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ACETURATO DE DIMINAZENO LIPOSSOMAL NO TRATAMENTO DA INFECÇÃO POR *Trypanosoma evansi*: TESTES *IN VITRO* e *IN VIVO*

TESE DE DOUTORADO

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Santa Maria, RS, Brasil

2014

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Camila Belmonte Oliveira

Tese apresentada ao Curso de Doutorado do Programa de Pós-Graduação em Medicina Veterinária, Área de Concentração em Medicina Veterinária Preventiva, da Universidade Federal de Santa Maria (UFSM, RS), como requisito parcial para obtenção do grau de **Doutor em Medicina Veterinária**

Orientadora: Prof.ª Silvia Gonzalez Monteiro

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Elaborada por
Camila Belmonte Oliveira

Como requisito parcial para obtenção do grau de
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RESUMO

Tese de Doutorado
Programa de Pós-Graduação em Medicina Veterinária
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ACETURATO DE DIMINAZENO LIPOSSOMAL NO TRATAMENTO DA INFECÇÃO POR *Trypanosoma evansi*: TESTES *in vitro* E *in vivo*

AUTOR: CAMILA BELMONTE OLIVEIRA
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Data e Local da defesa: Santa Maria, 02 de julho de 2014.

Este estudo teve como objetivo desenvolver e testar lipossomas de aceturato de diminazeno em testes *in vitro* e *in vivo* visando o controle de *Trypanosoma evansi*. O teste *in vitro* foi realizado em meio de cultura nas concentrações de 0,25, 0,5, 1, 2 e 3 µg/mL de aceturato de diminazeno convencional (C-DMZ) e lipossomal (L-DMZ). Para os testes *in vivo* foram utilizados 114 ratos (*Rattus norvegicus*) divididos em seis grupos (A, B, C, D, E e F) em dois experimentos, um para avaliar a eficácia e outro para analisar os parâmetros bioquímicos e histológicos. O grupo A foi utilizado como controle (animais sadios), B (animais infectados e não tratados), C (animais infectados e tratados com aceturato de diminazeno lipossomal com dose única – 3,5 mg/kg^{-1}), D (animais infectados e tratados com aceturato de diminazeno convencional com dose única – 3,5 mg/kg^{-1}), E (animais infectados e tratados com aceturato lipossomal por 5 dias consecutivos com a dose de 3,5 mg/kg^{-1}/dia) e grupo F (animais e infectados tratados com aceturato convencional por 5 dias consecutivos com a dose de 3,5 mg/kg^{-1}/dia). O teste *in vitro* com o lipossoma de aceturato de diminazeno mostrou uma maior mortalidade dose-dependente do *T. evansi* em meio de cultura se comparado ao medicamento comercial e os parasitos morreram mais rapidamente que nos grupos de aceturato de diminazeno convencional e controle. Nos resultados dos testes *in vivo*, a eficácia do aceturato de diminazeno lipossomal e convencional nos diferentes protocolos terapêuticos foram similares. A análise dos parâmetros bioquímicos e histológicos realizados no 7º e 40º pós-tratamento revelaram alterações nas enzimas hepáticas e renais, além de modificações na estrutura dos órgãos, principalmente nos animais tratados com lipossomas na maior dosagem. Os resultados obtidos neste estudo demonstraram que as formulações lipossomais podem ser uma nova alternativa para o tratamento das tripanossomoses. Futuras pesquisas poderiam ser realizadas para melhorar o carreamento e a direção dos lipossomas para uma maior eficácia.

Palavras-chave: Nanoestruturas. Tripanossomoses. Diamidinas.

ABSTRACT

Doctoral Thesis
Programa de Pós-Graduação em Medicina Veterinária
Universidade Federal de Santa Maria

LIPOSOMAL DIMINAZENE ACETURATE OF THE TREATMENT OF INFECTION *Trypanosoma evansi*: *IN VITRO* AND *IN VIVO*

AUTHOR: CAMILA BELMONTE OLIVEIRA
ADVISOR: DR.A SILVIA GONZALEZ MONTEIRO
Date and Place of defense: Santa Maria, July 2th, 2014.

The aim of this study was to develop and to evaluate the therapeutic efficacy of liposomes diminazene aceturate and *in vitro* and by using mice experimentally infected with *Trypanosoma evansi*. *In vitro* tests were performed in culture medium at concentrations of 0.25, 0.5, 1, 2 and 3 mg/ml of diminazene aceturate convetional (C-DMZ) and liposomal (L-DMZ). A total of 114 rats (*Rattus norvegicus)* were used of *in vivo* test. These rats were divided into 6 groups (A, B, C, D, E and F). Group A served as a negative control (uninfected and untreated). Rats in Group B served as a positive control and were infected with *T. evansi*. Animals in Group C were infected and treated with L-DMZ (single dose, 3.5 mg/kg^{-1}), Group D was composed with infected and treated with C-DMZ animals (single dose, 3.5 mg/kg^{-1}), Group E infected treated with L-DMZ animals for 5 consecutive days (3.5 mg/kg^{-1}/dia) and Group F infected treated with C-DMZ animals for 5 consecutive days (3.5 mg/kg^{-1}/dia). *In vitro*, a dose-dependent trypanocidal effect of L-DMZ was observed against the parasite. *In vivo*, the efficacy of L-DMZ and C-DMZ in different therapeutic protocols was similar. The analysis of biochemical and histological parameters on the 7th and 40th post-treatment revealed alterations in liver and kidney enzymes, and histological alterations in the structure of organs, especially in the animals treated with L-DMZ at 5 consecutive days. The results of this study showed that liposomal formulations may be a new alternative for the treatment of tripanosomoses, but future research could be undertaken to improve the conduction of liposomes and direction for greater efficiency.

Keywords: Nanostructures. Tripanossomoses. Diamidines.

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1 INTRODUÇÃO

Os fármacos comumente empregados no tratamento nas tripanossomoses são suramine, aceturato de diminazeno, quinapiramina, melarsoprol, cloridrato de homidium e cloridrato de isometamidium. Estes eliminam os tripanossomas da corrente sanguínea algumas horas após sua administração. No entanto, estes princípios ativos, comercializados com diferentes nomes comerciais, não apresentam a eficácia curativa em grande número dos casos, ocorrendo reincidência da parasitemia após o término do período residual do fármaco (em média de sete dias). Esta reincidência está relacionada com a passagem dos tripanossomas pela barreira hematoencefálica (BHE) e consequentemente ao cérebro, local de refúgio do *T. evansi* durante o período residual do fármaco (LONSDALE-ECCLES & GRAB, 2002; MASOCHA et al., 2007).

A evasão do parasito para o SNC impossibilita uma maior eficácia de fármacos tripanocidas. Este fato estaria relacionado ao tamanho da molécula do aceturato e as suas características hidrofílicas. A passagem de fármacos hidrofílicos para o cérebro é ainda um grande desafio para o tratamento de doenças que envolvem o SNC. Várias pesquisas têm sido realizadas explorando a nanotecnologia e a BHE, principalmente no tratamento para neoplasias, para aumentar a concentração e a biodisponibildade dos fármacos em tumores no SNC, através da utilização de nanopartículas lípidicas que facilitam a passagem pela BHE (SILVA, 2010).

Para melhorar a absorção e o carreamento dos fármacos nos tecidos, a nanotecnologia surge como uma alternativa para a utilização em várias doenças. Tröster et al. (1990) demonstraram que nanopartículas de polimetilmetacrilato (PMMA) atingiram uma elevada concentração em cérebros de ratos após a administração intravenosa. Yang et al. (1999) testaram nanopartículas de poli(butil)cianoacrilato (PBCA) e do antineoplásico camptotencina e obtiveram uma maior concentração no encéfalo de camundongos.

Estudos com sistemas nanoestruturados para o controle de tripanossomas são escassos, Olbrich et al. (2002) desenvolveram um conjugado lipídico (polisorbato 80) associado com aceturato de diminazeno contra *Trypanosoma brucei* e observaram a diminuição do potencial citotóxico através da análise de proteínas do

soro de ratos e em cultura celular e uma maior capacidade de atuação no cérebro. Em outro estudo, Kroubi et al. (2010) desenvolveram uma nanopartícula catiônica porosa com núcleo oleoso ($_{70}$DGNP+) de diminazeno que se mostrou mais estável e observaram uma mortalidade mais rápida em testes *in vitro* contra o *T. brucei*.

Nas doenças parasitárias, os lipossomas já foram estudados nas infecções por *Toxoplasma gondii*, *Trypanosoma cruzi* e *Trypanosoma brucei* (SOUTO-PADRON et al., 1984; TACHIBANA et al., 1988). Yongsheng et al., (1996) pesquisaram a ação do aceturato de diminazeno encapsulado com o lipossoma em camundongos infectados com *T. evansi* e observaram um aumento do tempo de sobrevivência dos animais tratados, mas não foi realizado um estudo sobre a toxicidade do fármaco e exames mais sensíveis para comprovação da cura dos animais. Nesse contexto, justifica-se a busca por uma nova alternativa para o controle de *T. evansi* em animais, utilizando a nanotecnologia nos fármacos disponíveis para o tratamento de hemoparasitoses, com a intenção de melhorar a distribuição do fármaco nos tecidos, facilitar a passagem pela barreira hematoencefálica e diminuir a sua toxicidade.

2 CAPÍTULO 1 – REVISÃO DE LITERATURA

2.1 *Trypanosoma evansi*

Os tripanossomas são micro-organismos pertencentes ao reino Protista, filo Protozoa, subfilo Sarcomastigophora, superclasse Mastigophora, classe Zoomastigophora, ordem Kinetoplastida, família Trypanosomatidae, gênero *Trypanosoma*. Os tripanossomas podem ser distribuídos em duas seções, a Salivaria (transmitidos pela inoculação de vetores) e a Stercoraria (transmitidos pela contaminação da pele ou das mucosas do hospedeiro com as fezes do vetor) (HOARE, 1972).

O *Trypanosoma evansi,* pertencente à seção Salivaria, possui somente a forma tripomastigota e a sua morfologia apresenta forma alongada com núcleo evidente, ausência de cinetoplasto e membrana ondulante. A sua estrutura celular é composta por flagelo; corpo basal ou blefaroplasto, local onde se insere o flagelo; núcleo com cromatina; retículo endoplasmático; complexo de Golgi; mitocôndria; corda paraxial; microtúbulos; ribossomos e membrana ondulante, que pode ser esticada pelo movimento do flagelo (HOARE, 1972).

O *T. evansi* foi o primeiro tripanossoma patogênico descoberto em 1880 por Griffith Evans, que encontrou organismos móveis no sangue de cavalos e camelos doentes (MAUDLIN et al., 1982). A sua transmissão é mecânica e as formas sanguíneas são transferidas diretamente de um mamífero para outro por insetos hematófagos, por exemplo, os insetos das famílias Tabanidae (*Tabanus* sp.) e Muscidae (*Stomoxys* sp.) ou artificialmente por agulhas contaminadas com sangue infectado. Em contraste com a transmissão cíclica, que pode ser tão longa quanto à vida do vetor, a capacidade de transmissão mecânica do protozoário é curta (até seis horas), pois, depende da sobrevivência dos parasitos nas peças bucais do vetor (SILVA et al., 2002).

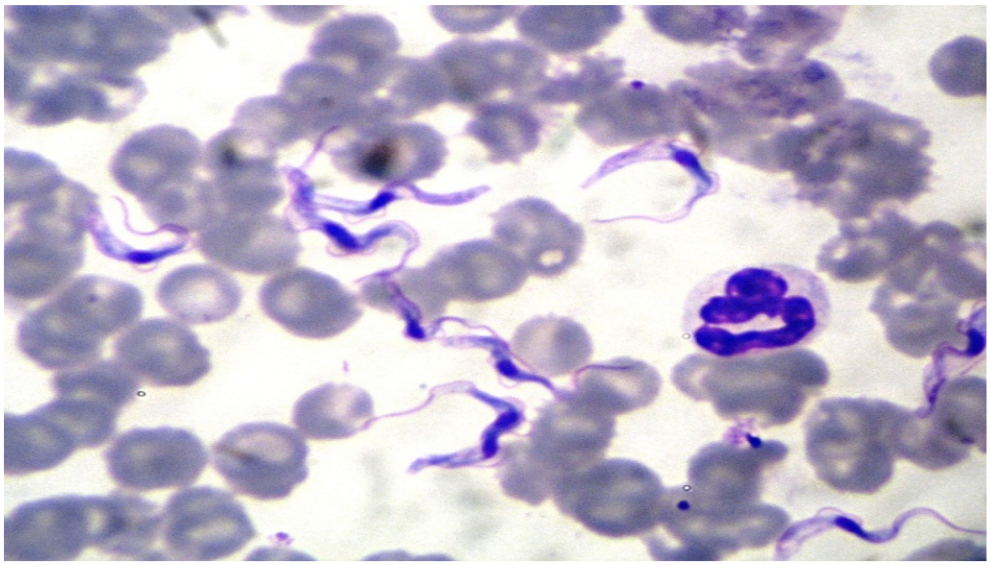

Figura 1 – Formas tripomastigotas de *T. evansi* em esfregaço sanguíneo de ratos infectados experimentalmente, visualizados em microscópio ótico no aumento de 100x.

Fonte: A autora

Na América do Sul, este flagelado pode também ser transmitido por morcegos hematófagos (*Desmodus rotundus*), onde os parasitos podem se multiplicar e sobreviver por um longo período. Dessa maneira, morcegos hematófagos atuam tanto como vetores como reservatórios (HOARE, 1972; URQUHART et al., 1996). Os carnívoros também podem se infectar através da ingestão de animais infectados por *T. evansi.* (URQUHART et al., 1996; BARRIGA, 1997; HERRERA et al., 2004). Esta doença é comumente denominada “Surra” (HOARE, 1972), “Derrengadera” (HOARE, 1972; LEVINE, 1973), “Mal das Cadeiras” (SILVA et al., 1995) ou “Peste Quebra-bunda”.

Vários mamíferos podem ser acometidos pelo protozoário, como equinos (SEILER et al., 1981), asininos (TUNTASUVAN et al., 2003), bovinos (TUNTASUVAN & LUCKINS, 1998), búfalos (TUNTASUVAN et al., 1997), veados (TUNTASUVAN et al., 2000), cães (COLPO et al., 2005), capivaras (*Hydrochaeris hydrochaeris*) (FRANKE et al.; 1994), quatis (*Nasua nasua*) (HERRERA, 2001), marsupiais (*Monodelphis* sp.), tatus (*Euphractus* sp.) (HERRERA et al., 2004), suínos (TUNTASUVAN et al., 2003), camelos (DELAFOSSE & DOUTOUM, 2004) , elefantes (LEVINE, 1973) e humanos (JOSHI et al., 2005).

No Brasil, o *T. evansi* afeta principalmente equinos e a prevalência desta hemoparasitose possuem variações de uma região para outra (HERRERA et al., 2004). A doença é enzoótica no Pantanal mato-grossense, onde assume grande importância em equinos, pois esses animais são amplamente usados para o manejo de bovinos (SILVA et al., 1995; AQUINO et al., 1999). Podem ocorrer surtos esporádicos nesta região com alta morbidade em certas sub-regiões ou estar ausentes em outras (DÁVILA et al., 1999). As capivaras são consideradas reservatórios pelos achados clínicos e laboratoriais (FRANKE et al., 1994).

O relato de animais infectados por *T. evansi* no Rio Grande do Sul é recente em cães (COLPO et al., 2005, FRANCISCATO et al., 2007) e equinos (CONRADO et al., 2005; RODRIGUES et al., 2005, ZANETTE et al., 2008). O surgimento de casos no sul da América do Sul sugere que estudos sobre o agente e a doença, bem como o diagnóstico devem ser aprofundados em áreas não endêmicas desta enfermidade.

A patogenicidade deste tripanossoma no hospedeiro varia de acordo com a espécie animal, cepa do flagelado, fatores inespecíficos afetando o animal como outras infecções e estresse e condições epizootiológicas locais (HOARE, 1972). O *T. evansi* é inoculado principalmente por insetos hematófagos, penetra na pele, multiplica-se no local da picada invade a corrente sanguínea e o sistema linfático, causando febre recurrente e induzindo uma resposta inflamatória (CONNOR & VAN DEN BOSSCHE, 2004). A parasitemia aumenta e é acompanhada por respostas febris, que são seguidas por períodos aparasitêmicos e afebris. Os picos de parasitemia ocorrem devido a variações antigênicas na superfície do parasito. Conforme os anticorpos são produzidos, há eliminação do clone corrente, mas sucessivos novos padrões de antígenos de superfície são gerados para evadir a resposta do hospedeiro (LUCAS et al., 1992).

Este flagelado tem afinidade pelos tecidos devido às alterações inflamatórias, degenerativas e necróticas resultantes da invasão dos tripanossomas nos espaços vasculares (LOSOS & IKEDE, 1972). Dessa maneira, os sinais clínicos dependem da distribuição dos parasitos e da gravidade das lesões induzidas nos diferentes órgãos e tecidos. Estes flagelados podem invadir o sistema nervoso central (SNC) levando a uma lesão progressiva (GIBSON, 1998) e induzir lesões na barreira hematoencefálica (BHE), que irão provocar edema e pequenas hemorragias. O edema vasogênico (aumento de água e outros constituintes do plasma no encéfalo

causado pela lesão nos elementos vasculares do encéfalo) geralmente ocorre nos estágios finais da infecção (PHILIP et al., 1994).

A multiplicação deste protozoário ocorre principalmente nos espaços teciduais extracelulares. Eventualmente, o parasito é encontrado nos espaços vasculares e, provavelmente, utiliza esta rota apenas como transporte. Sinais clínicos neurológicos parecem ocorrer com a presença dos tripanossomas no líquido cefalorraquidiano (LCR). Nesses casos, os tripanossomas induzem uma resposta inflamatória nos nervos espinhais, mas com pouco envolvimento do SNC (RADOSTITS et al., 2002; LUCKINS et al., 2004).

Os sinais clínicos comumente observados na infecção por este flagelado são: febre intermitente, anemia, edema das patas traseiras e das partes baixas do corpo, perda de pelos, fraqueza progressiva, perda de condição corporal e inapetência (LEVINE, 1973). Os animais afetados agudamente podem morrer dentro de dias, semanas ou poucos meses, mas infecções crônicas podem durar anos (BRUN et al., 1998).

Os casos mais graves ocorrem em equinos e cães, enquanto casos crônicos são comumente observados em búfalos e bovinos, que podem não apresentar sinais clínicos. Equinos experimentalmente infectados apresentam febre intermitente nos últimos estágios da doença, com temperatura constantemente elevada, além de emaciação das patas traseiras, do escroto, de mamas, de abdômen e tórax. A taquicardia e a taquipnéia ocorrem nos períodos febris e nos estágios finais e a dispneia poucos dias antes da morte. As mucosas conjuntivas inicialmente congestas tornam-se amarelas e no estágio final da doença apresentam-se pálidas e com petéquias. Ainda pode ocorrer lacrimejamento e descarga nasal aquosa. (FRAZER & SIMONDS, 1909)

Além dos sinais clínicos supracitados, outros sinais clínicos são relatados, como: letargia, depressão, fraqueza progressiva, inapetência, anemia acentuada, conjuntivite, tosse, abortos, aumento dos linfonodos superficiais e edema submandibular (SEILER et al., 1981; TUNTASUVAN & LUCKINS, 1998; MARQUES et al., 2000), distúrbios locomotores caracterizados por relutância em se mover, ataxia, fraqueza, paresia e incoordenação dos membros pélvicos; o equino pode assumir posição de cão-sentado (SEILER et al. 1981; MARQUES et al., 2000).

Nos bovinos, búfalos e veados, os sinais clínicos são semelhantes aos observados em equinos (FRAZER & SIMONDS, 1909; TUNTASUVAN et al., 2000),

mas desenvolvem um curso clínico cuja duração depende da condição do animal. Geralmente o curso é crônico, mas animais em mau estado corporal apresentam doença aguda, e aqueles em bom estado corporal podem não desenvolver doença clínica (FRAZER & SIMONDS, 1909). Bovinos e bubalinos podem abortar do meio até o final da gestação, podendo haver também retenção de placenta, febre alta e diminuição na produção de leite (TUNTASUVAN & LUCKINS, 1998).

Em cães descreve-se conjuntivite, febre, conjuntivas pálidas, emaciação progressiva e aumento dos linfonodos palpáveis. O apetite pode permanecer inalterado e a emaciação deve ser relacionada a outras causas (SILVA et al., 1995; COLPO et al., 2005). Animais silvestres, como capivaras e quatis podem desenvolver uma forma crônica da doença (NUNES et al., 1993; HERRERA et al., 2001). Sinais clínicos geralmente não são observados nas capivaras (FRANKE et al., 1994), mas quando ocorrem, se caracterizam por caquexia e andar cambaleante (NUNES et al., 1993). Os quatis desenvolvem emaciação progressiva, aumento dos linfonodos palpáveis, depressão e letargia (HERRERA et al., 2001). Suínos podem ter inapetência, febre intermitente, petéquias nas orelhas, patas e escroto. Algumas fêmeas abortam e alguns animais podem desenvolver sinais neurológicos, como convulsão, morte, andar em círculos, excitação, saltos, comportamento agressivo, decúbito lateral, incoordenação e paresia dos membros pélvicos, opistótono, convulsão e, finalmente morte. Em alguns bovinos que morreram com sinais clínicos neurológicos os parasitos foram encontrados no líquido cefalorraquidiano (LCR) (TUNTASUVAN & LUCKINS, 1998).

2.2 Tratamento

O tratamento das tripanossomoses é baseado dos fármacos suramine, diminazeno, quinapiramina, melarsoprol, homidium e isometamidium. A escolha da droga, as doses, e a rota de aplicação dependem da espécie animal, do manejo a ser empregado e da quimiosensibilidade da cepa de tripanossoma. A resistência aos fármacos ocorre frequentemente e pode restringir seu uso (BRUN et al., 1998).

O tratamento das tripanossomoses pode ser curativo (utilizando um fármaco com pouca ou nenhuma ação residual) ou preventivo. A diferença entre cura e prevenção vai depender do fármaco que está sendo usada e, em alguns casos, da dosagem que está sendo administrada (PEREGRINE, 1994). As drogas curativas são usadas quando a incidência é baixa e poucos casos ocorrem em um rebanho. A profilaxia ou prevenção é necessária quando os animais estão sob constante risco e quando a enfermidade ocorre em um alto nível durante o ano (SILVA et al., 2002).

O aceturato de diminazeno é comumente empregado no controle do *T. evansi* nos animais domésticos, pois apresenta um maior índice terapêutico na maioria das espécies domésticas. Este fármaco tem atividade contra tripanossomas que são resistentes a outros medicamentos e apresenta baixa incidência de resistência (PEREGRINE & MAMMAM, 1993).

Na América do Sul, nem todos os compostos tripanocidas estão disponíveis comercialmente. No Brasil e Argentina, apenas o aceturato de diminazeno é comercializado no tratamento preventivo e curativo das tripanossomoses africanas (DÁVILA et al., 2000).

2.3 Aceturato de diminazeno

O aceturato de diminazeno (DMZ) é uma diamidina aromática (Fig. 2) que possui atividade tripanocida, babesicida e bactericida, principalmente para *Brucella* sp. e *Streptococcus* sp.. Os fabricantes indicam uma dose única de 3,5mg kg^{-1} para equinos, bovinos, ovinos e caninos, ocorrendo o desaparecimento dos sinais clínicos em 24 horas (BRENDER et al., 1991). Atua contra tripanossomas que são resistentes a outros tripanocidas usados em bovinos e apresenta uma baixa incidência de resistência. O mecanismo de ação do DMZ não é bem compreendido, mas sabe-se que interfere na glicólise anaeróbica e síntese de DNA e RNA dos

parasitos. Também pode interferir na atividade das isoenzimas e da Ca^{++}-ATPase. (PEREGRINE, 1994).

Estudos realizados com o uso deste medicamento na dose de 3,5 mg/Kg em equinos e mulas experimentalmente infectados com *T. evansi*, demonstraram eficácia na primeira aplicação retirando os parasitos do sangue periférico; porém, em um segundo tratamento, 50% dos equinos e 25 % das mulas continuaram positivos. Esse medicamento demonstrou toxicidade leve ou acentuada nos equinos e mulas após a administração (TUNTASUVAN et al., 2003).

Após o tratamento com o princípio ativo, o reaparecimento dos tripanossomas na corrente circulatória pode ser atribuído à sobrevivência dos parasitos resistentes aos fármacos tripanocidas, ou resultante do escape do parasito no líquido cerebroespinhal (MONZON et al., 2003), já que esses medicamentos tripanocidas, com exceção do melarsoprol, não ultrapassam a BHE (JENNINGS & GRAY, 1983; GIBSON, 1998).

O Diminazeno é comercializado em combinação com antipirina, que é um estabilizador que prolonga a atividade do composto em solução. Populações de *T. congolense* e *T. vivax* sensíveis ao produto são eliminadas por administração intramuscular em uma dosagem de 3,5 mg/kg. Este tripanocida subsequentemente veio a ser o produto mais utilizado nas tripanossomoses dos animais domésticos devido a um número de fatores: apresenta o mais alto índice terapêutico do que os outras fármacos na maioria das espécies domésticas; possui atividade contra tripanossomas que são resistentes à outros fármacos usados em bovinos e apresenta uma baixa incidência de resistência (PEREGRINE E MAMMAN,1993).

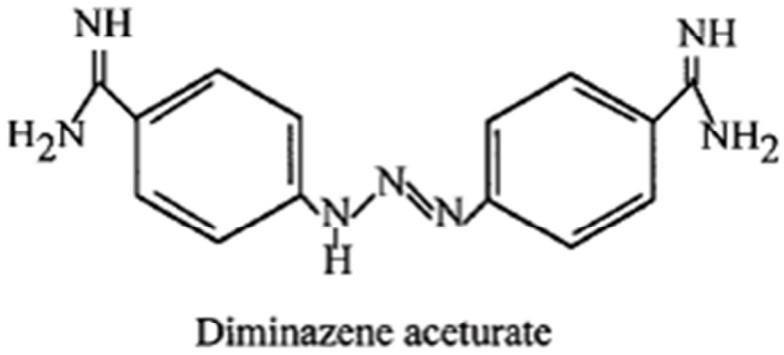

Figura 2 – Estrutura química do aceturato de diminazeno (C14H15N7 · 2C4H7NO3)
Fonte: www.sigmaaldrich.com.

2.4 Nanotecnologia

A nanotecnologia é o estudo que abrange diversas áreas do conhecimento e está impulsionando a pesquisa e o desenvolvimento de novas tecnologias no âmbito farmacêutico. O número de publicações envolvendo produtos nanoestruturados cresce exponencialmente a cada ano, comprovando que a nanotecnologia está em grande expansão. Entre estas publicações, destacam-se o desenvolvimento de sistemas inovadores de liberação de fármacos e outras substâncias ativas de interesse (SOPPIMATH et al., 2001). Sistemas nanoestruturados consistem em sistemas coloidais dispersos de diâmetro particular submicrométrico (< 1 μm) capazes de carrear fármacos e classificam-se em nanocápsulas e nanoesferas, de acordo com o método de obtenção.

Os sistemas coloidais incluem as emulsões submicrômicas, nanoesferas, nanocápsulas, lipossomas e complexos lipídicos que são capazes de atuar como veículos para fármacos lipofílicos e hidrofílicos. Os sistemas nanoestruturados podem ser administrados pelas vias intravenosa, subcutânea, intramuscular, ocular, oral e tópica. A principal vantagem destes sistemas é a capacidade de modificar consideravelmente a penetração intracelular das substâncias a eles associadas. Porém, muitas questões ainda encontram-se sem respostas no que diz respeito ao destino intracelular das nanopartículas, provavelmente devido à grande variedade de linhagens celulares e polímeros existentes (ALLEMÁNN et al.,1992; ALVAREZ-ROMÁN et al., 2001; COUVREUR et al., 1995).

Estes sistemas têm sido desenvolvidos visando um grande número de aplicações terapêuticas, sendo planejados, principalmente, para administração parenteral, oral ou oftálmica. Uma das áreas mais promissoras na utilização das nanopartículas é a vetorização de fármacos anticancerígenos (PUISIEUX et al., 1986) e de antimicrobianos (FRESTA et al., 1995) principalmente através de administração parenteral, almejando uma distribuição mais seletiva dos mesmos e, assim, um aumento do índice terapêutico. Com relação à administração oral de nanopartículas, as pesquisas têm sido direcionadas especialmente a diminuição dos efeitos colaterais de certos fármacos, destacando-se os anti-inflamatórios não esteroides, como o diclofenaco (GUTERRES et al., 2001), os quais causam frequentemente irritação à mucosa gastrintestinal, proteção de fármacos lábeis no trato gastrintestinal (JUNG et al., 2000) e administração oftálmica (LOSA et al.,

1993), visando a diminuição dos efeitos colaterais devido à absorção sistêmica dos fármacos. Dentre os sistemas nanoestruturados, destacam-se as nanopartículas poliméricas e os lipossomas (AMMOURY et al*.,* 1993)

2.5 Sistemas nanoestruturados

Os sistemas coloidais incluem as emulsões submicrômicas, nanoesferas, nanocápsulas, lipossomas e complexos lipídicos e são capazes de atuar como veículos para fármacos lipofílicos e hidrofílicos. Os sistemas nanoestruturados podem ser administrados pelas vias intravenosa, subcutânea, intramuscular, ocular, oral e tópica. A principal vantagem destes sistemas é a capacidade de modificar consideravelmente a penetração intracelular das substâncias a eles associadas.

2.6 Lipossomas

Os lipossomas são vesículas constituídas de uma ou mais bicamadas fosfolipídicas orientadas em torno de um compartimento aquoso (Fig. 3) e servem como carreadores de fármacos, biomoléculas ou agentes de diagnóstico. Eles apresentam diversas propriedades biológicas como a biodegradabilidade e biocompatibilidade e são constituídos de lipídios sintéticos ou naturais em suas membranas (UHUMWANGHO & OKOR, 2005).

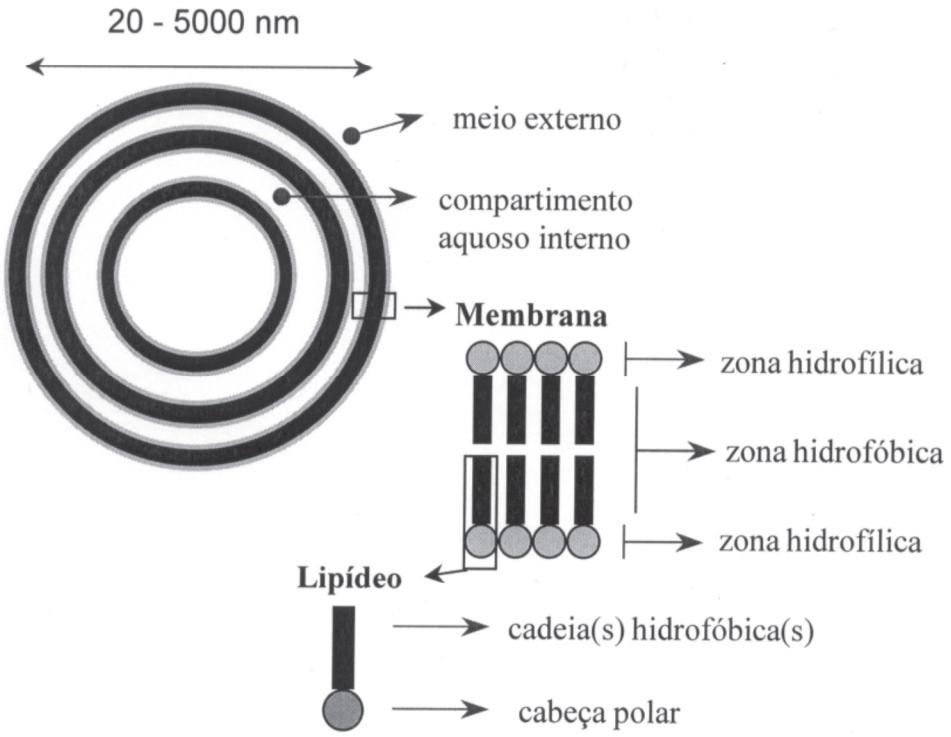

Figura 3 – Características estruturais dos lipossomas
Fonte: Adaptado de FRÉZARD et al.,2005.

2.6.1 Histórico

Os primeiros estudos com lipossomas surgiram de pesquisas realizadas por Alec Bangham há 50 anos com sua observação inicial de que fosfolipídeos em soluções aquosas podem formar estruturas fechadas em bicamadas. Neste mesmo período, Paul Ehrlich propôs pela primeira vez um sistema direcionado de transporte de fármacos, este modelo ficou conhecido por “Bala Mágica de Ehrlich” (*Ehrlich’s Magic Bullet*), o fármaco era ligado ao transportador, direcionando-o ao tecido alvo. Na década de 60, Bangham e colaboradores (1965) demonstraram a capacidade das vesículas lipídicas artificiais oferecerem barreiras para a difusão de solutos e, posteriormente, este sistema foi denominado lipossoma (SANTOS & CASTANHO, 2002).

Somente na década de 1970, Gregory Gregoriadis propôs a utilização de lipossomas como sistema transportador de fármacos, sugerindo que a liberação do fármaco resultava da difusão acelerada através da membrana lipossomal. Esta liberação do conteúdo lipossomal está baseada no fato que o lipossoma perde toda

ou parte de sua estabilidade em diferentes meios e como resultado expõe seu conteúdo para o meio externo (ANDERSON & OMRI, 2004). A utilização de lipossomas como carreadores não obteve sucesso em seus primeiros resultados, devido à instabilidade físico-química e biológica das vesículas, além da baixa eficiência na encapsulação de fármacos. Mas a utilização desta vesícula lipídica foi melhorada através de pesquisas básicas que permitiram o aumento da estabilidade e o entendimento das características físico-químicas e interação com fluidos biológicos (BATISTA et al., 2007)

2.6.2. Características

Os lipossomas são constituídos na sua estrutura básica por fosfolipídeos (sintéticos ou naturais), esteróis e um antioxidante (VEMURI & RHODES, 1995). Os lipídeos mais utilizados nas formulações são os que apresentam uma forma cilíndrica como as fosfatidilcolinas, fosfatidilserina, fosfatidilglicerol e esfingomielina, que tendem a formar uma bicamada estável em solução aquosa. As fosfatidilcolinas são as mais empregadas em estudos de formulação de lipossomas, pois apresentam grande estabilidade frente a variações de pH ou da concentração de sal no meio.

Quanto ao tamanho, as vesículas unilamelares podem ser pequenas ou grandes, sendo caracterizadas como lipossomas unilamelares pequenos - SUV (small unilamellar vesicles) e lipossomas unilamelares grandes - LUV (large unilamellar vesicles). Com relação ao método de preparação, os lipossomas podem ser caracterizados como: VER (vesículas obtidas por evaporação em fase reversa), FPV (vesículas obtidas em prensa de French) e EIV (vesículas obtidas por injeção de éter) (VEMURI & RHODES, 1995; LASIC, 1998).

Esta nanoestrutura pode encapsular agentes farmacêuticos hidrossolúveis e insolúveis em seu compartimento interno e em sua membrana, respectivamente. Os agentes incorporados em lipossomas ficam protegidos dos processos metabólicos que eventualmente possam degradá-los na circulação sanguínea antes de atingir seu alvo, com redução das reações indesejáveis (TORCHILIN et al., 2005). Muitos estudos demonstram a melhora da eficácia em tratamentos com lipossomas, além da redução da toxicidade sistêmica de fármacos, especificamente como

transportadores de fármacos antifúngicas, antitumorais e antimicrobianos (SCHWENDENER, 2007).

A sua estabilidade pode ser afetada por fatores químicos, físicos e biológicos como já supracitado, como por exemplo, após a administração intravenosa, os lipossomas convencionais são rapidamente capturados pelo sistema fagocitário mononuclear e para evitar essa captura foram desenvolvidos lipossomas com a superfície modificada contendo componentes hidrofílicos que permitam a liberação seletiva do fármaco nos sítios alvos (FRÉZARD & SCHETTINI, 2005).

Os métodos de preparação de lipossomas incluem hidratação de um filme lipídico seguida de sonicação ou extrusão para redução do tamanho das vesículas. Os lipossomas são caracterizados quanto ao tamanho, composição química das vesículas e conteúdo do material encapsulado (BATISTA et al., 2007).

2.6.3 Tipos de Lipossomas

2.6.3.1 Lipossomas convencionais

São compostos de fosfolipídeos e colesterol, além de um lipídeo com carga negativa ou positiva para evitar a agregação das vesículas, aumentando a estabilidade em suspensão. Os lipossomas convencionais *in vivo* são reconhecidos pelo sistema fagocitário mononuclear, sendo, então, rapidamente removidos da circulação (VEMURI & RHODES, 1995).

2.6.3.2 Lipossomas de longa circulação

Podem ser obtidos por diferentes métodos, incluindo o revestimento da superfície lipossômica com componentes hidrofílicos naturais como o monossialogangliosideo e fosfatidilinositol, ou de polímeros hidrofílicos sintéticos, especificamente os polietilenoglicóis (PEG) (SAGRISTÁ et al., 2000; TORCHILIN, 2005). A camada hidrofílica superficial destes polímeros aumenta o tempo de circulação dos lipossomas *in vivo* prevenindo o reconhecimento, inibindo a captura pelas células do sistema fagocitário mononuclear (NEEDHAM et al., 1992).

2.6.3.3 Lipossomas sítio-específicos

A superfície dos lipossomas pode ser modificada através da escolha de lipídeos que permitam a conjugação de uma variedade de elementos de reconhecimento, como por exemplo, os anticorpos, glicopeptídeos, polissacarídeos, proteínas virais e lecitinas (EDWARDS & BAEUMNER, 2006). Vários tipos podem ser listados como lipossomas sitio-específicos como, os imunolipossomas e os virossomas.

2.6.3.4 Lipossomas polimórficos

São aqueles que se tornam reativos devido à mudança na sua estrutura desencadeada por uma alteração de pH, temperatura ou carga eletrostática, como os lipossomas termo sensíveis e catiônicos.

2.6.4 Métodos de Preparação de Lipossomas

Alguns métodos são preconizados para a preparação dos diferentes tipos de lipossomas. A maioria dos métodos inclui a dissolução dos lipídeos em solvente orgânico, seguido da evaporação do solvente (formação do filme lipídico), e formação da dispersão de lipossomas multilamelares. O fármaco a ser encapsulado pode ser incorporado na solução tampão (hidrofílico) ou dissolvido na mistura lipídica (lipofílicos).

A partir da dispersão de MLV's, diferentes métodos são utilizados para produzir dispersões homogêneas de SUV's e LUV's, podendo-se empregar processos mecânicos, eletrostáticos ou químicos. Os mais frequentes são os processos mecânicos, em que estão incluídos: extrusão através de membranas de policarbonato, prensa de French ou uso de homogeneizador/ microfluidificador e a sonicação (LASIC, 1993).

2.7 Aplicações Terapêuticas

2.7.1 Emprego na microbiologia

A utilização de lipossomas na terapia antimicrobiana vem sendo tema de vários estudos (FLORINDO et al., 2009; GRECO et al., 2012). Há algumas vantagens na terapia destes medicamentos nas doenças infecciosas e parasitárias, uma delas é a tendência dos lipossomas de ser capturado pelo sistema fagocitário mononuclear (SFM) o que pode ser uma vantagem no tratamento de variedade de doenças infecciosas intracelulares. Labana et al. (2002) avaliaram a eficácia de lipossomas contendo isoniazida e rifampicina contra a tuberculose com doses de 12 e 10 mg/kg em camundongos infectados com *Mycobacterium tuberculosis* e observaram que a formulação exibiu liberação controlada dos fármacos no plasma durante 5 dias, com a presença do fármacos no pulmão, fígado e baço 7 dias após a administração. Além deste resultado os autores demonstraram a redução da carga micobacteriana nos órgãos afetados. Nas infecções por *Streptococcus pneumoniae* e *Klebsiella pneumoniae* causadores de doenças respiratórias, já foram testados ceftriaxona, ciprofloxacino e gentamicina, comprovando-se uma maior eficácia da forma lipossomal dos antimicrobianos na comparação com a forma livre (SCHIFFELERS et al., 2001; ELLBOGEN et al., 2003). Em *Pseudomonas aeruginosa* foi utilizado à gentamicina lipossomal e os resultados também foram satisfatórios (MUGABE et al., 2006).

2.7.2 Emprego na parasitologia

Nas doenças parasitárias, os lipossomas já foram estudados nas infecções por *Toxoplasma gondii*, *Trypanosoma cruzi*, *Trypanosoma brucei, Schistossoma mansoni e Leishmania sp.* (SOUTO- PADRON et al., 1984; TACHIBANA et al., 1988, PAPAGIANNAROS et al., 2005, SANTOS-MAGALHÃES et al., 2010). No estudo realizado por Melo et al. (2003) foi observado a habilidade dos lipossomas em aumentar a eficácia do tartarato de antimônio e potássio contra o trematoda *Schistosoma mansoni*, onde a formulação lipossomal apresentou redução significativa no número de helmintos (82%) e promoveu a redução na toxicidade do tartarato de antimônio e potássio. Mourão et al. (2005) avaliaram a eficiência do

praziquantel (PZQ) para o mesmo parasito supracitado em camundongos e observaram que os lipossomas contendo PZQ foram mais eficazes na redução de vermes comparada com o fármaco livre.

O uso de lipossomas já foi estudado em protozooses como a Leishmaniose. Schettini et al, (2006) testaram lipossomas de antimoniato de meglumina em cães com nanoestruturas que possuíam direcionamento para a leishmaniose visceral e alta retenção tecidual de antimônio. Badiee et al., (2007) encapsularam uma proteína (rLmSTH) da *L. major* em lipossomas e verificaram a redução no número de parasitos vivos e o aumento na produção de anticorpos em camundongos imunizados com a vacina lipossômica. A eficácia de antimoniais pentavalentes encapsulados em lipossomas foi observada no tratamento da leishmaniose visceral em hamsters e camundongos infectados por *L. donovani*. Este aumento da eficácia do antimônio é atribuído à farmacocinética dos lipossomas que são capturados por órgãos como fígado, baço, medula óssea e células (os macrófagos teciduais) nas quais se localizam os parasitos causadores da doença possibilitando uma maior eficácia em parasitoses intracelulares (FRÉZARD *et al*., 2005).

O desenvolvimento de técnicas avançadas de encapsulação e adição de fármacos antimoniais mais eficazes, como o antimoniato de meglumina, têm aumentado significativamente a eficácia das terapias contra leishmaniose (FRÉZARD et al., 2000).

Nas tripanossomoses, os lipossomas já foram alvo de vários estudos, pesquisas realizadas por Gruenberg et al., (1978) com o *Trypanosoma brucei*, flagelado responsável pela “Doença do Sono” em humanos na década de 70 observaram a interação de lipossomas com o protozoário e as membranas celulares para o auxilio em futuras pesquisas com esta nanoestrutura. Antimisiaris et al. (2003) e Zagana et al. (2007) avaliaram a eficácia de lipossomas contra *T. brucei*, mas o último estudo citado obteve melhores resultados modificando a composição das vesículas, sendo observado uma melhor atividade intrínseca contra os parasitos *in vitro*. Em um estudo realizado com o mesmo parasito, Tachibana et al., (1988) avaliaram a atividade citolítica de lipossomas de esterealamina e fosfatidilcolina *in vitro* e obtiveram resultados satisfatórios, pois este lipossomas apresentaram atividade tripanocida. Papagiannaros et al. (2005) testaram lipossomas de hexadecilfosfocolina/gema de ovo e fosfofatidilcolina/ esterarilamina (HePC/EPC/SA) com miletfosina, fármaco utilizado como antiprotozoário e antimicrobiano, contra *T.*

brucei e *L. donovani* e observaram um aumento da mortalidade de *T. brucei in vitro* de um dos compostos formados (HePC/ gema de ovo/ fosfatidilcolina - 10:10:0.1).

A nanotecnologia é também empregada nas pesquisas para *Trypanosoma cruzi* (ROMERO & MORILLA, 2010). Estudos iniciados na década de 80 por Yoshihara et al., (1987) com lipossomas de esterealamina e fosfatidilcolina contra as formas evolutivas do flagelado (epimastigota, triposmastigota e amastigotas), demonstraram uma maior mortalidade da forma tripomastigota, sugerindo que a interação das vesículas com o parasito depende da carga eletrostática do parasito e do lipossoma (SOUZA *et al*., 1977). Morilla et al.(2004) estudaram a cinética do benzimidazole lipossomal em camundongos infectados, esta droga é utilizada na fase aguda da doença de Chagas e provoca grande toxicidade.

As pesquisas realizadas com lipossomas, aceturato de diminazeno e *T. evansi* são escassas, Yongsheng et al., (1996) pesquisaram a ação do aceturato de diminazeno encapsulado em lipossomas de fosfatidilcolina/colesterol/estearilamina em camundongos infectados com *T. evansi* e observaram um aumento do tempo de sobrevivência dos animais tratados. Há algumas pesquisas com nanopartículas e diminazeno contra o *T. brucei*. Em estudos realizados por Kroubi et al. (2010) houve o desenvolvimento de uma nanopartícula ($_{70}DGNP_{+}$) estável e testaram contra *T. brucei in vitro*, e foi observado o aumento da eficácia em dose dependente. Nesta mesma linha Olbrich et al. (2002) desenvolveram uma nanopartícula conjugada com lipídios para potencializar o transporte de fármacos hidrofílicos para o cérebro, mas este estudo teve como objetivo avaliar a toxicidade das nanopartículas de diminazeno.

2.7.3 Toxicidade e lipossomas

A farmacocinética e a farmacodinâmica de um composto ativo é um parâmetro importante para determinação da sua eficácia terapêutica. A utilização de medicamentos implica em ações desejáveis e indesejáveis, neste caso, os efeitos adversos. Estes efeitos estão ligados à concentração e persistência dos fármacos nos vários compartimentos do organismo, além do local de absorção até os locais onde a sua ação será exercida e a passagem de variadas barreiras lipídica formada pelas membranas celulares (VASIR et al., 2005).

Os lipossomas funcionam como transportadores de fármacos que podem alterar a liberação e concentração plasmática, além de aumentar a biodisponibilidade, diminuir os efeitos tóxicos e a dose terapêutica do medicamento. Embora os lipossomas sejam constituídos por moléculas biocompatíveis, algumas alterações podem ser observadas na sua administração. Isto ocorre tanto em doses maiores ou nas administrações contínuas e podem causar efeitos adversos. Muitos destes estão ligados a metabolização do lipídio que é capturado pelo sistema retículo endotelial (Lasic, 1998) havendo o acúmulo de lipossomas no fígado, principalmente nas células não parenquimatosas (Qi et al., 2005). Além da metabolização, os lipídios podem apresentar toxicidade inerente, especialmente a esfingomielina e a esterilamina e produzir peróxidos lipídicos tóxicos (SAETERN et al., 2005).

A utilização de lipossomas e seus efeitos adversos, principalmente pelo acúmulo e excreção das vesículas têm sido alvo de estudos. Segal et al.(1974) provaram através da microscopia eletrônica e marcador lipossomal (NBT), que ocorre depósito desta nanoestrutura em células do sistema retículo-endotelial, demonstrando que a captação hepática de lipossomas foi confirmada a partir da observação das células de Kupffer, dado já relatado por Gregoriadis e Ryman (1972). Este depósito em alguns órgãos pode ser atribuído à interação dos lipossomas com as células e modificação das propriedades físico-químicas nas membranas celulares, acompanhado de alterações das enzimas como a (alcalino fosfatase e γ-glutamil), ALT e glutamato desidrogenase (VAIL et al., 2004). Em outro estudo realizado por Tikhonov et al.(2011) na incorporação de antimicrobianos em lipossomas, foi observado a redução da toxicidade em doses terapêuticas mais baixas e uma mudança na distribuição e prolongamento do efeito terapêutico. Esses mesmos autores observaram que a utilização dos antimicrobianos lipossomais diminuiu o efeito destes fármacos nas enzimas hepáticas e atribuiram este achado à liberação lenta das vesículas lipidicas, fazendo com que a concentração de fármacos antibacterianos, em células de tecido não atingam um nível tóxico.

Outros estudos abordam alterações em órgãos como o fígado, baços e rim na utilização de lipossomas. Chamilos et al. (2007) avaliaram os achados histopatológicos hepáticos na autópsia de pacientes com doenças hematológicas malignas e infecções fúngicas tratados com lipossomas de anfotericina B e os resultados das alterações histopatológicas encontradas no fígado (92%) não foram

associados ao uso da formulação lipidica. Resultados similares foram encontrados por Omri et al. (2003) que testaram “archeossomes”, lipossomas feitos a partir de um ou mais éteres lipídicos polares (Patel and Sprott, 1999) pela via endovenosa em camundongos. Em doses mais elevadas, foi observado o aumento de tamanho do baço e expansão moderada da polpa vermelha com aumento das células hematopoiéticas, mas não houve sinais clínicos de intoxicação.

Além das alterações hepáticas e renais já abordadas, os nanomedicamentos intravenosos (lipossomas, micelas e outras nanopartículas a base de lipídeos) causam reações de hipersensibilidade aguda com manifestações respiratórias, cutâneas e na hemodinâmica (SZEBENI et al., 2007). Estes eventos podem acontecer pela ativação do sistema do complemento (C5a e C3a) sobre as partículas lipídicas e consequentemente a desgranulação de mastócitos, basófilos e outras células inflamatórias em suínos, cães e ratos.

Nas protozooses, uma forma lipossomada de anfotericina B está sendo amplamente pesquisada para o tratamento da Leishmaniose e os resultados mostraram a redução da toxicidade (nefrotoxicidade) se comparados com a forma convencional (RATH et al, 2003).

3 CAPÍTULO 2 – Liposomes produced by reverse phase evaporation: *in vitro and in vivo* efficacy of diminazene aceturate against *Trypanosoma evansi*

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SUMMARY

This study aimed to develop and test the *in vitro* and *in vivo* effectiveness of diminazene aceturate encapsulated into liposomes (L-DMZ) on *Trypanosoma evansi*. To validate the *in vitro* tests with L-DMZ, the efficacy of commercial formulation of diminazene aceturate (C-DMZ) was also assessed. The tests were carried out in culture medium for *T. evansi*, at concentrations of 0.25, 0.5, 1, 2 and 3 µg/mL of L-DMZ and C-DMZ. A dose-dependent effect was observed for both formulations (L-DMZ and C-DMZ), with the highest dose-dependent mortality of trypomastigotes being observed at 1 and 3h after the onset of tests with L-DMZ. The results of *in vivo* tests showed the same effects in the animals treated with L-DMZ and C-DMZ in single dose of 3.5 mg/kg^{-1} and for 5 consecutive days (3.5 mg/kg^{-1}/day). It was possible to conclude that *T. evansi* showed greater *in vitro* susceptibility to L-DMZ when compared to C-DMZ. *In vivo* test, suggest that treatment with the L-DMZ and C-DMZ showed similar efficacy *in vivo*. It was clearly demonstrated the potential of the formulation developed in this study, increasing the efficacy of the treatment against trypanosomiasis DMZ, but more studies are needed to increase the effectiveness *in vivo*.

Keys words: Nanotechnology, Diamidines, Trypanosomosis.

INTRODUCTION

Trypanosoma evansi is a flagellate parasite of domestic and wild animals (Silva et al. 2002), but it rarely parasites humans (Joshi *et al*. 2005). This protozoan has wide geographical distribution, which allows it to be found in countries of Africa, Europe, Asia, Central and South America (Silva *et al*. 2002). In Brazil, the most commonly drug used to treat trypanosomiasis in domestic animals is the diminazene

aceturate (DMZ), which has trypanocidal, bactericidal and babesicide activity, of which manufacturers indicate a single dose of 3.5 mg kg^{-1} for horses, cattle, sheep, and dogs, resulting in suppression of clinical signs within 24 hours (Brender *et al*. 1991). The mechanism of action of the DMZ is not well understood, but it is known that it interferes with anaerobic glycolysis and synthesis of DNA and RNA of the parasites, as well as in the activity of enzymes, such as topoisomerases, nucleases, and Ca^{++}-ATPase (Peregrine, 1994).

DMZ is capable to provide an elimination of the trypanosomes in bloodstream few hours after administration (Peregrine and Mamman, 1993). However, has no curative efficacy in many clinical situations, sometimes occurring relapses of parasitaemia. This situation usually happens when the trypanosomes pass through blood-brain barrier, finding accommodation in the central nervous system (CNS), refuge/privilege area for T*. evansi* during the residual period (21 days) of the drug in the circulation. DMZ do not cross the blood brain barrier in amounts sufficient to eliminate all the parasites (Lonsdale-Eccles and Grab, 2002; Masocha *et al*. 2007).

In order to seek alternative treatments, the nanotechnology is a science that is driving the researches to the development of new pharmaceutical technologies. Among the nanostructured systems stand out the liposomes, which are vesicles that consist of one or more phospholipid bilayers that surround an aqueous compartment, serving as drug carriers, biomolecules or diagnostic agents (Batista *et al*. 2007). They are used as an alternative in reducing systemic toxicity, cytotoxicity to normal cells, side effects associated with chemotherapy, since they are able to change the pharmacokinetics and biodistribution of antineoplastic drugs (Mamot *et al*. 2003; Immordino *et. al*. 2007).

The treatment of trypanosomosis is based on specific drugs such as suramin, diminazene, quinapyramine, melarsoprol, homidium and isometamidium. In South America, the availability of trypanocidal compounds is limited, since some of them are not commercially available. In Brazil and Argentina, for example, only diminazene aceturate is marketed (Dávila *et al*. 2000). However, it is well known that it is, sometimes, ineffective, with recurrence of parasitemia in many of the animals infected with *T. evansi* and treated with DMZ. Therefore, this study aimed to develop a liposome formulation containing diminazene aceturate, as well as to evaluate its trypanocidal activity as a nanoencapsulated product, through the test of susceptibility *in vitro* and *in vivo.*

MATERIAL AND METHODS

Reagents

The reagents used for the preparation of culture medium, except for antibiotics and DMZ, were obtained from Sigma Chemical Co (St. Louis, MO, USA). DMZ (Ganazeg®) was purchased from Novartis, São Paulo, Brazil. Soybean phosphatidylcholine (100 %) was obtained from Idealfarma (São Paulo, Brazil), while polysorbate 80 was supplied by Henrifarma (São Paulo, Brazil) and cholesterol was donated by Cristália (São Paulo, Brazil). Potassium phosphate monobasic and sodium phosphate dibasic were purchased from Vetec (Rio de Janeiro, Brazil), and ethyl acetate was obtained from F. Maia (São Paulo, Brazil). HPLC grade methanol and acetonitrile were acquired from Tedia (São Paulo, Brazil).

Liposomes preparation

Liposomes were produced in batches of 100 mL, in triplicate, employing the method of evaporation in reverse phase (Mertins *et al*. 2005), according to the composition shown in Table 1. The phospholipid (phosphatidylcholine from soybean lecithin), cholesterol and alpha-tocopherol were dissolved in ethyl acetate with the aid of ultrasound. Later, an aqueous solution containing diminazene aceturate and polysorbate 80 was slowly added to the organic phase, leading to formation of two phases. This dispersion was subjected to ultrasonication aiming the formation of reversed micelles which were concentrated by evaporation at reduced pressure until the obtaining of an organogel. The dispersion was subjected to ultrasonication for 5 minutes. In this method, it is necessary to add an initial aqueous portion to the formation of reverse micelles, and another portion in the final step for forming the liposomal vesicles. The reverse micelles are formed immediately when phosphatidylcholine is dissolved in a non-polar medium, due to interactions between the polar heads of phosphatidylcholine, which can interact with each other or with the water molecules after the first water addition. Since the subsequent addition lead to a unidimensional growth of spherical reverse micelles for long cylinders called worm-like micelles due to the hydrogen bonds between phosphate groups of phosphatidylcholine and water. After reaching a maximum length, extended micelles begin a process of aggregation forming a thermoreversible organogel. In the reverse phase evaporation method, after evaporation of the organic solvent, all other components are in the organogel. Dispersion of the organogel in pure water under shaking leads to nanovesicle formation.

Finally, the remaining aqueous solution containing surfactant and the drug were added to the organogel and, under high agitation on rotary evaporator, liposomes

were formed. The size distribution of the vesicles was standardized through the technique of high pressure homogenization (two cycles, at 250 bar) (High-Pressure Homogenizer, Panda 2K, Niro-Soavi, Italy) followed by a sequential filtration on cellulose acetate membranes with porosity of 0.45 µm and 0.22 µm (Milex GV PVDF, Millipore Ireland Ltd, Ireland). This formulation was called DMZ-L. Blank liposomes were also prepared according to the same procedure described above (however, without DMZ in their formulation) as a parameter of comparison. This formulation was called B-L. All batches were stored in dark temperature controlled to maintain the stability of the formulations.

Drug content and encapsulation efficiency

Drug content was assayed by high performance liquid chromatography (HPLC) according to a previously described analytical method (Atsriku *et al*. 2002). The chromatography system consisted of a Luna RP-18 column (250 x 4, 6 mm 5 µm, Phenomenex, Torrance, USA) and a Shimadzu Instrument (LC-10AVP Pump, UV-Vis SPD-10AVP Module, LC Solution software, Shimadzu, Tokyo, Japan). The mobile phase at flow rate of 0.8 mL min $^{-1}$ consisted of acetonitrile-methanol and ammonium formate buffer (20 mM, pH 4.0) at proportion 10:10:80% (v/v/v). The sample was prepared dissolving the liposomes (50 µL) in 5 mL of mobile phase before analysis (dilution factor of 100 times). The volume injected was 20 µL and the drug was detected at 254 nm. The method was linear (r^2=0.9986) in the range of 5-20 µg ml^{-1}, accurate (recovery: 100 ± 2%) and precise (R.S.D.: 1.10% for repeatability and 0.06% for intermediate precision). The specificity was tested in presence of liposomal formulation components (without the drug), where the results demonstrated that these factors did not has influence on the drug assay. Figure 1 shows chromatogram

obtained from the standard solution at the concentration of 10.00 µg ml^{-1}. Encapsulation efficiency was determined by an ultrafiltration-centrifugation technique. Free drug was separated from liposomes using a filter unit (Ultrafree- MC® 10,000 MW, Millipore, Bedford, USA) submitted to a centrifugation at 5000 rpm for 10 minutes. Afterwards, the drug content was determined in the ultrafiltrate by HPLC. Encapsulation efficiency (%) was calculated by the difference between the total and free drug concentrations.

Particle size analysis

Particle size and Span values were firstly determined by laser diffraction (Mastersizer, Malvern Instruments, Worcestershire, UK) over the volume of the particles ($D_{4,3}$). Span values are a measure of the width of the size distribution. Afterwards, the mean particle size and polydispersity index (PDI) were determined by photon correlation spectroscopy (PCS) (Zetasizer® Nanoseries, Malvern Instruments, Worcestershire, UK) after dilution (1:500 v/v) of the liposome dispersion with purified water. pH values were determined by potentiometry directly in the dispersion using a calibrated potentiometer (Denver UB-10, Santo André, Brazil).

***Trypanosoma evansi* isolate**

T. evansi was originally isolated from a dog naturally infected (Colpo *et al*. 2005), and kept cryopreserved under laboratory environment. Initially, two *Wistar* rats (R1 and R2) were infected intraperitoneally with blood (cryopreserved in liquid nitrogen) containing 10^6 parasites/animal for *in vitro* tests.

Culture medium

Culture medium for *T. evansi* was formulated according to the method of Baltz (1985), modified by Dalla Rosa *et al*. (2012). Once prepared, it was stored under refrigeration (10 ºC) until the onset of the experiment. For that, 10 mL of medium was separated in a test-tube, with addition of 1 µl/mL of hypoxanthine 50 mM (dissolved in NaOH, 0,1M) and 2µl/mL of 2-mercaptoethanol 1,2 mM. After this procedure, the enriched culture medium was led to a laboratory stove (37 ºC at 5% of CO_2), where it was equilibrated for 2 hours prior to testing.

Obtention of Trypanosomes

When the R1 rat was under high parasitaemia (10^7 trypanosomes/µL), it was anesthetized with isoflurane inside an anesthetic chamber to perform collection of blood samples EDTA (ethylenediamine tetraacetic acid 10%). 200 µL of blood was employed to trypanosomes separation, diluted (1 v/v) in 200 µl of stabilized culture medium. It was, then, stored in microtubes and centrifuged at 400g during 10 minutes. The supernatant, where were the parasites and few red blood cells, was removed after centrifugation, with the protozoan collected and placed on the culture medium. Then, the count of trypomastigotes was performed using a *Neubauer* chamber, based on the methodology applied by Gillingwater *et al*. (2010) with modifications.

Bioassays *in vitro*

Culture medium with the parasites was distributed in microliter plates (270µl/well). Later, 0.25, 0.5, 1, 2 and 3 µg/mL of liposomal diminazene aceturate (L-DMZ) and conventional diminazene aceturate (C-DMZ) - unencapsulated, were

added. Conventional C-DMZ was prepared by the dilution of the commercial drug (Ganazeg® - Novartis, Barueri, SP, Brazil) in dimethylsulfoxide (DMSO) (Gomes-Cardoso *et al*. 1999). Thus, to validate the tests with C-DMZ, a control group was used (10 µL de DMSO). The control group to validate the test with the liposomal formulation (L-DMZ) was composed by lipid vesicles without diminazene aceturate, characterized as the blank liposomes (B-L). After 1, 3, 6 and 12 hours from the onset of the experiment, the counting of living parasites was performed in *Neubauer* chamber (Baltz *et. al*. 1985). All the tests were carried out in triplicate.

Animals

This study used 42 *Wistar* rats (*Rattus norvegicus*), males at 60 days of age and around 170 grams of weight. The animals were kept in cages with four animals each in an experimental room with temperature, humidity (23ºC; 70% UR) and luminosity (12 hours light/dark) controlled. They were fed with commercial ration and water *ad libitum*. A period of 10 days was set as the adaptation period, in which they were submitted to clinical evaluation and antiparasitic treatment (pyrantel pamoate and praziquantel). The procedure was approved by the Animal Welfare Committee of Ethics in Animal Experimentation of Federal University of Santa Maria (UFSM), number 041/2011.

Animal Inoculation

Animals from groups B, C, D, E, F e G were inoculated intraperitoneally with 0.1 mL of blood (from a rat previously infected with *T. evansi*) diluted in saline solution at 37 ºC. The inoculum was quantified in a *Neubauer* chamber, with the parasite density of 10^6 parasites/ µL.

Evaluation of infection

Parasitaemia was estimated daily by microscopic examination of smears. Each slide was mounted with blood collected from the tail vein (Da Silva *et al*. 2006), stained by *Romanowsky* method, and visualized at a magnification of 1000 x.

Treatments

The rats were divided into seven groups of six animals each (A, B, C, D, E, F and G). The efficacy of liposomal diminazene aceturate (L-DMZ) was evaluated through the longevity, when compared with the treatment with conventional diminazene aceturate (C-DMZ). Thus, Group A was composed by healthy or uninfected animals (negative control); Group B was used as a positive control (not-treated); in Group C the animals were infected and after treated with L-DMZ (3.5 mg/Kg^{-1}); Group D was composed by animals infected and treated with C-DMZ (3.5 mg/Kg^{-1}) in a single dose; In Group E, the infected animals were treated with L-DMZ during 5 days (3.5 mg/Kg^{-1}/day); Group F represented the animals that were infected and treated with C-DMZ (following the same protocol); and in Group G, the infected animals were treated with blank liposomes (B-L) for 5 days. The same volume was used in the groups E and F (1 mL).

The treatments started 24 hours post-inoculation (PI), when the parasitaemia was estimated from 0 to 1 trypomastigotes/field. L-DMZ, C-DMZ and B-L were administered intraperitoneally.

PCR detection of *T. evansi* in brain and blood

Blood and brain samples were collected in animals of groups E and F, since the animals of these groups did not show trypanosomes in their blood smears on the

40th day PI. For this, the animals were anesthetized with inhaled anesthetic (isoflurane). Blood was taken by cardiac puncture and the following volumes were utilized: 1 mL was stored in microtubes with EDTA 10%. Blood and brain samples were preserved in ethanol (1:1), in order to perform the technique of Polymerase Chain Reaction (PCR), carried out to detect the presence or absence of the parasite in CNS (VENTURA *et al*. 2002), proving the effectiveness of the treatment.

Data analysis

The results of tests were analyzed by ANOVA followed by *Duncan* test ($P > 0.05$). Statistical analyzes were performed using *GraphPad Prism* 4.00 for Windows (GraphPad Software, San Diego, CA, USA).

RESULTS

Physicochemical characterization of liposomes

Liposome formulations were analyzed by laser diffractometry in order to attest the presence of particles only at the nanoscale. Granulometric profiles of formulations containing or not the DMZ are showed in Figure 2. Mean particle size was 120 ± 3 nm and 123 ± 2 nm for DMZ-L and B-L, respectively. Span values were lower than 2 (0.88 and 0.92, respectively). Drug content assayed by HPLC was 1.02 ± 0.22 mg/ml. Encapsulation efficiency (%) calculated by the difference between the total and free drug concentrations was 53 ± 10 %. Further characterization was carried out to determine the particle size and polydispersity index by photon correlation spectroscopy and pH. Results are presented in Table 2.

***In vitro* assay**

A dose-dependent effect of both formulations tested was reached, as shown in Figure 3. However the L-DMZ led to a greater mortality of trypanosomes in a dose-dependent effect when compared to the C-DMZ, since at 1, 2 and 3µg mL^{-1} of L-DMZ was capable to cause the mortality of all the parasites after 1 hour of the assay (figure 3b). The same effect was not observed when the equal concentrations of C-DMZ were used (Fig. 3a).

Three hours after the onset of the experiment almost all the parasites subjected to C-DMZ were dead, exception at 0.25 µg mL^{-1}. However, at 6 hours of assay, the encapsulated drug killed all trypanosomes, differently of the control group used for test validation, where the parasites were kept alive until 12 h (Fig. 3a).

Elapsed the same 3 hours of assay, there were no alive trypanosomes at all the concentrations of L-DMZ tested, when compared with the control group (Fig. 3b). When only the diluents (liposome dispersion) were used, there was an initial reduction of parasites living during the first hour of the experiment; however, this number was kept constant for 12 hours, with a similar pattern as observed for the control group.

***In vivo* assay**

Result of longevity and *in vivo* test are shown in table 3. A single dose of 3.5 mg/kg^{-1} of liposomal and conventional diminazene aceturate (group C e D) controlled the infection, but a recurrence of parasitaemia was observed 26 and 31 days later, respectively.

Treatment with L-DMZ and C-DMZ at a dose of 3.5 mg/kg^{-1} for 5 consecutive days showed the absence of the parasite movement during the experiment. Specific

PCR assays from blood and brain of these animals were negative for the presence of *T. evansi*. Animals treated with the solution containing blank liposomes showed no statistical difference when compared with the infected rats (Group B).

DISCUSSION

There are many ongoing studies using the nanotechnology, especially on development of drugs for treatment of diseases caused by micro-organisms, and the liposomes have been suggested as effective carriers of antiprotozoal drugs (Alving, 1986). Liposomes composed of stearylamine/phosphatidylcholine (SA/PC) showed cytolytic activity against *Trypanosoma cruzi*, *T. brucei* and *Toxoplasma gondii* (Souto-Padron *et al*. 1984; Tachibana *et al*. 1988).

During our study, liposomes containing diminazene aceturate were produced, presenting vesicles with monomodal particle and size distribution in the nanometer scale, according to the analysis by laser diffraction. The exclusively nanometric population shows the suitability of the composition employed. There was not verified the presence of micrometric clusters, suggesting that none of the components is above the concentration required. Moreover, the absence of micrometric particles suggests that the drug is associated with the liposomes and/or dissolved in the external medium, excluding the possibility of precipitation in the external medium.

The determination of the size of the vesicles through the technique of photon correlation spectroscopy is more accurate for the particles/vesicles in the nanometer scale. The results from these analyses support those obtained by laser diffraction, showing vesicles with an average diameter close to 100 nm and low index of polydispersity. This low value of polydispersity index reflects the narrow distribution of vesicle sizes. The DMZ used in the formulation followed the therapeutic suggested

value (1.0 mg/mL) with low variation among inter-batch, demonstrating the accuracy and reproducibility of the preparation. Furthermore, although the DMZ is a compound with hydrophilic characteristics, the encapsulation efficiency was around 50%. It can be explained by the ability of liposomes in retaining hydrophilic drugs into the central aqueous phase or between its bilayers (Polozova *et al*. 1999).

Although the particle diameter and measures of uniformity of particle size distribution have not been altered by the presence of the drug, when compared formulations DMZ-L and B-L, the same cannot be stated to pH values. While the pH of the formulation containing no drug (B-L) was near neutrality, the addition of DMZ (DMZ-L) led to a significant reduction of the pH value. This decrease in pH could be explained by the 50% fraction of the drug that was not associated to the liposomes, but dissolved in the external aqueous phase, as previously shown by the results of encapsulation efficiency. The pH of the DMZ is around 5.8 to 6.5, the minimum value we have obtained an approximate value of the liposome (L-DMZ). This pH value may interfere with the in vivo response, since it can influence the stability of the nanostructure (Campbell *et al*. 2004), but the coating of a lipid vesicle could also protect against degradation in acidic medium. To confirm this finding we would accomplish more studies on the degradation of L-DMZ in acidic medium.

The interaction between the parasite and the liposome depends upon several factors, including the size of the liposome, its physicochemical characteristics, the surface charge and fluidity of the phospholipid membrane (Lopez-Berestein, 1987). In this study, the *in vitro* efficacy of L-DMZ was higher than the C-DMZ, leading to a faster mortality of protozoan incubated with L-DMZ. The greater efficacy of the liposome has been demonstrated in other studies with *T. cruzi* and *Leishmania major* (Yoshihara *et al*. 1987; Badiee *et al*. 2007). This result might be attributed to a higher

affinity of the liposomes by the parasite, as already suggested by Kroubi *et al*. (2010). These authors tested porous nanoparticles with lipid core ($_{70}$DGNP^{+}) of diminazene aceturate, observing greater interaction of these particles with the parasite. This increase in the interaction was explained by the different charges present, since, while the nanoparticles had a positive charge, the outer surface of trypanosomes has a negative charge, promoting an electrostatic attraction.

The difference of electric charges is also indicated by Yoshihara *et al*. (1987), when they assessed the *in vitro* efficacy of liposomes with stearylamine (SA-liposomes) against *T. cruzi*. The authors observed a rapid mortality of protozoa, as well as differences between the forms of the parasite. The trypomastigotes has a higher susceptibility, since their surface has more negative charge than epimastigotes and amastigotes (Romero and Morillo, 2010). However, there was no addition of component in the formulation prepared in the present study that could provide a positive charge to the liposomes. Thus, the hypothesis of an electrostatic interaction between the liposomes and the parasites, which would be an explanation to the higher efficacy obtained during the *in vitro* tests, can be discarded. In the liposome developed in this study, the addition of phosphatidylcholine of soybean lecithin may influence its surface potential. This component allows the liposome becomes negatively charged as phosphatidylserine or phosphatidylglycerol (Lopez-Berestein, 1987). Particularly, in this case, this greater effectiveness could be explained by the greater absorption of L-DMZ, due to the reduced size of lipid vesicles through purinergic receptors when compared to the C-DMZ. The P2 receptor of the parasite is involved in the transport of nucleosides and trypanocidal drugs (Anene *et al*. 2001). This might have led to a greater accumulation of L-DMZ by pathogen (Gillingwater *et al*. 2010).

In *vivo* test it was possible to observe that treatment with a single dose controlled the parasitemia, but it did not provide an effective and curative treatment with both C-DMZ e L-DMZ. The therapeutic protocol of 3.5 mg/kg^{-1} is the recommended protocol, however this lack in efficacy was already observed in other studies with dogs, horses and rodents (Tuntasuvan *et al*. 2003; Colpo *et al*. 2005; Doyle *et al*. 2007). In the case of L-DMZ, our data differ from the results of Yongsheng *et al*. (1996) who tested liposomal diminazene in mice and observed a greater longevity of the animals, with a dose 12x higher than the therapeutic recommendation. Additionally, the negative charges of the liposomes are differently developed in this study. The results observed in animals treated with liposomes could be attributed to the low stability of liposome, influenced by chemical, physical and biological agents post administration, since these nanostructures can be captured by the mononuclear phagocytic system, decreasing the active contact with the parasite. Frézard and Schettini (2005) affirmed that liposomes, when administered intravenously, are naturally captured by the macrophages of the reticuloendothelial system, particularly from the liver, spleen and bone marrow. In this study, the drug was administered intraperitoneally and could have been an increased concentration in the mentioned organs, reducing its local concentration, allowing less direct contact with the parasite, and therefore, providing a lower bioavailability of liposomal diminazene in places with high concentration of the parasite.

Another factor to be considered is that the molecules of the drug, in the therapeutic dose, did not cross the blood brain barrier (BBB) (Kaminsky and Brun, 1998) even nanostructured. The groups treated with the therapeutic dose for five days had curative efficacies with both, L-DMZ as C-DMZ. This treatment protocol demonstrates greater efficiency due the passage of these drugs molecules through the BBB,

leading to the higher concentration of an active ingredient, and thus, eliminating the trypanosomes in CNS (Zanette *et al*. 2008; Howes *et al*. 2011).

CONCLUSION

In conclusion, the liposomes developed by reverse phase evaporation have suitable characteristics as a nanotechnological formulation, allowing suggesting they could be alternative approaches for the administration of diminazene aceturate in chemotherapy of infectious diseases, especially trypanosomes. Furthermore, our results demonstrate that L-DMZ have greater efficacy *in vitro* against the *T. evansi*, when compared to conventional drug formulation (unencapsulated). *In vivo* test, these data suggest that treatment with the encapsulated and conventional drug showed similar efficacy *in vivo*. It was clearly demonstrated the potential of the formulation developed in this study, increasing the efficacy of the treatment against trypanosomiasis DMZ, but more studies are needed to increase the effectiveness *in vivo*. Subsequent studies will be performed to assess the *in vivo* course of these formulations, evaluating their potential for targeting and crossing of the blood-brain barrier.

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Legends of figures

Figure 1.Chromatogram obtained from standard solution at the concentration of 10 µg mL^{-1}.

Figure 2. Granulometric distribution of profiles obtained by laser diffractometry of (A) Blank liposomes and (B) Diminazene-loaded liposomes.

Figure 3. Trypanocidal activity in culture medium of diminazene aceturate in its conventional (a) and liposomal (b) forms on *Trypanosoma evansi*. The analyses were performed at 1, 3, 6 and 12 hours post treatment. In the same column, within the circle results not statistically different from each other in Duncan test ($P > 0.05$).

Table 1 – Evaluation of composition of liposomes (n = 3)

	DMZ-L	B-L
Soybean phosphatidylcholine	1.2 g	1.2 g
Cholesterol	0.06 g	0.06 g
Vitamin E	0.72 µg	0.72 µg
Polysorbate 80	0.8 g	0.8 g
Diminazene diaceturate	0.1 g	----
Ethyl acetate	40 mL	40 mL
Water	100 mL	100 mL

Table 2 – Physicochemical characteristics of diminazene aceturate-loaded liposomes (DMZ-L) and blank liposomes (B-L) (n=3).

Formulation	Particle size – PCS (nm)	Polydispersity index	pH (mV)
DMZ-L	96.5 ± 0.3	0.095	4.57 ± 0.04
B-L	112.4 ± 1.6	0.080	7.55 ± 0.02

Table 3 – Mean and standard deviation of the longevity, mortality and success of therapy using treatment with conventional diminizane aceturate (C-DMZ) and liposomal diminazene aceturate (L-DMZ) in rats experimentally infected with *Trypanosoma evansi*.

Groups (n=6)	Longevity (days)	Mortality (Dead/group)	*Therapeutic success (%)
A	40.0[a] (±0.0)	0/6	-
B	7.5[c] (± 1.64)	0/6	-
C	26,4[d] (± 7.63)	6/6	0.0
D	31.0[b] (± 7.34)	1/6	16.6
E	40.0[a] (±0.0)	0/6	100.0
F	40.0[a] (± 0.0)	0/6	100.0
G	40.0[a] (±0.0)	0/6	0.0

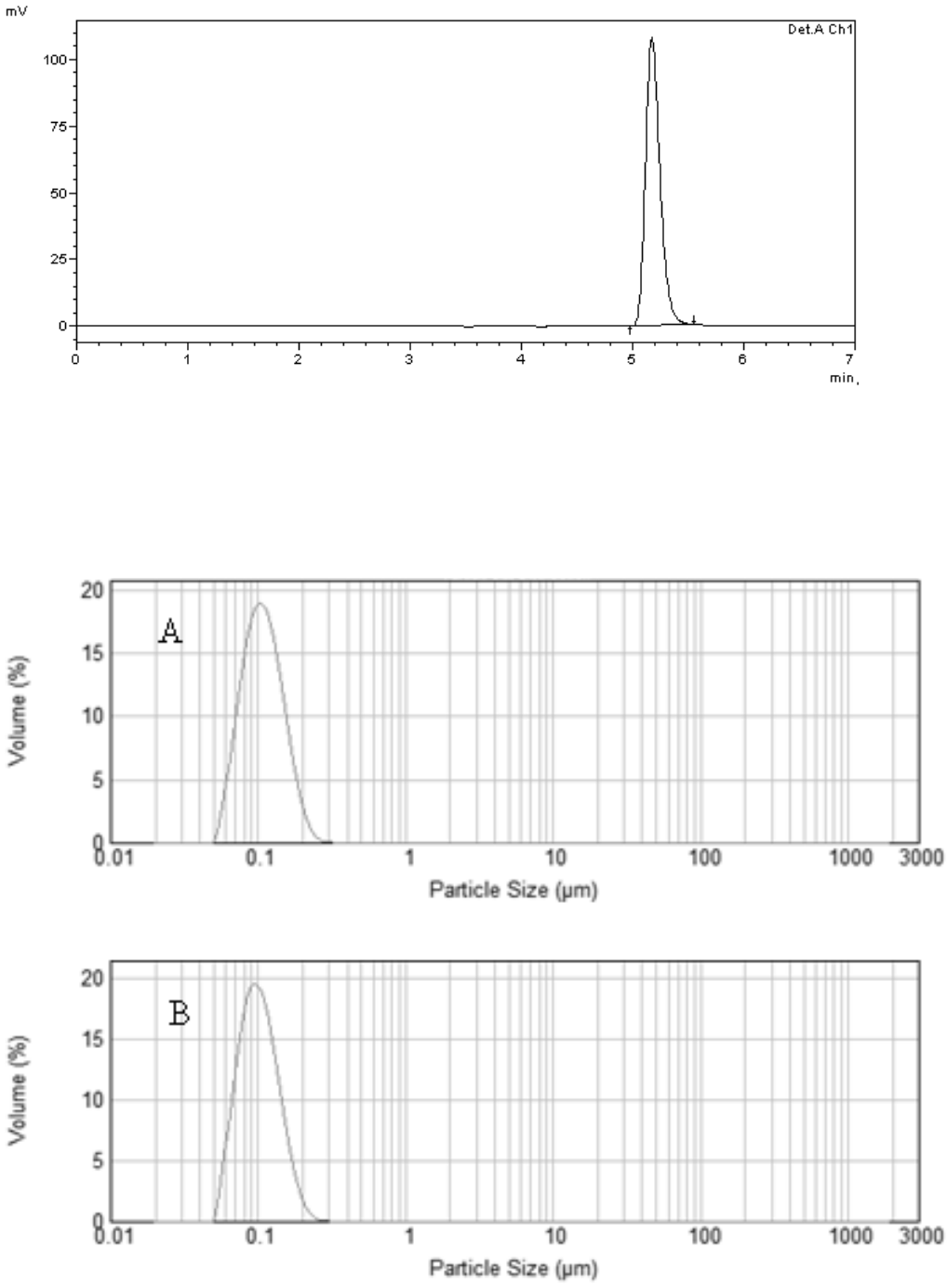

Figure 1 – Granulometric distribution of profiles obtained by laser diffractometry of (A) Blank liposomes and (B) Diminazene-loaded liposomes

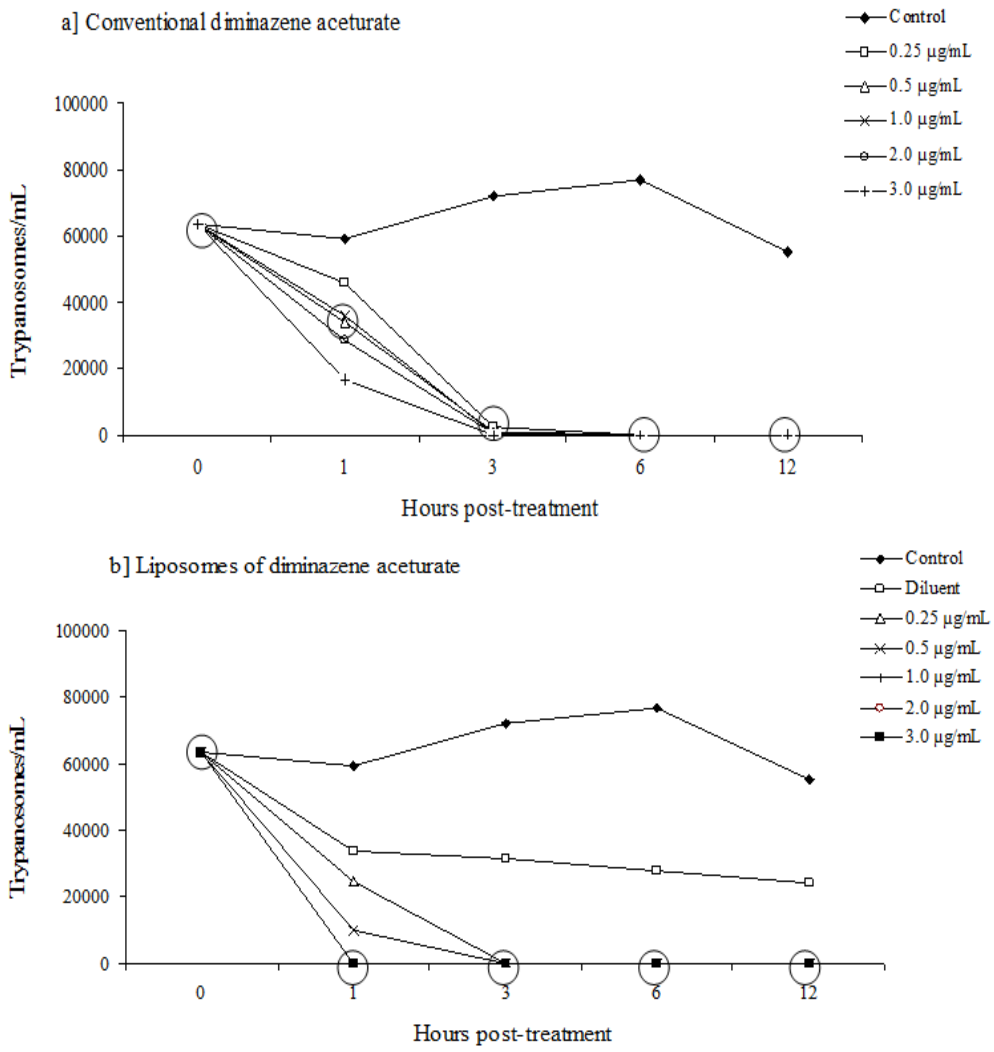

Figura 2 –Trypanocidal activity in culture medium of diminazene aceturate in its conventional (a) and liposomal (b) forms on *Trypanosoma evansi*. The analyses were performed at 1, 3, 6 and 12 hours post treatment. In the same column, within the circle results not statistically different from each other in Duncan test (P> 0.05)

4 CAPÍTULO 3 – Diminazene aceturate liposome: morphometry and influence on biochemical liver, kidney and spleen of rats infected with *T. evansi*

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Diminazene aceturate liposome: morphometry and influence on biochemical liver, kidney and spleen of rats infected with *T. evansi*

Abstract

The aim of this study was to evaluate the effect of treatment with liposomal (L-DMZ) and conventional (C-DMZ) diminazene aceturate formulations on hepatic and renal functions of rats experimentally infected with *Trypanosoma evansi*. For this purpose 72 Wistar rats (*Rattus norvegicus*) were divided into six groups (A, B, C, D, E and F). Each group was subdivided into other two subgroups in order to assess biochemical and histological results on the 7th and 40th days post-treatment. Treatments were carried out based on two different therapeutic protocols: L-DMZ and C-DMZ at 3.5 mg/kg^{-1}, single dose (groups C and D); and five successive doses within intervals of 24 hours (groups E and F). Groups A and B, corresponded to uninfected and infected (without treatment) animals, respectively. The sample´s collections were held on days 7 and 40 post-infection (PI) for assessment of hepatic alkaline phosphatase (AP), alanine transferase (ALT), albumin, gamma glutamil transferase (GGT) and renal functions (creatinine and urea). Additionally, histology of fragments of liver, kidney and spleen was performed. Animals in group B showed a significant increase in AP, GGT, ALT and urea, when compared with group A. On the 7th day PI, the biochemical analyzes showed a reduction (P<0.05) of AP and GGT, while levels of urea were increased in groups C, D, E, F. on 40th PI, ALT was increased in these same groups, when compared with group A. On histopathology, it was possible to observe changes on liver samples, on the 7th day PI, especially regarding to the area and density of hepatocytes, on groups D and F. Renal analysis showed changes in glomerular space, glomerular and corpuscular areas, in group E. Therefore, these results allowed us to conclude that treatment with L-DMZ and C-DMZ led to variable

biochemical changes, which set the liver and kidney functions of treated animals, since the main histopathology alterations were observed in animals treated with liposomes, at their higher dosages. Then, treatments with L-DMZ and C-DMZ, in five consecutive doses, were effective, but they were followed by liver toxicity.

Keywords: diamidines. Lipid vesicles. Toxicity.

1. Introduction

Trypanosoma evansi is a protozoan parasite of mammals and its most common clinical signs are the intermittent fever, anemia, edema, progressive weakness, loss of appetite, and body condition (Levine, 1973). Acutely affected animals may die within days, weeks or a few months, but chronic infections may last for years (Brun et al., 1998).

Diminazene Aceturate (DMZ) is a drug commonly used in Brazil for the treatment of trypanosomosis in domestic animals [27, 31]. The intramuscular dose of 3.5 mg/kg^{-1} is recommended, in order to reach a high treatment efficacy, except for horses and canines [24]. However, DMZ presents reports of resistance and ineffectiveness against different isolates [10], as well as, it is considered as toxic to camels, dromedaries, dogs and horses [34, 20]. This toxicity is attributable to its residual period, and because it easily spreads among various tissues and organs [26].

The safe use of any drug depends on the evaluation of its efficacy, as well as its side effects. The analysis of these effects usually is accomplished through the measurement of several parameters, such as the pharmacokinetics, pharmacodynamics and organic biodistribution. These factors can be influenced by drug administration conveyor systems, which can change the release and maintenance of plasma concentrations of carrier's molecules, increasing their bioavailability and decreasing their toxic effects. These drug carriers may also act as drug vectors, leading them to specific targets, and thereby, reducing the dose required for expressing functionality [18].

The liposomes are used as an alternative transport system in order to reduce the systemic toxicity, mainly because these molecules modify the pharmacokinetics

and biodistribution of drugs [21]. A recent study showed that L-DMZ has curative effectiveness in treating rats experimentally infected with *T. evansi* [25]. In this context, the aim of this study was to evaluate the biochemical and histological parameters of rats experimentally infected with *T. evansi* and treated with DMZ, conventional and encapsulated in liposomes, in order to evaluate the toxicity of these formulations

2. Material and Methods

2.1. Reagents

The reagents used for the preparation of culture medium, except for antibiotics and diminazene aceturate - DMZ, were obtained from Sigma Chemical Co (St. Louis, MO, USA). DMZ (Ganazeg®) was purchased from Novartis, São Paulo, Brazil. Soybean phosphatidylcholine (100 %) was obtained from Idealfarma (São Paulo, Brazil), while polysorbate 80 was supplied by Henrifarma (São Paulo, Brazil) and cholesterol was donated by Cristália (São Paulo, Brazil). Potassium phosphate monobasic and sodium phosphate dibasic were purchased from Vetec (Rio de Janeiro, Brazil), and ethyl acetate was obtained from F.Maia (São Paulo, Brazil). HPLC grade methanol and acetonitrile were acquired from Tedia (São Paulo, Brazil).

2.2. Liposomes preparation

Liposomes were produced in batches of 100 mL, in triplicate, employing the method of evaporation in reverse phase [23]. The phospholipid (phosphatidylcholine from soybean lecithin), cholesterol and alpha-tocopherol were dissolved in ethyl acetate with the aid of ultrasound. Later, an aqueous solution containing diminazene aceturate and polysorbate 80 was slowly added to the organic phase, leading to

formation of two phases. This dispersion was subjected to ultrasonication aiming the formation of reversed micelles which were concentrated by evaporation at reduced pressure until the generation of an organogel. Finally, the remaining aqueous solution containing surfactant and the drug were added to the organogel and, under high agitation on rotary evaporator, occurred the formation of liposomes. The distribution by size of the vesicles was standardized through the technique of high pressure homogenization (two cycles, at 250 bar) (High-Pressure Homogenizer, *Panda* 2K, Niro-Soavi, Italy) and a sequential filtration on cellulose acetate membranes with porosity of 0,45 µm and 0,22 µm (Milex GV PVDF, *Millipore Ireland Ltd*, Ireland) for standardization of size distribution. This formulation received the name L-DMZ. All batches were stored room at temperature, under dark environment.

2.3. *Trypanosoma evansi* isolate

T. evansi was originally isolated from a dog naturally infected [7], kept cryopreserved under laboratory environment. Initially, two *Wistar* rats (R1 and R2) were infected intraperitoneally with blood (cryopreserved in liquid nitrogen), in order to obtain inoculum for all the experimental animals.

2.4 Animals & Experimental Design

Seventy two (72) male Wistar rats (*Rattus norvegicus*), 60 days old and weighing in average 170 grams, composed our experimental animals. The animals were kept in cages with temperature, humidity and light controlled (23°C, 70% RH and 12h light/dark). They received, daily, food and water *ad libitdum*, and went through an adjustment period of 10 days. During this period the rats were evaluated

in their physical and parasitological aspects, receiving antiparasitic drugs (pyrantel pamoate and praziquantel).

The animals were divided into six groups (A, B, C, D, E and F), and each group was once again divided into other two subgroups, for biochemical and histological analysis, to be performed on the 7th (subgroups A1, B1, C1, D1, E1, F1) and 40th (subgroup A2, B2, C2, D2, E2, F2) days after treatment.

2.5 Treatments

The treatments were held, as follow: groups C and E were treated with liposomal diminazene aceturate (L-DMZ) at 3.5 mg/kg^{-1} in a single dose (group C), or during five consecutive (group E). Groups D and F were treated with conventional diminazene aceturate (C-DMZ) at 3.5 mg/kg^{-1} in a single dose (group D), and during for five consecutive days (group F). Groups A and B did not receive treatment, representing the negative control and positive control, respectively.

The treatment started within 24 hours post-inoculation, when the animals were showing low parasitemia (0 to 1 trypomastigotes/field). L-DMZ and C-DMZ were administered intraperitoneally.

2.6 Infection´s evaluation

Parasitemia was evaluated daily until the 1st sample´s collection (7th day), and every two days until the 2nd period (40th day). It was held through blood smears, microscopically observed [32], after stain through Romanowsky technique, and visualized at a magnification of 1000 x.

2.7 *T. evansi* inoculation

Animals of groups B, C, D, E and F were inoculated intraperitoneally with 0.2 mL of fresh blood, obtained from rats previously infected with *T. evansi*. It represented a dose of approximately of 10^6 parasites/ animal.

2.8 Sample´s collection

Blood samples were drawn on the 7th (subgroups A1, B1, C1, D1, E1 and F1) and 40th (subgroups A2, B2, C2, D2, E2 and F2) days PI, and the serum was separated for chemical analysis. Fragment samples of liver, kidney and spleen were removed for histological analysis. All the collection procedures followed the ethic and welfare requirements for animal experimentation.

2.9 Biochemical Analysis

In order to assess hepatic and renal functions, it was performed analysis of enzymatic activity and catabolite´s assessments, on all the groups. Activity of alanine transferase (ALT), gamma glutamil transferase (GGT), levels of albumin, as well as the catabolite's of urea and creatinine, were assessed through a semiautomatic analyzer (TP Analyzer Plus®, Thermoplate-China), using commercial kits (Diagnostic Labtest® SA, Lagoa Santa, MG, Brazil). All the tests were performed in triplicate.

2.10 Histopathological and morphometry analysis

Histological analyzes were carried out in order to investigate the possible tissue damages in animals infected and treated with L-DMZ and C-DMZ. Samples of liver, kidney, spleen and brain were fixed in alcohol 70%, for 24 hours, dehydrated in alcohol series', diaphanized in xylene, and embedded in paraffin. The blocks were

trimmed, providing samples sections of 6 mm thick, in microtome Easy Path EP-31-20094. The slides were stained with hematoxylin-eosin (HE), and subsequently photographed at 40x (liver), 10x (kidney) and 4x (spleen) in five random fields for morphometric.

Morphometry was performed using the *software* ImagePro Plus®. On each blade were photographed several random fields (05 in the liver, 12 renal corpuscles in 06 fields in the kidney and spleen). The Image-Pro Plus software was used to measure the different morphometric parameters studied, namely, cell density and hepatocyte nuclear area in the liver, corpuscular, capsular and glomerular area in the kidney and spleen.

2.11 Data analysis

The results of *in vitro* tests and longevity were analyzed by ANOVA followed by *Duncan* test ($P > 0.05$). Statistical analyzes were performed using GraphPad Prism 4.00 for Windows (GraphPad Software, San Diego, CA, USA).

3. Results

3.1 Biochemical Analysis

The results of biochemical analyzes on the 7th day pi are shown in Table 1. ALP activity was lower ($P<0.05$) in groups C, D, E and F, when compared with groups A and B. Creatinine levels were increased in group B and C when compared with the other groups. GGT activity and concentration of urea were higher in group B (infected), C, D, E, F (treated groups), when compared with group A. Furthermore, it was observed statistical difference between the groups treated with and the group infected and untreated. Albumin level was significantly higher ($P<0.05$) in groups C

and D, when compared with groups A, E and F. ALT only showed an activity change in group B, while in the other groups it did not show statistical difference on the 7th day post-treatment; However, on the 40th day PI, it was observed an ALT increase in groups treated with L-DMZ and C-DMZ, when compared with group A.

Serum biochemical parameters of rats infected with *T. evansi* and treated with L-DMZ and C-DMZ, 40 days post-treatment, are shown in Table 2. AP and GGT activities were higher in group F, compared with group A and group E. On the other hand, urea levels increased groups E and F, when compared with compared to group A.

3.2 Histopathological analysis

3.2.1 Morphometry

The results of the histological morphometric analyzes are shown in Table 3, Figure 1 and 2. On liver morphometry, 3570 hepatocytes were counted. These cells had their nuclear areas measured. On renal tissue, 348 corpuscles were measured in their total area, besides their glomerular and capsular space. In the spleen, it was measured the ratio between white and red pulp areas, area of lymph node and its germinal center.

3.2.2 Liver´s samples

Area of hepatocyte´s nucleus, in the same area groups: group F (L-DMZ/5 days) showed the highest values, with statistical difference ($P<0.05$) when compared with group E (C-DMZ/5 days) and group A. These results demonstrate that animals treated with L-DMZ had showed bulkier areas of hepatocytes. Analysis of hepatocyte density showed a significant difference on the 7th day post-treatment, between

uninfected and infected-L-DMZ treated (single dose). L-DMZ-treated group, under single dose, reached different results when compared with 5 doses-L-DMZ and C-DMZ groups, since these groups showed increased hepatocytes density areas. In the comparison between treatment groups, there was difference between C-DMZ and L-DMZ in single dose. The animals treated with C-DMZ, in both treatment protocols, showed a significant increase in the density of hepatocytes, when compared with L-DMZ. This same change was not observed on at 40th day post-treatment. However, if evaluation period are compared, there was no difference between animals treated with C-DMZ an L-DMZ with the highest dose on the 7^{th} and 40^{th} day post-treatment, resulting in an increased area with reduced density of hepatocytes.

3.2.3 Kidney´s samples

Kidney sample´s showed significantly larger glomeruli areas ($P<0.05$) in animals treated during five consecutive days with L-DMZ on the 7^{th} day post-treatment, when compared with uninfected animals, infected, infected and treated with C-DMZ in single dose, L-DMZ in single dose, and C-DMZ for five consecutive days (group F). The same difference was observed in the groups evaluated on the 40^{th} day post-treatment.

Corpuscular evaluations also showed a significant increase in the gromeruli area in animals treated during 5 consecutive days with L-DMZ, on 7^{th} day post-treatment, when compared with uninfected animals, infected, infected and treated, C-DMZ and L-DMZ in a single dose, and the ones treated with C-DMZ during 5 consecutive days. The same difference was observed in animals treated with both, L-MDZ and C-DMZ, evaluated on the 40^{th} day post-treatment.

3.2.4 Spleen´s samples

In morphometry of the nodule´s area, it was observed differences, especially if the treated animals are compared with uninfected. Nodular area of animals of groups C, D and E presented significantly smaller values ($P<0.05$) than the values of group A. Between groups of animals treated with the same protocol of C-DMZ and L-DMZ, using a single dose, threre wewe no statistical difference. However, at the highest dose, there was a significant differences ($P<0.05$), with D group presenting higher values than group E. This result was also observed on the 40th day post-treatment.

Analyzes of germinal center area showed significant differences ($P<0.05$) among groups C, D, and F on the 7^{th} day post-treatment, showing lower values. Between the groups treated with the same protocol, it was observed, between groups E and F, that the animals treated with L-DMZ showed higher values than the ons treated with C-DMZ, on the 7^{th} and 40^{th} day post-treatment.

4. Discussion

The therapeutic protocols employed showed different efficacy [25], without differences on the biochemical analyzes. However, the histological analysis showed that animals treated with L-DMZ, at its highest dose, showed hepatic, renal and splenic tissue alterations. Similar results were reported by [12] testing diminazene aceturate in mice infected with *T. evansi*. These authors used doses of 10 and 20 mg/kg, reaching a total protozoan elimination, but with a slow tissue recovery and persistence of tissue injury in different organs.

Nanotechnology is a wide alternative for use in the disease treatments, improving the drug absorption and transport into the tissues. The decrease of the side effects of certain drugs has been a target of several researches, for example,

studies on the side effects of nonsteroidal anti-inflammatory [16]; studies in protection of labile drugs in the gastrointestinal tract [17], and studies on ophthalmic administration [19], among others. It is known that the incorporation of antimicrobial agents, into liposomes, helps reduce the toxicity and the therapeutic doses, besides the change in its biodistribution, and the prolongation of the therapeutic effect [1]. In our study were observed, on the 7^{th} and 40^{th} day post-treatment, increased activity of hepatic enzymes and histopathologic alterations in both protocols, using C-DMZ and L-DMZ. This result does not match with the information mentioned above, may suggesting a higher toxicity of the active ingredient, due its higher absorption when as liposome-drug, and used in animals treated with the highest dose, when compared with the commercial product.

Groups treated with L-DMZ showed increased GGT and ALT activity, and as well as increased urea and creatinine levels, demonstrating that L-DMZ formulation was able to changes the hepatic and renal functioning, especially if the histopathological changes were also considered. These results may suggest that liposomal lipid vesicle acts preferably on organs, such as the liver, spleen and, also, on the bone marrow [29]. The liposomes accumulate in some organs, such as liver, and they can interact with the cells, by modifying the cell membranes physicochemical properties. This is followed by significant changes in activity of the enzymes located in the plasma membrane (AP and GGT), cytosol (ALT and AST) and mitochondrial matrix (GLDH) [35].

The reduction of AP on the 7th day post-treatment, of uninfected and infected animals, demonstrated the absence of any damage on liver in treated animals, unlike the results obtained in infected animals, which were euthanized under high parasitemia, corroborating other previous studies [2, 4, 9]. These results were the

same on 40th day, but at this period, it was also observed an albumin reduction in the animals treated with a single of both protocols (L-DMZ and C-DMZ), when compared with the uninfected animals. Thus, it is believed that, at this time, this decrease is linked to liver failure [3].

The influence of treatment with L-DMZ on biochemical parameters and histological analyzes can be mediated by several factors, such as the liposome load, which in this study was negative. This may provide a greater accumulation of these nanostructures in the liver and spleen, unlike positively charged liposomes, which primarily accumulate in the lung. Besides this factor, particles smaller than 100 nm are able to evade the phagocytic cells, accumulating on liver [28].

The creatinine concentration was increased in group B, suggesting a slight deficiency in glomerular filtration rate and renal functionality [14]. GGT also showed increased activity in groups B, C, D, E, and F on 7th day post-treatment, remaining elevated until the 40th day post-treatment in group F. This result suggests some level of changes in the liver, especially linked to hepatic tissue damage (as observed in the histology), since the parenchymal liver cells are also in charge for the detoxification of drugs and toxins [33]. Moreover, GGT is a biomarker of hepatic dysfunction and inflammation, with the main reason for its increase related with poisonings and substance abuse, obstruction of the biliary tract, liver inflammation (hepatitis) and medication use [3].

Urea levels were higher on the 7th day in the treated groups, and also, in the groups of infected animals, when compared with the group of uninfected animals. This change was also observed in the groups treated for five consecutive days with L-DMZ and C-DMZ on the 40th day post-treatment. Moreover, a statistical difference between the infected and treated groups was observed. This result suggests some

level of renal dysfunction, since there was an increase in the levels of creatinine and blood urea. ALT showed an alteration only in group B, since for the other groups no statistical differences were observed between them. ALT usually indicates liver disease caused by hepatocellular disease, hepatic necrosis, biliary obstruction, poisoning and parasitic infections. This finding was already reported in several studies with *T. evansi* [2, 5, 9]

Increased ALT values were observed on the 40th day post-treatment, on the groups treated with L-DMZ and C-DMZ under the same therapeutic protocol, when compared with group A, may suggesting a liver damage. The increased serum ALT levels may be related to necrosis of the hepatocyte membrane or hypoxia, since the ALT in chronic liver disease may be normal, decreased or increased slightly [22]. In this study the animals did not show typical clinical manifestations of liver disease, such as diarrhea, vomiting, anorexia, depression, jaundice and abdominal pain.

Most of the changes observed in animals treated with L-DMZ may be associated with liposomal metabolism, that when captured by the macrophages of the mononuclear phagocyte system, especially the liver, spleen and bone marrow [13] causes damage in liver tissue. In this study the drug was administered intraperitoneally, differently of the method used in study mentioned above; however, it is possible that an increase in the concentration of this drug occurred on the organs.

The changes reported in the liver of groups treated for 5 days with L-DMZ, in biochemical and histological parameters, can be attributed to the accumulation of these liposomes in this organ, especially in non-parenchymal cells, since the liver is composed predominantly of parenchymal cells with a small proportion of Kupffer cells, part of the reticuloendothelial system, and with highly phagocytic activity [15].

Another factor, which may have contributed to the changes already reported, is the liposome´s composition used. It is known that nanostructures with soybean sterylglucoside (SG) may accumulate in hepatocytes [30]. Liposomes of smaller diameter are directed, primarily, to the hepatocytes, mainly because they are small, passing through the fenestrae of sinusoids, which have an average diameter of 0.1 µm. The larger liposomes have restricted access to hepatocytes and are preferably captured by the Kupffer cells [18].

The parameters assessed in the morphometry of the kidneys showed significant changes, probably related with the renal function. The changes observed on the 7th day, in animals treated during 5 days with L-DMZ, suggested an increase in the filtration rate. This result was verified at the corpuscle area, capsule glomerular and capsular space. These structures are involved in some important kidney functions, representing a physical, and being in charge of glomerular filtration. Thus, our findings may suggest that the glomerular filtration rate was increased in the groups previous mentioned. This increase can be attributed to rapid excretion, by filtration of some aggressive and hazardous substances to the organism.

In the spleen, the observation of reduction of the germinal and nodular center areas may be related to the residue of active ingredient and lipid vesicles of liposomes. This change was observed in animals treated with C-and L-DMZ DMZ, no difference in the animals treated with the highest dose in both the 7th and in the 40th post-treatment day. The pharmacokinetics of diminazene and liposomes in rabbits, reporting DMZ residues in the spleen of animals treated with liposomes [36]. This fact might explain the increase in the germinal and nodular center area in animals treated with L-DMZ, when compared with those treated with C-DMZ. These residues could lead to spleen and liver damage [11].

Conclusion

In our study, the animals treated with L-DMZ showed alterations in both treatment protocols in biochemical parameters, with reduction of AP and increase of GGT, albumin and ALT, as well as an increase in the levels of urea on the 7th and 40th day post-treatment. In the histological changes, it was observed changes in the organs structure, suggesting the ability of liposomes to activate macrophages and increase the metabolism and excretion of lipid vesicles; thereby, causing changes in spleen, liver and kidneys.

Ethics Committee: This study was approved by the Ethics and Animal Welfare at the Universidade Federal de Santa Maria (UFSM), protocol number 87/2010.

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Table 1 – Comparison of serum biochemical parameters of rats infected with *T. evansi*, and treated with liposomal (L-DMZ) and conventional (C-DMZ) diminazene aceturate on the 7th day post-treatment.

Groups	Biochemical Parameters					
	PA	**Albumin**	**Creatinine**	**GGT**	**Urea**	**ALT**
A	606,6^{b}(±62,3)	1,91^{b}(±0,09)	0,78^{bc}(±0,07)	6.75^{c}(±1,47)	37.2^{c}(±4,25)	40^{b}(±5,16)
B	852,5^{a} (±35)	2,1^{b}(±0,28)	1,33^{a}(±0,37)	10.60^{ab} (±0,7)	61.04^{a}(±4,92)	163^{a}(±41)
C	431.6^{cd}(±65,04)	2,7^{a} (±0,27)	1,06^{ab}(±0,48)	11.80^{ab} (±2,7)	48.42^{b}(±6,66)	55^{b}(±4,08)
D	383.2^{d}(±16,44)	2,8^{a}(±0,28)	0,78^{bc}(±0,05)	9.28^{a} (±1,34)	47.10^{b}(±4,14)	41^{b}(±4,16)
E	509.9^{c}(±78,26)	1,9^{b}(±13)	0,66^{c}(±0,11)	12.65^{ab}(±2,74)	47.17^{b}(±4,86)	41,2^{b}(±4,6)
F	394.6^{d}(±36,43)	2,0^{b}(±0,38)	0,74^{c}(±0,06)	11.18^{ab} (±3,1)	48.32^{b}(±2,59)	36,4^{b}(±6,22)

* Same letters at the same column are not statistically different. Significance level of 5% was considered in the t-test. Group A: uninfected and untreated animals; Group B: animals infected with *T. evansi* and untreated; Group C: animals infected with *T. evansi* and treated with liposomal diminazene aceturate (3.5 mg/kg^{-1}); Group D: animals infected with *T. evansi* and treated with conventional diminazene aceturate (3.5 mg/kg^{-1}); Group E: animals infected with *T. evansi* and treated with liposomal diminazene aceturate during 5 days (3.5 mg/kg^{-1}); Group F: animals infected with *T. evansi* and treated with conventional diminazene aceturate for 5 days (3 5 mg/kg^{-1}).

Table 2 – Comparison of serum biochemical parameters of rats infected with *T. evansi*, and treated with liposomal (L-DMZ) and conventional (C-DMZ) diminazene aceturate on the 40th day post-treatment.

Groups	Biochemical Parameters			
	PA	**GGT**	**Urea**	**ALT**
A	563.46[b] (±62,3)	6.75[b] (±1,47)	37.19[b] (±4,25)	40[b] (±5,16)
E	552.6[b] (±78,26)	8.03[b] (±2,74)	49.09[a] (±4,86)	50[a] (±4,6)
F	715.06[a] (±36,43)	11.66[a] (±3,19)	46.98[a] (±2,59)	48.8[a] (±6,22)

* Same letters at the same column are not statistically different. Significance level of 5% was considered in the t-test. Group A: uninfected and untreated animals; Group E: animals infected with *T. evansi* and treated with liposomal diminazene aceturate during 5 days (3.5 mg/kg^{-1}); Group F: animals infected with *T. evansi* and treated with conventional diminazene aceturate for 5 days (3, 5 mg/kg^{-1}).

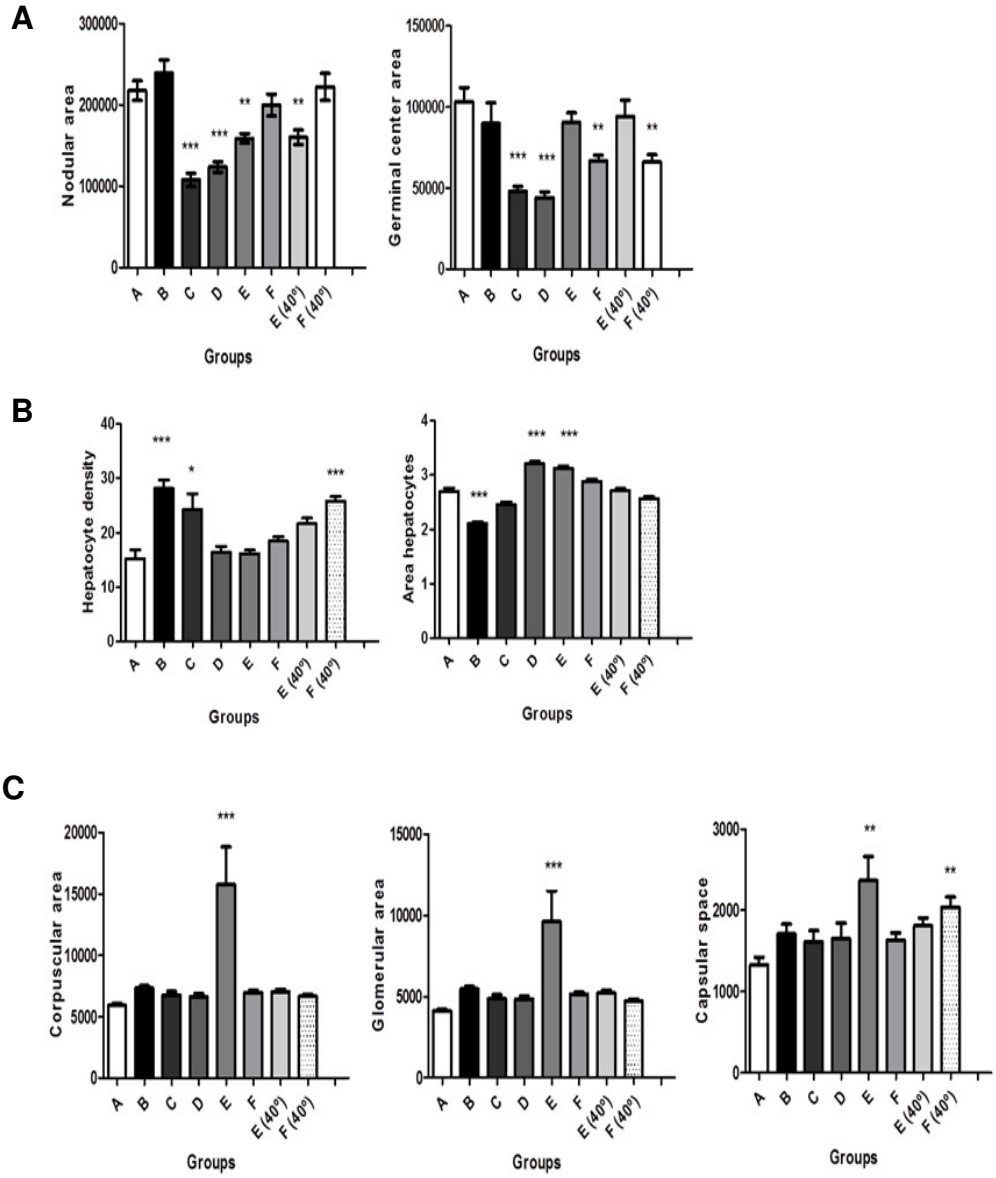

Figure 1 – Histological evaluation of groups A, B, C, D, E, and F on the 40th day post-treatment. (A) Spleen; (B) Liver and (C) Kidney. * Represents significant difference between groups (P<0, 05). The columns represent the mean ± are standard deviation, n = 6 (Bonferroni's test).

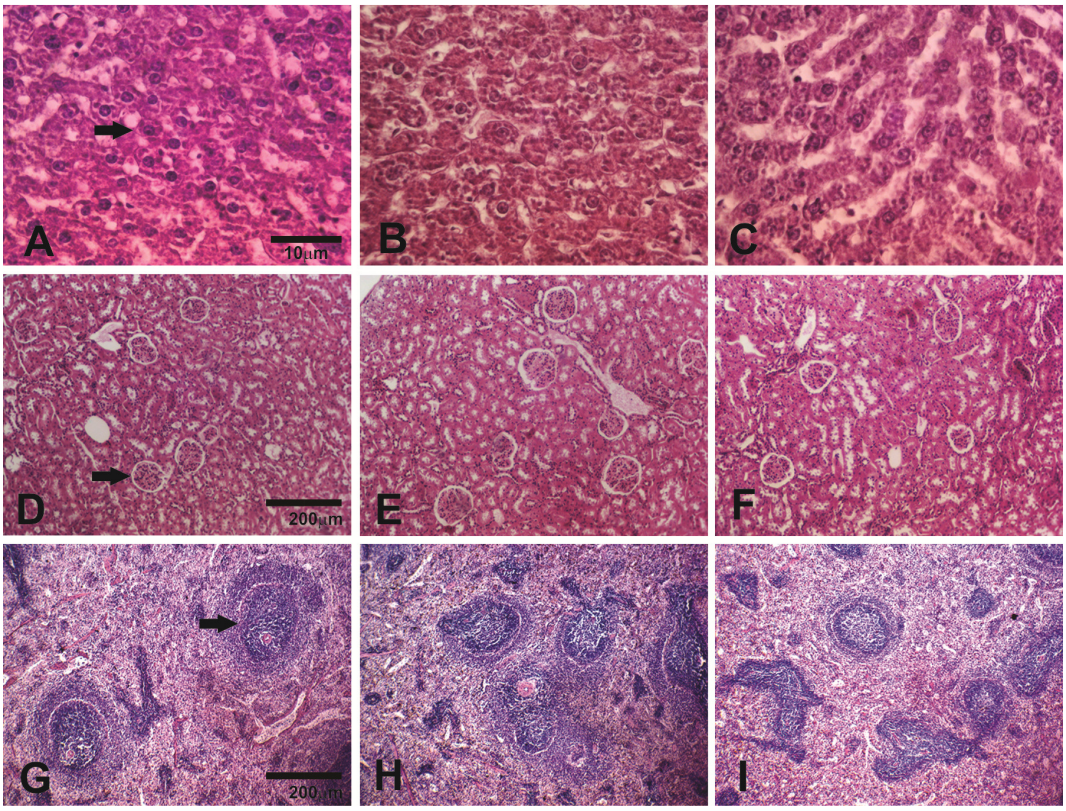

Figure 2 – Photomicrographs of liver tissue indicating the density and areas of hepatocytes nuclei; uninfected group (A); group treated with conventional diminazene aceturate (C-DMZ) during for 5 days; (B) treated with liposomes diminazene aceturate (L-DMZ) during 5 days (C). Photomicrographs of the renal cortex showing the corpuscular area, glomeruli and the capsular space; Healthy group (D); treated with C-DMZ during 5 days (E); and treated with L-DMZ during 5 days (F). Photomicrographs of splenic pulp showing areas of red pulp areas and of the lymph nodes of the white pulp; Healthy group (G); treated with a single dose of C-DMZ (H); and treated with a single dose of L-DMZ (I). The arrows indicate the quantified structures.

5 CONSIDERAÇÕES FINAIS

Neste estudo conclui-se que os lipossomas de aceturato de diminazeno desenvolvidos por evaporação em fase inversa têm características adequadas de uma formulação nanoestruturada podendo assim ser utilizados para o estudo da atividade tripanocida. Em nosso estudo, os resultados obtidos demonstraram uma maior eficácia dos lipossomas *in vitro* se comparados com o medicamento convencional, fato este que pode ser atribuído a uma maior absorção da molécula nanoestruturada, já que ela é menor que o medicamento convencional. No teste *in vivo*, a eficácia estre as duas formulações foi similar na dose terapêutica, ocorrendo à recidiva da parasitemia após o período residual do medicamento antiprotozoário. Na maior dose houve a eficácia curativa nos grupos tratados com aceturato de diminazeno lipossomal e convencional.

Além da eficácia, avaliamos as alterações provocadas pelas duas formulações e os protocolos terapêuticos, onde foi observado que os animais tratados com os lipossomas mostraram alterações em ambos os protocolos de tratamento nos parâmetros bioquímicos, com redução da enzima fosfatase alcalina e aumento da gama glutamil transferase, albumina e alanina aminotransferase, bem como um aumento dos níveis de ureia nos dois períodos do experimento (7º e 40º dia pós-tratamento). Nas análises histológicas, ocorreram alterações na estrutura de órgãos, como o fígado, rim e baço o que sugere a capacidade dos lipossomas para ativar macrófagos e aumentar o metabolismo e excreção das vesículas lípidicas.

Nossos dados sugerem que a utilização do aceturato de diminazeno lipossomal possui potencial na eficácia curativa desta tripanossomose, mas altera a estrutura dos tecidos de órgãos responsáveis pela metabolização e excreção dos fármacos. Para melhorar a absorção e o carreamento de fármacos nos tecidos, é necessário que mais pesquisas sejam realizadas para que o princípio ativo, que possui características hidrofílicas, tenha capacidade curativa em menores doses, tenha um melhor direcionamento para passagem pela barreira hematoencefálica, aumentando dessa forma a concentração do farmaco no SNC, já que o parasito se esconde nesse local evadindo o sistema imune.

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