antileishmanial. Nesse cenário a miltefosina se destacou pelos seus resultados satisfatórios e alto percentuais de cura no tratamento da leishmaniose (SUNDAR et al., 2002).

Somente após uma década de uso da miltefosina como antileishmanial, a OMS o incluiu na lista de medicamentos essenciais (FDA, 2013). No Brasil, em 2010, o Ministério da Saúde aprovou o uso da miltefosina para o tratamento de leishmaniose tegumentar. Entretanto, a incorporação do medicamento pelo Sistema Único de Saúde (SUS) não foi implementada

(BRASIL, 2016). Em 2015, a Fundação Oswaldo Cruz emitiu um parecer técnico científico recomendando o uso da miltefosina para tratamento de leishmaniose cutânea, devido apresentar eventos adversos de menor gravidade comparado à meglumina e por ser uma opção por via oral (FIOCRUZ, 2015). Após esse parecer técnico, novamente o Ministério da Saúde recomendou a incorporação da miltefosina para o tratamento de pacientes com leishmaniose tegumentar (BRASIL, 2016). Todavia, até o momento, a miltefosina não está incluído na Relação Nacional de Medicamentos Essenciais (Rename) e nem possui registro na Anvisa.

Após uma década de uso da miltefosina no subcontinente indiano para tratamento de LV, verificou-se uma diminuição nas taxas de cura e o dobro de aumento nas taxas de recaída. Na Índia, Bangladesh e Nepal, cerca de 7% a 10% dos pacientes desenvolveram recaída clínica de LV no prazo de 6 meses após o tratamento com miltefosina, com taxas de recaída atingindo 20% no Nepal após acompanhamento de 12 meses (SUNDAR et al., 2012; RIJAL et al., 2013; DORLO et al., 2014). Uma das hipóteses para o surgimento de resistência do parasita aa miltefosina pode estar relacionada à eliminação da droga que é extremamente lenta, podendo ainda ser detectada no plasma humano seis meses após o final do tratamento (DORLO et al., 2012).

Ainda assim, foi notória uma melhora no quadro geral da doença nesses países, pois num intervalo de sete anos (2010-2017) cinco estados do norte da Índia registraram mais de 80% de redução de novos casos de LV, sem ocorrência de óbitos (SINGH et al., 2016; SINGH; SINGH, 2019); Bangladesh atingiu metas de eliminação da doença em 90% das regiões; e, no Nepal, a eliminação foi alcançada a nível distrital (SINGH et al., 2016).

Para LC e LMC, a miltefosina demonstrou eficácia variável. Resultados obtidos na Colômbia mostrou taxas de cura maior que 80% para *L. panamensis* (SOTO et al., 2004). Mais tarde, outro estudo realizado também na Colômbia encontrou taxas de cura de 69,8% a 70% e uma maior proporção de casos infectados por *L. braziliensis* (VELEZ et al., 2010). Na Guatemala obteve-se taxas de cura de 50% para *L. braziliensis* e *L. mexicana* (SOTO et al., 2004). Na Bolívia, onde os casos de LC são predominantemente causados por *L. braziliensis*, a miltefosina mostrou 80% de taxa de cura (SOTO et al., 2008). No Brasil, a taxa de resposta para *L. braziliensis* foi de 75% (MACHADO et al., 2010) e 71,4% para *L. guyanensis* (CHRUSCIAK-TALHARI et al., 2011). Na Alemanha foi encontrado taxa de cura de 63% em 8 casos de leishmaniose cutânea importados causadas por *L. braziliensis* em viajantes provenientes da Bolívia, Costa Rica, Peru, Equador e Brasil (HARMS et al., 2011).

Sobre os efeitos adversos da miltefosina, os mais comumente relatados estão relacionados ao trato gastrointestinal como anorexia, náuseas, vômitos e diarreia (van THIEL et al., 2010). Pandey et al. (2013) relataram um caso de pancreatite aguda fatal, com aumentos nos níveis de aminotransferase e creatinina sérica, porém essa alteração é considerada ligeira, reversível e dependente da dose. Há ainda estudos relatando risco teratogênico, sendo, portanto, contra-indicado no período gestacional (SINDERMANN; ENGEL, 2006). Durante a amamentação a miltefosina também não é recomendado por não haver dados sobre o risco para a criança (SCHLOSSBERG; SAMUEL, 2011).

O mecanismo de ação da miltefosina não está totalmente esclarecido. Mas sabe-se que ao entrar na corrente sanguínea, a miltefosina fica sob a forma micelar (ALONSO et al., 2012) ou se liga à proteína albumina, disponível no plasma, que atua como um reservatório para a droga (RAKOTOMANGA et al., 2005). Ocorre então, um equilíbrio entre as moléculas da miltefosina que se ligam à albumina e àquelas que se ligam à membrana plasmática (PÉREZ-VICTORIA et al., 2006b) (Figura 5), onde serão solvatadas/particionadas, especialmente nas camadas lipídicas ricas em colesterol (MALTA de SÁ et al., 2015).

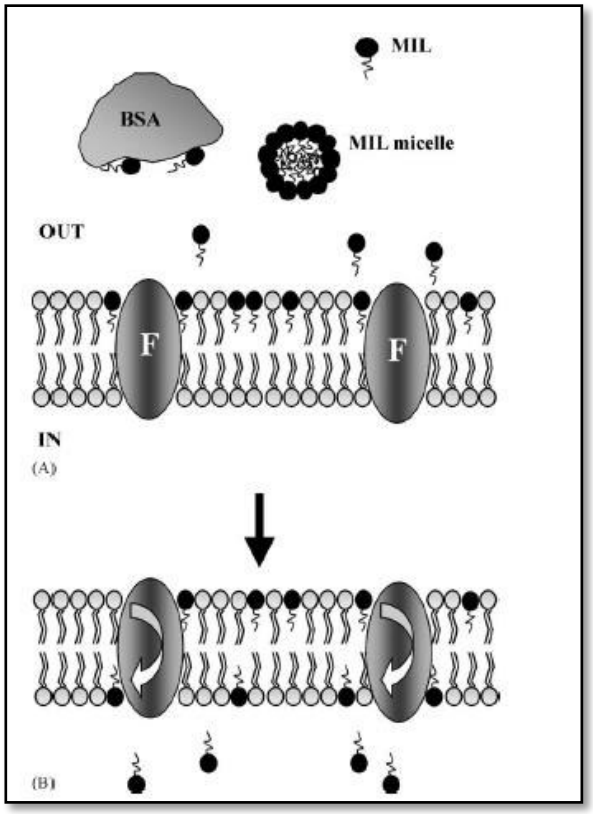


Figura 5. Ligação e captação da miltefosina.

LEGENDA: (A) Ligação do fármaco ao folheto externo da membrana de plasma. A miltefosina fora da célula fica sob a forma micelar ou é recrutado pela albumina sérica bovina, que atua como um reservatório para a droga. (B) A miltefosina ligado às membranas celulares é internalizado através da proteína flipase (F) num movimento chamado flip-flop.

FONTE: Pérez-Victoria et al. (2006b).

A droga penetra na membrana celular do macrófago por miscibilidade, através do seu componente lipídico e interage com esteróis e com cadeias laterais hidrofóbicas na interface lipídio-proteína (RAKOTOMANGA et al., 2004; MOREIRA et al., 2013). Neste momento a metabolização da miltefosina pode ser iniciada na membrana pela ação da fosfolipase D, que converte a miltefosina em colina, a qual é incorporada nos tecidos, e hexadecanol, que é oxidado a ácido palmítico (FDA, 2013). Porém, enquanto permanecer na bicamada lipídica, a miltefosina pode fazer modificações *turnover* na membrana celular (van der LUIT et al., 2007; JIMÉNEZ-LÓPEZ et al., 2010).

Nas membranas, a ação primária da miltefosina é a inibição da síntese de fosfatidilcolina por meio da inativação da CDP:colina (citidina 5'-difosfocolina) pela Via Kennedy (RAKOTOMANGA et al., 2007). A Via Kennedy é a principal via para produção de fosfatidilcolina a partir da colina, sendo predominante em eucariotos superiores (CARRASCO et al., 2008; JIMÉNEZ-LÓPEZ et al., 2010).

Além de interferir na síntese de fosfatidilcolina, a miltefosina prejudica a entrada do colesterol no retículo endoplasmático, levando a uma depleção de colesterol nessa organela e, consequentemente, a uma desregulação da biossíntese de colesterol (JIMÉNEZ-LÓPEZ et al., 2010; MALTA de SÁ et al., 2015). Tanto a inibição da síntese de fosfatidilcolina (WRIGHT; HOWE; ZAREMBERG, 2004), quanto o estresse no retículo endoplasmático (NIETO-MIGUEL et al., 2007), tem sido relacionados à indução de apoptose pela miltefosina. Outras evidências sugerem que esta classe de drogas interfere também com vias de sobrevivência bioquímica (tais como a Akt-mTOR) e em proteínas envolvidas na transdução de sinal, tais como a proteína quinase C, fosfolipase C e fosfolipase D (van BLITTERSWIJK; VERHEIJ, 2013; PACHIONI et al., 2013).

A miltefosina também se acumula na membrana dos parasitas (VINCENT et al., 2014), na qual interage através da modulação da superfície celular e ativação de fosfolipase (SINGH et al., 2014). Semelhante ao que ocorre nos macrófagos, a droga afeta a membrana celular dos

parasitas através da redução no teor de fosfatidilcolina e no aumento de fosfatidiletanolamina e lisofosfatidilcolina (RAKOTOMANGA et al., 2007).

A leishmania tem alta capacidade de internalizar a miltefosina, especialmente devido à ação de proteínas transportadoras da miltefosina (PÉREZ-VICTORIA et al., 2003), identificadas em algumas espécies do parasita, tais como em *L. donovani* (LdMT/LdRos3) (PÉREZ-VICTORIA et al., 2006b), *L. braziliensis* (LbMT/LbRos3) (SANCHEZ-CANETE et al., 2009) e *L. infantum* LiMT/LiRos3 (MONDELAERS et al., 2016). Após internalizado, a miltefosina pode induzir danos às organelas celulares (SANTA-RITA et al., 2000; CROFT et al., 2003).

Alguns estudos têm demonstrado uma correlação entre a acumulação da miltefosina dentro do parasita e sua eficácia antileishmanial (PÉREZ-VICTORIA et al., 2006a; SHAW et al., 2016). Dessa forma, os mecanismos que inibem a internalização da miltefosina ou aumentam o seu efluxo, conduziria a uma falha do tratamento (RAI et al., 2013; RIJAL et al., 2013), como demonstrado com a inativação das proteínas de membrana LdMT e LdRos3 (SEIFERT et al., 2007).

2.4 Mutagenicidade

O material genético da maioria dos organismos vivos, apesar de encontrar-se no interior das células protegido por membranas, imerso no citoplasma e com uma maquinaria de reparo, não está livre de sofrer alterações, ou seja, mutações (LIRA, 2007). Estas alterações podem ser decorrentes de processos celulares normais (mutações espontâneas) ou devidas à exposição do organismo a agentes químicos ou físicos (mutações induzidas) (GRIFFITHS, 2013).

Estamos constantemente em contato com agentes mutagênicos, entre eles a radiação solar, poluentes presentes no ar e na água ou mesmo elementos presentes em nossa dieta (GRIFFITHS, 2013). Esses agentes induzem diferentes tipos de lesões no DNA da célula, as quais podem não ser reparadas ou reparados erroneamente. Lesões reparadas erroneamente levam a efeitos biológicos tais como morte celular, aberrações cromossômicas, mutações, entre outras (NATARAJAN; PALITTI, 2008). Além disso, considerando que 80% dos agentes mutagênicos são potencialmente causadores de câncer, estudos de mutagenicidade de medicamentos em célula de mamíferos são importantes para o conhecimento de seus efeitos durante o tratamento de pacientes e para estudos de terapia combinada.

Dentre os ensaios de mutagênese, o ensaio do cometa ou Single Cell Gel Electrophoresis (SCGE), é amplamente empregado para avaliar dano ao DNA e reparação em células eucarióticas. É um método muito útil para estudar a genotoxicidade e antigenotoxicidade, *in vivo* e *in vitro*, de células expostas a uma variedade de agentes químicos e físicos, pois podem ser observadas lesões genômicas diretas (TICE et al., 2000).

Basicamente existem dois protocolos principais para a execução do teste padrão: a versão neutra, conforme o método original aprimorado por Ostling e Johanson em 1984, no qual se utiliza eletroforese em tampão de pH entre 7 e 8 para detecção de quebras duplas nas fitas de DNA e ligações cruzadas DNA-DNA, DNA-proteína, DNA-xenobiótico (OSTLING; JOHANSON, 1984); e a versão alcalina, que realiza eletroforese em pH maior que 13, desnaturando o DNA, podendo detectar quebras de fita dupla, fita simples, sítios álcali-lábeis, sítios de reparo por excisão incompletas, danos oxidativos em bases do DNA, crosslinks em DNA-DNA, DNA-proteína e DNA-xenobiótico (BOLOGNESI et al., 2004; WITTE et al., 2007).

A versão alcalina do ensaio do cometa é utilizada para estudos de toxicogenética devido as suas peculiaridades e vantagens quando comparado a outros testes para detecção de substâncias genotóxicas. O teste é utilizado para detectar lesões genômicas que, após serem processadas, podem resultar em mutações. Diferente das mutações, as lesões detectadas por este teste são passíveis de reparo. Desta maneira, o teste pode também ser utilizado para estudos de reparo do DNA, trazendo informações importantes sobre a cinética e o tipo de lesão reparada (GONTIJO; TICE, 2003).

Uma nova versão do ensaio do cometa, bastante utilizada nos últimos anos, utiliza enzimas de restrição que removem bases oxidadas do DNA. As mais usadas são a formamidopirimidina glicosilase (FPG) que removem purinas oxidadas e a endonuclease III (ENDOIII) que removem pirimidinas oxidadas. Através dessa modificação do ensaio do cometa é possível verificar se uma substância ou droga causa estresse oxidativo com oxidação de bases púricas, pirimídicas ou ambas (de JESUS et al., 2018; MOREIRA et al., 2017).

Outro teste bastante utilizado em ensaios de mutagênese é o teste do micronúcleo. Diferente do ensaio do cometa, cujo dano é considerado genotóxico, ou seja, passível de correção pelo sistema de reparo do DNA, o teste do micronúcleo é considerado um teste mutagênico, pois as mutações estão fixadas. Este teste foi criado para identificar danos no DNA a partir da análise e quantificação de estruturas citoplasmáticas, denominadas de micronúcleo, encontrados em populações celulares em divisão (SCHMID, 1975). Micronúcleos são

corpúsculos celulares que não foram incorporados no interior do núcleo durante a divisão celular, permanecendo no citoplasma (BOMBAIL et al., 2001). É semelhante em aparência ao núcleo celular e geralmente é resultado de quebras cromossômicas, gerando fragmentos sem centrômero (ALBERTINI et al., 2000).

Uma das principais vantagens do teste do micronúcleo é a sua simplicidade e facilidade de execução. Segundo Mersch et al. (1996), a observação de micronúcleos em organismos expostos a determinados compostos, pode ser uma resposta entre a atividade genotóxica e os mecanismos de defesa fisiológicos como a metabolização, eliminação de células afetadas, dentre outros. Entretanto, este teste não pode ser aplicado em populações de células que não estejam em divisão ou em que células onde os mecanismos de divisão celular não sejam conhecidos ou controlados (FENECH, 2000).

Embora a miltefosina estivesse sendo extensivamente estudado nos últimos anos, são escassos dados sobre seu efeito sobre o material genético. A única descrição de possível efeito mutagênico da droga encontra-se em um relatório, no qual foram realizados sete testes de mutagenicidade. Destes, apenas um mostrou positividade, no entanto, não é detalhada a metodologia utilizada nesses testes (FDA, 2014), de modo a não ficar claro se os testes realizados foram testes *in vitro* ou *in vivo*, em procarioto ou eucarioto, e as condições experimentais utilizadas.

2.5 Morte celular

Os tipos de morte celular são classificados de acordo com critérios morfológicos, enzimáticos (sem envolvimento de nucleases ou com o envolvimento de distintas classes de proteases, como caspases, catepsinas e transglutaminase) e aspectos funcionais (morte programada ou acidental, fisiológico ou patológico) (KROEMER et al., 2009; GALLUZZI; KROEMER, 2008). A seguir, serão explanados os tipos de morte celular por apoptose e necrose.

2.5.1 Apoptose

A molécula de DNA sofre constante agressão. Uma variedade de compostos físicos e químicos, sejam eles endógenos ou exógenos, assim como próprios erros nos processos de metabolismo de DNA, geram diariamente milhares de lesões na sua estrutura (BATISTA, 2008). Quando há um excesso de danos ao DNA, de modo que o sitema não seja capaz de corrigi-los,

é acionado um mecanismo celular de morte a fim de que mutações não sejam replicadas, denominado apoptose (GRIVICICH; REGNER; ROCHA, 2007).

De modo geral, a apoptose é um fenômeno bastante rápido e pode ser reconhecido por características morfológicas muito marcantes e coordenadas: ocorre uma retração da célula que perde aderência à matriz extracelular e células vizinhas. As organelas celulares mantêm a sua morfologia, com exceção, em alguns casos, das mitocôndrias, que podem apresentar ruptura da membrana externa. A cromatina sofre condensação e se concentra junto à membrana nuclear, que se mantém intacta. A seguir, a membrana celular forma prolongamentos (blebs) e o núcleo se desintegra em fragmentos envoltos pela membrana nuclear. Os prolongamentos da membrana celular aumentam de número e tamanho, rompem, originando estruturas contendo o conteúdo celular. Estas porções celulares envoltas pela membrana celular são denominados corpos apoptóticos, que são rapidamente fagocitados por macrófagos e removidos sem causar um processo inflamatório (OUYANG et al., 2012).

Alguns estudos demonstraram a indução de apoptose pela miltefosina em células de mamíferos (WRIGHT; HOWE; ZAREMBERG, 2004; ZUO et al., 2011) e em formas amastigotas (VERMA; DEY, 2004) e promastigotas (PARIS et al., 2004; MARINHO et al., 2011) de leishmania. Entretanto, como esta família de compostos induz apoptose, tanto em células de parasitas quanto de mamíferos, ainda não está totalmente esclarecido. A hipótese mais prevalente é a inibição da síntese de fosfatidilcolina, um elemento essencial para a síntese e a integridade das membranas celulares e uma fonte de moléculas sinalizadoras (WRIGHT; HOWE; ZAREMBERG, 2004; JIMÉNEZ-LÓPEZ et al., 2010). A diminuição da síntese de fosfatidilcolina, pode levar à diminuição da síntese de esfingomielina e, consequentemente, ao aumento dos níveis de ceramida, um conhecido indutor de apoptose (WRIGHT; HOWE; ZAREMBERG, 2004).

A miltefosina também induz apoptose por disfunção mitocondrial relacionado à inibição do citocromo c oxidase, que é liberado no citoplasma após a ruptura da membrana mitocondrial externa por estímulos apoptóticos e é uma característica importante da apoptose metazoária (VERMA et al., 2007; LUQUE-ORTEGA; RIVAS, 2007; ZUO et al., 2011); e pela via PI3K/AKT através da dimunuição da proteína AKT, que é importante para a via de sinalização intracelular da fosfatidilinositol-3-quinase/AKT, caminho essencial para a sobrevivência celular (CHAKRABANDHU et al., 2008; VINCENT et al., 2014).

2.5.2 Necrose

A necrose é um processo que envolve a perda da integridade da membrana celular, com inchaço citoplasmático, formação de vacúolos, retículo endoplasmático inchado, distenção ou rompimento mitocôndrial, lise dos lisossomos e liberação do conteúdo citoplasmatico para o tecido circundante levando a inflamação (KUROSAKA et al., 2003; GOLSTEIN; KROEMER, 2007; OUYANG et al., 2012).

Durante muito tempo a necrose foi considerada como um mecanismo de morte celular acidental. Atualmente está claro que ela pode ocorrer de forma regulada, a partir de um mecanismo chamado necroptose, cujo papel é importante em processos fisiológicos e patológicos (FULDA, 2013). O termo necroptose tem sido utilizado como um sinônimo de necrose programada que depende da atividade da Proteína Quinase e da Interação com Receptor-1 (RIPK1) (KROEMER et al., 2009).

Marcadores clássicos de apoptose, como condensação de cromatina e fragmentação nucleossomal de DNA não são vistos em morte via necrose (BROWN; ATTARDI, 2005). Entretanto, semelhantes à apotose, células necróticas externalizam a fosfatidilserina antes da permeabilização da membrana plasmática, promovendo, assim, o seu reconhecimento pelos fagócitos (GALLUZZI; KROEMER, 2008).

2.6 Estresse oxidativo e defesa antioxidante

O estresse oxidativo resulta do desequilíbrio entre espécies reativas de oxigênio (ROS), de nitrogênio (RNS) e de outras espécies reativas com o sistema de defesa antioxidante, seja por geração excessiva de radicais livres ou pelo detrimento da velocidade de remoção desses, induzindo danos às biomoléculas (BARBOSA et al., 2010; D'AMICO et al., 2013).

A miltefosina induz a produção de ROS (VINCENT et al., 2014; CARNIELLI et al., 2014), que está relacionado à atividade antiliehsmanial da droga (HORTA et al., 2012), mas também à capacidade de causar danos ao DNA (MOREIRA et al., 2011). O dano mais importante causado por ROS no DNA é a oxidação das bases guanina, adenina e timina (GARRE et al., 2011). A oxidação da guanina é o dano melhor caracterizado produzindo 8-oxoguanina (8-oxoG) no DNA e 8-hidroxi-guanina (8-OH-dGTP) em nucleotídeos livres. A 8-oxoG ocasiona transversões do tipo G:C para T:A nas fitas do DNA, que ocorrendo em oncogenes ou genes supressores de tumor pode levar à carcinogênese (GARRE et al., 2011; NASCIMENTO et al., 2017)

O sistema de excisão de bases (BER) é a via primária de reparo do DNA conhecido por atuar principalmente em modificaçõs causadas por agentes endógenos (BERRA; MENCK; Di MASCIO, 2006). Ele tem papel fundamental na preservação da integridade do DNA, corrigindo bases oxidadas submetidas à ação deletéria de ROS. Os principais genes do sistema BER são *OGG1*, *MUTHY* e *NUDT1*. Os genes *MUTHY* e *OGG1* codificam DNA-glicosilases que têm como função remover as bases oxidadas erroneamente pareadas, assegurando a manutenção da integridade das fitas do DNA (NASCIMENTO et al., 2017). O gene *NUDT1* age indiretamente no sistema BER e codifica uma enzima que hidrolisa 8-oxo-dGTP para 8-oxo-dGMP, previnindo, assim, a incorporação de 8-oxoG no DNA durante a replicação (BURTON; RAI, 2015).

O gene *hOGG1* (8-oxoguanina DNA glicosilase 1) localiza-se no braço curto do cromossomo 3 (3p26.2). É formado por 12 éxons e codifica a enzima 8-oxoguanina glicosilase. Estima-se que essa enzima contenha 424 aminoácidos em sua molécula e peso molecular de 47kD (NASCIMENTO et al., 2017). A enzima transcrita promove a hidroxilação das ligações glicofílicas formadas entre a 8-oxoG e a fração de açúcar da base nitrogenada, removendo a molécula da 8-oxoG e formando um sítio apurínico na fita do DNA que, após a excisão, é preenchido com a base nitrogenada correta. Assim, o gene *OGG1* evita transversões do tipo G:C para T:A. Por seu papel fundamental no sistema BER, a ocorrência de mutações e polimorfismos em *OGG1* são considerados eventos que aumentam a susceptibilidade a várias formas de câncer (ZOU et al., 2016). Os polimorfismos mais comuns (S326C e R46Q) diminuem ligeiramente a capacidade da enzima em suprimir mutações (GARRE et al., 2011; KABZINSKI et al., 2014).

O gene *hMUTYH* (muty DNA glicosilase) localiza-se no braço curto do cromossomo 1 (1p 32-34) e possui 16 éxons. Ele codifica uma DNA-glicosilase formada por 535 aminoácidos, com peso molecular de 39 kD, denominada MUTHY-glicosilase. Essa enzima age removendo as bases de adenina mal pareadas com a 8-oxoG. A enzima corrige este erro para que não sejam fixadas mutações por transversões do tipo G:C para T:A (NASCIMENTO et al., 2017). O polimorfismo comum do gene *MUTYH*, Gly396Asp, também está associado com risco de câncer (GARRE et al., 2011).

O gene *NUDT1* (nudix hidrolase 1), também conhecido como *MTH1* (human MutT Homolog 1) localiza-se no cromossomo 7 (7p22.3) e possui seis éxons. Em mamíferos, a enzima é um importante desintoxicante dos precursores da oxidação do DNA porque hidrolisa os nucleotídeos livres 8-oxo-dGTP, 8-oxo-dATP e 2-OH-dATP, formando monofosfatos,

evitando, assim, a incorporação incorreta desses trifosfatos de nucleosídeos de purina no DNA e a subsequente ocorrência de transversões A:T para C:G ou G:C para T:A (BURTON; RAI, 2015). O gene *NUDT1* é antimutagênico e suprime a disfunção celular e/ou a morte induzida pelo estresse oxidativo. Consequentemente, sua deficiência aumenta a suscetibilidade das células à disfunção mediada pelo dano oxidativo. A enzima NUDT1 é encontrada principalmente no citoplasma, mas também no núcleo e nas mitocôndrias, com níveis mais altos de expressão em tecidos como o timo, testículo e embrião. O polimorfismo mais comum desse gene (Asp142Asp) resulta na produção de uma isoforma adicional e mais longa (p26) e está relacionado ao risco de desenvolvimento de câncer (GARRE et al., 2011).

Para controlar a formação de espécies reativas, as células possuem dois sistemas de defesa antioxidante: enzimático e não enzimático. O primeiro é representado, principalmente, pelas enzimas antioxidantes: superóxido dismutase (SOD), que são metaloproteínas que catalisam a dismutação do superóxido (O^{2-}) em oxigênio (O_2) e peróxido de hidrogênio (H_2O_2); catalase (CAT) que atua na decomposição de H_2O_2 em O_2 e H_2O; e, glutationa peroxidase (GPx), que atua sobre peróxidos em geral (MARÍN et al., 2006; VASCONCELOS et al., 2007; CAYMAN CHEMICAL, 2018). O sistema antioxidante não enzimático é formado por diversas substâncias, como a glutationa (GSH), ácido úrico, β-caroteno tocoferóis (vitamina E), ácido ascórbico (vitamina C) e outros (VASCONCELOS et al., 2007; NIMSE; PAL, 2015).

O ácido ascórbico é um importante antioxidante, cuja atividade tem sido associada ao combate a diversos tipos de câncer, doenças cardiovasculares e outras doenças associadas ao estresse oxidativo e, embora os seres humanos sejam incapazes de sintetizar esse antioxidante, ele participa de diversas atividades fisiológicas, como: síntese do colágeno; reciclagem da vitamina E; diminuição do colesterol e resistência a infecções, modulando a atividade imunológica (CERQUEIRA et al., 2007; GRANGEIRO et al., 2013). Por outro lado, é reconhecido que o ácido ascórbico pode apresentar efeito tóxico quando em excesso, passando a ter ação pró-oxidante (HALLIWELL, 2001; KATO et al., 2014).

A propriedade antioxidante do ácido ascórbico em eliminar radicais livres sugere que essa molécula pode modular danos oxidativos ao DNA em células de mamíferos. Sabendo que a miltefosina produz ROS (VINCENT et al., 2014; CARNIELLI et al., 2014) e que esse radicais estão associados à atividade antileishmanial da droga (HORTA et al., 2012), é interessante investigar se esse antioxidante tem capacidade de diminuir os danos causados pela miltefosina, sem interferir na eficácia da droga como antileishmanial.

3 OBJETIVOS

3.1 Objetivo Geral

Investigar se o antileishmanial miltefosina induz danos genéticos em células de mamíferos *in vitro* e *in vivo*, bem como avaliar a capacidade do antioxidante ácido ascórbico em modular esses danos.

3.2 Objetivos específicos

- Avaliar o efeito genotóxico e mutagênico da miltefosina em leucócitos humanos *in vitro*;
- Verificar se a miltefosina induz morte celular (apoptose e necrose) em leucócitos humanos *in vitro;*
- Avaliar o potencial genotóxico e mutagênico da miltefosina em camundongos Swiss (tratamento de dose única) *in vivo*;
- Averiguar se a miltefosina induz danos no DNA por oxidação de suas bases nitrogenadas;
- Avaliar o potencial genotóxico e mutagênico da miltefosina em camundongos Balb/c (tratamento de doses repetidas) infectados com *L. infantum*;
- Verificar o efeito do antioxidante ácido ascórbico nos animais infectados com *L. infantum* e tratados com miltefosina;
- Verificar a carga parasitária nos animais infectados com *L. infantum* e tratados com miltefosina e ácido ascórbico;
- Verificar a atividade das enzimas antioxidantes SOD, CAT e GPx nos animais infectados com *L. infantum* e tratados com miltefosina e ácido ascórbico;
- Verificar a expressão diferencial dos genes de reparo *NUDT1*, *OGG1* e *MUTHY* em animais infectados com *L. infantum* e tratados com miltefosina e ácido ascórbico

4 MATERIAL E MÉTODOS

Esta tese foi desenvolvida com base no seguinte desenho experimental: inicialmente foram realizados testes de viabilidade celular em leucócitos humanos para determinação das concentrações da miltefosina a serem utilizadas nos ensaios *in vitro*. Uma vez determinadas as concentrações, foram realizados os ensaios de genotoxicidade e de morte celular. Nesses ensaios, a miltefosina revelou seu potencial genotóxico e mutagênico por oxidação de bases do DNA, bem como sua capacidade de induzir apoptose e necrose (resutados descritos no capítulo 1).

A partir desses reultados, investigou-se a capacidade do antioxidante ácido ascórbico em proteger o DNA do estresse causado pela miltefosina. Para isso, utilizou-se duas linhagens de camundongos com diferentes backgrounds, Swiss e Balb/c (não susceptível e susceptível à infecção por leishmania, respectivamente), sob tratamento agudo (simultâneo, pré e pós tratamento com ácido ascórbico) e crônico (tratamento simultâneo com miltefosina e ácido ascórbico), e diferentes vias de administração (oral e intraperitoneal). Assim, foram realizados ensaios de antigenotoxicidade para verificar se o ácido ascórbico era capaz de reverter os danos genômicos induzidos pelo antileishmanial. Além disso, foi avaliada a resposta celular às agressões do antileishmanial em animais tratados. Para tanto, foi medida a atividade de enzimas antioxidantes no soro, bem como foi determinada a carga parasitária por meio do ensaio de diluição limitante (resutados descritos no capítulo 2).

Finalmente, foram realizados ensaios de expressão diferencial dos principais genes relacionados ao sistema de reparo do DNA para avaliar possíveis mecanismos moleculares envolvidos na resposta celular ao estresse oxidativo e lesões genômicas induzidas pelo antileishmanial mltefosine (resutados descritos no capítulo 3).

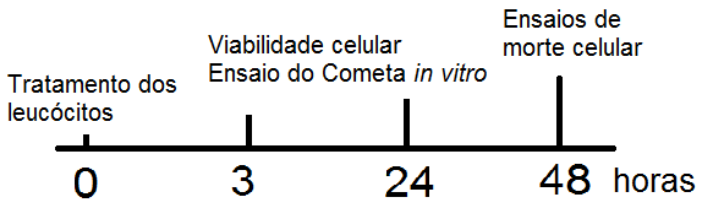
Abaixo são descritos os métodos utilizados em cada ensaio experimental.

4.1 Ensaios *in vitro*

Para obtenção dos leucócitos foram coletados 20ml de sangue periférico em tubos heparinizados, de seis doadores voluntários, três de cada sexo, entre 19 e 30 anos, saudáveis, não fumantes, sem uso de medicamentos e não expostos a tratamento quimioterápico ou por radiação nos últimos seis meses (Parecer Consubstanciado do Comitê de Ética nº 1.284.493-UFMA, ANEXO A). As células foram separadas com Ficol Paque™ Plus, posteriormente diluídas uniformemente em 5 ml de meio de cultura RPMI e imediatamente tratadas.

O tratamento com miltefosina aconteceu no tempo 0 (início da cultura das células) e as células ficaram expostas ao medicamento por 3 e 24 horas para os ensaios do cometa e viabilidade celular e 48 horas para os ensaios de morte celular (Figura 6). O controle negativo consistiu de meio RPMI 1640 e o controle positivo de 10 µL de peróxido de hidrogênio (H_2O_2) 10V (30 mg/mL), que agiu por 10 minutos.

Figura 6. Esquema representando os tempos e ensaios realizados.



4.1.1 Viabilidade celular (ELIA et al., 1993)

A viabilidade celular foi determinada adicionando 10 µl de azul de trypan (0,4%) em 10 µl da suspensão celular. Essa mistura foi gotejada em uma lâmina de microscopia e as células viáveis e as não viáveis foram imediatamente diferenciadas e contadas em microscópio de fluorescência (BX51/BX52-Olympus; filtro 516-560nm; barreira de filtro de 590nm, objetiva de 40x). Foram analisadas 100 células de cada grupo de tratamento e considerou-se o índice de viabilidade mínimo de 70% de células viáveis.

As concentrações da miltefosina (Impavido®) foram definidas através de testes piloto. Inicialmente foram utilizadas 5 concentrações (100 µg/ml, 50 µg/ml, 25 µg/ml, 10 µg/ml e 1,0 µg/ml) para avaliação da viabilidade dos leucócitos acima de 70%. Somente a concentração 1,0 µg/ml manteve a viabilidade celular desejada, a partir da qual foram definidas as concentrações para este estudo: C1 (0,25 µg/ml), C2 (0,5 µg/ml) e C3 (1,0 µg/ml).

4.1.2 Ensaio do cometa (SINGH et al., 1988; TICE et al., 2000)

Após 3 e 24 h de cultura, 100 µl da suspensão celular foi adicionada a 200 µl de agarose baixo ponto de fusão (0,5%). Esta mistura foi aplicada a duas lâminas pré-tratadas com agarose ponto de fusão normal (1,5%). Em seguida, lamínulas (24 x 60 mm) foram sobrepostas nessas lâminas e refrigeradas por 5 min. Após a solidificação, as lâminas foram imersas em solução de lise (2,5 M de NaCl, 100 mM de EDTA, 10 mM de Tris, 10% de dimetilsulfóxido, 1% de

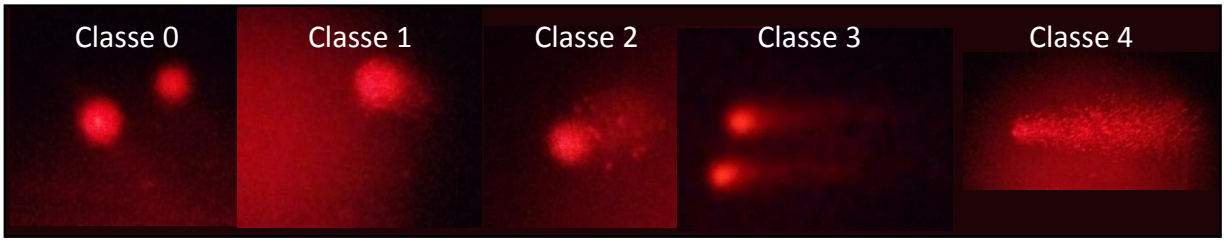
Triton X-100, pH 10) e refrigeradas overnight a 4ºC. Posteriormente, as lâminas foram incubadas em solução alcalina (NaOH 10 M, 0,2 M EDTA e água destilada, pH 13) durante 20 minutos a 4ºC, seguida por eletroforese a 25 V e 300 mA durante 30 min. As lâminas foram neutralizadas (0,4 M de Tris/HCl, pH 7,5), secadas à temperatura ambiente e fixados em etanol 100% por 4 minutos. Em seguida, as lâminas foram coradas com 30 µL de brometo de etídio (20 µg/mL), cobertas com lamínula (24 x 60 mm) e analisadas sob microscópio de fluorescência (Olympus BX61). Todos os procedimentos foram executados sem a incidência direta de luz sobre as preparações.

Cem nucleoides foram analisados por animal considerando o tamanho e a quantidade de DNA presente na cauda do cometa. Os danos foram classificados em 5 níveis: Classe 0 - nenhum dano (<5%); Classe 1 - baixo nível de danos (5-20%); Classe 2 - nível moderado de danos (20-40%); Classe 3 - alto nível de danos (40-94%); Classe 4 - dano total (> 95%) (Figura 7) (SPEIT; HARTMANN, 1995).

O escore de danos foi obtida através da multiplicação do número de nucleoides em cada classe pelo respectivo valor da classe, usando a equação:

Escore = [(0 x n0) + (1 x n1) + (2 x n2) + (3 x n3) + (4 x n4)] / total do número de células

Figura 7. Diferentes classes de danos no DNA de animais tratados com miltefosina.



FONTE: O autor.

4.1.3 Ensaio de morte celular (DIVE et al., 1992, com modificações)

Após 48 horas de cultura, 50 µL da suspensão celular foi corada com 2,5 mL de uma mistura de corantes fluorescentes (25 µL de iodeto de propídio 5 µL/mL, 50 µL de diacetato de fluoresceína 15 µg/ml, 10 µL de Hoechst 3334 2µg/ml e 15 µl de PBS, pH 8) incubou-se a 37 °C durante 5 min em banho-maria. Dessa suspensão, 15µL foram colocados em uma lâmina, que foi posteriormente coberta por uma lamínula (24 x 60 mm). A contagem das células foi

feita em microscópio de fluorescência (Olympus BX61) utilizando filtro triplo (DAPI, Iodeto de propídio e FITC- Isotiocianato de Fluorceína).

Para cada grupo de tratamento foram realizadas seis repetições, sendo analisados 500 células por animal, totalizando 3000 células analisadas por grupo, conforme fórmula abaixo. As células foram classificadas como normais (núcleos esféricos corados em azul e, citoplasma em verde), necróticas (núcleo esférico corado em vermelho) e apoptóticas (núcleo corado em azul com corpos apoptóticos e, citoplasma verde) utilizando microscópio de fluorescência (filtro triplo) (HASHIMOTO et al., 2003).

$$\text{Células apoptóticas} = \frac{\text{nº total de células apoptóticas}}{\text{nº total de células contadas}} \times 100$$

4.2 Ensaios *in vivo*

4.2.1 Animais

Foram utilizados camundongos machos (n = 5 por grupo) Swiss *Mus musculus*, provenientes do Biotério Central da Universidade Federal do Maranhão (Maranhão, Brasil) e camundongos Balb/c *Mus musculus* provenientes do Biotério da Universidade de Campinas (São Paulo, Brasil) com peso médio corporal de 30g e cerca de 60 dias de vida. Os animais foram mantidos em caixas de policarbonato em condições controladas: temperatura em torno de 21 ± 2ºC e ciclo alternado de claro/escuro de 12 horas. Este projeto foi aprovado pelo Comitê de Ética em Experimentação Animal (CEUA) da UFMA (Protocolos nº. 23115-009229/2014-21 - ANEXO B e nº 23115.029807/2018-70 - ANEXO C).

4.2.2 Parasitas

Formas promastigotas de *L. infantum* (MHOM/BR/1970/BH46) foram cultivadas em meio Schneider (Sigma, St Louis, MO, USA), suplementado com 10% de soro fetal bovino inativado (GIBCO) e acondicionadas em estufa a 27°C. Para os ensaios, os camundongos Balb/c foram infectados na pata traseira direita com 10^7 promastigotas em fase estacionária de *L. infantum*. A infecção foi mantida por 28 dias e no 29° dia foram realizados os tratamentos com o antileishmanial.

4.2.3 Doses e tratamento

Inicialmente foram realizados ensaios de dose única, nos quais as doses da miltefosina (Impavido®) foram determinadas com base na dose recomendada para tratamento humano de leishmaniose, segundo a OMS, ou seja, 2,5 mg/kg durante 28 dias, totalizando 70mg/kg. Assim, foram administradas três doses por gavagem, a saber: D1 (35 mg/kg); D2 (70 mg/kg); D3 (140 mg/kg). O grupo controle negativo recebeu água destilada por via oral e, o grupo controle positivo foi tratado com ciclofosfamida (50 mg/kg), por via intraperitoneal (Capítulo 1).

Em outro ensaio, os animais foram divididos em dois grupos. 1) camundongos Swiss sem infecção que receberam dose única (70 mg/kg) da miltefosina (Chemical Cayman Company, MI, USA, lote 0463792-1) conforme descrito no parágrafo anterior (CASTELO BRANCO et al., 2016); 2) camundongos Balb/c infectados que receberam miltefosina (Impavido®) na dose de 2,5 mg/kg durante 28 dias; e, outro grupo, recebeu 5,0 mg/kg durante 14 dias, todos por gavagem. Para avaliar o efeito protetor do ácido ascórbico (Sigma-Aldrich, St. Louis, MO, Lote 69085) sobre os danos causados pela miltefosina, os animais em tratamento agudo receberam o antioxidante (30, 60 e 120mg/kg, via oral e intraperitoneal) simultaneamente com o antileishmanial. Além disso, a dose intermediária do ácido ascórbico foi administrada 24 horas antes da miltefosina (grupo pré-tratamento), bem como 24 horas após (grupo pós-tratamento). Em relação ao efeito do ácido ascórbico no tratamento crônico, os camundongos infectados receberam, simultaneamente, 2,5 mg/kg da miltefosina e 15 mg/kg de ácido ascórbico por gavagem, diariamente, por 28 dias. Foram utilizados também os grupos controle negativo (água destilada), controle infectado (animais apenas com a infecção por *L. infantum*) e controle positivo (miltefosina) (Capítulo 2).

Por fim, no terceiro ensaio, os animais (*Mus musculus* Balb/c) foram divididos em cinco grupos de tratamento: Grupo NC – controle negativo (animais não infectados que receberam água destilada); Grupo IC – controle infectado (animais infectados que receberam água destilada); Grupo MT – animais infectados com *L. infantum* tratados com miltefosina (30 mg/Kg/dia durante 28 dias, gavagem); Grupo MT + AA – animais infectados com *L. infantum* tratados com miltefosina (30 mg/Kg/dia durante 28 dias, via oral) mais ácido ascórbico (30 mg/Kg/dia durante 28 dias, via i.p.); Grupo AA – animais infectados tratados com ácido ascórbico (30 mg/Kg/dias durante 28 dias, via i.p.). Para a quantificação da carga parasitária foi incluído um grupo de animais (Grupo IC-i), sacrificados 28 dias após a infecção. A dose utilizada da miltefosina foi baseada na conversão da dose humana, recomendada pela OMS, para a dose animal (Nair e Jacobe, 2016). Para isso, multiplicou-se a dose da miltefosina (2,5

mg/Kg/dia) pela constante da dose equivalente para camundongo (12,3), obtendo-se 30 mg/Kg/dia. A dose de 30 mg/Kg via i.p. do ácido ascórbico foi baseada em um estudo anterior, onde detectou-se seu feito antioxidante (CASTELO BRANCO et al., 2019). Ambas as drogas foram dissolvidas em água destilada (Capítulo 3) (Tabela 1).

4.2.4 Ensaio do cometa (SINGH et al., 1988; TICE et al., 2000)

O ensaio do cometa alcalino convencional foi realizado em células do sangue periférico dos camundongos. Após 24 horas do tratamento foi feita uma pequena excisão na extremidade da cauda dos animais para coletar 5µL de sangue periférico, os quais foram misturados com 100µL de agarose de baixo ponto de fusão (0,5%). Em seguida, essa mistura foi transferida para uma lâmina pré-revestida com agarose de ponto de fusão normal (1,5%). Os passos subsequentes foram similares ao descrito anteriormente para Ensaio do Cometa *in vitro*.

Para o ensaio do cometa modificado utilizou-se as enzimas formamidopirimidina glicosilase (FPG) e endonuclease III (ENDOIII). Após o período na solução de lise, as lâminas foram lavadas, três vezes de 5 minutos cada, com tampão (40 mM HEPES, 0,1M KCl, 0,5mM EDTA, 0,2 mg/mL BSA, pH 8 com KOH). As enzimas foram diluídas conforme recomendação do fabricante e acrescentadas nas lâminas (50 µl de ENDO III e 75 µl de FPG). Posteriormente, foram incubadas por 30 minutos (FPG) e 45 minutos (ENDO III) a 37ºC (New England Biolabs®). As lâminas foram lavadas uma vez com tampão PBS e colocadas em cuba de eletroforese conforme as demais etapas do ensaio do cometa descritas anteriormente.

Tabela 1. Grupos experimentais utilizados para avaliação do potencial toxicogenético da miltefosina e efeito protetor do ácido ascórbico.

Grupo	Agente Químico	Doses (respectivamente)	Via de Administração
Capítulo 1			
Ensaios *in vitro*			
CN	água destilada	----	----
CP	H_2O_2 (10 vol)	30 mg/mL	----
C1	miltefosina (Impavido®)	0,25 µg/mL	----
C2	miltefosina (Impavido®)	0,5 µg/mL	----
C3	miltefosina (Impavido®)	1,0 µg/mL	----
Ensaios in vivo			
CN	água destilada		gavagem
CP	ciclofosfamida	50 mg/kg	ip
D1	miltefosina (Impavido®)	35 mg/kg	gavagem
D2	miltefosina (Impavido®)	70 mg/kg	gavagem
D3	miltefosina (Impavido®)	140 mg/kg	gavagem
Capítulo 2			
Swiss (tratamento de dose única)			
NC	água destilada	----	gavagem
AA	ácido ascórbico	60 mg/kg	gavagem
MT	miltefosine	70 mg/kg	gavagem
So30	ácido ascórbico + miltefosine	30 mg/kg + 70 mg/kg	gavagem
So60	ácido ascórbico + miltefosine	60 mg/kg + 70 mg/kg	gavagem
So120	ácido ascórbico + miltefosine	120 mg/kg + 70 mg/kg	gavagem
PRE	ácido ascórbico (24h antes) + miltefosine	60 mg/kg + 70 mg/kg	gavagem
POS	miltefosina (24h antes) + ácido ascórbico	70 mg/kg + 60 mg/kg	gavagem
Si30	ácido ascórbico + miltefosine	30 mg/kg + 70 mg/kg	ip + gavagem
Si60	ácido ascórbico + miltefosine	60 mg/kg + 70 mg/kg	ip + gavagem
Si120	ácido ascórbico + miltefosine	120 mg/kg + 70 mg/kg	ip + gavagem
Balb/c (tratamento de doses repetidas)			
NC	água destilada	--- (28 dias)	gavagem
IC	água destilada	--- (28 dias)	gavagem
MT5.0	miltefosina (Impavido®)	5,0 mg/kg (14 dias)	gavagem
MT2.5	miltefosina (Impavido®)	2,5mg/kg (28 dias)	gavagem
AA	ácido ascórbico	15 mg/kg (28 dias)	gavagem
MT+AA	ácido ascórbico + miltefoine (Impavido®)	15 mg/kg + 2,5mg/kg (28 dias)	gavagem
Capítulo 3			
NC	água destilada	--- (28 dias)	gavagem
IC	água destilada	--- (28 dias)	gavagem
IC-i	água destilada	--- (28 dias)	gavagem
MT	miltefoine (Impavido®)	30 mg/kg (28 dias)	gavagem
MT+AA	ácido ascórbico + miltefoine (Impavido®)	30 mg/kg cada (28 dias)	gavagem + ip
AA	ácido ascórbico	30 mg/kg (28 dias)	ip

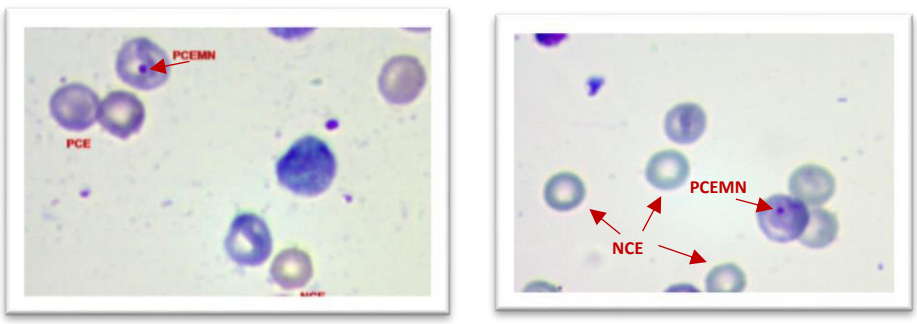
4.2.5 Ensaio do micronúcleo (SCHMID, 1975)

O ensaio do micronúcleo foi realizado em células da medula óssea de camundongos. Foram utilizados os mesmos animais e tratamentos para o ensaio do cometa. Resumidamente, após a coleta de sangue para a realização do teste do cometa, os animais foram eutanasiados por overdose anestésica (xilazina - 60 mg/Kg e ketamina 300 mg/Kg). Em seguida, os fêmures foram removidos, retirada toda a musculatura e cortadas as epífises. A medula óssea foi removida por aspiração com soro bovino fetal. A suspensão celular foi centrifugada por 5 min a 1000 rpm. O sobrenadante foi descartado e com o restante do material, confeccionou-se o esfregaço em lâmina. O material foi fixado em metanol absoluto, por 10 minutos, e em seguida as lâminas foram coradas com o kit hematológico Panótico (Laborclin), de forma a diferenciar eritrócitos policromáticos (PCE) dos eritrócitos normocromáticos (NCE).

Para investigar os micronúcleos, 2000 eritrócitos policromáticos por animal foram analisados em aumento de 1000x em microscópio óptico (Carl Zeiss Primo Star) (Figura 8). A razão PCE/NCE (eritrócitos policromáticos e eritrócitos normocromáticos) foi calculada utilizando 1000 eritrócitos por animal para estimar a toxicidade da miltefosina e do ácido ascórbico.

A porcentagem de redução de danos causados pela miltefosina modulada pelo ácido ascórbico foi calculada de acordo com Waters et al. (1990) usando a seguinte fórmula: % Redução = (CP-CT/CP-CN) x 100, onde CP corresponde à média de danos do grupo tratado apenas com miltefosina, CT é a média de danos observada nos grupos tratados com ácido ascórbico mais miltefosina e, CN corresponde à média de danos observada no controle negativo (água destilada).

Figura 8. Formação de micronúcleo pelo antileishmanial miltefosina.



Fonte: O autor.

4.2.6 Dosagem de enzimas antioxidantes

A dosagem das enzimas antioxidantes SOD, GPx e CAT foi realizada a partir do plasma dos animais, seguindo-se as recomendações do fabricante (Cayman Chemical, Ann Arbor, MI, USA).

4.2.7 Ensaio de diluição limitante (RODRIGUES et al., 2009)

Resumidamente, o baço e o linfonodo foram colhidos assepticamente e homogeneizados individualmente em 1,0 ml de meio Schneider's Drosophila (Sigma-Aldrich, EUA) suplementado com 10% de soro fetal bovino inativado (GIBCO). Este ensaio foi realizado em duplicata. A suspensão com o órgão homogeneizado foi diluída em 1:2 no primeiro poço, contendo 100 µl do meio, totalizando um volume de 200 µl de suspensão. Do primeiro poço retirou-se 100 µl da suspensão e continuou-se a diluição de 1:2 até o 12º poço da placa de 96 poços. Após 14 dias, analisou-se a amostra de cada poço e definiu-se como *positivo* ou *negativo* dependendo da presença ou ausência de promastigotas no poço. O título final foi definido como a diluição mais elevada para a qual o poço continha pelo menos um parasita ativo. O número de parasitas por grama do órgão homogeneizado foi calculado como se segue: a recíproca do último título positivo pelo total do volume homogeneizado do órgão x fator de diluição dividido pelo peso em gramas do órgão homogeneizado. A carga parasitária viável foi expressa como o número de leishmania por grama de órgão homogeneizado.

4.2.8 Expressão gênica por PCR em tempo real (LIVAK; SCHMITTGEN, 2001).

O RNA foi extraído do fígado dos animais (100 mg) usando Trizol® (Invitrogen) de acordo com as recomendações do fabricante. A transcrição reversa foi realizada utilizando-se o kit High Capacity cDNA Reverse Transcription Kit™ (ThermoFisher™). A reação foi feita de acordo com a especificação do fabricante. O cDNA obtido foi diluído em água destilada estéril na razão 1:5.

As reações de PCR quantitativa em tempo real foram realizadas em sistema TaqMan Universal PCR Master Mix (Applied Biosystems, EUA). Utilizou-se 2 µL de cDNA de cada amostra mais 8 µL do mix (2,5 µL de nuclease free water, 5,0 µL de Taqman Universal PCR Master Mix e 0,5 µL de cada primer), com volume total de 10 µL por reação. Todas as reações foram feitas em triplicata. As condições de amplicação seguiram as recomendações do fabricante.

Em cada ensaio, utilizou-se como amostra de referência o *pool* de DNA dos animais do grupo CN (grupo não infectado e não tratado). Os dados foram normalizados com o gene gliceraldeído-3-fosfato-desidrogenase (*GAPDH*) amplificado em cada ensaio para o cálculo da expressão relativa dos genes de reparo *OGG1, MUTYH, NUDT1* (ThermoFisher™).

4.3 Análise Estatística

A normalidade dos dados foi analisada pelo teste de Kolmogorov-Smirnov. Quando paramétricos, os ensaios com dados numéricos (ensaio de viabilidade celular, ensaio do cometa, dosagem de enzimas antioxidantes, ensaio de diluição limitante e expressão gênica) foram submetidos à análise de variância 1 critério (ANOVA), seguido do pós-teste de Tukey; quando não paramétricos, os ensaios com dados numéricos foram submetidos ao teste de Kruskal-Wallis, seguido pelo pós-teste de Dunn. A comparação entre o escore de danos nos testes do cometa alcalino convencional e modificado pelas enzimas FPG e ENDO III foi realizada pelo teste T de Student pareado. Os ensaios do micronúcleo e morte celular foram analisados pelo teste do qui-quadrado. Todos os dados foram analisados utilizando o software GraphPad Prism 6.01, com valores considerados significativos quando $p < 0,05$.

5 RESULTADOS

CAPÍTULO 1

The antileishmanial drug miltefosina (Impavido®) causes oxidation of DNA bases, apoptosis, and necrosis in mammalian cells. **Mutation Research - Genetic Toxicology and Environmental Mutagenesis, 806:34-9, 2016.**

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The antileishmanial drug miltefosine (Impavido®) causes oxidation of DNA bases, apoptosis, and necrosis in mammalian cells

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ABSTRACT

Miltefosine was developed to treat skin cancer; further studies showed that the drug also has activity against *Leishmania*. Miltefosine is the first oral agent for treating leishmaniasis. However, its mechanism of action is not completely understood. We have evaluated the induction of DNA damage by miltefosine. Cytotoxicity and genotoxicity (comet assay) tests were performed on human leukocytes exposed to the drug *in vitro*. Apoptosis and necrosis were also evaluated. *In vivo* tests were conducted in Swiss male mice (*Mus musculus*) treated orally with miltefosine. Oxidation of DNA bases in peripheral blood cells was measured using the comet assay followed by digestion with formamidopyrimidine glycosylase (FPG), which removes oxidized guanine bases. The micronucleus test was performed on bone marrow erythrocytes. Miltefosine caused DNA damage, apoptosis, and necrosis *in vitro*. Mice treated with miltefosine showed an increase in the DNA damage score, which was further increased following FPG digestion. The micronucleus test was also positive.

1. Introduction

Miltefosine (hexadecylphosphocholine) was developed as an anticancer drug. The antileishmanial activity of this agent was discovered through *in vitro* and *in vivo* tests [1]. Miltefosine has been in use since 2002 as the first and only orally administered drug to treat leishmaniasis, in India, South America, and Germany [2]. In 2011, miltefosine was included as an antileishmanial drug in the World Health Organization list of essential medicines [3].

Miltefosine is a simple, very stable, relatively safe, and highly effective drug. Cure rates in India are 97% for *Leishmania donovani*, 91% for *L. panamensis* in Colombia, and 53% for *L. braziliensis* and *L. mexicana* in Guatemala [4]. In Brazil, the response rate for *L. braziliensis* is 75% [5] and 71.4% for *L. guyanensis* [6].

Miltefosine belongs to the class of zwitterionic surfactants; it is structurally similar to biological membrane phospholipids [7]. It interacts with protozoan cell membranes, then quickly reaches subcellular membranes, where it can affect metabolism [8,9]. Hence, the cell membrane is considered the main site of action; mitelfosine is inserted in the phospholipid membrane by miscibility and through interaction with membrane sterols [10]. The drug is transported into the cell through a protein complex in the parasite's plasma membrane [3].

The use of miltefosine for the treatment of leishmaniasis is considered very beneficial due to its lower toxicity and side effects compared to those of pentavalent antimonials and also due to its easier administration [11]. Effective oral medication against leishmaniasis is critical because this disease is prevalent in poor regions, such as India, Bangladesh, Nepal, and Brazil; oral administration would increase adherence to the treatment [12].

Miltefosine is well absorbed orally and is rapidly distributed throughout the body. It accumulates in the liver, spleen, kidneys, lungs, and adrenal glands; it is slowly metabolized by liver phospholipases, and urinary excretion is minimal [13,14]. Prolonged treatment (28 d) is required and the drug has a long half-life (approximately 150 h) [15].

Pentavalent antimonial agents are the most commonly used antileishmanial drugs worldwide, despite studies that have

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reported their high toxicity [16,17], genotoxicity, and mutagenicity [18].

The Food and Drug Administration (FDA) described possible mutagenic effects of miltefosine, based on seven mutagenicity tests. Only one of the tests gave positive results [19]. The objective of the present study was to evaluate the interaction of miltefosine with the genetic material.

2. Materials and methods

Miltefosine capsules (Impavido®–50 mg) were kindly provided by Prof. Dr. Carlos Costa from the Leishmaniasis Laboratory (Laboratório de Leishmanioses – [LabLeish]) of the Federal University of Piauí (UFPI), Brazil. For the assays, capsules were dissolved in distilled water. All concentrations were previously assayed for cellular viability based on the World Health Organization recommended dose of 2.5 mg/kg/day.

2.1. In vitro assays

All *in vitro* assays were carried out on peripheral blood leukocytes obtained from three male and three female volunteers. Inclusion criteria were: 19–30 years of age, healthy, non-smoker, medication-free, and chemotherapy- or radiation therapy-free for the last six months (Ethics Committee Opinion n° 1.284.493-UFMA). Peripheral blood (20 mL) was collected from each subject in heparinized tubes; leukocytes were obtained using Ficol Paque™ Plus. Cells were uniformly diluted in RPMI culture medium, 5 mL, exposed to the drug immediately (time 0), and harvested after 3 and 24 h (for the comet assay and cell viability) and after 48 h for the cell death assays. For the negative control, cells were grown in RPMI 1640 medium alone. For the positive control, cells were treated with H_2O_2, 10 vol (30 mg/mL), 10 μL, for 10 min.

2.1.1. Cell viability

Cell viability was determined in a pilot test (100, 50, 25, and 10 μg/mL) by adding trypan blue (0.4%, 10 μL) to cell suspension, 10 μL. One hundred cells were counted for each treatment and the cultures had to have >70% viable cells to be used [20]. We performed cytotoxicity tests at both exposure times (3 and 24 h). The final concentrations established were C1 (0.25 μg/mL), C2 (0.5 μg/mL), and C3 (1.0 μg/mL).

2.1.2. Comet assay

Leukocytes were grown for 3 and 24 h in PB-MAX™ medium (Gibco® Life Technologies™). Then, low-melting-point agarose (0.5%, 200 μL) was added to cell suspension, 100 μL. This mixture was dropped on slides pre-treated with normal melting point agarose (1.5%) and cooled for 5 min. After solidification, the slides were immersed in lysis buffer (2.5 M NaCl, 100 mM EDTA, 10 mM Tris, 10% dimethyl sulfoxide, 1% Triton X-100, pH 10.0) and refrigerated overnight at 4 °C. Subsequently, the slides were incubated in alkaline solution (10 M NaOH, 0.2 M EDTA, and distilled water, pH 13.0) for 20 min at 4 °C. Electrophoresis was performed for 25 min/25 V (0.72 V/cm)/300 mA. Slides were neutralized (0.4 M Tris/HCl, pH 7.5), dried at room temperature (22–25 °C) and fixed in 100% ethanol for 4 min. Then, slides were submitted to 20 μg/mL ethidium bromide [21,22] and analyzed under a fluorescence microscope (Olympus BX61).

One hundred nucleoids were analyzed by considering size and amount of DNA present in the comet tail. The damage was classified into five levels: Class 0, no damage (<5%); Class 1, low damage (5–20%); Class 2, moderate damage (20–40%); Class 3, high damage (40–94%); Class 4, total damage (>95%) [23]. The damage score was obtained by multiplying the number of nucleoids in each class by the respective class value, according the equation:

$$\text{Score} = [(0 \times n_0) + (1 \times n_1) + (2 \times n_2) + (3 \times n_3) + (4 \times n_4)]/\text{total number of cells}$$

2.1.3. Cytotoxicity assay

After 48 h culture, an aliquot (50 μL) of cell suspension was stained with 2.5 μL of a fluorescent dye mixture (5 μg/mL propidium iodide, 15 μg/mL fluorescein diacetate, 2 μg/mL Hoechst 3334 and 15 μL PBS, pH 8.0) and then incubated at 37 °C for 5 min [24]. Six repetitions were performed for each treatment; 500 cells from each animal were analyzed using a fluorescence microscope (triple-band filter), totaling 3000 cells per treatment group. Cells were classified as normal (spherical nuclei stained in blue and cytoplasm stained in green), necrotic (spherical nuclei stained in red), or apoptotic (blue-stained nucleus with apoptotic bodies and green cytoplasm) [25].

2.2. In vivo assays

2.2.1. Animals

The Animal Ethics Committee of the Federal University of Maranhão (Protocol no. 23115-009229/2014-21) approved all experimental procedures. Male Swiss *Mus musculus* mice (n = 25) weighing 30 g were obtained from the Animal Breeding Unit (Federal University of Maranhão, São Luís, MA, Brazil). The animals were kept in a well cross-ventilated room at 21 ± 2 °C with relative humidity 44–56% and light and dark cycles of 12 h. They had access ad lib to sterilized food and water.

2.2.2. Doses and treatment

To perform *in vivo* tests, miltefosine (Impavido®) doses were established based on the recommended dose for treating leishmaniasis, according to the WHO, which is 2.5 mg/kg over 28 d, for cumulative dose = 70 mg/kg. Thus, each group of animals received an acute treatment at one of three doses (35; 70; and 140 mg/kg). All treatments were administered orally in volumes of 0.1 mL/10 g of body weight. The negative control group received distilled water and the positive control group received cyclophosphamide (50 mg/kg) intraperitoneally. The animals were euthanized 24 h after receiving the drug.

2.2.3. Comet assay

The standard alkaline comet assay was performed using peripheral blood cells. After 24 h treatment, a small incision was made on the extremity of the animal's tail to collect 5 μL peripheral blood, which was mixed with 100 μL low-melting-point agarose (0.5%). Then, this mixture was transferred to slides with normal-melting-point agarose (1.5%). The subsequent steps were as previously described for the comet assay *in vitro*.

The comet assay was followed by FPG (formamidopyrimidine-DNA glycosylase) digestion to test whether miltefosine induces DNA damage by base oxidation. The FPG FLARE™ kit (Trevigen, Gaithersburg, MD) was used following the protocol described by Gajski et al. [26]. After the lysis step, the slides were immersed in 1 × FLARE TM (Trevigen) for 30 min. Then, each slide was treated with 150 μL FPG solution, covered with a coverslip, and incubated at 37 °C for 45 min in a moist chamber. The coverslips were then removed and the electrophoresis, neutralization, fixation, staining, and analysis steps were performed as previously described for the *in vitro* comet assay.

2.2.4. Micronucleus test

The micronucleus test was performed in bone marrow cells collected from the same animals used for the comet assay. Briefly,

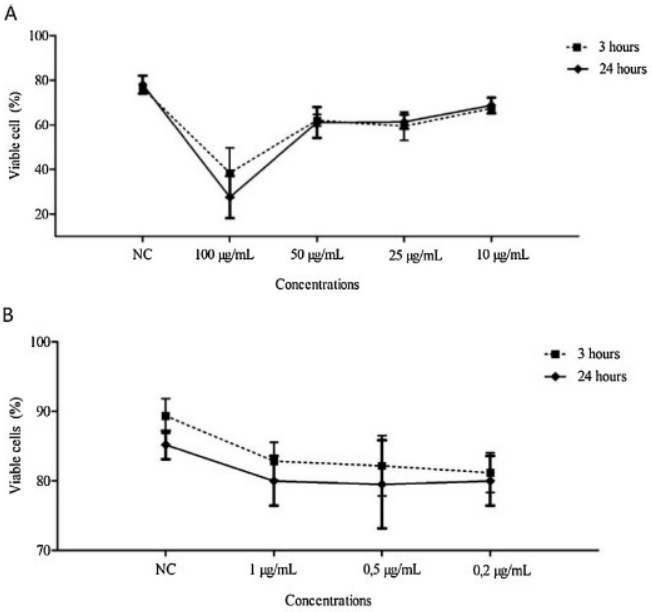

Fig. 1. Cell viability (%) detected in human leukocytes (n = 6 volunteers) *in vitro* treated with different concentrations of miltefosine (Impavido®) for 3 and 24 h. A: concentrations lower than 70% viability; B: concentrations higher than 70% viability. NC: negative control (water).

Table 1
DNA damage scores (mean ± SD) and frequency of comet classes detected in human leukocytes treated with different concentrations of miltefosine (Impavido®) *in vitro* for 3 and 24 h.

Groups	Class (%)					Scores Mean ± SD
	0	1	2	3	4	
3h						
NC	86.5	7.5	2.0	1.8	2.2	0.26 ± 0.01^{a}
PC	0.0	0.0	0.2	11.6	88.2	3.88 ± 0.06^{b}
C1	79.0	12.8	3.6	1.6	3.0	$0.37 \pm 0,17^{a}$
C2	80.2	10.5	3.3	3.0	3.0	0.38 ± 0.18^{a}
C3	80.5	9.2	3.2	2.8	4.3	0.41 ± 0.15^{a}
						F = 762.35
						p < 0.0001
24h						
NC	86.7	7.8	1.7	1.8	2.0	0.25 ± 0.15^{a}
PC	0.0	0.2	0.7	10.3	88.8	3.88 ± 0.08^{b}
C1	61.1	20.2	5.0	7.5	6.2	0.83 ± 0.28^{c}
C2	58.7	18.4	3.7	10.7	8.5	0.77 ± 0.28^{c}
C3	64.9	13.1	4.5	9.0	8.5	0.83 ± 0.23^{c}
						F = 259.14
						p < 0.0001

Different letters denote statistical difference among groups ($p < 0.01$ by Tukey test).
F refers to the ANOVA's statistic.
NC: negative control (water); PC: positive control (cyclophosphamide 50.0 mg/kg); C1: 0.25 µg/mL; C2: 0.5 µg/mL; C3: 1.0 µg/mL.

after collecting blood, the animals were euthanized by anesthesia overdose (xylazine, 1 mL/kg and ketamine, 0.1 mL/kg). Next, femurs and muscles were removed, and epiphyses were cut. The bone marrow was removed by aspiration with fetal bovine serum. The cell suspension was centrifuged for 5 min at 1000 rpm. The supernatant was discarded and a slide smear was made with the remaining material. The material was fixed in absolute methanol for 10 min, and then stained with May Grunwald-Giemsa diluted in a phosphate buffer (pH 6.8). This stain differentiates young or polychromatic erythrocytes (PCEs) (light, purplish blue) from normochromatic erythrocytes (NCEs) (light red) [27].

The toxicity of miltefosine (Impavido®) was estimated using 1000 erythrocytes per animal by calculating the PCE/NCE ratiMoreover, 2000o. Moreover, 2000 PCE for each animal were analyzed to determine the frequency of micronucleated cells.

2.3. Statistic analysis

The normality of the data was analyzed by the Shapiro-Wilk test. The nonparametric Kruskal-Wallis test was applied to analyze the comet assay data, followed by Tukey's test. The comparison between the damage score obtained from both the comet assays

Table 2
DNA damage scores (mean ± SD) and frequency of comet classes detected by the alkaline comet assay in peripheral blood of Swiss *Mus musculus* mice treated with different doses of miltefosine (Impavido®) for 24 h.

Groups	Classes (%)					Scores Mean ± SD
	0	1	2	3	4	
NC	50.0	37.4	5.8	3.2	3.6	0.73 ± 0.1^{a}
PC	30.2	23.8	18.4	11.8	15.8	$1.59 \pm 0.3^{b*}$
D1	21.6	19.4	13	12.4	34.4	$2.20 \pm 0.2^{c**}$
D2	19.0	19.8	12	11.4	37.2	$2.27 \pm 0.1^{c**}$
D3	18.2	19.2	12.0	13.6	37.0	$2.32 \pm 0.2^{c**}$
						F = 54.1067
						p < 0.0001

Different letters denote statistical difference among groups ($p < 0.05$ by Tukey test; * $p < 0.05$, ** $p < 0.01$).
F refers to the ANOVA's statistic.
NC: negative control (water), CP: positive control (cyclophosphamide 50 mg/kg), D1: 35 mg/kg, D2: 70 mg/kg, D3: 140 mg/kg.

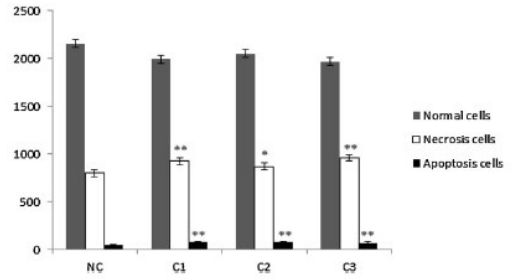

Fig. 2. Cell death induced by miltefosine (Impavido®) in human leukocytes from volunteers (n = 6) treated for 48 h *in vitro*.
$\chi 2$ test * $p < 0.05$; ** $p < 0.0001$.
NC: negative control (culture medium RPMI 1640), C1: 0.25 μg/mL, C2: 0.5 μg/mL, C3: 1.0 μg/mL.

(standard and FPG version) was performed by Student-T test. The micronucleus and the cell death assays were analyzed by Chi-square test ($p < 0.05$) [28].

3. Results

3.1. Cell viability

Viability tests were initially performed in cells treated with miltefosine at concentrations of 100, 50, 25, and 10 μg/mL, which revealed cell viability lower than 70% (Fig. 1A). The final concentrations were established as 0.25, 0.5, and 1.0 μg/mL, which revealed no cytotoxicity after 3 h and 24 h of exposure, compared to the negative control. The viability values were 82.8%, 82.2%, 81.2% and 89.3%, respectively. Similarly, 24 h exposure to miltefosine resulted in 80.0%, 79.5%, 80.0%, and 85.8% viability, respectively (Fig. 1B).

3.2. Miltefosine (Impavido®) induces DNA damage in vitro

Table 1 shows the damage scores obtained from the comet assay. Significant differences in the damage scores were not observed at 3 h for any concentrations (0.25, 0.5, and 1.0 μg/mL). However, when cells were exposed for 24 h, there was a significant increase in the damage scores at all concentrations tested ($p < 0.01$) compared to the negative control, providing evidence of miltefosine genotoxicity.

3.3. Miltefosine (Impavido®) induces cell death by necrosis and apoptosis

Significant increases in the frequencies of necrotic and apoptotic cells were observed for all miltefosine (Impavido®) concentrations (0.25, 0.5, and 1.0 μg/mL) when compared to the negative control group ($p < 0.05$); Fig. 2. However, no differences were observed among the concentrations.

3.4. Miltefosine (Impavido®) induces DNA damage in vivo

Table 2 shows the DNA damage scores calculated from the standard comet assay data. Miltefosine was genotoxic to all groups (35, 70, and 140 mg/kg) ($p < 0.01$), but significant differences were not observed among the three doses. The miltefosine damage score was

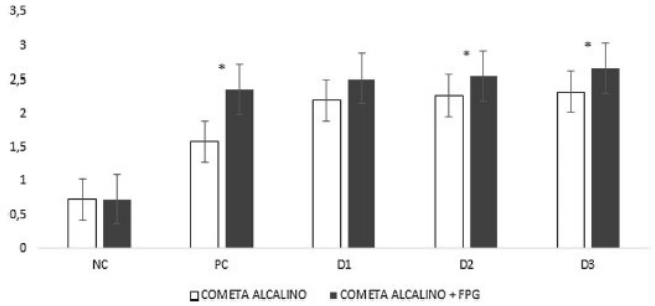

Fig. 3. DNA damage scores detected by conventional alkaline comet assay and the comet sssay followed by Formamidopyrimidine-DNA glycosylase (FPG) digestion (n = 5 animals per group).
*t test $p < 0.05$
NC: negative control (water), PC: positive control (cyclophosphamide 50.0 mg/kg), D1: 35 mg/kg, D2: 70 mg/kg, D3: 140 mg/kg.

Table 3
Frequency of micronucleated polychromatic erythrocytes (MNPCE) in bone marrow of swiss mice (n = 5 animals per group) treated intraperitoneally with miltefosine (Impavido®).

Grupos	PCE	PCEMNs	‰ MÉDIA ± DP	PCE/NCE
NC	10000	26	2.6 ± 0.04	1.03
PC	10000	202	20.2 ± 0.31*	0.65
D1	10000	94	9.4 ± 0.32*	1.14 ns
D2	10000	95	9.5 ± 0.40*	1.01 ns
D3	10000	91	9.1 ± 0.32*	0.86 ns

$\chi 2$ test * $p < 0.0001$ compared to the negative control group.
NC: Negative control (water), PC: positive control (50.0 mg cyclophosphamide/kg) D1: 35 mg/kg, D2: 70 mg/kg, D3: 140 mg/kg; ns: no significant difference compared with NC.

also significantly higher than that for the positive control group (cyclophosphamide).

3.5. Miltefosine (Impavido®) induces DNA damage by oxidation of its bases

Based on the previous results, the comet assay with Fpg digestion was performed. The data showed an increase in DNA damage in the positive control and at the two higher doses of miltefosine ($p < 0.05$). On the other hand, there was no difference for the negative control (Fig. 3).

3.6. Miltefosine (Impavido®) induces micronuclei in vivo

The PCE/NCE ratio demonstrates that miltefosine (Impavido®) was not cytotoxic at any of the three doses ($p > 0.05$). The frequencies of micronuclei in the negative and positive controls were within the expected range. A significant increase ($p < 0.05$) in PCEMN is observed at the three miltefosine doses when compared to the negative control. Statistically-significant differences were not observed among the miltefosine doses (Table 3).

4. Discussion

Our data demonstrate that miltefosine (Impavido®) is genotoxic. The *in vitro* comet assay indicates that DNA damage did not occur during the 3 h treatment time at any concentrations (0.25, 0.5, and 1.0 μg/mL). However, cells exposed to all miltefosine concentrations for 24 h had significant damage compared to the negative control. High frequencies of necrotic and apoptotic cells were observed after 48 h drug exposure. These findings corroborate the results of Jiménez-Lopez et al. [29], who observed cytostatic and apoptotic activity in HepG2 cells (human hepatoma) treated with miltefosine. These authors [30] suggested that programed cell death processes may be related to specific changes in lipid metabolism. This hypothesis was confirmed when the authors observed that miltefosine alters cholesterol metabolism, which prevents its transport from the plasma membrane to the endoplasmic reticulum. They concluded that miltefosine leads to apoptosis due an accumulation of compounds in membrane regions rich in cholesterol and sphingolipids.

The strong interaction of miltefosine with bilayers rich in cholesterol was also noted by Sá et al. [31]. The authors confirmed that cholesterol depletion in the endoplasmic reticulum inhibits cholesterol esterification and, hence, signaling cell death by apoptosis. These studies support the findings of Li et al. [32], who demonstrated that cholesterol-depleting agents induce apoptosis in cancer cells, which have lipid regions rich in cholesterol. Considering that miltefosine is a cholesterol-depleting agent, these data may suggest the possible efficacy of miltefosine as an antitumor agent.

We also noted a high frequency of necrotic cells. According to Elmore [33], this type of cell death is related to direct damage inflicted on the cell membranes when their integrity is broken. Furthermore, the necrosis is also related to the removal of cancer cells [34,35], which once again supports the action of miltefosine as an antitumor agent.

Our results also demonstrated increases of genomic instability in mice receiving miltefosine at all doses (35, 70 and 140 mg/kg), revealing a damage score higher than the positive control treated with cyclophosphamide (50 mg/kg), a known mutagen and carcinogen. In addition, the *in vivo* comet assay followed by formamidopyrimidine DNA glycosylase (FPG) digestion was performed to determine the mechanism by which this damage occurs. FPG is a repair endonuclease which recognizes oxidized purine bases, particularly 7,8-dihydro-8-oxoguanine (8-oxoG). FPG removes the products of guanine oxidation, causing more DNA fragmentation and an increased damage score compared to the standard comet assay [36]. At 70 and 140 mg/kg miltefosin, a significant increase in damage was seen with FPG treatment, indicating that the drug causes DNA oxidation. Our data suggest that cell death induced by miltefosine may result from DNA damage due to oxidation of purine bases.

Genomic lesions induced by the accumulation of 8-oxoG are usually repaired by base excision, which functions to maintain the integrity of the genome and reduce the rate of mutations [37]. However, if damage levels are high and DNA is not repaired, mutations may become permanent. DNA damage activates checkpoint regulators, such as *p53*, *SMC1*, *FANCD2*, *BRCA1*, that promote interruption of the cell cycle and apoptosis. Impairment of any of these regulators increases mutagenesis, which can lead to carcinogenesis [38]. In this study, we also noted a high frequency of micronucleated cells following miltefosine treatment. This suggests that the damage observed in the comet assay was not repaired and, therefore, the resulting mutations were fixed.

In summary, our findings demonstrate genotoxic and mutagenic effects of miltefosine. We also suggest that the mechanism of genotoxic action for miltefosine may be purine base oxidation. Other authors have reported that miltefosine leads to production of reactive oxygen species in yeast [39]. Our data indicate cell death by necrosis, which can be explained by the action of miltefosine on lipid membranes, as proposed by other authors. These results provide insight into the mechanisms of action of miltefosine and may assist the development of strategies to reduce its side effects and improve treatment efficacy.

The present study is the first to assess the genotoxic and mutagenic effects of miltefosine in animals treated with an acute dose. Pre-clinical studies are needed to evaluate the genotoxic effects of this drug under chronic-use conditions. Since miltefosine is already being used to treat leishmaniasis, monitoring its possible genotoxicity in patients is recommended.

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CAPÍTULO 2

Ascorbic acid reduces the genetic damage caused by miltefosina (hexadecylphosphocholine) in animals infected by *Leishmania* (*Leishamnia*) *infantum* without decreasing its antileishmanial activity. **IJP: Drugs and Drug Resistance 9:8–15, 2019.**

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Ascorbic acid reduces the genetic damage caused by miltefosine (hexadecylphosphocholine) in animals infected by *Leishmania (Leishamnia) infantum* without decreasing its antileishmanial activity

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ABSTRACT

Leishamaniasis is a neglected disease caused by over 20 *Leishmania* species, occurring in more than a hundred countries. Miltefosine (hexadecylphosphocholine) is the single oral drug used in treatment for leshmaniases, including cases of infections resistant to pentavalent antimony. Our group has recently demonstrated the ability of miltefosine to cause genomic lesions by DNA oxidation. Acknowledging that antioxidant compounds can potentially modulate Reactive Oxygen Species (ROS), our study verified whether ascorbic acid reduces the genotoxic and mutagenic effects caused by miltefosine, and whether it interferes with drug efficacy. For this purpose, uninfected Swiss mice received simultaneous (single dose treatment) miltefosine and ascorbic acid (gavage and intraperitoneally), besides pre and post treatments (ascorbic acid 24 h before and after drug administration); furthermore, Balb/c mice infected with *Leishmania infantum* received miltefosine plus ascorbic acid (repeated doses treatment). We conducted comet assays, micronucleus tests, dosages of superoxide dismutase enzyme and parasitic burden by the limiting dilution assay. We observed that ascorbic acid administered intraperitoneally displayed a protective effect over damage caused by miltefosine. However, this effect was not not observed when the same doses were administered via gavage, possibly due to low serum levels of this antioxidant. Ascorbic acid's protective effect reinforces that miltefosine damages DNA by oxidizing its nitrogenous bases, which is reduced by ascorbic acid due to its ability of protecting genetic material from the action of ROS. Therefore, our results show that this drug is efficient in reducing parasitic burden of *L. infantum*.

1. Introduction

Leishmaniasis comprise a group of non-contagious, highly neglected, parasitic and chronic infectious diseases caused by protozoa of the genus *Leishmania* (Brasil et al., 2017). There are three main forms of the disease: cutaneous leishmaniasis, the most common form, which causes skin lesions; mucocutaneous leishmaniasis, which leads to partial or total destruction of mucous membranes; and visceral leishmaniasis, which is fatal if not treated appropriately. Visceral leishmaniasis is broadly distributed and recorded in 102 countries, with over 90% of the cases occurring in the following countries: Bangladesh, Brazil, Ethiopia, India, South Sudan and Sudan. We estimate that around 700 thousand to 1 million new cases of this disease surface every year, out of which 500 thousand correspond to the visceral form of leishmaniasis, generating 20 to 30 thousand deaths a year (WHO, 2017).

Treatment for leishmaniasis is very restricted, consisting mostly of two pharmacological lines: antimonials (meglumine antimoniate and sodium stibogluconate) and non-antimonials (pentamidine, amphotericin B, paromomycin and miltefosine). Pentavalent antimonials are still commonly used (Almeida and Santos, 2011), even if several studies have already declared them highly toxic (Demicheli et al., 2002; Kato et al., 2014) and mutagenic (Lima et al., 2010; Cantanhêde et al., 2015; Moreira et al., 2017; de Jesus et al., 2018).

In the past decade, a non-antimonial drug has gathered attention in treatment of leishmaniasis: miltefosine (hexadecil 2- (trimethylazionyl) ethyl phosphate). Also known as hexadecylphosphocholine, miltefosine was created in the United States, in the 1980's, originally for treatment of skin cancer. Following studies demonstrated that it also presented antileishmanial activity and, in 2002, countries such as India, Nepal and Bangladesh introduced it in treatment of leishmaniasis (Moore and

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Lockwood, 2010). In 2011, miltefosine (Impavido®) was included in a list of essential medications by the World Health Organization as an antileishmanial drug and is currently the single antileishmanial drug of oral administration (FDA, 2013), prominent for being an alternative in the maintenance of treatment, including cases of infections resistant to conventional therapy with the pentavalent antimonial (Sundar and Chatterjee, 2006).

Recently, our laboratory demonstrated the ability of this antileishmanial to cause apoptosis, necrosis and genomic lesions with oxidation of purine bases in mammalian cells (Castelo Branco et al., 2016). Oxidation of bases occurs due to the oxidative stress caused by the imbalance between the Reactive Oxygen Species (ROS) and the antioxidant defense system, with a predominance of ROS (Vasconcelos et al., 2007).

To control the formation of ROS and other reactive species, cells have an antioxidant defense system classified as enzymatic and non-enzymatic. The enzymatic system is represented mainly by the antioxidant enzymes: superoxide dismutase (SOD), which are metalloproteins that catalyze the dismutation of superoxide (O_2^-) in oxygen (O_2) and hydrogen peroxide (H_2O_2); catalase (CAT) that acts on the decomposition of H_2O_2 into O_2 and H_2O; and glutathione peroxidase (GPx), which acts on peroxides in general (Marín et al., 2006; Vasconcelos et al., 2007). The non-enzymatic antioxidant system consists of several substances, such as glutathione (GSH), the main intracellular antioxidant compound, β-carotene tocopherols (vitamin E), ascorbic acid (vitamin C) and others (Vasconcelos et al., 2007; Nimse and Pal, 2015).

Ascorbic acid is an important antioxidant in the combat against certain types of cancer, cardiovascular diseases and other diseases associated with oxidative stress (Harrison, 2012). Even though humans are unable to synthesize this antioxidant, it participates in several physiological activities, such as: collagen synthesis; ability to recycle vitamin E; promoting cholesterol reduction and resistance to infections by participating in the immunological activity of leukocytes (Cerqueira et al., 2007; Grangeiro et al., 2013). On the other hand, it is also known that ascorbic acid can be toxic in large quantities, instead taking pro-oxidant action, which actually favors the occurrence of oxidative stress (Halliwell, 2001; Kato et al., 2014).

The antioxidant property of ascorbic acid in eliminating free radicals suggests that this molecule can modulate oxidative damage to DNA in mammalian cells. Thus, this study investigated the ability of ascorbic acid to decrease the genotoxic and mutagenic effects caused by miltefosine in the *in vivo* system, with and without *Leishmania infantum* infection, as well as to verify whether it interferes with the antileishmanial action of the drug.

2. Material and methods

2.1. General study design

To evaluate the capacity of ascorbic acid to protect the DNA damage caused by miltefosine, two strains of mice presenting different genetic backgrounds, Swiss and Balb/c (non-susceptible and susceptible to *Leishmania* infection), were treated with both compounds using single (simultaneous, pre and post treatment) and repeated doses. Thus, the effect of ascorbic acid on DNA damage was assessed under different conditions.

Firstly, considering that miltefosine causes genomic instability and mutations in uninfected Swiss mice (Castelo-Branco et al., 2016), the ability of ascorbic acid to protect the DNA was assessed by comet assay and micronucleus test. For that, Swiss mice received single dose of both compounds in simultaneous, pre and post treatments. After 24 h, peripheral blood was obtained from the tail of each animal to perform the comet assay. Thereafter, the animals were euthanized by anesthetic overdose (Xylazine - 60 mg/kg and Ketamine - 300 mg/kg) and then the femurs were removed to carry out micronucleus test.

Secondly, to evaluate the effects of ascorbic acid in infected animals, Balb/c mice were treated using repeated doses, according to the World Health Organization for human treatment. Toxicogenetic tests were performed by comet and micronucleus assays, and the parasite burden was determined in lymph node and spleen by limiting dilution. Moreover, considering previous data that showed miltefosine causes oxidation of DNA bases (Castelo-Branco et al., 2016), enzyme superoxide dismutase activity was measured in serum. Briefly, on 14th and 28th days after treatments, peripheral blood was obtained from the tail of each animal to perform comet assay. Then, the animals were anesthetized (Xylazine-20 mg/kg and Ketamine-100 mg/kg) to collect bood for the measurement of superoxide dismutase activity. Therefore, the femurs were removed to carry out micronucleus test and, finally, the spleen and lymph node were removed to determine parasite burden by limiting dilution assay.

2.2. Animals

Male Swiss *Mus musculus* mice (n = 5/group) were obtained from the Central Bioterium of the Federal University of Maranhão – UFMA (Maranhão, Brazil) and Balb/c *Mus musculus* mice were obtained from the Bioterium of the University of Campinas (São Paulo, Brazil), all weighting 30 g and aging around 60 days. Animals were kept in polycarbonate boxes under controlled conditions: temperature around 21 ± 2 °C and alternated 12 h light-dark cycle. Our project has been approved by the Animal Experimentation Ethics Committee (CEUA) of UFMA (nº 23115.009229/2014-21).

2.3. Parasites

Promastigotes of L. *infantum* (MHOM/BR/1970/BH46) were cultured in Schneider's medium (Sigma, St Louis, MO, USA), which was supplemented with 10% Heat-Inactivated Fetal Bovine Serum (GIBCO) at 27 °C. To perform the tests, Balb/c mice were infected in the right hind footpad with 10^7 promastigotes in stationary phase of *L. infantum*, as mentioned by de Jesus Pereira et al. (2015) and Martins et al. (2015). The infection was maintained for 28 days (Marques-da-Silva et al., 2005; Serafim et al., 2010) and on the 29th day the antileishmanial treatments were performed.

2.4. Drugs and treatment

Assays with miltefosine were conducted under different treatment conditions. For this purpose, animals were divided into two groups: 1) uninfected Swiss mice receiving a single dose (70 mg/kg) of miltefosine (Chemical Cayman Company, MI, USA, batch 0463792-1) as previously described (Castelo Branco et al., 2016); 2) infected Balb/c mice which received miltefosine (Impavido®) at 2.5 mg/kg for 28 days, as recommended by the World Health Organization for human treatment; and another group that received 5.0 mg/kg for 14 days. In both assays, the drug was dissolved in distilled water and administered via gavage. Miltefosine capsules (batch 1C2130A) were gently conceded by Prof. Dr. Carlos Costa from Federal University of Piauí, Brazil.

To evaluate protective effect of ascorbic acid (Sigma-Aldrich, St. Louis, MO, batch 69085) over DNA damage induced by miltefosine, Swiss mice received single doses (30, 60 and 120 mg/kg) of ascorbic acid simultaneously to the antileishmanial (70 mg/kg). In order to evaluate the effect of the ascorbic acid exposure route, one group of these animals received the antioxidant via gavage (So30, So60 and So120 groups) and another group received via intraperitoneal (Si30, Si60 and Si120 groups). Besides, intermediate dose (60 mg/kg) was administered 24 h before (pre treatment) and 24 h after (post treatment) miltefosine administration. The doses of ascorbic acid were based on previous study (de Jesus et al., 2018). The negative control (NC) group was administered distilled water.

Concerning the effect of ascorbic acid in repeated dose treatment,

Table 1
Experimental groups used to evaluate protective effect of ascorbic acid over genetic damage induced by miltefosine.

Group	Chemical Agents	Doses (respectively)	Exposure route
Swiss (acute treatment)			
NC	distilled water	–	gavage
AA	ascorbic acid	60 mg/kg	gavage
MT	miltefosine	70 mg/kg	gavage
So30	ascorbic acid + miltefosine	30 mg/kg + 70 mg/kg	gavage
So60	ascorbic acid + miltefosine	60 mg/kg + 70 mg/kg	gavage
So120	ascorbic acid + miltefosine	120 mg/kg + 70 mg/kg	gavage
PRE	ascorbic acid (24 h before) + miltefosine	60 mg/kg + 70 mg/kg	gavage
POS	miltefosine (24 h before) + ascorbic acid	70 mg/kg + 60 mg/kg	gavage
Si30	ascorbic acid + miltefosine	30 mg/kg + 70 mg/kg	i.p. + gavage
Si60	ascorbic acid + miltefosine	60 mg/kg + 70 mg/kg	i.p. + gavage
Si120	ascorbic acid + miltefosine	120 mg/kg + 70 mg/kg	i.p. + gavage
Balb/c (repeated doses treatment)			
NC	distilled water	— (28 days)	gavage
IC	distilled water	— (28 days)	gavage
MT5.0	Impavido®	5.0 mg/kg (14 days)	gavage
MT2.5	Impavido®	2.5 mg/kg (28 days)	gavage
AA	ascorbic acid	15 mg/kg (28 days)	gavage
MT + AA	Impavido® + ascorbic acid	2.5 mg/kg + 15 mg/kg (28 days)	gavage

infected Balb/c mice received, simultaneously, 2.5 mg/kg miltefosine and 15 mg/kg ascorbic acid every day for 28 days; another group received only ascorbic acid in the same dose and in the same period of time. The dose of ascorbic acid was based on study of Kato et al. (2014). Negative control (distilled water) and infected control (animals with *L. infantum* infection) were also used. Treatment groups and doses are listed in Table 1.

2.5. Assays

2.5.1. Comet assay

It was collected from the tail of each animal approximately 5 µl peripheral blood and mixed with 100 µl low melting point agarose (0.5%). This mixture was then applied to pre-gelled slides with regular melting point agarose (1.5%). Then, coverslips were superimposed on these slides and refrigerated for 5 min. After solidification, slides were immersed in cold lysis solution (2.5M NaCl; 100 mM EDTA; 10 mM Tris (pH 10); 10% DMSO and 1% Triton X-100) and refrigerated for overnight at 4 °C. Slides were then incubated for 20 min in alkaline buffer (10M NaOH, 0.2M EDTA and distilled water, pH 13.0) followed by electrophoresis for 25 min at 25 V (0.72 V/cm)/300 mA. Slides were neutralized (0.4M Tris/HCl, pH 7.5), dried at room temperature and fixed with absolute ethanol for 4 min (Singh et al., 1988; Tice et al., 2000). Slides were stained with ethidium bromide (20 µg/ml) and analyzed by fluorescence microscopy (Olympus BX61).

For each animal, we analyzed 100 nucleoids, considering their size and quantity of DNA in the comet. DNA damage was classified in five levels: Class 0: no damage (< 5%); Class 1: low damage (5–20%); Class 2: medium damage (21–40%); Class 3: high damage (41–94%) e Class 4: total damage (> 95%). Damage scores were calculated by multiplying the number of nucleoids in each class by their respective class value, using the following equation:

Score = [(0 x n0) + (1 x n1) + (2 x n2) + (3 x n3) + (4 x n4)] / total cell number

2.5.2. Micronucleus test

The femurs were removed for extraction of the bone marrow using 1 ml fetal bovine serum. Cell suspension was centrifuged for 5 min at 1000 rpm and the supernatant was discarded. Slide smearing (Schmid, 1975) was conducted with the remaining material. Slides were stained using hematological dye Panoptic kit (Laborclin, Pinhais, Paraná, Brasil) to differentiate polychromatic erythrocytes (PCE) from normochromatic erythrocytes (NCE).

To investigate micronucleus, 2000 polychromatic erythrocytes were analyzed for each animal in optical microscope magnification of 1000x (Carl Zeiss Primo Star). The ratio PCE/NCE (polychromatic erythrocytes and normochromatic erythrocytes) was calculated using 1000 erythrocytes for each animal to estimate toxicity for miltefosine and ascorbic acid.

Percentage of reduction in DNA damage induced by miltefosine, modulated by ascorbic acid, was calculated according to Waters et al. (1990) using the following formula: % Reduction = (CP – CT/CP - CN) x 100, where CP corresponds to the average damage of the group treated only with miltefosine, CT is the average damage observed in groups treated with ascorbic acid plus miltefosine and CN is the average damage observed in the negative control (distilled water).

2.5.3. Limiting dilution assay

Parasitic burden of infected animals was determined by the limiting dilution assay. Organs were collected and homogenized individually in 1.0 ml Schneider's Drosophila medium (Sigma-Aldrich, USA) supplemented with 10% inactivated fetal bovine serum (GIBCO, USA). The suspension with the homogenized organ was diluted 1:2 in the first well, which contained 100 µl of the medium, making up a total volume of 200 µl of suspension. From the first well, 100 µl of the suspension was withdrawn and the 1:2 dilution continued until the 12th well of the 96-well plate. This assay was conducted in duplicates. After 14 days, the sample from each well was analyzed and defined as positive or negative depending on the presence or absence of promastigotes in the well. The final titre was defined as the highest dilution for which the well contained at least one active parasite. The number of parasites per gram of the homogenate was calculated as follows: the reciprocal of the last positive titre by the total homogenized volume of the organ x dilution factor divided by the weight in grams of the homogenized organ. The viable parasitic burden was expressed as the number of leishmania per gram of homogenized organ (Rodrigues et al., 2009).

2.5.4. Superoxide dismutase activity

To analyze Superoxide Dismutase (SOD) activity, we collected blood from infected animals via cardiac puncture. Blood remained at rest for 30 min at 25 °C. It was then centrifuged at 2,000 g for 15 min at 4 °C and the serum was withdrawn. Subsequently, the Superoxide Dismutase Assay Kit (Chemical Cayman, MI, USA) was employed following the manufacturer's instructions.

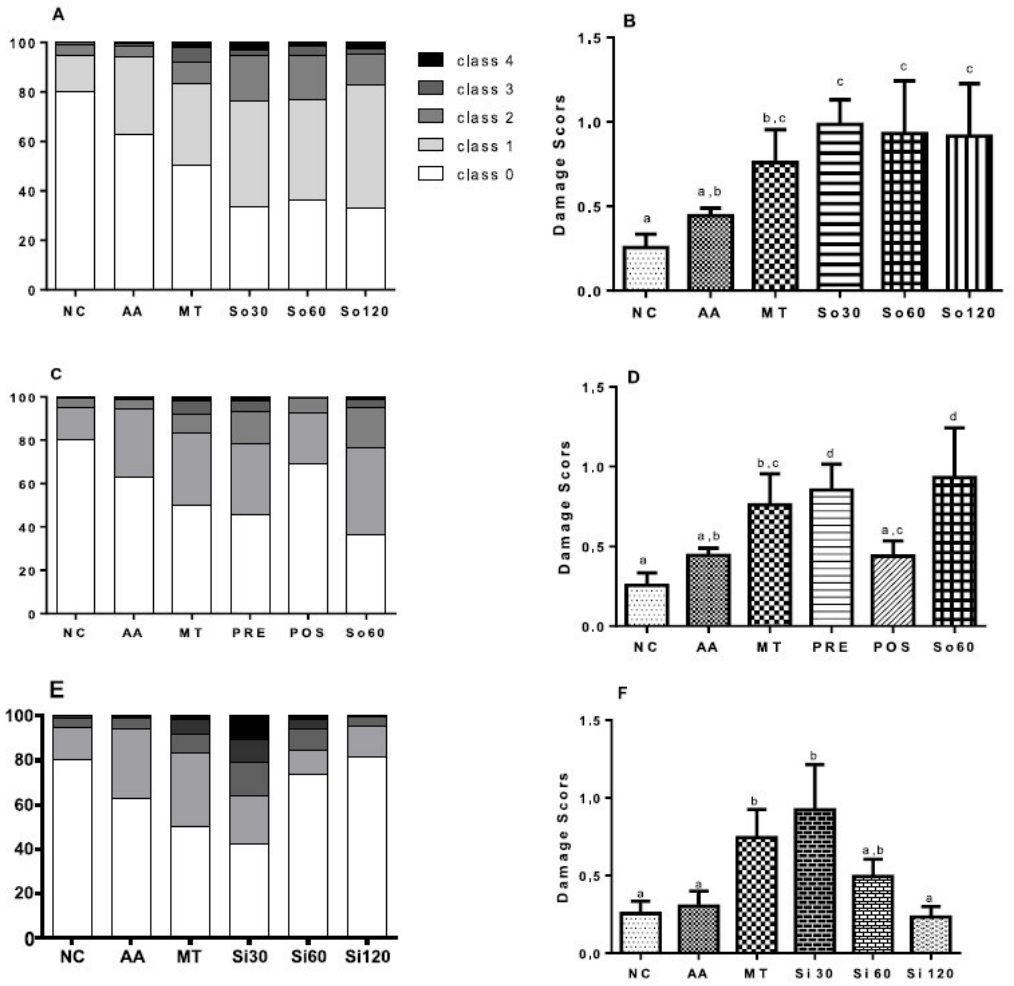

Fig. 1. Analysis of the antigenotoxic activity of ascorbic acid administered by gavage and intraperitoneally in Swiss mice on the genotoxic damages induced by the acute treatment with miltefosine (Chemical Cayman).

2.6. Statistical analysis

Data normality was assessed by Kolmogorov-Smirnov test. Numeric data obtained by comet assays, citotoxicity, parasitic burden and SOD Activity had a normal distribution and were submitted to one-way analysis of variance (ANOVA), followed by Tukey's *ad hoc* test. Micronucleus data were submitted to chi-square test (χ^2). All data were analyzed using GraphPad Prism 6.01 software, with values considered significant when $p < 0.05$.

3. Results

Through the comet assay, our results show that ascorbic acid did not have a protective effect over damage caused by miltefosine at the dose of 70 mg/kg (MT) when animals were treated via gavage and simultaneously (30, 60 and 120 mg/kg) (Fig. 1A and B). There was also no reduction in damage when ascorbic acid was administered 24 h prior to the MT group (PRE). However, the group treated with ascorbic acid 24 h after treatment with miltefosine (POS) showed reduction of damage near baseline (Fig. 1C and D).

The PCE/NCE ratio revealed that miltefosine and ascorbic acid were not cytotoxic at any of the doses tested ($p > 0.05$). When analyzing the antimutagenic activity of the ascorbic acid administered via gavage, none of the treatments (So30, So60, So120, PRE and POS) showed a protective effect of this antioxidant on DNA damage caused by miltefosine (Table 2).

When the same doses were tested intraperitoneally, we observed the protective effect of ascorbic acid over genotoxic damage caused by miltefosine, so that the higher dose (120 mg/kg) significantly reduced DNA damage at baseline, even though damage reduction could be observed from the intermediate dose (Fig. 1E and F).

The antimutagenic activity of ascorbic acid when administered intraperitoneally in mice was also analyzed. The same groups (Si60 and Si120) showed a significant decrease in micronuclei frequency, with 70.4% and 77.8% reduction, respectively, revealing the protective effect of ascorbic acid at these doses when administred intraperitoneally. The PCE/NCE ratio again demonstrated that miltefosine and ascorbic acid were not cytotoxic at any of the doses tested ($p > 0.05$), as shown in Table 3.

Based on these results, we sought to investigate whether this

Table 2
Analysis of the protective effect of orally administered ascorbic acid in Swiss mice on the mutagenic damage induced by acute treatment with miltefosine (Chemical Cayman).

GROUPS	PCEMNs	‰ MEDIA ± SD	PCE/NCE
NC	26	2.6 ± 0.42[a]	1.00
AA	25	2.5 ± 0.35[a]	1.16 [ns]
MT	53	5.3 ± 1.40[b]	0.87 [ns]
So30	50	5.0 ± 2.15[b]	1.14 [ns]
So60	37	3.7 ± 0.91[b]	1.32 [ns]
So120	40	4.4 ± 2.31[b]	0.73 [ns]
PRE	44	4.4 ± 0.65[b]	1.16 [ns]
POS	45	4.5 ± 0.79[b]	1.17 [ns]

Different letters denote statistical difference between the groups by χ^2 test.
[ns] absence of statistical difference when compared to the negative control group by ANOVA, followed by Tukey's *ad hoc* test.
NC: negative control (distilled water); **AA**: ascorbic acid (60 mg/kg); **MT**: miltefosine (70 mg/kg); **So30, So60** and **So120**: groups treated simultaneously with miltefosine (70 mg/kg) and ascorbic acid via gavage at the doses of 30 mg/kg, 60 mg/kg and 120 mg/kg, respectively; **PRE**: Group pretreated (24 h) with ascorbic acid (60 mg/kg) and later with miltefosine (70 mg/kg); and, **POS**: Group treated with miltefosine (70 mg/kg) and later (24 h) with ascorbic acid (60 mg/kg). To determine micronuclei frequency, 2000 polychromatic erythrocytes were counted per animal, and 1000 cells were counted for polychromatic and normochromatic erythrocytes ratio (PCE/NCE).

Table 3
Protective effect of ascorbic acid administered intraperitoneally in Swiss mice on the mutagenic damage induced by acute treatment with miltefosine (Chemical Cayman).

GROUPS	PCEMNs	‰ MEDIA ± SD	% DAMAGE REDUCTION	PCE/NCE
NC	26	2.6 ± 0.42[a]	–	1.00
AA	25	2.5 ± 0.35[a]	–	1.16 [ns]
MT	53	5.3 ± 1.40[b]	–	0.87 [ns]
Si30	49	4.9 ± 1.08[b]	–	1.30 [ns]
Si60	34	3.4 ± 0.96[a]	70.4	1.32 [ns]
Si120	32	3.2 ± 1.20[a]	77.8	1.28 [ns]

Different letters denote statistical difference between the groups by χ^2 test.
[ns] absence of statistical difference when compared to the negative control group by ANOVA, followed by Tukey's *ad hoc* test.
NC: negative control (distilled water); **AA**: ascorbic acid (60 mg/kg); **MT**: miltefosine (70 mg/kg); Simultaneous treatment with miltefosine (70 mg/kg) and ascorbic acid via i.p. at the doses of 30 mg/kg (**Si30**), 60 mg/kg (**Si60**) and 120 mg/kg (**Si120**). To determine micronuclei frequency, 2000 polychromatic erythrocytes were counted per animal, and 1000 cells were counted for polychromatic and normochromatic erythrocytes ratio (PCE/NCE).

protective effect of ascorbic acid also occurred in animals infected with *L. infantum*. Our results showed that in both doses tested (5.0 and 2.5 mg/kg/day), miltefosine (Impavido®) causes genomic lesions ($p < 0.01$). The genotoxic effect of miltefosine is observed both at the lowest dose with higher exposure (2.5 mg/kg for 28 days) and at twice the dose and half the treatment time (5.0 mg/kg for 14 days). It is also observed in Fig. 2 that animals receiving daily doses of miltefosine (2.5 mg/kg) and ascorbic acid (15 mg/kg) had a reduction in the damage score, reaching the basal levels of the negative control ($p > 0.05$). Table 4 shows that these same animals presented no increase in micronucleus frequency, revealing that at the tested doses miltefosine does not induce mutations. Likewise, neither *L. infantum* infection nor tested doses of miltefosine and ascorbic acid were able to alter the activity of the SOD enzyme ($p > 0.05$) (Fig. 3).

The action of miltefosine (Impavido®) on parasitic burden in animals infected with *L. infantum* is shown in Fig. 4, which shows that miltefosine (Impavido®) was effective in reducing the parasitic burden on the lymph node ($p < 0.0001$) by 86% and 88% at doses of 5 mg/kg and 2.5 mg/kg, respectively. When ascorbic acid was administered concurrently with antileishmanial miltefosine (Impavido®) (MT + AA), we found that there was no interference in drug efficacy, resulting in 81% reduction in parasite burden. Animals treated only with ascorbic acid (AA) also had a significant reduction in the parasitic burden of the lymph node (84%). In the spleen, only the 5.0 mg/kg dose (MT5.0) was effective in reducing parasitic burden, resulting in 99% *Leishmania* reduction ($p < 0.05$).

4. Discussion

Many studies have investigated ascorbic acid's potential in modulating the harming effects of free radicals through its antioxidant activity, helping to prevent or delay the development of certain cancers (da Mata et al., 2016), cardiovascular diseases (Zhang et al., 2014) and other diseases related to oxidative stress (Harrison, 2012), in addition to aging (Monacelli et al., 2017).

Taking into consideration that our previous study demonstrated that miltefosine can induce oxidation in the nitrogenous bases of DNA (Castelo Branco et al., 2016), this work evaluated the ability of ascorbic acid to reduce the genetic damage caused by this antileishmanial. For this, the erythropoietic cytotoxicity of miltefosine and ascorbic acid was initially evaluated, in which no change in PCE/NCE ratio was observed in any of the treatment groups, which shows that the doses studied were not cytotoxic.

Firstly, the antioxidant effect of ascorbic acid was evaluated for both vias, gavage and i.p. routes, when administered simultaneously with miltefosine in uninfected animals. The results revealed that ascorbic acid does not present antigenotoxic properties for any of the doses tested when given via gavage. On the other hand, through via i.p., highest doses (60 and 120 mg/kg) presented a protective effect over DNA, corroborating with other studies that reported the antigenotoxic effect of ascorbic acid when administered intraperitoneally (Devi and Latha, 2011; García-Rodríguez et al., 2016; de Jesus et al., 2018). These findings are justified by the fact that intravenous administration of ascorbic acid produces plasma concentrations of 30–70 times higher than gavage doses (Padayatty et al., 2004; Chen et al., 2007; Kato et al., 2014).

Besides simultaneous treatment, we also analyzed two other forms of animal treatment with ascorbic acid: pre and post treatment. In the pre treatment group, no protective effect of ascorbic acid was observed, which may be related to the low plasma concentration added to the short half-life of this antioxidant, which is of approximately 10 h (Schwedhelm et al., 2003). Studies demonstrating the antimutagenic capacity of ascorbic acid in pre treatment have administered the antioxidant more than once before treatment and simultaneously with the drug (Giri et al., 1998; Vijayalaxmi and Venu, 1999; Cheng et al., 2003; Franke et al., 2005; Roy et al., 2008). On the other hand, in the post treatment group there was a reduction in genotoxic damage (Fig. 1D), which approached baseline control, with an increase in class 0 score (Fig. 1C). However, in this group, the frequency of micronucleated cells was not reduced, which may be related to the low serum level of ascorbic acid in this exposure route.

Based on the results obtained in uninfected animals, the ascorbic acid response was investigated in animals infected with *L. infantum* under repeated doses treatment with miltefosine (Impavido®). We observed that, although there was no significant difference between the damage scores (Fig. 2), ascorbic acid (15 mg/kg/day) showed a slight reduction in the damage caused by the drug (2.5 mg/kg/day). In addition, corroborating with single dose treatment data, in the repeated doses treatment the miltefosine (Impavido®) was genotoxic, although it was not mutagenic at the doses studied (5.0 and 2.5 mg/kg/day) (Table 4). Both damage (genotoxic and mutagenic) induced by miltefosine (Impavido®) were demonstrated by our group in single dose tretament. We have suggested that miltefosine (Impavido®) is a drug that damages DNA by oxidative stress, with guanine oxidation being the most common damage, forming 8-oxo-7,8-dihydroguanine (Castelo

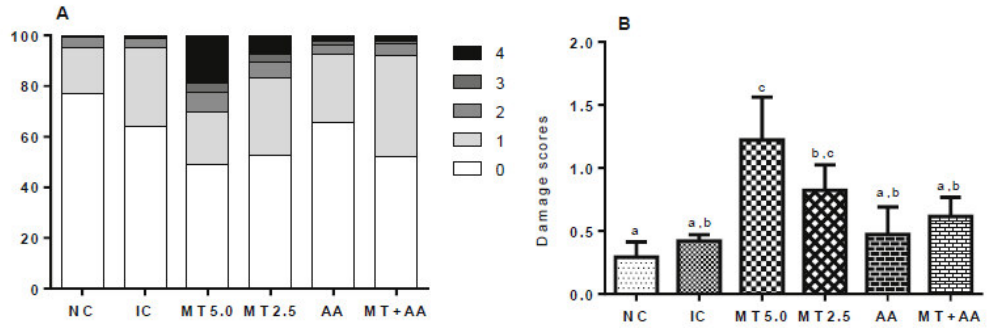

Fig. 2. Analysis of the antigenotoxic activity of ascorbic acid in Balb/c mice infected with *L. infantum* on the genotoxic damages induced by the cronic treatment with miltefosine (Impavido®).
Different letters denote statistical difference between the groups by ANOVA, followed by Tukey's *ad hoc* test.
A: Frequency of nucleoid classes observed by the Comet Assay; **B:** and their respective damage scores.
NC: negative control (distilled water); **IC**: infected control; **MT5.0**: treatment with miltefosine (5.0 mg/kg for 14 days); **MT2.5:** treatment with miltefosine (2.5 mg/kg for 28 days); **AA**: treatment with ascorbic acid (15 mg/kg for 28 days); **MT + AA**: treatment with ascorbic acid + miltefosine (15 mg/kg + 2.5 mg/kg for 28 days, respectively).

Table 4
Percentage of micronucleated cells in the bone marrow of Balb/c mice, infected with L. infantum, in chronic treatment with orally introduced miltefosine (Impavido®) and antioxidant effect of ascorbic acid.

GROUPS	PCEMNs	‰ MEDIA ± SD	PCE/NCE
NC	27	2.7 ± 0.89[a]	0.99
IC	31	3.1 ± 1.92[a]	1.14 [ns]
MT5.0	35	4.4 ± 1.50[a]	1.05 [ns]
MT2.5	28	2.8 ± 2.30[a]	1.27 [ns]
AA	28	3.5 ± 2.16[a]	1.06 [ns]
MT + AA	22	2.8 ± 2.08[a]	1.09 [ns]

[a] Absence of statistical difference between the groups by χ^2 test.
[ns] absence of statistical difference ($p > 0.05$) compared to the negative control group by ANOVA, followed by Tukey's *ad hoc* test.
NC: negative control (distilled water); **IC**: infected control; **MT5.0:** treatment with miltefosine (5.0 mg/kg for 14 days); **MT2.5**: treatment with miltefosine (2.5 mg/kg for 28 days); **AA**: treatment with ascorbic acid (15 mg/kg for 28 days); **MT + AA**: treatment with miltefosine + ascorbic acid (2.5 mg/kg + 15 mg/kg for 28 days, respectively). To determine micronuclei frequency, 2000 polychromatic erythrocytes were counted per animal, and 1000 cells were counted for polychromatic and normochromatic erythrocytes ratio (PCE/NCE).

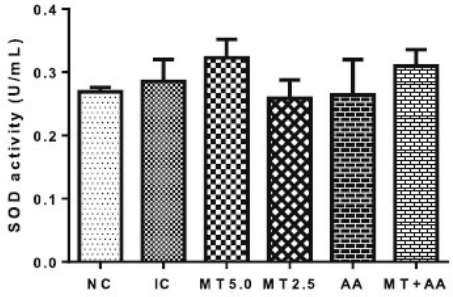

Fig. 3. Activity of antioxidant enzyme Superoxide dismutase (SOD).

Branco et al., 2016).

In response to this stress and to maintain genomic stability, cells activate DNA repair processes and transcription factors, which in turn modulate ROS-scavenging expression levels. Among them, the 8-oxoguanine glycosylase (OGG1), muty DNA glycosylase (MUTYH) and homologous mutT 1 (MUTH1) enzymes, which constitute the 8-oxoG repair pathway (Barnes and Lindahl, 2004), are activated, as well as the transcription factor Yap1, which positively regulates several genes related to the antioxidant defense (catalase, superoxide dismutase and glutathione peroxidase) (Rowe et al., 2008). Thus, our data suggest that genomic damage induced by miltefosine (Impavido®) may have been efficiently corrected by the DNA repair cell system, probably because the doses in the animals were lower than the equivalent therapeutic doses used in humans, as reported by Nair and Jacob (2016). In addition, we did not observe an increase in the activity of the SOD enzyme (Fig. 3), supporting the lack of mutagenic effect of this antileishmanial, including in the group with the infection alone (IC). Similar results were obtained by Moreira et al. (2017) who also found that the infection alone does not affect the levels of this enzyme.

Considering that some antileishmanial drugs exert their activity through the induction of oxidative stress (Basu et al., 2006; Castelo Branco et al., 2016; Moreira et al., 2017), we also evaluated whether the antioxidant action of ascorbic acid interferes with the antileishmanial activity of miltefosine. Our results have shown that the concurrent administration of ascorbic acid and miltefosine (Impavido®) did not interfere in the drug efficacy, as we have verified a reduction of 81% in the lymph node parasitic burden (Fig. 4). Similar results were observed in mice treated with antileishmanial Glucantime® and treated with the same dose of ascorbic acid used in this study (Kato et al., 2014); furthermore, the authors still observed that the antioxidant improved the drug's efficacy in suppressing parasites. It is also interesting to highlight that in the present study we also observed that infected animals treated only with ascorbic acid also had a significant reduction of *L. infantum* in the lymph node (84% reduction), showing that this antioxidant contributes to the fight against infection (Fig. 4).

In infected animals treated only with the drug, we found that, even at a dose lower than the equivalent human dose, miltefosine (Impavido®) was effective in reducing the parasite burden of *L. infantum*, with a reduction of 86% (at a dose of 5.0 mg/kg) and 88% in the lymph node (2.5 mg/kg) and 99% in the spleen (5.0 mg/kg) (Fig. 4). Other studies have also shown the efficacy of miltefosine action on the parasite burden in the spleen of *L. infantum* infected animals, with a reduction of 70 and 75% (10 and 20 mg/kg respectively for 5 days) (Kuhlencord et al., 1992), 78% (20 mg/kg for 5 days) (Le Fichoux et al., 1998) and 86% (20 mg/kg for 10 days) (Reimão et al., 2015). Therefore, our data show that lower doses of miltefosine during 14 days of treatment have equal or better efficacy in reducing parasitic burden.

In summary, our data showed that ascorbic acid was able to reduce the parasite load, besides protecting the mammalian DNA against

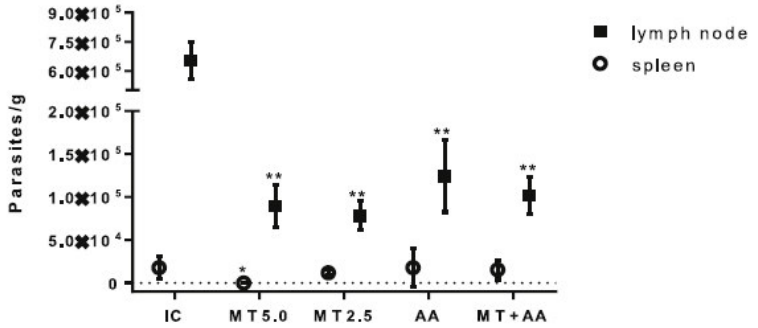

Fig. 4. Effect of ascorbic acid on the parasite load of Balb/c mice infected with L. infantum and treated with miltefosine (Impavido®) and ascorbic acid.

genotoxic damage caused by the antileishmanial, opening perspectives for future studies in order to minimize the harmful effects on DNA, besides being potentially capable of reducing infection.

5. Conclusion

Our data show that ascorbic acid reduces the genetic damage induced by miltefosine, without decreasing its antileishmanial activity. In addition, the results reveal that miltefosine is effective in reducing the parasitic burden of *L. infantum* at lower doses than others previously reported.

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CAPÍTULO 3

Ácido ascórbico modula atividade da enzima SOD e do gene de reparo *OGG1* em camundongos infectados com *L. infantum* e tratados com miltefosina

(a submeter)

Ácido ascórbico modula atividade da enzima SOD e do gene de reparo *OGG1* em camundongos infectados com *L. infantum* e tratados com miltefosina

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RESUMO

As leishmanioses compreendem um grupo de doenças infecto parasitárias e crônicas não contagiosas, altamente negligenciadas, ocasionadas por protozoários do gênero *Leishmania*. A leishmaniose visceral tem atualmente ocorrência descrita em 102 países. O Brasil se destaca com altas taxas de casos dessa doença, sendo registrado somente em uma década (2008 a 2017), 37.639 casos e 2.735 óbitos. A miltefosina é antileishmanial de uso oral usado desde 2002 em países como Índia, Bangladesh e Nepal. Demonstramos inicialmente que a miltefosina causa lesões genômicas em células de mamíferos com oxidação de bases do DNA e que esse dano pode ser modulado pelo ácido ascórbico. Assim, este trabalho verificou como se comportam as enzimas antioxidantes SOD, CAT e GPx, bem como os genes do sistema de reparo *OGG1*, *MUTHY* e *NUDT1,* em animais infectados com *Leishmania infantum* sob tratamentos com miltefosina e ácido ascórbico. Nossos resultados reafirmaram a genotixicidade da miltefosina e revelaram a oxidação de bases púricas e pirimídicas. O ácido ascórbico também atuou como genotóxico, causando estresse oxidativo por oxidação de bases púricas somente. Porém, o ácido ascórbico não interferiu na eficácia da miltefosina e modulou positivamente o sistema de reparo antioxidante contra os radicais livres produzidos pela miltefosina.

Palavras-chave: Leishmaniose, ácido ascórbico, enzimas antioxidantes, expressão gênica.

1. Introdução

As leishmanioses compreendem um grupo de doenças infecto parasitárias e crônicas não contagiosas, ocasionadas por protozoários do gênero *Leishmania*. Tratam-se de patologias com distintas manifestações clínicas, sendo reconhecidas quatro formas principais: leishmaniose cutânea (LC), leishmaniose mucocutânea (LMC), leishmaniose visceral (LC, também conhecida como calazar) e leishmaniose dérmica pós calazar (LDPC) (WHO, 2016).

A LV tem atualmente ocorrência descrita em 102 países, sendo 94% dos casos no Brasil, Etiópia, Índia, Quênia, Somália, Sudão do Sul e Sudão (WHO, 2019). O Brasil se destaca com altas taxas de casos dessa doença, onde registrou-se, somente em uma década (2008 a 2017), 37.639 casos e 2.735 óbitos (Brasil et al., 2019).

Um dos medicamentos recomendados pela OMS para o tratamento de leishmanioses no Brasil é o antimoniato N-metil glucamina (Brasil, 2014), embora estudos tenham relatado sua alta toxicidade (Demicheli et al., 2002; Kato et al., 2014), bem como sua capacidade de induzir danos genotóxicos e mutagênicos (Lima et al., 2010; Moreira et al., 2017; de Jesus et al., 2018). Além disso, foi demonstrado vários mecanismos de resistência do parasita ao antimônio, como diminuição da internalização do fármaco, deleção ou expressão reduzida do gene *AQP1*, redução biológica diminuída de SbV para SbIII e aumento dos níveis de tripanotiona (Ponte-Sucre et al., 2017).

Diante de um cenário parecido, com resistência ao tratamento com N-metil-glutamina, desde 2002 países como Índia, e, posteriormente, Bangladesh e Nepal começaram a utilizar a miltefosina para tratamento antileishmanial (Sundar e Olliaro, 2007; Singh et al., 2016). A descoberta da miltefosina como agente antileishmanial (Impavido®) foi considerado um grande avanço para o tratamento da leishmaniose, por ser uma droga oral e efetiva, inclusive em casos de infecções resistentes à terapia convencional com antimonial pentavalente (Sundar et al., 2002).

Devido a falta de estudos sobre o efeito genotóxico da miltefosina, nosso laboratório investigou como essa droga interagia com o DNA. Assim, foi demonstrado que a miltefosina (Impavido®) causa lesões genômicas em células de mamíferos, com oxidação de bases púricas do DNA (Castelo Branco et al., 2016). A oxidação de bases é decorrente do estresse oxidativo e é causada pelo desequilíbrio entre espécies reativas de oxigênio (ROS) e o sistema de defesa antioxidante, danificando as principais biomoléculas como proteínas, lipídios (Bento et al., 2013) e o DNA (de Jesus et al., 2018; Castelo Branco et al., 2016).

O dano mais importante causado por ROS no DNA é a oxidação da guanina, produzindo 8-oxoguanina (8-oxoG) no DNA e 8-hidroxi-guanina (8-OH-dGTP) em nucleotídeos livres. A 8-oxoG ocasiona transversões do tipo G:C para T:A nas fitas do DNA, que em oncogenes ou genes supressores de tumor pode levar à carcinogênese (Garre et al., 2011; Nascimento et al., 2017).

Para prevenir mutações por transversões, o DNA possui um sistema de reparo que retira as bases pareadas erroneamente, chamado sistema de excisão de bases (BER). O sistema BER é a via primária de reparo do DNA (Berra et al., 2006) e seus principais genes são *OGG1* (8-oxoguanina DNA glicosilase 1), *MUTHY* (muty DNA glicosilase) e *NUDT1* (nudix hidrolase 1). Os genes *MUTHY* e *OGG1* codificam DNA-glicosilases que têm como função remover as bases oxidadas erroneamente pareadas, evitando mutações por transversões do tipo G:C→T:A (Nascimento et al., 2017). O gene *NUDT1* age indiretamente no sistema BER e codifica uma enzima que hidrolisa 8-oxo-dGTP para 8-oxo-dGMP, previnindo, assim, a incorporação de 8-oxoG no DNA durante a replicação (Burton e Rai, 2015).

A produção de ROS pode também ser equilibrada pela existência de defesas antioxidantes, incluindo enzimas que removem ROS (superóxido dismutase, catalase, peroxidase, etc.), proteínas que sequestram íons de metais de transição (ferritina, transferrina), peptídeos de baixo peso molecular e cofatores (glutationa, NADPH, tiorredoxina, etc.) e agentes dietéticos de baixo peso molecular solúveis em água que limpam espécies reativas de oxigênio e nitrogênio (vitamina E, vitamina C, beta-caroteno, etc.) (Vasconcelos et al., 2007; Nimse e Pal, 2015).

Nosso grupo tem demonstrado que o ácido ascórbico (vitamina C) é um importante antioxidante contra o estresse oxidativo causado por drogas antileishmaniais, sem diminuir a eficácia da droga (de Jesus et al., 2018; Castelo Branco et al., 2019). Sabe-se que o ácido ascórbico participa da neutralização de formas reativas de oxigênio e nitrogênio que surgem no metabolismo celular. Através da doação de hidrogênio, ele neutraliza os radicais hidroxila de curta duração, oxi-álcool, superóxido e nitrogênio, com a formação de radicais ascórbidos estáveis e não reativos (Konopacka, 2004). Porém, dependendo do ambiente fisiológico, o ácido ascórbico pode agir como antioxidante (doador de elétrons) ou pró-oxidantes (receptor de elétrons) (Harréus et al., 2005).

Sabendo que a miltefosina induz estresse oxidativo e visando analisar a participação de vitaminas antioxidantes na manutenção da integridade do genoma celular, este trabalho

verificou como se comportam as enzimas antioxidantes SOD, CAT e GPx, bem como os genes do sistema de reparo *OGG1*, *MUTHY* e *NUDT1,* em animais infectados com *Leishmania infantum* sob tratamentos com miltefosina e ácido ascórbico.

2 MATERIAL E METODOS

2.1 Animais

Foram utilizados camundongos machos (n = 5 por grupo) Balb/c *Mus musculus* provenientes do Biotério da Universidade de Campinas (São Paulo, Brasil) com peso médio corporal de 30g e cerca de 60 dias de vida. Os animais foram mantidos em caixas de policarbonato em condições controladas: temperatura em torno de 21 ± 2ºC e ciclo alternado de claro/escuro de 12 horas. Este projeto foi aprovado pelo Comitê de Ética em Experimentação Animal (CEUA) da UFMA (nº 23115.029807/2018-70).

2.2 Parasitas

Formas promastigotas de *L. infantum* (MHOM/BR/1970/BH46) foram cultivadas em meio Schneider (Sigma, St Louis, MO, USA), o qual foi suplementado com 10% de soro fetal bovino inativado (GIBCO) e acondicionadas em estufa a 27°C. Para os ensaios, os camundongos foram infectados na pata traseira direita com 10^7 promastigotas em fase estacionária de *L. infantum*. A infecção foi mantida por 28 dias e no 29° dia foram realizados os tratamentos com o antileishmanial.

2.3 Tratamento

Os animais foram divididos em cinco grupos de tratamento: Grupo NC – controle negativo (animais não infectados que receberam água destilada); Grupo IC – controle infectado (animais infectados que receberam água destilada); Grupo MT – animais infectados com *L. infantum* tratados com miltefosina (30 mg/Kg/dia durante 28 dias, via oral); Grupo MT + AA – animais infectados com *L. infantum* tratados com miltefosina (30 mg/Kg/dia durante 28 dias, via oral) mais ácido ascórbico (30 mg/Kg/dia durante 28 dias, via i.p.); Grupo AA – animais infectados tratados com ácido ascórbico (30 mg/Kg/dias durante 28 dias, via i.p.). Para a quantificação da carga parasitária foi incluído um grupo de animais (Grupo IC-i), sacrificados 28 dias após a infecção.

A dose utilizada da miltefosina (Impavido® - lote 1C2130A) foi baseada na conversão da dose humana, recomendada pela Organização Mundial de Saúde (WHO), para a dose animal

(Nair e Jacobe, 2016). Para isso, multiplicou-se a dose da miltefosina (2.5 mg/Kg/dia) pela constante da dose equivalente para camundongo (12.3), obtendo-se 30 mg/Kg/dia. A dose de 30 mg/Kg via i.p. do ácido ascórbico (Sigma-Aldrich, St. Louis, MO, lote 69085) foi baseada em um estudo anterior, onde detectou-se seu feito antioxidante (Castelo Branco et al., 2019). Ambas as drogas foram dissolvidas em água destilada.

2.4 Ensaios

Ao final de 28 dias de tratamento os animais foram submetidos aos ensaios. Inicialmente realizou-se o teste do cometa para avaliacao genotóxica. Posteriormente os animais foram anestesiados com xilazina (20 mg/Kg) e ketamina (100 mg/Kg) para coleta do sangue por punção cardíaca, seguindo-se os ensaios para dosagem de enzimas antioxidantes, avaliação da carga parasitária e expressão gênica, conforme descrito a seguir.

2.4.1 Ensaio do cometa (Singh et al. 1988; Tice et al. 2000)

Para a análise da genotoxicidade da miltefosina e do ácido ascórbico foi realizado o ensaio do cometa com sangue da cauda dos animais e misturado com 100 µl de agarose baixo ponto de fusão (0,5%), sendo essa mistura transferida para uma lâmina pré-revestida com agarose com ponto de fusão normal (1,5%). Em seguida, lamínulas foram sobrepostas nessas lâminas e refrigeradas por 15 min. Após a solidificação, as lâminas foram imersas em solução de lise (2,5 M de NaCl, 100 mM de EDTA, 10 mM de Tris, 10% de dimetilsulfóxido, 1% de Triton X-100, pH 10) e refrigeradas overnight a 4ºC. Posteriormente, as lâminas foram incubadas em solução alcalina (NaOH 10 M, 0,2 M EDTA e água destilada, pH 13) durante 20 minutos a 4ºC, seguida por eletroforese a 25 V e 300 mA durante 30 min. As lâminas foram neutralizadas (0,4 M de Tris/HCl, pH 7,5), secadas à temperatura ambiente e fixadas em etanol 100% por 4 minutos. Em seguida, as lâminas foram coradas com 20 µg/mL de brometo de etídio e analisadas em microscópio de fluorescência (Olympus BX61).

Para o ensaio do cometa modificado utilizou-se as enzimas formamidopirimidina glicosilase (FPG) e endonuclease III (ENDOIII), após o período na solução de lise, as lâminas foram lavadas, três vezes de 5 minutos cada, com tampão (40 mM HEPES, 0,1M KCl, 0,5mM EDTA, 0,2 mg/mL BSA, pH 8.0 com KOH). As enzimas foram diluídas conforme recomendação do fabricante e acrescentadas nas lâminas (50 µl de ENDO III e 75 µl de FPG). Posteriormente, foram incubadas por 30 minutos (FPG) e 45 minutos (ENDO III) a 37ºC (New

England Biolabs®). As lâminas foram lavadas uma vez com tampão PBS e colocadas em cuba de eletroforese, seguindo-se as demais etapas do ensaio do cometa descritas anteriormente.

Duzentos nucleoides foram analisados por animal considerando o tamanho e a quantidade de DNA presente na cauda do cometa. Os danos foram classificados em 5 níveis: Classe 0 - nenhum dano (<5%); Classe 1 - baixo nível de danos (5-20%); Classe 2 - nível moderado de danos (20-40%); Classe 3 - alto nível de danos (40-94%); Classe 4 - dano total (> 95%) (Speit e Hartmann, 1995). O escore de danos foi obtido através da multiplicação do número de nucleoides em cada classe pelo respectivo valor da classe, usando a equação:

Escore = [(0 x n0) + (1 x n1) + (2 x n2) + (3 x n3) + (4 x n4)] / total do número de células

2.4.2 Dosagem de enzimas antioxidantes

A dosagem das enzimas antioxidantes SOD, GPx e CAT foi realizada a partir do plasma dos animais, seguindo-se as recomendações do fabricante (Cayman Chemical, Ann Arbor, MI, USA).

2.4.3 Ensaio de Diluição Limitante (Rodrigues et al., 2009)

Resumidamente, o baço e o linfonodo foram colhidos assepticamente e homogeneizados individualmente em 1.0 ml de meio Schneider's Drosophila (Sigma-Aldrich, EUA) suplementado com 10% de soro fetal bovino inativado (GIBCO). Este ensaio foi realizado em duplicata. A suspensão com o órgão homogeneizado (25 µl) foi diluída em 1:8 no primeiro poço, contendo 175 µl do meio, totalizando um volume de 200 µl de suspensão. Do primeiro poço retirou-se 100 µl da suspensão e realizou-se a diluição de 1:2 até o 12º poço da placa de 96 poços. Após 14 dias, analisou-se a amostra de cada poço e definiu-se como *positivo* ou *negativo* dependendo da presença ou ausência de promastigotas no poço. O título final foi definido como a diluição mais elevada para a qual o poço continha pelo menos um parasita ativo. O número de parasitas por grama do órgão homogeneizado foi calculado como se segue: a recíproca do último título positivo pelo total do volume homogeneizado do órgão x fator de diluição dividido pelo peso em gramas do órgão homogeneizado. A carga parasitária viável foi expressa como o número de leishmania por grama de órgão homogeneizado.

2.4.4 Expressão gênica por PCR em tempo real (Livak e Schmittgen, 2001).

O RNA foi extraído do fígado dos animais (100 mg) usando Trizol® (Invitrogen) de acordo com as recomendações do fabricante. A transcrição reversa foi realizada utilizando-se o kit High Capacity cDNA Reverse Transcription Kit™ (ThermoFisher™). A reação foi feita de acordo com a especificação do fabricante. O cDNA obtido foi diluído em água destilada estéril na razão 1:5.

As reações de PCR quantitativa em tempo real foram realizadas em sistema TaqMan Universal PCR Master Mix (Applied Biosystems, EUA). Utilizou-se 2 µL de cDNA de cada amostra mais 8 µL do mix (2,5 µL de nuclease free water, 5,0 µL de Taqman Universal PCR Master Mix e 0,5 µL de cada primer), com volume total de 10 µL por reação. Todas as reações foram feitas em triplicata. As condições de amplicação seguiram as recomendações do fabricante.

Em cada ensaio, utilizou-se como amostra de referência o *pool* de DNA dos animais do grupo CN (grupo não infectado e não tratado). Os dados foram normalizados com o gene gliceraldeído-3-fosfato-desidrogenase (*GAPDH*) amplificado em cada ensaio para o cálculo da expressão relativa dos genes de reparo *OGG1, MUTYH, NUDT1* (ThermoFisher™).

2.5 Análise Estatística

A normalidade dos dados foi analisada pelo teste de Kolmogorov-Smirnov. Os dados numéricos obtidos pelos ensaios do cometa, ensaio de diluição limitante, dosagem de enzimas antioxidantes e expressão gênica foram submetidos à análise de variância 1 critério (ANOVA), seguido do pós-teste de Tukey. Os dados do cometa seguido de digestão enzimática foram submetidos ao teste T pareado. Todos os dados foram analisados utilizando o software GraphPad Prism 6.01, com valores considerados significativos quando $p < 0.05$.

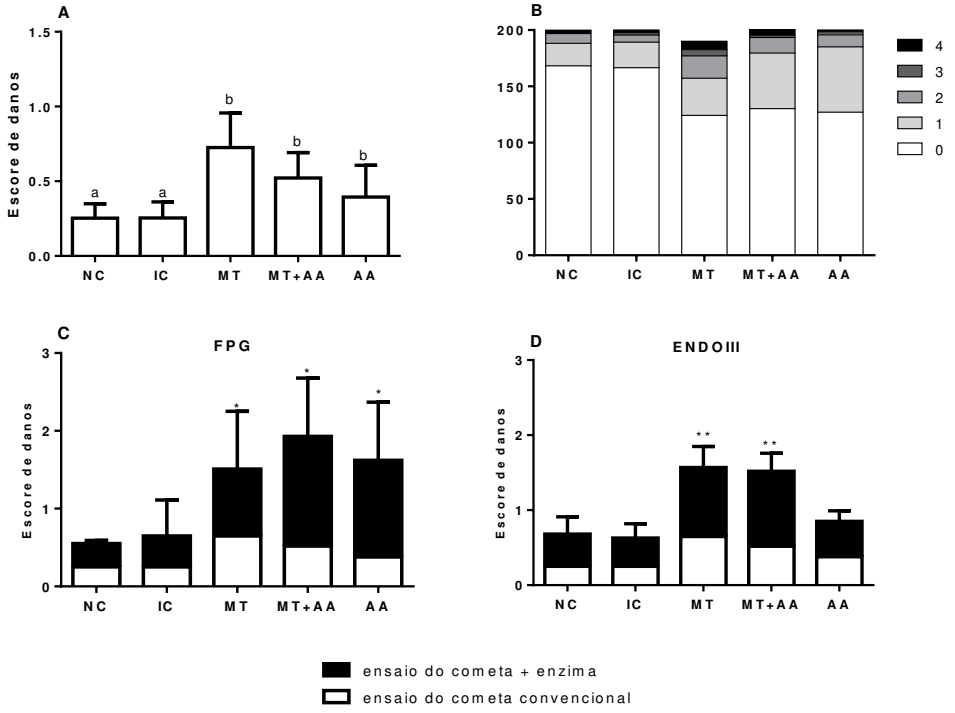
3 RESULTADOS

Miltefosina causa oxidação de purinas e pirimidinas

O ensaio do cometa convencional mostrou que a miltefosina, assim como o ácido ascórbico, apresentam ação genotóxica ($p < 0.05$) nas condicões experimenatis testadas (Figura 1A). Nesses grupos verificou-se principalmente aumento de danos de classes 2 e 3 (Figura 1B).

No ensaio do cometa modificado, a adição de ambas as enzimas de reparo, FPG e ENDO III, revelou danos oxidativos causados pela miltefosina ($p < 0.05$), inclusive na presença do ácido ascórbico (Figura 1C e 1D). Observa-se também que os animais tratados somente com ácido ascórbico induzem danos oxidativos apenas na presença da enzima FPG.

Figura 1. Escore de danos no DNA detectado pelo ensaio do cometa convencional (A e B) e pelo ensaio do cometa seguido da digestão das enzimas FPG (C) e ENDO III (D) em camundongos Balb/c infectados com *L. infantum* e tratados com miltefosina e ácido ascórbico.



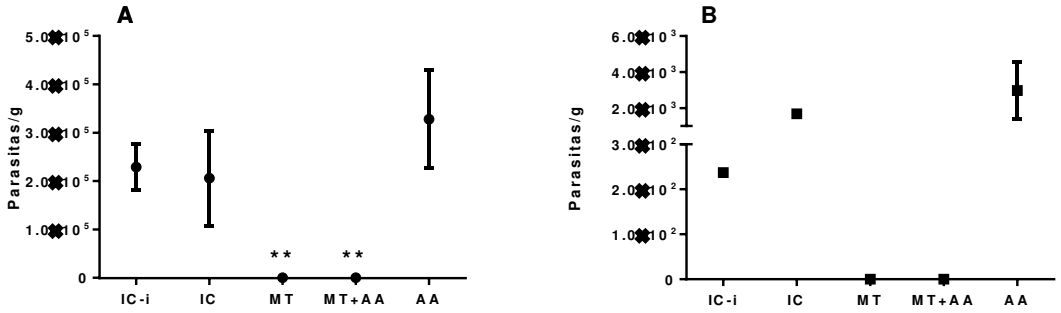
LEGENDA: A: Frequência de classes de nucleoides observadas pelo ensaio do cometa; B: Ensaio do cometa sem adição de enzima. ANOVA seguido do pós teste de Tukey.
C e D: Ensaio do cometa com adição das enzimas FPG e ENDO III, respectivamente. Teste T pareado.
* $p < 0.05$; ** $p < 0.01$; Letras diferentes denotam diferença estatística ($p < 0.05$),
NC: controle não infectado e não tratado; IC: controle infectado com *L. infantum*; MT: grupo tratado com miltefosina (30 mg/Kg durante 28 dias); MT + AA: grupo tratado com miltefosina e ácido ascórbico (30 mg/Kg durante 28 dias cada); AA: grupo tratado com ácido ascórbico (30 mg/Kg durante 28 dias).

O ácido ascórbico não interfere na eficácia da miltefosina

Também demonstramos que tanto nos animais tratados somente com miltefosina quanto nos animais tratados simultaneamente com miltefosina e ácido ascórbico houve redução

significativa da carga parasitária no linfonodo, enquanto no baço essa redução não foi estatisticamente significativa (Figura 2).

Figura 2. Efeito do ácido ascórbico na carga parasitária do linfonodo (A) e baço (B) de camundongos Balb/c infectados com *L. infantum* tratados com miltefosina.

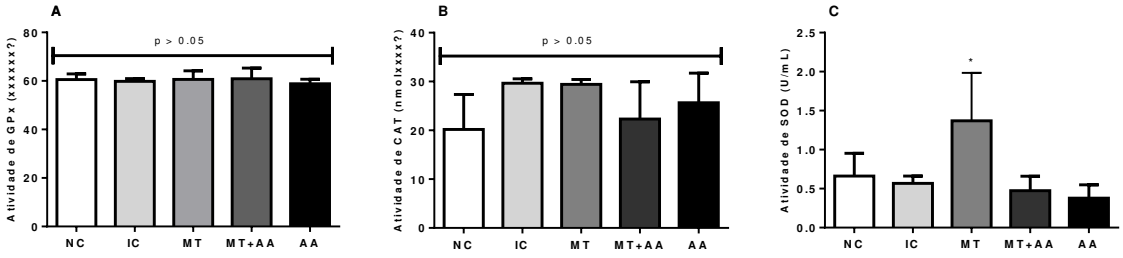


LEGENDA: ** $p < 0.01$, ANOVA seguido pelo pós-teste de Tukey.
IC-i: controle infectado com *L. infantum* (28 dias de infecção somente); IC: controle infectado com *L. infantum* mais 28 dias recebendo água destilada; MT: grupo tratado com miltefosina (30 mg/Kg durante 28 dias); MT + AA: grupo tratado com miltefosina e ácido ascórbico (30 mg/Kg durante 28 dias cada); AA: grupo tratado com ácido ascórbico (30 mg/Kg durante 28 dias).

Miltefosina aumenta a atividade da enzima SOD

A Figura 3 mostra que o antileishmanial miltefosina aumentou a atividade da enzima SOD ($p < 0.05$), enquanto o ácido ascórbico foi capaz de modular esse feito (Figura 3). Para as enzimas GPx e CAT não houve alteração da atividade enzimática em nenhum dos grupos ($p > 0.05$).

Figura 3. Efeito do ácido ascórbico na atividade das enzimas antioxidantes glutationa peroxidase (A), catalase (B) e superóxido dismutase (C) em camundongos Balb/c infectados com *L. infantum* tratados com miltefosina.



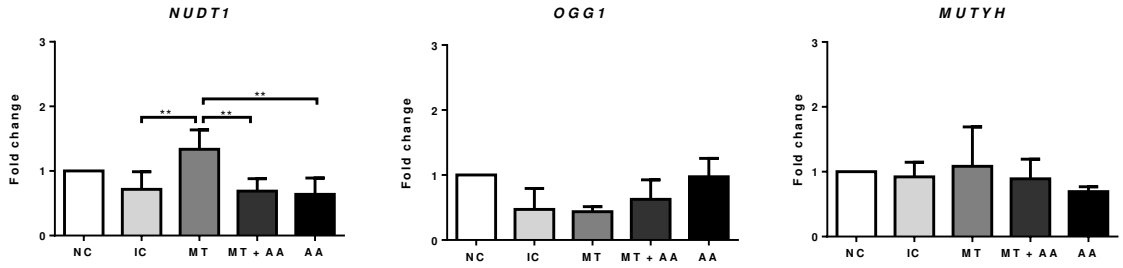
LEGENDA: * $p < 0.05$, ANOVA seguido pelo pós-teste de Tukey.

NC: controle não infectado e não tratado; IC: controle infectado com *L. infantum*; MT: grupo tratado com miltefosina (30 mg/Kg durante 28 dias); MT + AA: grupo tratado com miltefosina e ácido ascórbico (30 mg/Kg durante 28 dias cada); AA: grupo tratado com ácido ascórbico (30 mg/Kg durante 28 dias).

Miltefosina aumenta a atividade do gene de reparo *OGG1*

Os animais infectados e tratados com miltefosina apresentaram maior expressão do gene *OGG1* ($p < 0.001$). Interessantemente, quando esses animais foram tratados simutanemente com miltefosina e ácido ascórbico não foi observada alteração na expressão desse gene (Figura 4). Os genes *NUDT1* e *MUTHY* não apresentaram expressão diferenciada em nenhum dos grupos.

Figura 4. Expressão diferencial dos genes de reparo *NUDT1, OGG1* E *MUTHY* em camundongos Balb/c infectados com *L. infantum* tratados com miltefosina e ácido ascórbico.



LEGENDA: *** $p < 0.001$, ANOVA seguido do pós-teste de Tukey.
NC: controle não infectado e não tratado; IC: controle infectado com *L. infantum*; MT: grupo tratado com miltefosina (30 mg/Kg durante 28 dias); MT + AA: grupo tratado com miltefosina e ácido ascórbico (30 mg/Kg durante 28 dias cada); AA: grupo tratado com ácido ascórbico (30 mg/Kg durante 28 dias).

4 DISCUSSÃO

Nos últimos anos tem sido demonstrado o efeito genotóxico e mutagênico de drogas antileishmaniais. Nosso grupo mostrou que tanto o N-metil-glutamina (Glucantime®) (Lima et al., 2010; de Jesus et al., 2018; Moreira et al., 2017) quanto a miltefosina (Impavido®) (Castelo Branco et al., 2016) causam lesões genômicas. Além disso, demonstrou-se que essas lesões decorrentes de estresse oxidativo são passíveis de correção pelo sistema antioxidante não enzimático, como isoflavonas (Cantanhêde et al., 2015), genisteína ou ácido ascórbico (de Jesus et al., 2017; Castelo Branco et al., 2019). Neste trabalho, sob condição de tratamento múltiplo

e utilizando dose huamana equivalente, ratificamos os danos genotóxicos causados pela miltefosina em bases purinas do DNA (Castelo Branco et al., 2016), além de demonstrarmos que a miltefosina também causa oxidação de bases pirimidinas.

Mostramos também que o ácido ascórbico, na dose utilizada, apresentou atividade genotóxica. Outros autores já relataram que o ácido ascórbico pode ter propriedade pró-oxidante (Duarte e Lunec, 2005; Harréus et al., 2005; Ergul et al., 2010), o que justifica que neste trabalha não tenha sido observada sua atividade antigenotóxica. Sabe-se que o ácido ascórbico contribui para a formação do dano oxidativo pela redução de íons férrico Fe^{3+} para Fe^{2+} e Cu^{2+} a Cu^{1+}, o que, por sua vez, pode reduzir peróxido de hidrogênio (H_2O_2) a radicais hidroxila (OH-). No entanto, em geral, essas reações, conhecidas como reações de Fenton, quando mediadas pelo ácido ascórbico são controladas no corpo humano devido ao sequestro eficiente de ferro por proteínas como ferritina e transferrina (Duarte e Lunec, 2005). Dessa forma, o estresse oxidativo observado pode ter sido ocasionado pela alta dose administrada nos animais somado à capacidade natural destes em sintetizar esse antioxidante (Drouin et al., 2011). De todo modo, o ácido ascórbico não interferiu na eficácia da miltefosina no combate aos parasitas do linfonodo (Figura 2).

Outro dado importante demonstrado neste estudo é o aumento da atividade da enzima SOD causado pela miltefosina, sendo este diminuído significativamente na presença de ácido ascórbico (Figura 3). Sabe-se que a SOD tem papel crucial no controle do estresse oxidativo em células eucarióticas (Van Assche et al., 2011) e que sua atividade é aumentada na presença de radicais superóxido (O_2^-) (Ergul et al., 2010), transformando-os em oxigênio (O_2) e peróxido de hidrogênio (H_2O_2). Segundo Smirnoff (2018) o ácido ascórbico, em altas concentrações, na sua forma ionizada (ascorbato) reage com radicais superóxido complementando a SOD na remoção de superóxido *in vivo*, justificando nossos achados.

Para restabelecer o equilíbrio redox na célula, a enzima CAT e/ou GPx deveria transformar o H_2O_2 em $H_2O + O_2$. Porém, não houve aumento de atividade dessas enzimas em nenhum dos grupos, sugerindo que o H_2O_2 produzido após a reação de dismutação do radical O_2^- possa ter sido transformado, pelas reações de Fenton ou Haber-Weiss, em radical hidroxila (OH·), que é altamente reativo podendo causar danos em todos os tipos de biomoléculas, incluindo proteínas, lipídios e DNA e não apresenta defesa enzimática especializada (Ribeiro et al., 2008).

Além disso, não foi observada alteração em nenhuma dessas enzimas nos animais do grupo controle infectado, diferente dos resultados de Moreira et al. (2017) que mostrou que *L. infantum per se* alterou os níveis das três enzimas. Isso se deve talvez ao período de infecção dos animais antes de receber tratamento antileishmanial, 45 dias, enquanto neste trabalho foi de apenas 28 dias.

Por fim, verificamos aumento na expressão do gene de reparo *OGG1* no grupo de animais tratados com a miltefosina e a diminuição da expressão desse gene nos animais co-tratados com ácido ascórbico. Esse gene codifica uma proteína que tem como função remover 8-oxoG. A oxo-G facilmente pareia-se com a adenina, provocando durante a replicação transversões do tipo G:C→T:A (Nascimento et al., 2017). Corroborando nossos resultados, Tarng et al. (2004) relataram não apenas uma regulação positiva da expressão de mRNA de *hOGG1* 24 h após a administração de ácido ascórbico, mas também uma diminuição nos níveis de 8-oxo-dG.

Por outro lado, não houve expressão diferencial dos genes *NUDT1* e *MUTYH* (dados não mostrados). O gene *NUDT1* previne a incorporação de 8-oxoG no DNA durante a replicação (Burton e Rai, 2015), enquanto o gene *MUTYH* atua retirando a adenina pareada erroneamente a 8-oxoG. Como o gene *NUDT1* não atuou, a 8-oxoG incorporou-se no DNA, daí a regulação positiva da expressão do gene *OGG1*. Por sua vez, com o funcionamento correto do gene *OGG1*, as bases oxidadas 8-oxoG são retiradas, não permitindo que a adenina faça o pareamento incorreto, onde atuaria o *MUTYH*.

Com base no exposto, talvez a estratégia mais promissora para o tratamento da leishmaniose seja a combinação de múltiplas terapias para aumentar a eficiência, diminuir o tempo do tratamento e resistência aos medicamentos. Diversos estudos têm demonstrado que a miltefosina é um antileishmanial efetivo, enquanto o ácido ascórbico, na dose adequada, protege as células contra estressores causados pela miltefosina, atuando como potente antioxidante e aumentando a capacidade de reparo celular.

5 CONCLUSÃO

Neste trabalho demonstramos que a miltefosina causa danos ao DNA por oxidação de pirimídicas, além de bases púricas. O ácido ascórbico também atuou como genotóxico, causando estresse oxidativo por oxidação de bases púricas. Contudo o ácido ascórbico não

interferiu na eficácia da miltefosina e modulou positivamente a enzima antioxidante SOD e o gene de reparo *OGG1* contra os radicais livres produzidos pela miltefosina.

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6 CONSIDERAÇÕES FINAIS

No Brasil, o antimoniato de meglumina ainda é o medicamento de primeira escolha no tratamento das leishmanioses. Porém o aumento de casos de morbidade e mortalidade revelam as falhas de tratamento decorrentes do uso desse antimônio. A miltefosina é uma droga não antimonial, de uso oral, considerada eficaz para o tratamento das leishmanioses. Com apenas uma década e meia de uso, sua eficácia foi bastante relatada em diversos trabalhos no mundo, embora seus primeiros relatos de recidivas e resistências também. Contudo, não havia relatos sobre a interação desta droga com o material genético. Neste trabalho demonstramos que a miltefosina causa danos ao DNA por oxidação de bases púricas e pirimídicas, além de apoptose e necrose. Demonstramos ainda que o antioxidante ácido ascórbico consegue reparar esses danos, não interferindo na eficácia da droga, modulando positivamente a enzima antioxidante SOD e o gene de reparo *OGG1* contra os radicais livres produzidos pela miltefosina.

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ANEXOS

ANEXO A. Parecer consubstanciado do Comitê de Ética em Pesquisa Humana.

UNIVERSIDADE FEDERAL DO MARANHÃO/MA

PARECER CONSUBSTANCIADO DO CEP

DADOS DO PROJETO DE PESQUISA

Título da Pesquisa: TOXICOGENÉTICA DE MEDICAMENTOS ANTILEISHMANIAIS E ANTIHANSÊNICOS
Pesquisador: SILMA REGINA FERREIRA PEREIRA
Área Temática:
Versão: 2
CAAE: 48119215.6.0000.5087
Instituição Proponente: FUNDACAO UNIVERSIDADE FEDERAL DO MARANHAO
Patrocinador Principal: FUND DE AMPARO A PESQUISA AO DESEN CIENTIFICO E TECNOLOGICO DO MARANHÃO - FAPEMA

DADOS DO PARECER

Número do Parecer: 1.284.493

Apresentação do Projeto:
As leishmanioses e a hanseníase são endêmicas no Maranhão, sendo que os tratamentos dessas doenças são realizados por medicamentos recomendados pela OMS e Ministério da Saúde, apesar dos seus reconhecidos efeitos tóxicos. Os mecanismos de ação desses fármacos também não são completamente esclarecidos, inclusive a interação desses compostos com o material genético, apesar de que, desde a década de 1980, os
órgãos de saúde pública e as agências ambientais, em vários países industrializados, acrescentaram a mutagenicidade à lista das propriedades tóxicas a serem avaliadas antes que agentes químicos, aditivos alimentares e medicamentos fossem introduzidos no mercado uma vez que estudos mostram que cerca de 80 dos agentes mutagênicos são carcinogênicos. A carência de trabalhos que investiguem os danos genéticos que os antileishmaniais e antihansênicos podem causar nos pacientes reflete a falta de política das indústrias farmacêuticas em avaliar os efeitos colaterais de produtos utilizados no tratamento de doenças negligenciadas prevalentes em países em desenvolvimento. Tão pouco há investimento significativo na busca de novas drogas para tratamento dessas doenças. Considerando-se a escassez de trabalhos investigando os mecanismos pelos quais as drogas antileishmaniais e antihansênicas agem, bem como a crescente busca de medidas que reduzam os efeitos tóxicos desses medicamentos, propomos o

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desenvolvimento deste trabalho a fim de esclarecer o possível efeito genotóxico nos pacientes infectados e em
tratamento, no Estado do Maranhão, bem como avaliar se agentes reconhecidamente antimutagênicos, como a genisteína e a vitamina C, são capazes de agir como quimioprotetores sobre a genotoxicidade induzida por esses fármacos.

Objetivo da Pesquisa:

Objetivo Primário:

Avaliar a genotoxicidade e mutagenicidade de antileishmaniais (Glucantime e Metilfosine) e antihansênicos (Rifampicina, Dapsona e Clofazimina) de modo a determinar o potencial risco de indução de lesões genômicas em pacientes sob tratamento com esses medicamentos.

Objetivo Secundário:

- Analisar os efeitos da vitamina C sobre a modulação da genotoxicidade induzida pelo Glucantime® em camundongos.
- Avaliar o potencial genotóxico e mutagênico do fármaco miltefosina em cultura de células humanas in vitro.
- Analisar os efeitos genotóxicos e mutagênico do tratamento com poliquimioterapia MB e PB em pacientes com hanseníase.

Avaliação dos Riscos e Benefícios:

Riscos:

Este projeto confere riscos mínimos aos voluntários uma vez que será feita apenas uma punção venosa para retirada de 5ml de sangue. É garantida a manutenção do sigilo e da privacidade dos participantes da pesquisa durante todas as fases da pesquisa. Os resultados das análises genéticas para fins de divulgação científica sempre serão feitos considerando-se o grupo amostral, e não individualmente. Esses procedimentos garantem a
confidencialidade e a privacidade, a proteção da imagem e a não estigmatização dos participantes da pesquisa, garantindo a não utilização das informações em prejuízo das pessoas, inclusive em termos de auto-estima.

Benefícios:

- Conhecimento do potencial genotóxico e mutagênico de medicamentos utilizados no tratamento de doenças infecciosas endêmicas no Maranhão.
- Consolidação de Grupo de Pesquisa no Maranhão que se dedica a caracterização e avaliação de atividade biológica de compostos químicos fortalecendo cooperações científicas e transferência de tecnologias entre a Universidade Federal do Maranhão e outros centros do país.

UNIVERSIDADE FEDERAL DO MARANHÃO/MA

Continuação do Parecer: 1.284.493

Comentários e Considerações sobre a Pesquisa:

A pesquisa esta muito bem elaborada com solido suporte cientifico excelentes objetivos e materiais e métodos de excelente padrão.

Considerações sobre os Termos de apresentação obrigatória:

Todos os termos foram apresentados e estão de acordo com a resolução 466/12 do CNS.

Recomendações:

Não existem recomendações.

Conclusões ou Pendências e Lista de Inadequações:

Todas as pendências foram acatadas e corrigidas pela pesquisadora.

Considerações Finais a critério do CEP:

Este parecer foi elaborado baseado nos documentos abaixo relacionados:

Tipo Documento	Arquivo	Postagem	Autor	Situação
Informações Básicas do Projeto	PB_INFORMAÇÕES_BÁSICAS_DO_PROJETO_535372.pdf	06/10/2015 17:47:11		Aceito
Folha de Rosto	Folhaderosto.pdf	06/10/2015 17:39:55	SILMA REGINA FERREIRA	Aceito
Projeto Detalhado / Brochura Investigador	Projeto Fapema 2014 universal Plataforma Brasil.pdf	01/08/2015 10:30:23		Aceito
Projeto Detalhado / Brochura Investigador	Projeto Fapema 2014 universal Plataforma Brasil.doc	01/08/2015 10:29:39		Aceito
TCLE / Termos de Assentimento / Justificativa de Ausência	TCLE prof.docx	22/07/2015 13:36:21		Aceito
Outros	termo-de outorga - universal 2014.pdf	21/07/2015 11:14:35		Aceito
Outros	FORMULARIO CEUA 231150092292014 SILMA.pdf	11/06/2015 10:49:49		Aceito

Situação do Parecer:

Aprovado

Necessita Apreciação da CONEP:

Não

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UNIVERSIDADE FEDERAL DO MARANHÃO/MA

Continuação do Parecer: 1.284.493

SAO LUIS, 17 de Outubro de 2015

Assinado por:
FRANCISCO NAVARRO
(Coordenador)

Endereço: Avenida dos Portugueses, 1966 CEB Velho
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ANEXO B. Parecer consubstanciado do Comitê de Ética em Experimentação Animal: Toxicogenética de Medicamentos Antileishmaniais e Antihansêmicos

UNIVERSIDADE FEDERAL DO MARANHÃO COMITÊ DE ÉTICA EM EXPERIMENTAÇÃO ANIMAL	
PARECER CONSUBSTANCIADO INICIAL	Nº do parecer: 17
PROJETO DE PESQUISA TOXICOGENÉTICA DE MEDICAMENTOS ANTILEISHMANIAIS E ANTIHANSÊNICOS	Registro do CEUA: 17/14 Nº do Protocolo: 23115.009229/2014-21 Data de entrada no CEUA: 31/06/14 Parecer: APROVADO

I – Identificação

Título do projeto: TOXICOGENÉTICA DE MEDICAMENTOS ANTILEISHMANIAIS E ANTIHANSÊNICOS		
Identificação da equipe executora: Profa. Dra. Silma Regina Ferreira Pereira (coordenador) Colaboradores: Vanessa Ribeiro Moreira Patrícia Valeria Gomes Castelo Branco Rossy-Eric Pereira Soares Elisabeth Coelho Morais Luis Claudio Lima de Jesus Poena Pereira da Silva Antonio Augusto Lima Teixeira Junior		
Instituição onde será realizado: Universidade Federal do Maranhão		
Área temática: Ciências Biológicas	Multicêntrico:	Data de recebimento: 30/06/2014

Cooperação estrangeira:		Data de devolução 20/05/2015

II – Objetivos:

Avaliar a genotoxicidade e mutagenicidade de antileishmaniais (Glucantime e Metilfosine) e antihansenicos (Rifampicina, Dapsona e Clofazimina) de modo a determinar o potencial risco de indução de lesões genômicas em pacientes sob tratamento com esses medicamentos.

III – Sumário do projeto:

O projeto destaca duas doenças endêmicas no Estado do Maranhão, como leishmanioses e a hanseníase sendo o tratamento, mesmo reconhecido pela OMS, apresenta uma elevada toxicidade. Neste contexto o projeto se subdivide em um estudo clinico e pré-clinico, sendo este último com o objetivo de avaliar os efeitos de toxicidade gênica de drogas antileishmaniais (Glucantime e Metilfosine) e antihansenicos (Rifampicina, Dapsona e Clofazimina) através do tratamento de animais para posterior analise pelo teste de cometa e micronúcleo. O projeto propõe 14 ensaios subdivididos em 5 grupos contendo 5 animais/grupo, totalizando 350 camundongos Swiss.

IV – Comentário do relator frente à resolução Nº 779 - CONSEPE e complementares em particular sobre:

O projeto apresenta uma temática relevante, fundamentado em com uma proposta que colabora não só no aspecto científico, mas na melhoria do tratamento de doenças endêmicas no Estado do Maranhão. Os objetivos são claros, entretanto na descrição do método não fica claro, pelo contexto fundamentado, a importância de todos os grupos. Os controles negativo (água) e positivo (ciclofosfamida) são os mesmos para todos os grupos, não sendo claro a necessidade da repetição.

V – Pendências

VI – Recomendações

VII – Parecer consubstanciado do CEUA

São Luís, _____20_____/_____05_____/___2015_____

Profa Dra Lucilene Amorim Silva

ANEXO C. Parecer consubstanciado do Comitê de Ética em Experimentação Animal: Potencial Toxicogenético do Antileishmanial Miltefosina (hexadecilfosfocolina)

UNIVERSIDADE FEDERAL DO MARANHÃO
COMISSÃO DE ÉTICA NO USO DE ANIMAIS-CEUA
CIAEP: 01.0341.2014

CERTIFICADO

Certificamos que a proposta intitulada:" **POTENCIAL TOXICOGENÉTICO DO ANTILEISHMANIAL MILTEFOSINE (HEXADECILFOSFOCOLINA)", Processo nº 23115.029807/2018-70,** sob a responsabilidade da **Profa. Dra. Silma Regina Ferreira Pereira**, que envolve a produção, manutenção ou utilização de animais pertencentes ao filo Chordata, subfilo Vertebrata (exceto humanos), para fins de pesquisa científica (ou ensino) - encontra-se de acordo com os preceitos da Lei nº 11.794, de 8 de outubro de 2008, do Decreto nº 6.899, de 15 de julho de 2009, e com as normas editadas pelo Conselho Nacional de Controle de Experimentação Animal (CONCEA), e foi considerado **APROVADO** pela Comissão de Ética no Uso de Animais (CEUA - UFMA) da Universidade Federal do Maranhão.

FINALIDADE	( ) ENSINO (X) PESQUISA ( ) EXTENSÃO
Vigência da autorização	20/02/2019 a 20/02/2021
Espécie/linhagem/raça	Camundongos – *Mus musculus* – Balb/c
Nº de animais	25 animais
Peso/Idade	25 a 30g - 60 dias
Sexo	Machos
Origem	Centro Multidisciplinar para Investigação Biológica - CEMIB, Universidade de Campinas – UNICAMP.

Prof. Dr. Rafael Cardoso Carvalho
Presidente da Comissão de Ética no uso de Animais – CEUA/UFMA

ANEXO D. Co-autoria em artigo publicado: Genistein and Ascorbic Acid Reduce Oxidative Stress-Derived DNA Damage Induced by the Antileishmanial Meglumine Antimoniate

AMERICAN SOCIETY FOR MICROBIOLOGY | Antimicrobial Agents and Chemotherapy®

EXPERIMENTAL THERAPEUTICS

Genistein and Ascorbic Acid Reduce Oxidative Stress-Derived DNA Damage Induced by the Antileishmanial Meglumine Antimoniate

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ABSTRACT Meglumine antimoniate (Glucantime) is a pentavalent antimonial used to treat leishmaniasis, despite its acknowledged toxic effects, such as its ability to cause oxidative damage to lipids and proteins. Recently, our group demonstrated that meglumine antimoniate causes oxidative stress-derived DNA damage. Knowing that antioxidants modulate reactive oxygen species, we evaluated the capacity of genistein and ascorbic acid for preventing genotoxicity caused by meglumine antimoniate. For that, mice (n = 5/group) received genistein (via gavage) in doses of 5, 10, and 20 mg/kg for three consecutive days. After this period, they were treated with 810 mg/kg meglumine antimoniate via intraperitoneal (i.p.) route. Furthermore, mice (n = 5/group) simultaneously received ascorbic acid (i.p.) in doses of 30, 60, and 120 mg/kg and 810 mg/kg meglumine antimoniate. We also conducted post- and pretreatment assays, in which animals received ascorbic acid (60 mg/kg) 24 h prior to or after receiving meglumine antimoniate. Genomic instability and mutagenicity were analyzed through conventional comet assay and enzymatic assay using formamide pyrimidine DNA glycosylase (Fpg) enzyme, as well as the micronucleus test, respectively. Meglumine antimoniate induced an increase in the DNA damage after digestion with Fpg, reinforcing its mutagenic potential by oxidizing DNA bases, which was prevented by genistein. Similarly, ascorbic acid was capable of reducing mutagenic effects in simultaneous treatment as well as in posttreatment. Therefore, our results demonstrate that both compounds are efficient in preventing mutations in mammalian cells treated with meglumine antimoniate.

KEYWORDS antioxidants, antimutagenicity, soy isoflavone, vitamin C, pentavalent antimonial

Leishmaniases are chronic and noncontagious infectious diseases that are broadly distributed around the world and caused by more than 20 species of protozoans from the genus *Leishmania* (1). Clinical manifestations vary according to the parasite species causing the disease, with three main forms: (i) cutaneous leishmaniasis (CL), the most common form, which causes ulcers in the skin; (ii) mucocutaneous leishmaniasis (ML), which affects skin and mucous membranes, causing total or partial damage to affected areas; and (iii) visceral leishmaniasis (VL), also known as kala-azar, which is the most serious clinical form and affects internal organs, sometimes being fatal if not adequately treated (1, 2). Estimates of about 50,000 to 90,000 new cases of VL throughout the world are predicted every year. In 2015, over 90% of all cases were recorded in Brazil, Ethiopia, India, Kenya, Somalia, Sudan, and South Sudan. Regarding CL, about 0.6 to 1 million new cases occur yearly, of which 2/3 are from six countries located in South America, the Mediterranean bay, the Middle East, and Central Asia (1).

Despite its high incidence and alarming clinical consequences, leishmaniasis is still

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a neglected disease. Meglumine antimoniate (Glucantime) is one of the drugs most used to treat leishmaniasis by recommendation of the World Health Organization (3–5), even though its mechanisms of action are still unclear and its toxic effects are well known (6, 7). Among the main hypotheses on the mechanism of action of meglumine antimoniate, one suggests that Sb^{V} acts as a prodrug, which is metabolized and reduced to its more toxic trivalent form (Sb^{III}). In vertebrates, this reduction can be mediated by thiols, such as glutathione (GSH) and cysteine or cysteine-glycine, presented in the cytosol and phagolysosomes of the macrophages, respectively. On the other hand, the reduction can occur within the parasite through the trypanothione- and thiol-dependent reductase enzyme (8–10). Hence, the antimonial induces the production of reactive oxygen species (ROS) and, consequently, oxidative stress (10–14). This leads to disturbances in the normal redox state of the cell, damaging the main biomolecules such as proteins, lipids (15), and DNA (16).

To control generation of ROS, cells possess an elaborate enzymatic system involving many enzymes with antioxidant activities, such as glutathione peroxidase (GPx), superoxide dismutase (SOD), glutathione *S*-transferase (GST), and catalase (CAT), whose function is to the stop buildup of ROS in the organism and prevent these agents from causing damage to biomolecules (17, 18). Some nonenzymatic compounds also show antioxidant activity and are obtained mainly through diet, such as α-tocopherol (vitamin E), ascorbic acid (vitamin C), carotenoids, and flavonoids, as well as peptides with thiol groups, such as GSH, among others (19, 20). Interestingly, studies have demonstrated that antioxidant compounds such as ascorbic acid and genistein can also reduce mutations induced by different mutagens (21, 22).

Considering that our research group has demonstrated that meglumine antimoniate causes oxidation of nitrogenous bases on DNA and alters oxidant enzymes activities in BALB/c mice infected by *Leishmania* (*Leishmania*) *infantum*, leading to genomic instability and mutation fixation (23), and keeping in mind that antioxidant compounds neutralize deleterious actions of ROS on biomolecules (24, 25), the aim of this study was to evaluate the potential of two acknowledged antioxidants (genistein and ascorbic acid) in modulating genetic damage caused by the antileishmanial meglumine antimoniate.

RESULTS

Meglumine antimoniate induces oxidative stress-derived DNA damage. Figure 1 shows the frequencies of nucleoid classes observed by the conventional comet assay (Fig. 1A) and by comet assay followed by digestion using Fpg enzyme (Fig. 1B) in peripheral blood leukocytes from healthy mice treated with meglumine antimoniate. The frequency of the highest nucleoid classes (3 and 4) increased when comparing the group treated using meglumine antimoniate to the negative control (NC) ($P < 0.05$). There also was no significant difference between the positive control (PC) and the group treated with the antimonial, reiterating the drug's genotoxic potential (Fig. 1C). On the other hand, the comet assay followed by digestion with Fpg enzyme showed an increase in frequency for classes 2, 3, and 4 (Fig. 1B) compared to that found by conventional comet assay. This novel damage class distribution is reflected in DNA damage scores (Fig. 1C), in which the level of damage increased significantly after digestion with the endonuclease Fpg ($P < 0.001$), thus reinforcing that this antileishmanial causes damage to DNA by oxidation of bases.

Genistein and ascorbic acid reduce genotoxic damage caused by meglumine antimoniate. The comet assay in peripheral blood leukocytes of mice treated with genistein and meglumine antimoniate revealed an increase in the frequency of nucleoids 2, 3, and 4 in the group treated with meglumine antimoniate in relation to the vehicle control (VC) group ($P < 0.05$) (Fig. 2A). On the other hand, there is a significant difference ($P < 0.05$) between damage scores from the group receiving meglumine antimoniate (GLU group) (1.53 $\pm$ 0.30) and those obtained from groups pretreated with genistein at 5 mg/kg (1.11 $\pm$ 0.08), 10 mg/kg (0.9 $\pm$ 0.11), and 20 mg/kg (0.98 $\pm$ 0.06) (Fig. 2B). These results reveal the ability of genistein to reduce genomic instability

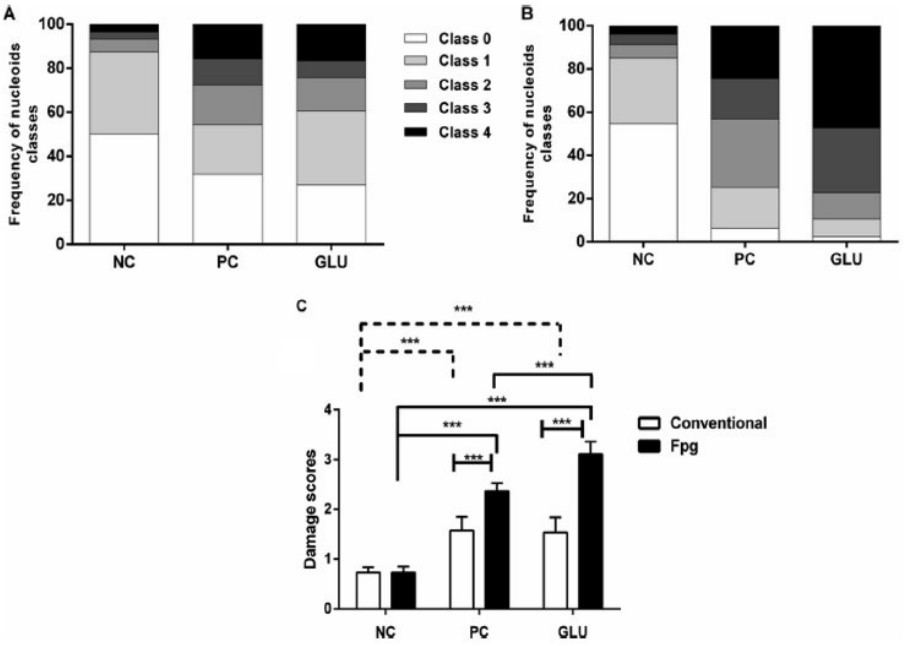

FIG 1 Frequencies of nucleoid classes observed in peripheral blood leukocytes from uninfected Swiss mice treated with meglumine antimoniate ($n = 5$ per group) by conventional comet assay (A) and comet assay followed by Fpg digestion (B) and their respective DNA damage scores (mean ± standard deviation [SD]) (C). Statistical differences were calculated using the Student *t* test (*, $P < 0.05$). NC, negative control (distilled water); PC, positive control (50 mg/kg cyclophosphamide); GLU, meglumine antimoniate (810 mg/kg).

induced by the antileishmanial, which showed a reduction of DNA damage of 51%, 78%, and 68%, respectively.

Regarding ascorbic acid, our data show an increase of class zero nucleoids in the groups treated simultaneously (S1, S2, and S3) (Fig. 3A) compared to the group treated with meglumine antimoniate ($P < 0.05$). Hence, damage scores from groups treated simultaneously with meglumine antimoniate and ascorbic acid (0.36 ± 0.09, 0.27 ± 0.11, and 0.39 ± 0.12) were significantly lower than those from the GLU group (0.70 ± 0.03) ($P < 0.01$), reaching a basal damage level similar to that for the negative control (0.25 ± 0.07) (Fig. 3B). Considering the percentage of DNA damage reduction (76%,

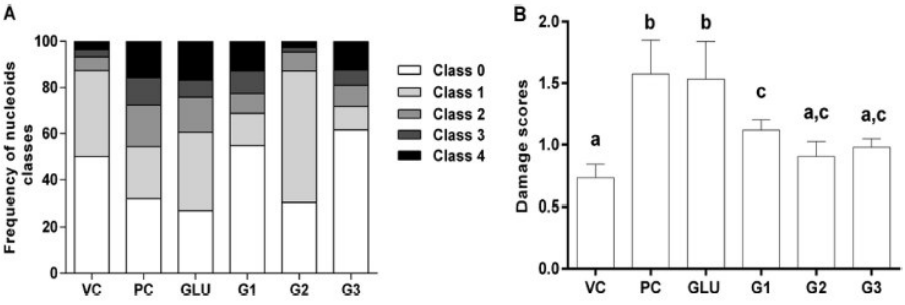

FIG 2 Frequency of nucleoid classes observed by comet assay in peripheral blood leukocytes of Swiss mice treated using genistein and meglumine antimoniate (A) and their respective damage scores (mean ± SD) (B) ($n = 5$ per group). Different letters (a, b, and c) indicate statistically significant differences ($P < 0.05$) by ANOVA followed by Tukey's *ad hoc* test. VC, vehicle control (1% DMSO); PC, positive control (50 mg/kg cyclophosphamide); GLU, meglumine antimoniate (810 mg/kg); G1, 5 mg/kg genistein plus 810 mg/kg meglumine antimoniate; G2, 10 mg/kg genistein plus 810 mg/kg meglumine antimoniate; G3, 20 mg/kg genistein plus 810 mg/kg meglumine antimoniate).

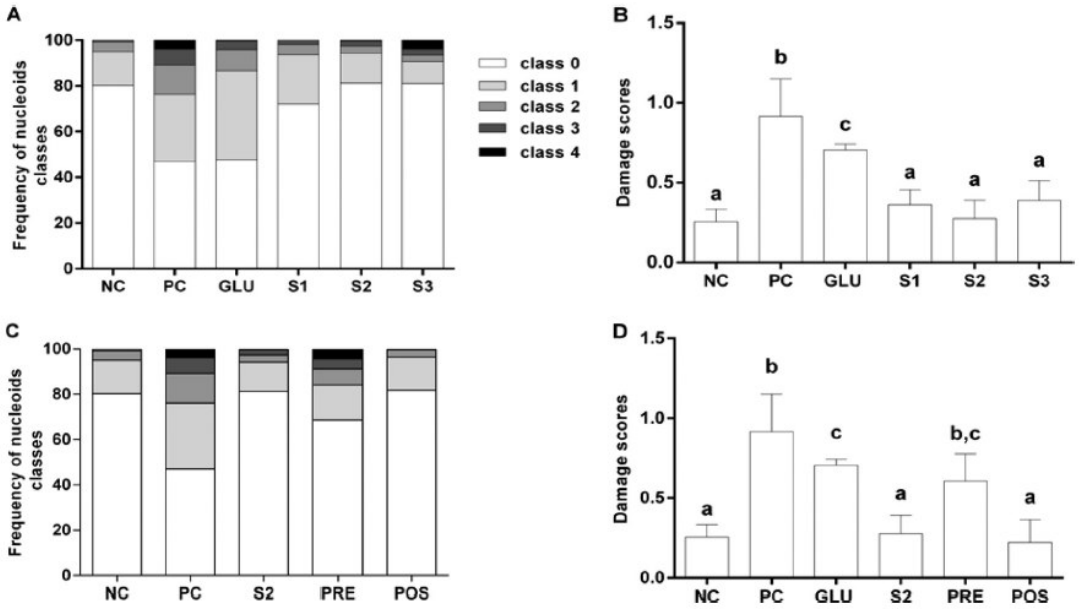

FIG 3 Frequencies of nucleoid classes and their respective DNA damage scores (mean ± SD) obtained by comet assay in peripheral blood leukocytes in Swiss mice treated with ascorbic acid and meglumine antimoniate. (A and B) Simultaneous treatment. S1, 30 mg/kg; S2, 60 mg/kg; S3, 120 mg/kg ascorbic acid plus 810 mg/kg meglumine antimoniate ($n = 5$ animals per group). (C and D) Pretreatment (60 mg/kg ascorbic acid 24 h prior to meglumine antimoniate [810 mg/kg]) and posttreatment (60 mg/kg ascorbic acid 24 h after meglumine antimoniate [810 mg/kg]) ($n = 5$ animals per group). Different letters (a, b, and c) indicate statistical difference ($P < 0.05$) by ANOVA followed by Tukey's *ad hoc* test.

95%, and 70%, respectively), we evaluated the effects of the intermediate dose (60 mg/kg) in simultaneous treatment, pretreatment, and posttreatment. The distribution of classes and respective damage scores are shown in Fig. 3C and D. Scores obtained from simultaneous treatment (0.27 ± 0.11) and posttreatment (0.22 ± 0.14) are significantly lower than those from the group treated with meglumine antimoniate (0.70 ± 0.03) and do not differ between each other or from those for the negative control. The procedures are thus equally efficient in reducing genomic damage caused by the antileishmanial. On the other hand, ascorbic acid administration prior to meglumine antimoniate did not present antigenotoxic action (score equal to 0.60 ± 0.16).

Genistein and ascorbic acid decrease the frequency of micronucleated cells. Meglumine antimoniate did not have a cytotoxic effect on erythrocytes since the ratio of polychromatic erythrocytes (PCE) to normochromatic erythrocytes (NCE) was similar to that for the control. The frequencies of micronuclei in the positive control (50 mg/kg) and in the meglumine antimoniate group (810 mg/kg) also confirmed the mutagenic effect of these compounds. On the other hand, the three genistein doses were capable of significantly decreasing the antileishmanial-induced frequency of micronucleated cells ($P < 0.001$), reaching the basal levels in the vehicle control (Table 1). Tables 2 and 3 show the data from animals treated with ascorbic acid and meglumine antimoniate in simultaneous treatment and under pre- and posttreatment conditions. For the groups treated simultaneously, the three ascorbic acid doses showed efficiency in reducing meglumine antimoniate-induced micronuclei (Table 2). Notably, the intermediate dosage was able to decrease the frequency of micronuclei and also showed the highest potential to reduce DNA damage. Based on our results from simultaneous treatment, the protective effect of ascorbic acid was also evaluated under pre- and posttreatment conditions with the intermediate dose. We observed a decrease in the frequency of micronucleated cells in posttreatment, which did not show any significant difference from the simultaneous treatment. On the other hand, pretreatment with

TABLE 1 Frequency of micronucleated polychromatic erythrocytes and PCE/NCE ratio in the bone marrow of Swiss mice treated with genistein and meglumine antimoniate and of their respective controls

Group[a]	No.	MnPCE, % (mean ± SD)[b]	PCE/NCE ratio[c]
VC	33	3.3 ± 0.50 a	0.99
PC	306	30.6 ± 4.04 b	0.93 (ns)
GLU	144	14.4 ± 1.43 c	0.96 (ns)
G1	60	6.0 ± 0.12 a	0.99 (ns)
G2	42	4.2 ± 0.07 a	1.00 (ns)
G3	33	3.3 ± 0.05 a	0.99 (ns)

[a]$n = 5$ animals/group; 2,000 erythrocytes per animal. VC, vehicle control (1%); PC, positive control (50 mg/kg cyclophosphamide); GLU, meglumine antimoniate (810 mg/kg); G1, genistein (5 mg/kg) plus meglumine antimoniate (810 mg/kg); G2, genistein (10 mg/kg) plus meglumine antimoniate (810 mg/kg); G3, genistein (20 mg/kg) plus meglumine antimoniate (810 mg/kg).
[b]MnPCE, micronucleated polychromatic erythrocytes. Different letters indicate a statistically significant difference ($P < 0.05$) by ANOVA followed by Tukey's *ad hoc* test; $F = 164.9$.
[c]ns, no statistically significant difference compared to VC.

ascorbic acid was not able to reduce the mutagenic effect of the antileishmanial (Table 3).

DISCUSSION

Many studies have proposed that meglumine antimoniate acts as a prodrug. This model suggests that the pentavalent antimonial (Sb^V) is converted into its more toxic trivalent form (Sb^{III}) by thiols (8, 9). Evidence indicates that Sb^{III} compromises the redox potential of the cell, leading to ROS production and, consequently, increasing the oxidative stress (10–12, 15). Hence, the reduction of Sb^{III} has been mentioned as one possible mechanism for the drug's toxicity and for its therapeutic activity. This hypothesis is corroborated by our previous study, in which it was demonstrated that meglumine antimoniate induces mutations *in vivo* but not *in vitro* (16). These data also support a previous study that related genetic risk and oxidative stress in workers exposed to antimony trioxide (26).

The association between genetic alterations and oxidative damage was reinforced by a study by Cantanhêde and collaborators (27), who demonstrated that soy isoflavones are effective in reducing genomic lesions as well as the frequency of micronucleated cells induced by meglumine antimoniate. More recently, this idea was supported by demonstrating that meglumine antimoniate causes genetic instability by oxidation of nitrogenous bases in infected BALB/c mice treated for 20 days (20 mg/kg/day) (23). The score of damage caused by the antileishmanial increased in the comet assay that uses Fpg repair endonucleases. This assay allows recognition and removal of products generated from the oxidation of purine bases, therefore increasing

TABLE 2 Frequency of micronucleated polychromatic erythrocytes and PCE/NCE ratio in the bone marrow of Swiss mice treated simultaneously with ascorbic acid and meglumine antimoniate and of their respective controls

Group[a]	No.	MnPCE, % (mean ± SD)[b]	PCE/NCE ratio[c]
NC	33	3.5 ± 0.50 a	1.00
PC	306	30.6 ± 4.04 b	0.93 (ns)
GLU	144	14.4 ± 1.43 c	0.96 (ns)
S1	76	7.6 ± 1.29 d	0.98 (ns)
S2	64	6.4 ± 1,19 a, d	0.93 (ns)
S3	103	10.3 ± 1.48 d	0.87 (ns)

[a]$n = 5$ animals/group; 2,000 erythrocytes per animal. NC, negative control (distilled water); PC, positive control (50 mg/kg cyclophosphamide); GLU, meglumine antimoniate (810 mg/kg); S1, ascorbic acid (30 mg/kg) plus meglumine antimoniate (810 mg/kg); S2, ascorbic acid (60 mg/kg) plus meglumine antimoniate (810 mg/kg); S3, ascorbic acid (120 mg/kg) plus meglumine antimoniate (810 mg/kg).
[b]MnPCE, micronucleated polychromatic erythrocytes. Different letters indicate a statistically significant difference ($P < 0.05$) by ANOVA followed by Tukey's *ad hoc* test; $F = 120.4$.
[c]ns, no statistically significant difference compared to NC.

TABLE 3 Frequency of micronucleated polychromatic erythrocytes and PCE/NCE ratio in the bone marrow of Swiss mice treated with ascorbic acid and meglumine antimoniate under simultaneous treatment, pretreatment, and posttreatment conditions, with their respective controls

Group[a]	No.	MnPCE, % (mean ± SD)[b]	PCE/NCE ratio[c]
NC	35	3.5 ± 0.50 a	1.00
PC	306	30.6 ± 4.04 b	0.93 (ns)
GLU	144	14.4 ± 1.43 c	0.96 (ns)
S2	64	6.4 ± 1.19 a, d	0.93 (ns)
PRE	110	11.0 ± 1.00 c, e	0.93 (ns)
POS	81	8.1 ± 0.96 d, e	0.95 (ns)

[a]n = 5 animals/group; 2,000 erythrocytes per animal. NC, negative control (distilled water); PC, positive control (50 mg/kg cyclophosphamide); GLU, meglumine antimoniate (810 mg/kg); S2, ascorbic acid (60 mg/kg) plus meglumine antimoniate (810 mg/kg); PRE, ascorbic acid (60 mg/kg) plus meglumine antimoniate (810 mg/kg); POS, meglumine antimoniate (810 mg/kg) plus ascorbic acid (60 mg/kg).
[b]MnPCE, micronucleated polychromatic erythrocytes. Different letters indicate a statistically significant difference ($P < 0.05$) by ANOVA followed by Tukey's *ad hoc* test; $F = 129.2$.
[c]ns, no statistically significant difference compared to NC.

DNA fragmentation and, consequently, the damage score detected by the conventional comet assay (28).

This present study focused on the capacity of recognized antioxidants to modulate DNA damage induced by meglumine antimoniate. First, our data show that pentavalent antimony causes damage to the DNA of mice treated intraperitoneally with a high dose and for a short period of time. These results support that the reduction of pentavalent antimony occurs, at least in part, in phagolysosomes of macrophages (8), since our experimental model allows a greater and faster exposure of the peritoneal macrophages. Furthermore, some studies found large amounts of residual Sb^{III} in pentavalent antimony, up to 15 to 30% (14, 29, 30). Hence, considering that animals received nearly 195 mg/kg of Sb^{III}, the genotoxicity observed in the present study could also be due to the high level of residual trivalent antimony in the commercial meglumine antimoniate.

Second, considering that the mutagenic effects of meglumine antimoniate are mediated by oxidative processes, here we investigated the capacity of two antioxidants, genistein and ascorbic acid, to reduce/prevent antileishmanial meglumine antimoniate-induced genetic damage. The first one, genistein, is a soybean-derived isoflavonoid that is considered an important agent in cancer prevention (31, 32); this effect is associated with its recognized antioxidant capacity (33–36).

Our data demonstrated that pretreatment with doses of 5 mg/kg, 10 mg/kg, and 20 mg/kg genistein was capable of decreasing meglumine antimoniate-induced genotoxicity, revealing that the pretreatment with genistein was able to modulate the reactive oxygen species generated by the antileishmanial meglumine antimoniate. We suggest that the genistein antimutagenic activity is correlated with its capacity to capture free radicals generated after metabolic activation from the pentavalent antimonial form to its trivalent form, thus protecting genetic material from oxidative damage. Corroborating this idea, Pugalendhi et al. (22) verified that a dose of 20 mg/kg genistein was able to protect from mutagenic effects induced by 7,12-dimethylbenzo[a]anthracene. Furthermore, Ali et al. (37) verified that genistein administration for 21 days reduced lipid peroxidation, protein carbonylation, and DNA damage induced by *N*-nitrosodiethylamine (0.1 mg/ml) in rat hepatocytes.

According to Ding and Liu (38), genistein acts by increasing the expression of genes involved in the detoxification of ROS, such as superoxide dismutase, glutathione peroxidase, and catalase. This hypothesis is supported by Suzuki et al. (39), who observed that genistein was able to significantly increase expression of the gene *GPx-1* in human prostate cancer cell lines (LNCaP and PC-3), contributing to antioxidant defense and inhibition of proliferation of these cells. Gong et al. (40) also verified an increase in the activity of these enzymes by genistein against cadmium-induced neurotoxic damage in Wistar mice, thus demonstrating the protective potential of this isoflavonoid and its capacity to maintain redox equilibrium.

Another possible mechanism to explain the antimutagenic effect of genistein is related to its ability to stimulate expression of genes involved in DNA repair, such as *BCRA1* (41) and *ATM* and *p53* (42). This mechanism was also reported by Song et al. (43), who studied the effect of genistein in preventing and repairing radiation-induced DNA damage in HL-7702 cells. The authors noted that genistein was able to increase expression of genes involved in DNA repair, such as *hHR23A*, *HUS1*, *RAD1*, and *RAD9*. Therefore, the data here presented support the hypothesis that the antileishmanial causes DNA damage by oxidation of its nitrogenous bases, which was reduced by genistein through its antioxidant activity.

Our data also revealed that ascorbic acid was able to reduce meglumine antimoniate-induced genomic damage, corroborating previous studies that observed a protective effect of ascorbic acid against mutagenic damage induced by other stressors such as cyclophosphamide (44), arsenic trioxide (45), 3,5-dimethylaminophenol (46), and vanadium pentoxide (47). Ascorbic acid is a nonenzymatic antioxidant obtained from the diet and is essential to humans due to its participation in several physiological activities (48). This vitamin promotes beneficial effects on health, since its antioxidant activity prevents development of certain types of cancer, cardiovascular diseases, and other illnesses related to oxidative stress (49–51). According to Vijayalaxmi and Venu (21), as well as Farghaly and Abo-Zeid (52), the antigenotoxic and antimutagenic abilities are due to its capacity to convert reactive compounds to less toxic and more easily excreted products, as well as to capture free radicals before they interact with other molecules, protecting them from oxidative damage.

Our data also agree with those of Kato et al. (14), who demonstrated that ascorbic acid promotes hepatoprotection in animals treated with meglumine antimoniate due its capacity to decrease the peroxidase enzyme activity. In addition, these authors showed that cotreatment with ascorbic acid and meglumine antimoniate did not interfere with the antileishmanial activity of the drug in mice infected by *Leishmania infantum*, suggesting that this antioxidant may be part of a therapeutic strategy effective against toxicity caused by meglumine antimoniate. Here, our data emphasize that this protection includes DNA protection against oxidative stress.

Besides its well-known ability to eliminate ROS (21, 46, 47), other studies suggest that ascorbic acid can act as a cofactor of transcription factors, upregulating genes involved in DNA repair (53–55). These authors demonstrated that ascorbic acid is able to increase expression of *SOD3* (encoding superoxide dismutase 3) and *OGG1* (encoding 8-oxoguanine DNA glycosylase) by activation of NRF2 (factor 2 nuclear transcription factor related to erythroid 2). Moreover, Alevva et al. (56) have verified that dietetic ingestion of ascorbic acid (600 mg/day) in cells from volunteers treated with hyperbaric oxygen (HBO) induced the expression of important genes for cell detoxification, such as *GSTZ1* (encoding glutathione *S*-transferase zeta 1), genes encoding multifunctional enzymes important to detoxification of electrophilic molecules, and *NUDT1* (encoding nucleoside diphosphate linked moiety X motif 1), a gene encoding 7,8-di-hydro-8-oxoguanine triphosphatase, which hydrolyzes oxidized purines. Additionally, Cooke et al. (57) observed a significant decrease of 8-oxo-G in DNA of cells from volunteers supplemented with ascorbic acid (500 mg/day), thus highlighting that ascorbic acid probably stimulates repairs to oxidized bases in DNA due to its redox properties.

Briefly, based on our data regarding antigenotoxic and antimutagenic effects detected in simultaneous treatment, we suggest that ascorbic acid, a strong reducing agent, acts by eliminating reactive oxygen species generated during conversion of pentavalent antimonial to its trivalent form, thus protecting genetic material from oxidative damage. Additionally, ascorbic acid administered 24 h after the antileishmanial was able to reduce DNA damage, suggesting that this antioxidant also acts by repairing damage, possibly by inducing expression of genes related to the DNA repair system, halting mutation fixation. Therefore, the protective effect of ascorbic acid against oxidative stress-derived DNA damage caused by meglumine antimoniate may be due to both mechanisms. Future studies must be performed to evaluate the

differential expression of genes involved in both molecular responses to oxidative DNA damage caused by meglumine antimoniate.

We have also shown that ascorbic acid (60 mg/kg) is not able to decrease the deleterious effects on genomic instability when administered 24 h prior to the mutagenic drug. This probably occurs due to ascorbic acid's half-life; it is eliminated from an organism 10 to 12 h after consumption (58, 59). In addition, we emphasize that the antigenotoxic effects of genistein and ascorbic acid were observed at all doses, but there was no dose-response effect. Many different factors can be related to this fact, such as the pathological and physiological conditions of the organism, pharmacokinetics and bioavailability of the drugs, dosage, route of administration, interaction with other compounds, and time of exposure (60, 61). In addition, antioxidants may not exhibit antimutagenic effects or even act as pro-oxidants (62–65). The literature regarding the effects of antioxidants is still controversial, so further investigations are necessary to better understand their interaction with meglumine antimoniate and its lack of dose-response effects.

In conclusion, since genistein and ascorbic acid, two recognized antioxidants, were able to reduce the DNA damage caused by meglumine antimoniate, the present data support that the antileishmanial induces oxidative stress-derived DNA damage by promoting overactivation of the SOD-CAT axis. Additionally, our data reinforce the protective potential of these two compounds against reactive oxygen species, opening up perspectives for novel therapeutic strategies to minimize the side effects caused by meglumine antimoniate, including protection against genotoxic damage.

MATERIALS AND METHODS

Animals. To evaluate the antigenotoxic effects of genistein and ascorbic acid on genetic damage induced by meglumine antimoniate, five Swiss *Mus musculus* mice (male, healthy, 30 g, 8 weeks old) were used per treatment group; they were obtained from the Central Bioterium of the Federal University of Maranhão (UFMA) (São Luís, Maranhão, Brazil). Animals were kept in polycarbonate boxes under controlled conditions: temperature around 21 ± 2°C, 12-h light-dark cycle, and water and food *ad libitum*. All procedures were done according to guidelines of the Brazilian College of Animal Experimentation (COBEA) and were approved by the Animal Experimentation Ethics Committee (CEUA-UFMA, no. 23115.012909-2013-41).

Chemical agents. Meglumine antimoniate (Glucantime) was from the pharmacy of the University Hospital Presidente Dutra (HUUFMA) (batch 246888; Sanofi-Aventis, São Paulo, Brazil), genistein (4′,5,7-trihydroxyisoflavone; molecular weight, 270.24 g/mol) was obtained from Sigma-Aldrich (St. Louis, MO, USA), and ascorbic acid was obtained from Dinâmica Química Contemporânea Ltd. (São Paulo, Brazil). For the positive control, we used the mutagen cyclophosphamide (batch 43H0269) obtained from Sigma-Aldrich (St. Louis, MO, USA).

Treatments and doses. The dosage for meglumine antimoniate was estimated from the maximum recommended dosage for treatment of patients with leishmaniasis, that is, 20 mg/Sb^V/kg/day for 20 days (16). Doses for cyclophosphamide, ascorbic acid, and genistein were based on work conducted by Cantanhêde et al. (27), Vijayalaxmi and Venu (21), and Pugalendhi et al. (22), respectively.

For this study, we opted for an acute treatment using 810 mg/kg body weight of meglumine antimoniate (16). To evaluate the occurrence of dose-dependent effects, we used three different doses of the antioxidants, as follows: ascorbic acid, 30 mg/kg, 60 mg/kg, and 120 mg/kg; genistein, 5 mg/kg, 10 mg/kg, and 20 mg/kg. The negative control (NC) group was administered distilled water. The vehicle control (VC) group received 1% dimethyl sulfoxide (DMSO), the same concentration used to dilute genistein. The positive control (PC) group was treated with a single dose of 50 mg/kg cyclophosphamide. The meglumine antimoniate group (GLU group) was treated with a single dose of 810 mg/kg meglumine antimoniate (Table 4).

To evaluate the protective effect of genistein, groups G1, G2, and G3 received 5 mg/kg, 10 mg/kg, and 20 mg/kg genistein, respectively, for three consecutive days. On the third day, animals received their respective doses of genistein together with 810 mg/kg of meglumine antimoniate. To evaluate the protective effect of ascorbic acid, three types of treatment were conducted: the pretreatment group (PRE) received a single dose of ascorbic acid (60 mg/kg) and 24 h later received a single dose of meglumine antimoniate (810 mg/kg); the posttreatment group (POS) received a single dose of meglumine antimoniate (810 mg/kg) and 24 h later received a single dose of ascorbic acid (60 mg/kg); and groups with simultaneous treatment (S1, S2, and S3) received simultaneously a single dose of 30 mg/kg, 60 mg/kg, and 120 mg/kg ascorbic acid, respectively, and 810 mg/kg meglumine antimoniate (Table 4).

Doses of meglumine antimoniate, cyclophosphamide, and ascorbic acid were administered intraperitoneally, standardized in solutions of 0.1 ml per 10 g body weight. Doses of genistein and 1% DMSO were administered via gavage.

Comet assay. For the comet assay (66, 67), after 24 h of treatment, peripheral blood (10 μl) was obtained and mixed with 100 μl low-melting-point agarose (0.5%). This mixture was then applied to

TABLE 4 Experimental groups and their respective treatments

Group	Chemical agent(s)	Dose (mg/kg)	Administration
Genistein			
VC	DMSO	1%	Gavage
PC	Cyclophosphamide	50	Intraperitoneal
GLU	Meglumine antimoniate	810	Intraperitoneal
G1	Genistein + meglumine antimoniate	5 + 810	Gavage + intraperitoneal
G2	Genistein + meglumine antimoniate	10 + 810	Gavage + intraperitoneal
G3	Genistein + meglumine antimoniate	20 + 810	Gavage + intraperitoneal
Ascorbic acid			
NC	Distilled water		
PC	Cyclophosphamide	50	Intraperitoneal
GLU	Meglumine antimoniate	810	Intraperitoneal
S1	Ascorbic acid + meglumine antimoniate	30 + 810	Intraperitoneal
S2	Ascorbic acid + meglumine antimoniate	60 + 810	Intraperitoneal
S3	Ascorbic acid + meglumine antimoniate	120 + 810	Intraperitoneal
PRE	Ascorbic acid + meglumine antimoniate	60 + 810	Intraperitoneal
POS	Meglumine antimoniate + ascorbic acid	810 + 60	Intraperitoneal

pregelled slides with high-melting-point agarose (1.5%). Slides, in duplicate, were covered with coverslips and refrigerated for 5 min. After solidification, slides were immersed in cold lysis solution (2.5 M NaCl, 100 mM Na_2EDTA, 10 mM Tris [pH 10], 10% DMSO, and 1% Triton X-100) and refrigerated for 12 h at 4°C. Slides were then incubated for 20 min in alkaline buffer (10 M NaOH, 0.2 M EDTA, and distilled water, pH 13), followed by electrophoresis for 20 min at 25 V (0.72 V/cm) and 300 mA. Slides were neutralized (0.4 M Tris-HCl, pH 7.5) for 15 min and fixed with absolute ethanol for 4 min. The slides were stained with ethidium bromide (20 μg/ml) and analyzed by fluorescence microscopy (BX51/BX52-Olympus; 516- to 560-nm filter, 40× lens). For each animal, we analyzed 100 nucleoids. Each nucleoid was classified according to DNA damage at five levels: class 0, no damage (<5%); class 1, low damage (5 to 20%); class 2, medium damage (21 to 40%); class 3, high damage (41 to 94%); and class 4, total damage (>95%). Damage scores were calculated by multiplying the number of nucleoids in each class by their respective class value (68).

The percentage of reduction in meglumine antimoniate-induced genotoxic damage was calculated as described by Waters et al. (69): percent reduction = (GLU − TG/GLU − NC) × 100, where GLU corresponds to the average damage in the group treated solely with meglumine antimoniate, TG is the average damage observed in groups treated using genistein with meglumine antimoniate or ascorbic acid with meglumine antimoniate, and NC corresponds to the average damage observed in the negative control or vehicle control group.

Comet assay followed by Fpg enzyme. To evaluate oxidative damage to DNA caused by meglumine antimoniate, we also conducted a comet assay using formamide pyrimidine DNA glycosylase (Fpg) (Fpg Flare assay kit; Trevigen, Gaithersburg, MD) (70). After lysis in lysis solution (2.5 M NaCl, 100 mM EDTA, and 10 mM Tris [pH 10] with 10% dimethyl sulfoxide and 1% Triton X-100), slides were dipped in 1× Flare buffer (Trevigen) for 30 min. Each slide was then treated with 150 μl Fpg enzyme solution (0.5 U), covered with coverslips, and incubated at 37°C for 45 min in humid chamber. After incubation, the coverslips were carefully removed, and electrophoresis, neutralization, fixation, staining, and data analysis were conducted as described above.

Micronucleus test. For the micronucleus test (71), after blood collection for the comet assay, animals were euthanized by anesthetic overdose (1 ml/kg xylazine and 0.1 ml/kg ketamine), and bone marrow was extracted using 1 ml fetal bovine serum. The cell suspension was centrifuged for 5 min at 1,000 rpm. The supernatant was discarded, and cell pellet smearing was conducted. Slides were stained using the hematological dye Panoptic (Laborclin) to differentiate polychromatic erythrocytes (PCE), colored in light purplish blue, from normochromatic erythrocytes (NCE), which stain in light red.

To investigate the micronucleus, 2,000 polychromatic erythrocytes were analyzed for each animal in an optical microscope at a magnification of ×1,000 (Carl Zeiss Primo Star). The PCE/NCE ratio was calculated using 300 erythrocytes from each animal to estimate toxicity for cyclophosphamide, meglumine antimoniate, and meglumine antimoniate associated with genistein and ascorbic acid.

Statistical analysis. Data normality was assessed by the Kolmogorov-Smirnov test. Data with a normal distribution were tested by analysis of variance (ANOVA) followed by Tukey's *ad hoc* test. A P value of <0.05 was considered significant. The comparison between the damage scores obtained from both the comet assays (standard and Fpg version) was performed by the Student t test. All data were evaluated using GraphPad Prism 6.

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ANEXO E. Co-autoria em artigo publicado: River Waters near agricultural sites in the Northeast Brazil (Maranhão State) cause genetic damage in Nile Tilapia (*O. niloticus*)

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River waters near to agricultural sites in the Northeastern Brazil (Maranhão State) cause genetic damage in Nile Tilapia (*Oreochromis niloticus*)

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(With 2 figures)

Munim River is a small basin (15,918.04 km², 320,000 inhabitants) in northeastern Brazil (Maranhão state), whose water quality has been threatened by pesticides, untreated domestic and industrial wastewater discharges, and solid waste disposal (Presoti and Cutrim, 2011). These pollutants may bring several problems to the aquatic biota, such as mutagenic and carcinogenic effects (Bolognesi and Hayashi, 2011) including risks to human health. Therefore, it is critical to understand the potential impacts of xenobiotics on this river basin, especially their genotoxic and mutagenic effects. We investigated the genotoxic and mutagenic potential of the Munim River waters to assess the impact of the presence of xenobiotics on the aquatic biota.

We collected water from two sampling sites (Figure 1) in September 2015 (dry season). As test organism, we used 40 juvenile Nile tilapia (*Oreochromis niloticus*) obtained from local fish farms, which were acclimatized to experimental conditions for 30 days in a 1,000-L (Liter) tank and maintained with constant filtration and aeration system. Fish were fed three times a day with commercial fish food at a proportion of 5% of their biomass. Four 80-L water glass tanks containing ten fish each were used, as follows: a) negative control (tap water from a well); b) positive control (intraperitoneal injection of cyclophosphamide at 50 mg/kg); c) Water from Sampling site 1; and d) Water from Sampling site 2. Fish were exposed to treatments for 72 hours without the provision of food in a static system with constant aeration (Ethics Committee Permit: CEUA # 23115.008075/2016-12).

We measured genotoxic and mutagenic effects of water samples on the erythrocytes from tilapias using the comet assay (Singh et al., 1988; Tice et al., 2000) and the micronucleus test (Hooftman and Raat, 1982), respectively. For comet assay, we acquired the damage scores by observing the number of nucleoids in each damage class (0 to IV) per sample under fluorescence microscopy. The damage score was calculated by multiplying the number of nucleoids in each class by the respective class value according to Speit and Hartmann (1995). DNA damage scores revealed a normal distribution (Shapiro-Wilk test) and were analyzed by ANOVA (analysis of variance) followed by Tukey test ($p < 0.05$). The frequency of micronuclei was determined by observing 1000 cells per sample under light microscope. Because data did not fit into normal distribution, we applied the Kruskal-Wallis test, followed by Student-Newman-Keuls test ($p < 0.05$).

Water from both sampling sites proved to be genotoxic and mutagenic to *O. niloticus*. Cells showed high DNA damage, showing high genotoxicity (Figure 2A), similar to the positive control ($p < 0.05$). We also found a significant increase in the frequency of micronuclei when all groups are compared to the negative control ($p < 0.05$) (Figure 2B).

Sampling sites are 181 km apart and showed the same level of DNA damage and mutagenic effects, revealing that harzadous xenobiotics are widely dispersed along the basin. Both sites are located in a region characterized by small subsistence agriculture and discharge of untreated sewage. The city of Chapadinha (Sample site 1) and others regions from Middle-Upper Munim drainage are also affected by large scale transgenic soybean cultures (Presoti and Cutrim, 2011) dependent on the use of the glyphosate herbicid, which has been shown to be genotoxic and mutagenic to fish, even at low concentrations (Moreno et al., 2014). Other studies have showed that several compounds, even in small concentrations, may be associated with genotoxicity and mutagenicity on aquatic organisms (Barberio et al., 2009). Some compounds, such as surfactants, phenols, and metals, together with untreated domestic and industrial wastewater discharges are also associated to genotoxic and mutagenic effects in fishes and might be present in the Munim waters (Steffens et al., 2015).

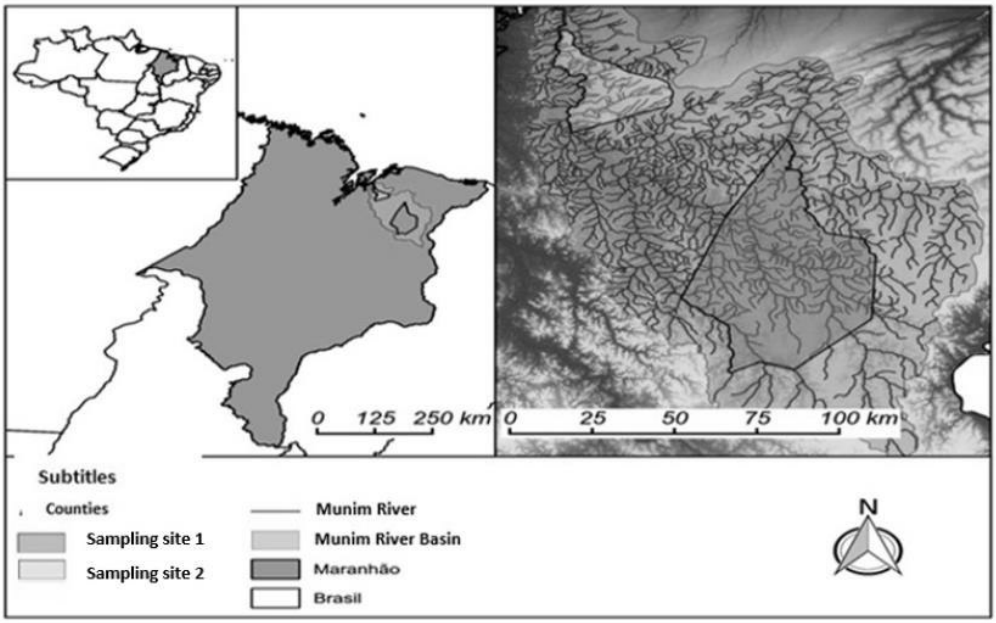

Figure 1. Location of Munim River Basin, in Maranhão State, and cities where water samples were collected. Sampling site 1: Chapadinha (3°50'47.69"S, 43°19'12.13"W), in the Upper-Middle Munim River; Sampling site 2: Cachoeira Grande (3°8'30.16"S, 43°54'19.86"W), in the Lower Munim River.

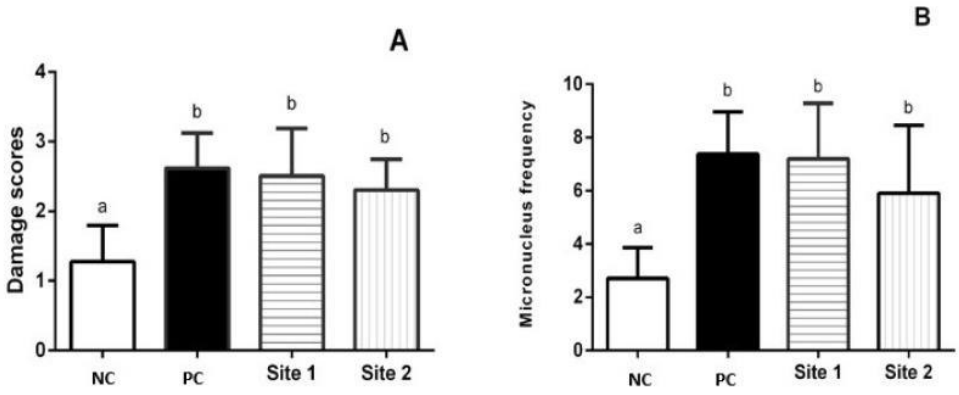

Figure 2. (A) Scores of DNA damage measured by the comet assay on erythrocytes of *O. niloticus* exposed to water samples from the Munim River for 72 h. Different letters indicate statistically significant differences between groups; (B) Frequency of micronuclei in erythrocytes of *O. niloticus* exposed to water samples from the Munim River for 72 h. Different letters indicate statistically significant differences between groups. NC: Negative Control (well water); PC: Positive Control (Cyclophosphamide at 50 mg/kg); Sampling site 1: Chapadinha; Sampling site 2: Cachoeira Grande.

This is the first time that genotoxic and mutagenic effects of waters from the Munim River are demonstrated. Our results suggest the presence of xenobiotic agents affecting the DNA of the aquatic biota. We emphasize that many other neotropical river basins, like Munim, are exposed to similar anthropogenic activities, thus we suggest the need for monitoring water quality (and sediment) in order to identify xenobiotics and its impacts on the biota, as well as on human populations who utilizes these hydric resources.

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ANEXO F. Co-autoria em artigo publicado: Soy isoflavones have antimutagenic activity on DNA damage induced by the antileishmanial Glucantime (meglumine antimoniate)

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RESEARCH ARTICLE

Soy isoflavones have antimutagenic activity on DNA damage induced by the antileishmanial Glucantime (meglumine antimoniate)

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Abstract

Isoflavones are phytoestrogens reported to be potent antioxidant agents. In contrast, the antileishmanial meglumine antimoniate has mutagenic activities. This study evaluated the ability of soy isoflavones to reduce DNA damage induced by meglumine antimoniate. Antimutagenic effects (by micronucleus test) were tested using Swiss mice divided into seven groups treated with meglumine antimoniate (425 mg/kg bw pentavalent antimony); cyclophosphamide (50 mg/kg bw); water (negative control); single isoflavones dose (1.6 mg/kg bw), and three groups received one dose of isoflavones via gavage (0.4 mg/kg bw, 0.8 mg/kg bw or 1.6 mg/kg bw) plus meglumine antimoniate via intraperitoneal, simultaneously. To evaluate antigenotoxicity (by Comet assay), each group with 10 animals received the above-mentioned control doses; single dose of isoflavones 0.8 mg/kg bw, and three groups received isoflavones (0.8 mg/kg bw) by gavage along with intraperitoneal meglumine antimoniate, which were treated with isoflavones 24 h before or after receiving meglumine antimoniate (pre-treatment and post-treatment, respectively) or simultaneously. Cells were harvested 24 h after the treatment, and the data were evaluated by ANOVA followed by Tukey's test ($p < 0.05$). The data from the simultaneous treatment by micronucleus test revealed that isoflavones (0.4 and 0.8 mg/kg) were able to reverse the mutagenic effect of Glucantime. Moreover, all regimes of the treatment with 0.8 mg/kg bw dose were able to reduce the genotoxicity caused by meglumine antimoniate. It is suggested that the protective effect of isoflavones against DNA damage is related to their ability to reduce oxidative stress caused by the trivalent Sb(III) metabolite of meglumine antimoniate.

Keywords

Comet assay, micronucleus test, mice

History

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Introduction

Isoflavones, which are abundant in soybeans, are phenolic chemical compounds belonging to the flavonoids class of plant secondary metabolites. Isoflavones display wide variation in their chemical structure, and the soybean alone has three types of isoflavones with four isomeric forms. Daidzein, genistein, and glycitein are present in greater proportions, and isoflavones also occur in the form of glycosides (Duncam et al., 2003). These compounds have structural and functional similarity to estradiol. Indeed, these compounds bind to the estrogen receptors, and many studies have evaluated their effects and benefits on health and hormone replacement therapy. However, the effectiveness of the phytohormonal therapy is still uncertain (Bolaños-Díaz et al., 2011; Carmignani et al., 2010; Levis et al., 2011; Molla et al., 2011; Szkutnik-Fiedler et al., 2010).

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Although epidemiological and clinical studies have been conducted to study the effects of isoflavones on diabetes, cancer, osteoporosis and cardiovascular diseases, further study is still needed to understand the mechanisms of action of these compounds and their safety for therapeutic use (Kalaiselvan et al., 2010). Studies have reported that flavonoids may have genotoxic properties (Fox et al., 2012; Lehmann et al., 2005) and may act as pro-oxidants (Michels et al., 2005; van Zanden et al., 2003), or they may have significant protective effects against oxidative damage (Hagen et al., 2012; Sato et al., 2011; Widyarini et al., 2012; Yen & Lai, 2003).

Glucantime® is a pentavalent antimoniate that presents antimony coordinated with several *N*-methyl-D-glucamine (meglumine antimoniate) salt molecules, and it coexists in equilibrium with complex structure oligomers. Meglumine antimoniate is soluble in water and slightly soluble in organic compounds (Roberts et al., 1998; Sereno et al., 2001). Even though pentavalent antimonials are the first choice of drug for the treatment of leishmaniasis, these compounds have limited efficacy. Treatment failures occur due to low dosage, interrupted treatment, as well as the emergence of resistant

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forms of *Leishmania*. Patients most commonly exhibit skeletal and muscle aches, nausea, vomiting, diarrhea, abdominal pain, headache, anorexia, asthenia, fever, fatigue, exanthema, erythema and urticaria. These patients may develop cardiac, liver and kidney abnormalities, and these effects usually manifest themselves at the end of the treatment (Oliveira et al., 2011; Sundar & Chakravarty, 2010; Valencia et al., 2012).

Despite being used for over six decades, the mechanism of action of pentavalent antimonials is unclear, and at least three possible mechanisms of action have been suggested (Haldar et al., 2011). According to the ''Prodrug Model'', the pentavalent antimony Sb(V) is reduced to trivalent form Sb(III) that exhibits antileishmanial activity. The second one is the ''Intrinsic Antileishmanial Activity Model'', in which there is an anti-leishmanial activity of the Sb(V) drug. This activity could occur by inhibiting the biosynthesis of macromolecules. Dimicheli et al. (2002) revealed the formation of complexes between Sb(V) and ribonucleosides, particularly in acidic medium, which could favor its action in the phagolysosome. The third possible mechanism is the ''Immune Activation Model'', acting both on the innate and adaptive immune responses. The production of reactive oxygen species (ROS) and nitric oxide (NO) is directly related to leishmanicidal action (Mookerjee Basu et al., 2006).

Our group (Lima et al., 2010) demonstrated the mutagenic activity of meglumine antimoniate *in vivo*; however, no genotoxic activity was observed *in vitro*. Thus, we suggest that meglumine antimoniate is a promutagen that requires metabolization to exert its mutagenic action. Because there is no significant investment by the pharmaceutical industry for the exploration of new drugs for neglected diseases, it is desirable to find strategies that may minimize the adverse effects of pentavalent antimonials in patients undergoing treatment. With the knowledge that some studies have shown that isoflavone has antioxidant activity and the potential to protect genetic material from damage caused by different agents, the present study evaluated the ability of soy isoflavones to protect DNA damage induced by meglumine antimoniate.

Material and methods

Animals

Swiss mice weighing 30 g that had been maintained for many generations in the Animal Breeding Unit (Federal University of Maranhão, Sao Luis, MA, Brazil) under standard conditions were used in each group. The animals were housed in a cross-ventilated room at 26 ± 2 °C, relative humidity of 44–56%, and light-dark cycles of 12 h. The mice had access to sterilized food and water *ad libitum*. All procedures described in this report were approved by the Ethics Committee on Animal Experimentation (CEUA-UFMA No. 23115.012909-2013-41).

Chemical agents and doses

Meglumine antimoniate (Glucantime) is available in 5 ml vials of injectable solution at 300 mg/ml containing 425 mg of pentavalent antimony in 1.5 g of N-methyl-glucamine antimoniate (CAS number 133-51-7; lot numbers 402, 513, 301, 927 and 55398). The dose of meglumine antimoniate was estimated from the recommended dosage to patients with leishmaniasis, i.e. 20 mg/kg/d of Sb(V) during 20 d. In the present study we chose an acute treatment using 425 mg/kg bw of pentavalent antimony (Lima et al., 2010), which corresponds to the total dosage used throughout the treatment.

Isoflavones were obtained from capsules containing 80 mg of dry extract from seeds of *Glycine max* (daidzein 32.12%, genistein 7.47%, daidzin 0.42%, genistin 0.16%, glycitin 0.16% and glycitein 0.03%), lot number 12030875G – PHARMANOSTRA, commercialized by Facial Farmácia de Manipulação (São Luis, Maranhão, Brazil) (compounding pharmacy authorized to manipulate medications of Group I/ raw materials, including materials of vegetable origin, Group III antibiotics, hormones, cytostatic drugs and substances subject to special control according to RDC-67/07). The doses (0.4 mg/kg bw, 0.8 mg/kg bw or 1.6 mg/kg bw) were established in preliminary tests by determining the ratio of polychromatic and normochromatic erythrocytes (PCE/NCE). The isoflavones were diluted in water.

Treatments

Isoflavones were administered by gavage, and the other treatments were administered intraperitoneally using the standard of 0.1 ml per 10 g of body weight. For the micronucleus test, 56 Swiss mice were distributed into seven experimental groups with 8 animals (four males and four females) per group, i.e. two positive control groups, one treated with 50 mg/kg bw of cyclophosphamide (PC-C) and the other with meglumine antimoniate (PC-G) corresponding to 425 mg/kg bw of pentavalent antimony, as described by Lima et al. (2010); a negative control group (NC) receiving only distilled water; one group receiving single dose of extract 1.6 mg/kg bw; and three groups received one dose of extract by gavage (0.4 mg/kg bw, 0.8 mg/kg bw or 1.6 mg/kg bw) plus meglumine antimoniate by intraperitoneal injection, simultaneously. The animals were sacrificed 24 h after the treatments.

For the Comet assay, the animals were distributed into seven experimental groups with 10 animals (five males and five females) per group: three groups correspond to the same controls mentioned above; one group received single dose of extract (0.8 mg/kg bw); one group was treated simultaneously with meglumine antimoniate (425 mg/kg bw, via intraperitoneal) plus extract (0.8 mg/kg bw, via gavage). In the pre- and post-treatment groups, the animals received extract 24 h before or after receiving meglumine antimoniate, respectively. After 24 h, the animals were sacrificed (Table 1).

Bone marrow micronucleus test

The mice were sacrificed and the bone marrow from each femur was extracted using 1 ml of fetal calf serum and centrifuged for 5 min (Heddle, 1973). The supernatant was discarded, and the cells were smeared onto slides. The tissues were then fixed with methanol and stained with Giemsa diluted in phosphate buffer (pH 6.8) (1:10). To investigate the micronucleated cells, 2000 polychromatic erythrocytes (PCEs) were analyzed per animal. To estimate the toxicity, the

Table 1. Experimental groups, chemical agents, doses, and administration routes used in this study.

Group	Chemical agent	Dose (mg/kg)	Administration route	Test
PC-C	Cyclophosphamide	50	Intraperitoneal	Comet assay Micronucleus test
PC-G	Glucantime	425	Intraperitoneal	Comet assay Micronucleus test
NC	Distilled water	–	Intraperitoneal	Comet assay Micronucleus test
Iso	Isoflavones	0.8	Gavage	Comet assay
		1.6	Gavage	Micronucleus test
[a]D1	Isoflavones	0.4	Gavage	Micronucleus test
	Glucantime	425	Intraperitoneal	
[a]D2	Isoflavones	0.8	Gavage	Comet assay
	Glucantime	425	Intraperitoneal	Micronucleus test
[a]D3	Isoflavones	1.6	Gavage	Micronucleus test
	Glucantime	425	Intraperitoneal	
Pre-treatment	Isoflavones	0.8	Gavage	Comet assay
	Glucantime	425	Intraperitoneal	
Post-treatment	Glucantime	425	Intraperitoneal	Comet assay
	Isoflavones	0.8	Gavage	

NC: negative control; PC-C: positive control with cyclophosphamide; PC-G: positive control with glucantime; Iso: isoflavones; D: Dose. The animals were sacrificed 24 h after the treatment.
[a]Simultaneous treatment.

PCE/NCE (polychromatic to normochromatic erythrocytes) ratio was calculated using 300 erythrocytes per animal.

Comet assay

The peripheral blood leukocytes used for the Comet assay were obtained by making a small cut in the tails of the mice (Singh et al., 1988). The pellet of two drops of blood was mixed with low-melting agarose (0.5%) and placed on slides containing normal melting point agarose. After gel solidification, the slides were immersed in cold lysing solution (2.5 M NaCl, 100 mM Na_2-EDTA, 10 mM Tris (pH 10), 10% dimethyl sulfoxide, and 1% Triton X-100) and refrigerated at 4 °C for 2 h. The slides were then incubated for 20 min in alkaline buffer of pH > 13 (10 M NaOH, 0.2 M EDTA, and distilled water). Electrophoresis was performed for 20 min at 25 V (0.72 V/cm)/300 mA, and the slides were neutralized (0.4 M Tris-HCl; pH 7.5) for 15 min and fixed in ethanol. The slides were then stained with ethidium bromide (20 µg/ml) and analyzed under a fluorescence microscope (BX51/BX52-Olympus; 516–560-nm filter, 590-nm barrier filter, 400 × magnification).

DNA damage was classified into five levels: Class 0 – no damage; Class 1 – low level of damage; Class 2 – medium level of damage; Class 3 – high level of damage; and Class 4 – completely damaged. The damage score was calculated by multiplying the number of nucleoids in each class by the respective class value (Speit & Hartmann, 1995).

Statistical analyses

For statistical analyses, the data were analyzed using the Bioestat 5.0 software (Belém, Pará, Brazil) (Ayres et al., 2005). The dependent variables of DNA damage scores in the Comet assay revealed a normal statistical distribution. For this reason, these data were analyzed using the parametric one-way ANOVA test followed by Tukey's test ($p < 0.05$). In contrast, the frequency of micronuclei in polychromatic erythrocytes did not reveal a normal statistical distribution. Therefore, these data were transformed into square root data for further analysis. Subsequently, the parametric one-way ANOVA test was applied followed by Tukey's test ($p < 0.05$).

The percentage of damage reduction was calculated according to Waters et al. (1990) using the formula: % Reduction = (PC-EG/PC-NC) × 100, where PC is the average damage in the positive control, EG is the average damage in the experimental groups treated with the positive control plus isoflavones, and NC is the average damage in the negative control.

Results

Isoflavones modulates DNA damage caused by meglumine antimoniate

Table 2 displays the frequency of micronucleated polychromatic erythrocytes (MN-PCE) in Swiss mice treated with isoflavones (gavage) and meglumine antimoniate (intraperitoneal) for 24 h. The frequency of micronucleated cells observed in the negative and positive controls are consistent with the treatment received by the animals, thus ensuring the reliability of the data presented for the other treatment groups. The mutagenic action of meglumine antimoniate is evident from the difference between the scores from the group treated only by meglumine antimoniate (16.5 ± 5.6) and the negative control (4.8 ± 2.1). There was no difference between males and females in any treatment group.

There were reduced frequencies of micronuclei for both the groups treated with meglumine antimonate plus isoflavones at 0.4 mg/kg bw (7.9 ± 2.3) and 0.8 mg/kg bw (8.8 ± 2.9) doses, compared to the frequency of micronucleated cells in the group treated only with meglumine antimoniate. Moreover, the frequencies of micronuclei in both the groups do not differ from micronuclei seen in the negative control. On the other hand, the group treated with meglumine antimoniate plus isoflavones at 1.6 mg/kg bw dose did not

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Table 2. Frequency of micronucleated polychromatic erythrocytes (MNPE) obtained by micronucleus test in Swiss mice treated with isoflavone and meglumine antimoniate.

Treatment	MNPE		Cytotoxicity PCE/NCE
	N	Average (‰) ± SD	
NC	77	$4.8^{a} \pm 2.1$	0.87
PC-C	681	$42.6^{b} \pm 18.3$	0.96^{ns}
PC-G	264	$16.5^{c} \pm 5.6$	0.92^{ns}
Iso	226	$14.1^{c,d} \pm 3.5$	0.98^{ns}
D1	126	$7.9^{a,d,} \pm 2.3$	0.96^{ns}
D2	141	$8.8^{a,d} \pm 2.9$	0.93^{ns}
D3	169	$10.6^{c,d,} \pm 3.8$	0.93^{ns}
		$F = 32.1395$; $p < 0.0001$	$F = 0.5836$; $p = 0.7435$

NC: negative control (distilled water); PC-C: positive control with cyclophosphamide (50 mg/kg bw); PC-G: positive control with Glucantime (425 mg/kg bw); Iso: isoflavones (1.6 mg/kg bw); D1: isoflavones (0.4 mg/kg bw) plus Glucantime (425 mg/kg bw); D2: isoflavones (0.8 mg/kg bw) plus Glucantime (425 mg/kg bw); D3: isoflavones (1.6 mg/kg bw) plus Glucantime (425 mg/kg bw). Different letters represent $p < 0.05$ by Tukey's test; ns: not significant when compared to the negative control by ANOVA. A total of 16 000 polychromatic erythrocytes were analyzed per treatment group ($N = 8$).

Table 3. DNA damage scores, nucleoid frequency and rate of damage reduction observed in Swiss mice treated with isoflavones and meglumine antimoniate by Comet assay.

Treatment	Class					Score	Reduction
	0	1	2	3	4	Average ± SD	(%)
NC	80.3	15.1	4	0.6	0	$0.25^{a} \pm 0.06$	–
PC–C	45.6	30.7	13.6	6.9	3.2	$0.91^{b} \pm 0.21$	–
PC-G	47.1	38.3	10.2	3.8	0.6	$0.72^{c} \pm 0.08$	–
Iso	80.8	11.5	5	2.7	0.2	$0.29^{a,e} \pm 0.06$	–
Simultaneous	71.4	20.9	4.9	2.2	0.6	$0.40^{d,e} \pm 0.07$	68
Pre-treatment	72.1	16.9	7.6	3.1	0.3	$0.43^{e,f} \pm 0.13$	65
Post-treatment	78.9	13.6	4.5	3.6	0	$0.32^{a,e} \pm 0.05$	82
						$F = 55.4364$	
						$p < 0.0001$	

Different letters represent $p < 0.001$ by Tukey's test. NC: negative control; PC-C: positive control: cyclophosphamide (50 mg/kg bw); PC-G: positive control: Glucantime (425 mg/kg bw); Iso: isoflavones (0.8 mg/kg bw). Simultaneous, Pre-treatment e post-treatments: isoflavones (0.8 mg/kg bw) plus Glucantime (425 mg/kg bw). All animals were treated over 24 h ($N = 10$ per treatment group).

further reduce the frequency of micronuclei. Furthermore, the mutagenic effect of this dose is evident since the frequency of micronucleated cells in the group treated only with this dose of isoflavone is significantly higher (14.1 ± 3.5) than the frequency observed in the negative control (Table 2).

Table 3 displays the DNA damage scores and the class frequencies detected by the Comet assay in peripheral blood lymphocytes of Swiss mice treated with isoflavones (gavage) and meglumine antimoniate (intraperitoneal) and their respective control groups. The damage score and frequency of nucleoids observed in the negative (0.25 ± 0.06) and positive (0.91 ± 0.21) controls are consistent with the treatment received by the animals.

The damage score (0.29 ± 0.06) in the group treated with the intermediate dose of isoflavones (0.8 mg/kg bw) displays no significant difference compared to the negative control, revealing the absence of genotoxicity at this dose. So, that dose was tested to its ability to modulate the DNA damage induced by meglumine antimoniate in three different treatments: simultaneous, pre- and post-treatment. In all treatments, the damage scores (0.40 ± 0.07, 0.43 ± 0.13, 0.32 ± 0.05, respectively) were significantly reduced compared to the group treated only with meglumine antimoniate (0.72 ± 0.08).

Discussion

In the present study, the *in vivo* genotoxic and mutagenic activity of meglumine antimoniate was confirmed using the Comet assay and micronucleus test, respectively. Our results further support the findings of Lima et al. (2010). Some possible mechanisms of action of pentavalent antimonials against *Leishmania* have been proposed (Haldar et al., 2011). The mechanism that our data supports is that meglumine antimoniate is a prodrug, Sb(V), that is required to be metabolized to act and that its trivalent form Sb(III) produces oxidative reactions that result in DNA damage. This conversion may occur in both the parasite (in its amastigote form) and in the macrophage. Frezard et al. (2001) suggested that this reduction occurs in macrophages by glutathione (GSH) mediation or by a related thiol compound. Cysteine (Cys) and cysteinyl glycine (Cys-Gly) are thiols that are predominant in the lysosomes and can also perform this reduction in mammalian cells. Moreover, trypanothione $T(SH)_2$ has a reducing activity inside the parasite (Frézard et al., 2009). Other studies have suggested the participation of parasite enzymes in this reduction (Denton et al., 2004; Zhou et al., 2004).

Our results revealed that depending on the dose used, soy isoflavones may have mutagenic action or can protect DNA from the damage caused by meglumine antimoniate. Previous studies have reported that effects of flavonoids are dose-dependent, so they may have genotoxic properties (Fox et al., 2012; Lehmann et al., 2005) acting as pro-oxidants by inhibiting enzymes involved in the antioxidant defense system, such as glutathione reductase and glutathione S-transferase (Ferguson, 2001; Kang & Liang, 1997; van Zanden et al., 2003). In addition, flavonoids can generate hydroxyl radicals by auto-oxidation, triggering lipid peroxidation and indirectly inducing oxidative stress in the cell (Michels et al., 2005).

On the other hand, other studies have cited the antioxidant properties of isoflavones, especially their free radical scavenging activity. It has been demonstrated their significant protective effects against oxidative damage induced by reactive nitrogen species (Yen & Lai, 2003) and ROS (Sato et al., 2011; Sierens et al., 2002; Widyarini et al., 2012). These effects are displayed by isoflavone compounds, as either single agents or combined agents. Iovine et al. (2011) demonstrated a synergistic effect of genistein and dadzein against UV-induced DNA damage.

Our data demonstrate that isoflavones were able to reduce genetic damage caused by Glucantime®. Oxidative damage to DNA is an inevitable event of cellular metabolism that can be controlled by antioxidant mechanisms inherent to the cell. However, oxidative stress occurs when there is a mismatch

between the generation of oxidative damage and the repair capacity of the cell. Oxidative damage to DNA is especially important because it produces genetic changes, such as point mutations, chromosomal abnormalities, microsatellite instability, loss of heterozygosity, which are related to the carcinogenic process.

We determined that isoflavones possess antimutagenic activity at 0.8 mg/kg bw by micronucleus test. Yen & Lai (2003) state that these compounds have the ability to decrease the content of nitrate, nitrite and nitrotyrosine in damaged cells. Our data also showed that the protective effect against the genotoxic effects of meglumine antimoniate (by Comet assay) occurred in all treatments (i.e. simultaneous, pre- and post-treatment).

The protective action of isoflavones has been associated with increased activity of antioxidant enzymes (Liu et al., 2005; Wei et al., 1995). Park et al. (2011) demonstrated that isoflavone metabolites increase the binding of transcription factors to promoter regions of antioxidant response elements, thereby increasing the expression of phase II antioxidant enzymes in astrocytes, such as heme oxigenase-1 (HO-1) and NAD(P)H quinone oxidoreductase 1 (NQO1). Thus, the production of ROS is inhibited, and there is a consequent inhibition of cell death. In another study, reduction in DNA oxidative damage and increase in *O*-β-*N*-acetyl-D-glucosaminidase enzymatic activity were observed in healthy women whose diets were supplemented with isoflavones (Erba et al., 2012). Ding & Liu (2011) suggested that genistein reduces the levels of oxidative stress and increases antioxidant enzymes such as superoxide dismutase, glutathione peroxidase and catalase, possibly via the MAPK (ERK1/2) pathway. Our antigenotoxicity and antimutagenicity results corroborate the protective action of isoflavones and their derivatives.

Some studies have suggested the ability of isoflavones in reducing the risk of cancer development (Chen et al., 2003; Jian, 2009; Kim et al., 2005; Luo et al., 2008; Sha & Lin, 2008; Somjen et al., 2012). As the dose and exposure time to isoflavones can interfere with their effects, several mechanisms, such as the inhibition of cell proliferation, inflammation, invasion, metastasis and activation of apoptosis, are related to the anticancer activity of these compounds (Romagnolo & Selmin, 2012).

Our data illustrate that isoflavones have the ability to reduce DNA damage, possibly by reducing oxidative stress. Therefore, we suggest that the anticarcinogenic property of isoflavones can also be related to their antimutagenic activity. However, more studies need to be performed to elucidate the molecular and cellular mechanisms of isoflavones that provide DNA protection and how isoflavones can reverse DNA-induced damage. Future studies will be performed to confirm our hypothesis, including analysis with the FPG (formamidopyrimidine DNA glycosylase), modified version of the Comet assay which detects oxidative DNA bases. Furthermore, it is necessary to assess whether increased antioxidant defenses could reduce or even eliminate the effect of the drug against the parasite. Thus, new studies are being conducted to determine the parasite load in infected animals that are treated with antioxidants and Glucantime.

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Declaration of interest

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The authors report no conflict of interest.

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ANEXO G. Apresentação de trabalhos em eventos científicos.

UNIVERSIDADE FEDERAL DO MARANHÃO
CENTRO DE CIÊNCIAS BIOLÓGICAS E DA SAÚDE
COORDENADORIA DO CURSO DE CIÊNCIAS BIOLÓGICAS
LABORATÓRIO DE GENÉTICA E BIOLOGIA MOLECULAR

LabGeM UFMA

Certificado

O *Laboratório de Genética e Biologia Molecular - LabGeM* da Universidade Federal do Maranhão certifica que PATRÍCIA VALÉRIA CASTELO BRANCO apresentou o trabalho intitulado **Avaliação do Potencial toxicogenético do antitumoral e antileishmanial miltefosine** durante o *II Workshop do LabGEM* nos dias 4 a 6 de março de 2015.

São Luís, 10 de março de 2015

Profa. Dra. Silma Regina Ferreira Pereira
Laboratório de Genética e Biologia Molecular

Prof. Dr. Luis Fernando Carvalho Costa
Coordenador do Curso de Ciências Biológicas

SEMANA NACIONAL DE CIÊNCIA E TECNOLOGIA NO MARANHÃO 2015 LUZ, CIÊNCIA E VIDA

SECRETARIA DA CIÊNCIA, TECNOLOGIA E INOVAÇÃO

GOVERNO DO MARANHÃO GOVERNO DE TODOS NÓS

Certificado

Certificamos que **PATRICIA VALERIA CASTELO BRANCO** apresentou um **POSTER** com o título **O ANTILEISHMANIAL MILTEFOSINE (IMPAVIDO®) INDUZ DANOS AO DNA IN VIVO** na Semana Nacional de Ciência e Tecnologia 2015 no Maranhão.

São Luis, 26 de Outubro de 2015.

CÓDIGO ÚNICO: TWpjd01BPT1zbmN0aQ

UBIRAJARA DO PINDARÉ ALMEIDA SOUSA
SECRETÁRIO DE ESTADO DA CIÊNCIA, TECNOLOGIA E INOVAÇÃO
REITOR PRÓ-TEMPORE DO IEMA

MutaGen - Brasil
ASSOCIAÇÃO BRASILEIRA DE MUTAGÊNESE E GENÔMICA AMBIENTAL

XII CONGRESSO DA MUTAGEN-BRASIL
(ANTIGA SBMCTA - SOCIEDADE BRASILEIRA DE MUTAGÊNESE, CARCINOGÊNESE E TERATOGÊNESE AMBIENTAL)
DE 28 A 30 DE JANEIRO DE 2016 | INDAIATUBA - SÃO PAULO

Certificado

PATRÍCIA VALÉRIA CASTELO BRANCO ARAÚJO

Apresentou no XII Congresso da MutaGen-Brasil, no período de 28 a 30 de janeiro de 2016, o painel: **EVALUATION OF THE GENOTOXICITY AND MUTAGENIC POTENTIAL OF ANTILEISHMANIAL MILTEFOSINE (HEXADECYLPHOSPHOCHOLINE)**

Indaiatuba, 30 de janeiro de 2016

Profª Drª Nadja C. de Souza Pinto
Presidente da MutaGen-Brasil

Prof. Dr. Fabio L. Forti
Secretário da MutaGen-Brasil

XIII CONGRESSO DA MUTAGEN-BRASIL
01 A 03 DE JUNHO DE 2017
RIBEIRÃO PRETO-SP

Realização
MutaGen-Brasil

CERTIFICADO

Certificamos que

PATRÍCIA VALÉRIA CASTELO BRANCO

Apresentou o trabalho

"VITAMIN C PROTECTS AGAINST OXIDATIVE DNA DAMAGE CAUSED BY ANTILEISHMANIAL MILTEFOSINE"

"Patrícia Valéria Castelo Branco, Hugo José Alves, Vera Maciel, Muryllo Santos Castro, Silma Regina Ferreira Pereira"

na Sessão de Pôsteres do XIII CONGRESSO DA MUTAGEN-BRASIL
no período de 01 a 03 de junho de 2017

Ribeirão Preto - SP, 03 de junho de 2017

Daisy Maria Favero Salvadori
Presidente da Associação Brasileira de Mutagênese e Genômica Ambiental

Raquel Alves dos Santos
Primeira Secretária

Apoio
CNPq
CAPES
FAPESP