UNIVERSIDADE FEDERAL DE SANTA MARIA
CENTRO DE CIÊNCIAS RURAIS
PROGRAMA DE PÓS-GRADUAÇÃO EM MEDICINA VETERINÁRIA

3'DEOXIADENOSINA E DEOXICOFORMICINA NO TRATAMENTO DE CAMUNDONGOS INFECTADOS EXPERIMENTALMENTE COM *Trypanosoma evansi*

TESE DE DOUTORADO

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Santa Maria, RS, Brasil

2014

3'DEOXIADENOSINA E DEOXICOFORMICINA NO TRATAMENTO DE CAMUNDONGOS INFECTADOS EXPERIMENTALMENTE COM *Trypanosoma evansi*

Luciana Dalla Rosa

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 de grau de
Doutor em Medicina Veterinária

Orientadora: Silvia Gonzalez Monteiro

Santa Maria, RS, Brasil

2014

Ficha catalográfica elaborada através do Programa de Geração Automática
da Biblioteca Central da UFSM, com os dados fornecidos pelo(a) autor(a).

Dalla Rosa, Luciana
3\'Deoxiadenosina e Deoxicoformicina no tratamento de camundongos infectados experimentalmente com Trypanosoma evansi / Luciana Dalla Rosa.-2014.
80 p.; 30cm

Orientadora: Silvia Gonzalez Monteiro
Tese (doutorado) - Universidade Federal de Santa Maria, Centro de Ciências Rurais, Programa de Pós-Graduação em Medicina Veterinária, RS, 2014

1. Cordicepina 2. Pentostatina 3. Adenosina 4. Tripanossomatídeo 5. Dose ideal I. Monteiro, Silvia Gonzalez II. Título.

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UNIVERSIDADE FEDERAL DE SANTA MARIA
CENTRO DE CIÊNCIAS RURAIS
PROGRAMA DE PÓS-GRADUAÇÃO EM MEDICINA VETERINÁRIA

A Comissão Examinadora, abaixo assinada, aprova a Tese de Doutorado

3'DEOXIADENOSINA E DEOXICOFORMICINA NO TRATAMENTO DE CAMUNDONGOS INFECTADOS EXPERIMENTALMENTE COM *Trypanosoma evansi*

Elaborada por
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como requisito parcial para obtenção do grau de
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Santa Maria, 01 de dezembro de 2014.

DEDICATÓRIA

As pessoas mais importantes da minha vida: meus pais

AGRADECIMENTOS

Aos meus pais, Roque e Jocélia, que foram, durante todos esses anos, um grande exemplo de força, coragem, amor, perseverança e energia infinita. E aos meus irmãos, Lucas e Marcos, pelo apoio e incentivo constantes em minha vida.

À professora Silvia Gonzalez Monteiro, minha orientadora, e junto dela, o professor Daniel Roulim Stainki. Obrigada por confiarem em mim e pela colaboração, fazendo eu chegar até aqui e ir muito além.

Ao Mauro, por estar sempre disposto a ajudar em qualquer situação e, principalmente, pelo seu apoio que me confortou no período de doutorado sanduiche. E também um muito obrigada cheio de saudades aos amigos que fiz em Salt Lake City: Diana, Oscar, Bia, Ricardo, Mocha, Camille, Leandro, Dennis, Fabinho, Marina, Osman, Cris, Vida, Andressa e Iverson.

Aos colegas do Laboratório de Parasitologia Veterinária, Aleksandro, Camila e Lucas que acompanharam toda minha tragetória no laboratório, e à Thirssa, Bianca, Matheus, Mariângela, Andressa, Luzia Cristina que compartilharam os prazeres e dificuldades, pela convivência agradável e agitada, pelo carinho e disponibilidade em sempre me ajudar.

À CAPES pela bolsa, possibilitando minha dedicação exclusiva ao doutorado e a possibilidade de realização do doutorado sanduiche.

Enfim, dirijo, igualmente, meu reconhecimento e agradecimento à Universidade Federal de Santa Maria, especificamente ao Programa de Pós-Graduação em Medicina Veterinária – PPGMV/UFSM, pela acolhida e por todo o apoio recebido, especialmente à Maria.

RESUMO

Tese de Doutorado
Programa de Pós-Graduação em Medicina Veterinária
Universidade Federal de Santa Maria

3'DEOXIADENOSINA E DEOXICOFORMICINA NO TRATAMENTO DE CAMUNDONGOS INFECTADOS EXPERIMENTALMENTE COM *Trypanosoma evansi*

AUTOR: LUCIANA DALLA ROSA
ORIENTADORA: SILVIA GONZALEZ MONTEIRO
Santa Maria, 01 de dezembro de 2014.

Trypanosoma evansi é um tripanossomatídeo patogênico de distribuição mundial, causador de grandes prejuízos econômicos, podendo afetar várias espécies de mamíferos, principalmente equinos, espécie na qual produz uma doença conhecida como "Mal das cadeiras". Muitos são os sinais clínicos e as patologias decorrentes da infecção por este parasito. A fase aguda da doença é caracterizada pelo surgimento de febre intermitente, edema subcutâneo, anemia progressiva, cegueira, letargia e alterações hemostáticas. Na fase crônica os animais apresentam caquexia, edema, incoordenação motora e paralisia de membros posteriores. O tratamento para essa infecção é medicamentoso, no entanto, os produtos químicos disponíveis possuem eficácia moderada e toxicidade, especialmente para os rins e fígado. Assim, é importante a pesquisa de terapias alternativas para o tratamento da doença causada pelo *T. evansi*. O objetivo deste estudo foi investigar a susceptibilidade do *T. evansi* à 3′-deoxiadenosina (cordicepina - análogo da adenosina) associada a deoxicoformicina (pentostatina - inibidor da adenosina deaminase (ADA) e análogo da desoxiadenosina) em camundongos infectados experimentalmente e verificar a influência desta terapia nos parâmetros hematológicos, bioquímicos, de atividade da ADA, marcadores de viabilidade e toxicidade celular e de estresse oxidativo e análise histopatológica. Essas análises foram divididas em três experimentos. O primeiro experimento demonstrou que a combinação de cordicepina (2 mg kg^{-1}) e pentostatina (2 mg kg^{-1}) foi 100% efetiva na cura dos animais infectados, mas esse tratamento ocasionou um aumento significativo nos níveis de enzimas hepáticas e produziu lesões histológicas no fígado e rins. O segundo experimento demonstrou uma redução nos níveis de proteínas plasmáticas totais nos roedores sadios e tratados com 1mg/kg de cordicepina ou 1mg/kg de pentostatina, tendo os animais pertencentes aos grupos tratados com pentostatina isolada ou associada a cordicepina, uma diminuição da atividade da ADA. O terceiro experimento mostrou que as combinações de cordicepina (2,0 mg kg^{-1}) e pentostatina (0,2; 0,5; 1,0; 2,0 mg kg^{1}) foram eficazes na cura de animais infectados, mas na dose mais alta (cordicepina 2mg kg^{-1} e pentostatina 2mg kg^{-1}), foi observado toxicidade elevada. A dose de cordicepina 2.0 mg kg^{-1} associada a pentostatina 0.2 mg kg^{-1} foi recomendada como opção terapêutica, com 100% de cura dos animais infectados experimentalmente sem apresentar toxicidade aos mesmos.

Palavras-chave: Cordicepina. Pentostatina. Adenosina. Tripanossomatídeo. Dose ideal.

ABSTRACT

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

3'DEOXYADENOSIN AND DEOXYCOFORMYCIN TREATMENT OF EXPERIMENTALLY INFECTED MICE WITH *Trypanosoma evansi*

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ADVISOR: SILVIA GONZALEZ MONTEIRO
Santa Maria, December 01st, 2014.

Trypanosoma evansi is a pathogenic trypanosomatid with world distribution, which may cause big economic losses and affect almost all mammalian species. One of the most affected species is horses, which may develop a disease known as Mal das cadeiras. There are several clinical signs and pathologies resulting from this parasite infection. The acute phase is characterized by intermittent fever, subcutaneous edema, progressive anemia, blindness, lethargy and hemostatic abnormalities. In the chronic phase, animals exhibit cachexia, edema, incoordination and paralysis. The most common treatment for this infection is the use of drugs; however, these drugs have a moderate efficacy, and they may present toxicity, especially to kidney and liver. Thus, it is important to study new therapies for the treatment of the disease caused by *T. evansi*. The aim of this study was to investigate the anti-trypanosomal effect of the treatment with 3'deoxyadenosine (cordycepin - adenosine analogue) combined with deoxycoformycin (pentostatin - inhibitor of the adenosine deaminase (ADA) enzyme and deoxyadenosine analogue) in mice experimentally infected with *T. evansi*. Furthermore, we also verified the influence of the therapy in the hematologic, biochemical and ADA activity parameters, makers of cell viability and toxicity, oxidative stress and histopathology analyses. These analyses were divided into three experiments. The first one showed that the combination of cordycepin ($2mgkg^{-1}$) and pentostatin ($2mgkg^{-1}$) was 100% effective in the *T. evansi*-infected groups; however, the treatment increased significantly the liver enzyme levels, which were accompanied by histological lesions in the liver and kidneys. The second experiment showed a reduction in the levels of plasma total protein in healthy mice and treated with 1mg/kg cordycepin or 1mg/kg pentostatin. The animals treated with pentostatin alone or associated with cordycepin showed an ADA activity significantly reduced. The third experiment showed that the combination of cordycepin (2.0 mg kg^{-1}) and pentostatin (0.2, 0.5, 1.0, 2.0 mg kg^{-1}) is effective in the clearance of *T. evansi*, although at higher concentrations (cordycepin 2mg kg-1 and pentostatin 2mg kg^{-1}), toxicity was observed. Therefore, the dose cordycepin 2.0 mg kg-1 in combination with pentostatin 0.2 mg kg^{-1} was recommended as a therapeutic option. This combination showed to be 100% effective in the experimentally infected animals and presented no toxicity to the animals.

Keywords: Cordycepin. Pentostatin. Adenosine. Trypanosomatid. Optimal dose.

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APRESENTAÇÃO

Os resultados que fazem parte desta tese estão apresentados sob a forma de artigos científicos publicados e submetidos nas revistas científicas: Parasitology, Experimental Parasitology e Pathology - Research and Practice disponíveis nos capítulos I, II e III. As seções Materiais e Métodos, Resultados, Discussão e Referências Bibliográficas encontram-se nos próprios artigos e representam a íntegra deste estudo. As REFERÊNCIAS BIBLIOGRÁFICAS se referem somente às citações que aparecem nos itens INTRODUÇÃO e REVISÃO BIBLIOGRÁFICA desta tese.

1 INTRODUÇÃO

Entre os tripanossomas patogênicos, o *Trypanosoma evansi* tem a mais ampla gama de hospedeiros e maior distribuição geográfica, sendo encontrado em todas as áreas tropicais e subtropicais do mundo. A transmissão deste protozoário ocorre principalmente por meio de moscas hematófagas (*Tabanus* spp. e *Stomoxys* spp.) e morcegos. Clinicamente, a infecção é caracterizada pela rápida perda de peso, vários graus de anemia, febre intermitente, edema dos membros posteriores, fraqueza progressiva e distúrbios locomotores. Na espécie equina, os sinais clínicos podem ser mais graves e levam, muitas vezes, ao óbito. Consequentemente, a enfermidade produzida por este parasito possui grande importância devido aos altos prejuízos econômicos, principalmente quando afeta animais de alto valor zootécnico.

No Brasil, há diversos relatos de casos de tripanossomose em diferentes espécies animais, demonstrando sintomatologia aguda e crônica. A partir do diagnóstico preciso da doença, o protocolo quimioterápico é empregado, mas sabe-se que muitas vezes esse protocolo não é realmente eficaz para o cura dos animais. Até o momento, muitas drogas têm sido usadas para o tratamento da tripanossomose, no entanto, a eficácia contra o parasito e a toxicidade para o hospedeiro, especialmente para os rins e fígado, têm provado ser problemática. Além disso, em alguns casos, tem sido relatada a resistência do parasito aos medicamentos. Assim, é importante investigar terapias alternativas para o tratamento da enfermidade causada pelo *T. evansi*.

Características do metabolismo desses protozoários podem ser exploradas como alvo terapêutico. O presente trabalho baseou-se na incapacidade dos tripanossomas de sintetizar purinas e da sua consequente dependência em utilizar o nucleosídeo dos fluidos corporais de seus hospedeiros. Dessa maneira, estudamos a utilização da Cordicepina, um análogo da adenosina, que pode metabolicamente substituir a adenosina e, consequentemente, interromper a síntese de RNA nucleolar; e a Pentostatina, um análogo da desoxiadenosina e um potente inibidor de adenosina deaminase, que leva ao acúmulo de adenosina devido a não degradação da adenosina em ionosina pela carência de adenosina deaminase e pode provocar a inibição da síntese de DNA e eventual morte celular.

Neste contexto, o objetivo do nosso trabalho foi avaliar a eficácia curativa e encontrar a dose ideal da combinação dos fármacos análogos da adenosine (cordicepina) e

desoxiadenosina (pentostatina) no tratamento de camundongos infectados experimentalmente por *Trypanosoma evansi*.

2 REVISÃO DE LITERATURA

2.1 *Trypanosoma evansi*

Trypanosoma evansi é um protozoário pertencente ao reino Protozoa, filo Euglenozoa, ordem Kinetoplastida, família Trypanosomatidae, seção Salivaria (SILVA et al., 2002). Foi descrito pela primeira vez em 1880 por Griffith Evans, um médico veterinário do exército do Reino Unido que, ao examinar ao microscópio lâminas com o sangue de equideos na Índia, observou o protozoário. Evans comprovou sua hipótese ao inocular o sangue de animais doentes em animais sadios e após seis dias observar os protozoários no sangue dos animais inoculados (FALLIS, 1986).

Este protozoário é classificado como monomórfico, ou seja, não passa por complexas mudanças morfológicas e bioquímicas, permanecendo em sua forma infectante (tripomastigota) durante toda sua vida (BRUN et al., 1998). Sua forma é basicamente lancetada e o corpo é alongado e achatado, com comprimento variando entre 24 ± 4µm (min 15µm, max 33µm) e largura entre 1,5 ± 0,5 µm. Há uma membrana ondulante que permeia toda a extensão do parasito, extremidades afiladas, um flagelo terminal e um núcleo central (BRUN et al., 1998; SILVA et al., 2002). O cinetoplasto pode ou não estar presente, dependendo da origem da cepa. Cepas brasileiras não possuem cinetoplasto (VENTURA et al., 2002) e as que possuem apresentam-no incompleto (BORST et al., 1987) (Figura 1).

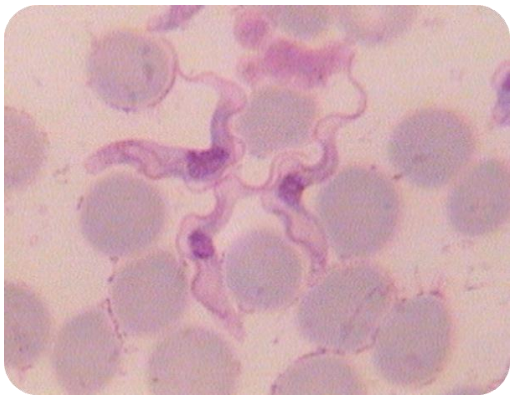

Figura 1 – *Trypanosoma evansi* encontrados em esfregaço sanguíneo de um camundongo experimentalmente infectado. Microscópio Olympus (Serie CX40), 1000X. Fonte: Arquivo pessoal

2.2 Ciclo Biológico

O ciclo de vida do *T. evansi* consiste da transmissão mecânica do protozoário de um hospedeiro infectado para outro não infectado. Os vetores são principalmente insetos hematófagos dos gêneros *Glossina* sp., *Tabanus* sp., *Stomoxys* sp. e também, na América Central e do Sul, os morcegos (*Desmodus rotundus*) (HOARE, 1972; LOSOS, 1980). Nestes vetores, o parasito não desenvolve nenhuma fase do ciclo (SILVA et al., 2002), os tripanossomas permanecem na probóscide dos vetores na forma tripomastigota e a transmissão depende diretamente da sobrevida destes no aparelho bucal dos insetos. Para que a transmissão seja realizada com sucesso, a alimentação do vetor no hospedeiro infectado deve ser interrompida, fazendo com que o inseto procure outro hospedeiro e inocule o parasito no mesmo. Quanto menor a diferença de tempo entre os repastos sanguíneos, maiores são as possibilidades de passagem do parasita para um novo hospedeiro (HOARE, 1972). Ainda, existe a possibilidade de transmissão oral em carnívoros que se alimentam da carcaça de animais infectados ou através de ferimentos ocasionados por brigas (RAMIREZ et al., 1979, BAZZOLI et al., 2002).

Os tripanossomas sobrevivem e multiplicam-se nos fluidos extracelulares de seus hospedeiros, especialmente no sangue, onde dividem-se assexuadamente por fissão binária (BRUN et al, 1998).

O *T. evansi* pode acometer um grande número de animais domésticos e selvagens, entre eles, cavalos, camelos, bovinos, gatos, caprinos, suínos, cães, búfalos, elefantes, capivaras, quatis, antas, tatus, marsupiais, zebuínos, veados e pequenos roedores silvestres (LEVINE, 1973; SILVA et al., 2002; ATARHOUCH et al., 2003; HERRERA et al., 2004). Para completar o quadro, o primeiro caso humano foi relatado em 2005 na Índia, onde um fazendeiro teve a parasitemia flutuante associada a episódios febris (JOSHI et al., 2005).

2.3 Distribuição da doença

De todos os tripanossomas patogênicos, *T. evansi* tem a mais ampla gama de hospedeiros e maior distribuição geográfica (DESQUESNES et al., 2013a), sendo encontrado em todas as áreas tropicais e subtropicais do mundo, podendo ocorrer na África, Índia,

Malásia, Indonésia, China, Rússia, Filipinas, América Central e América do Sul (Figura 2).

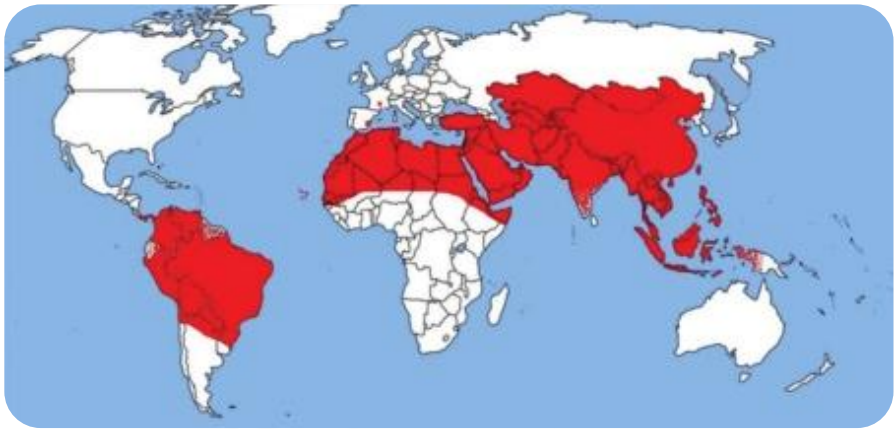

Figura 2 – Distribuição geográfica do *Trypanosoma evansi* no mundo. Fonte: Desquesnes et al., 2013a

Este protozoário teve sua origem no continente africano e foi introduzido nas Américas pelos primeiros colonizadores europeus. Estima-se que a chegada do *T. evansi* na América do Sul tenha ocorrido no final no século XIX com a importação de cavalos da Espanha (HOARE, 1972). Desde então, surtos ou casos isolados de tripanossomose em animais domésticos e silvestres têm sido relatados em diversas regiões brasileiras: no Rio Grande do Sul (COLPO et al., 2005; CONRADO et al., 2005; FRANCISCATO et al., 2007), Mato Grosso do Sul (MOREIRA e MACHADO, 1985; BRANDÃO et al., 2002), Santa Catarina (DA SILVA et al., 2008), Paraná (KUBIAK e MOLFI, 1954), Minas Gerais (NUNES et al., 2012) e no Pantanal, onde a doença é endêmica, com casos recorrentes (SILVA et al., 2002).

Provavelmente em locais onde o *T. evansi* foi estabelecido em um nível enzoótico a existência de uma grande variedade de reservatórios silvestres e domésticos, a capacidade de ser transmitido por vetores mecânicos inespecíficos presentes em todo o mundo e sua capacidade de difundir-se silenciosamente através de portadores saudáveis foram os motivos para a sua não erradicação. Capivaras (*Hydrochaeris hydrochaeris*), coatis (*Nasua nasua*) e morcegos hematófagos (*Desmodus rotundus*) são considerados os principais reservatórios silvestres (NUNES et al., 1993; SILVA et al., 2002).

2.4 Patogenia e Sinais Clínicos

O *T. evansi* inicia sua multiplicação no local da picada, na pele, invadindo a corrente sanguínea e o sistema linfático, levando a picos de febre 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).

Os sinais clínicos exibidos são muito variáveis, dependendo do hospedeiro, virulência da cepa e situação epidemiológica. Estas características fazem da tripanossomose não apenas uma doença multiespécie mas também uma doença polimórfica (DESQUESNES et al., 2013b). A doença causada pela infecção por *T. evansi* é mundialmente conhecida por "Surra", e, na América Latina, é também chamada de "Mal das Cadeiras".

Em infecções naturais e experimentais, observou-se que a tripanossomose causada pelo *T. evansi* pode apresentar um quadro clínico agudo e crônico. Geralmente, a fase aguda da infecção é caracterizada pelo surgimento de febre intermitente, edema subcutâneo, anemia progressiva, cegueira, letargia e alterações hemostáticas (GARDINER; MAHMOUD, 1990). Os animais afetados agudamente podem morrer dentro de semanas ou poucos meses. No entanto, as infecções crônicas podem durar anos (BRUN et al., 1998). Durante a fase crônica, estes flagelados podem invadir o sistema nervoso central (SNC) levando a uma lesão progressiva (GIBSON, 1998) e agravamento dos sinais clínicos, consequentemente observa-se nos animais infectados caquexia, edema, incoordenação motora e paralisia de posterior (BRANDÃO et al., 2002; SILVA et al., 2002; RODRIGUES et al., 2005). Os sinais neurológicos têm sido descritos na fase terminal da doença, principalmente em equinos, bovinos, veados e búfalos infectados naturalmente (TUNTASUVAN et al., 1997; TUNTASUVAN; LUCKINS, 1998; TUNTASUVAN et al., 2003; RODRIGUES et al., 2005).

Os sinais clínicos da infecção por *T. evansi* são em sua maioria inespecíficos, principalmente no início da doença (SILVA et al., 2002). O diagnóstico definitivo envolve a análise laboratorial, com identificação direta do agente ou usando ferramentas sorológicos para provar contato imunológico, e molecular.

2.5 Tratamento

O controle de doenças vetoriais é normalmente dividido em duas seções: controle de patógenos e controle de vetores. No caso da “Surra”, o controle é realizado, principalmente, com a utilização de tripanocida e métodos preventivos para proteção dos animais contra a infecção. 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 (DESQUESNES et al., 2013a).

O aceturato de diminazeno é comumente empregado no controle do *T. evansi* nos animais domésticos. Esse medicamento elimina os tripanossomas da corrente sanguínea algumas horas após sua administração. No entanto, este princípio ativo, comercializado com diferentes nomes comerciais, não apresenta a eficácia curativa em um grande número de 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 e consequentemente ao cérebro, local de refúgio do *T. evansi* durante o período residual do fármaco (LONSDALE-ECCLES; GRAB, 2002; MASOCHA et al., 2007). Além disso, a alta toxicidade para o hospedeiro (SPINOSA et al., 1999) e o uso indiscriminado e/ou errôneo destas drogas pode culminar em uma menor sensibilidade dos protozoários aos tratamentos disponíveis e o surgimento de cepas resistentes (JENNINGS et al., 1977).

Desta maneira é de grande importância a investigação de novas alternativas na terapêutica, como por exemplo, a associação de drogas a outros diferentes compostos.

2.6 3'Deoxiadenosina e Deoxicoformicina

Devido a incapacidade dos tripanossomas em sintetizar novas purinas e a consequente dependência destes nucleosídeos dos fluidos corporais de seus hospedeiros (ROTTENBERG et al., 2005; VODNALA et al., 2008) o potencial tripanocida da cordicepina (análogo da adenosina) foi observado desde a década de 1970 (WILLIAMSON, 1972; WILLIAMSON e MACADAM, 1976). No entanto, a administração da cordicepina não resultou em uma cura completa da infecção (AIYEDUN et al, 1973; DA SILVA et al., 2011). Por outro lado,

estudos (ROTTENBERG et al., 2005; VODNALA et al., 2008), mostraram que a cordicepina combinada a um inibidor da adenosina deaminase (ADA) teve efeito curativo na infecção por *T. brucei* em camundongos, na fase aguda e crônica da doença. Segundo esses autores, a eficácia do tratamento está relacionada com a proteção da cordicepina contra a enzima ADA, que é responsável pela desaminação do análogo da adenosina.

Assim constatou-se que a cordicepina e a pentostatina (inibidor da adenosina deaminase e análogo da desoxiadenosina) agem na via metabólica dos tripanosomas de uma maneira que as drogas atualmente disponíveis não fazem (ROTTENBERG et al., 2005). Na rota normal, a adenosina pode ser desaminada ada através da ADA tornando-se inosina, ou pode ser fosforilada através de quinases em adenosina mono (AMP), di (ADP) e tri fosfato (ATP) e inseridas pelas RNAs polimerases no RNA nascente. A adenosina pode, também, ser transformada em desoxiadenosina e essa ser fosforilada a desoxidenosina mono (dAMP), di (dADP) e tri fosfato (dATP) e ser inserida na cadeia de DNA. Ainda há uma terceira via que catalisa a ADP em dADP através da enzima ribonucleotídeo redutase (RNR). O produto final dessa rota, o dATP, atua como regulador alostérico da RNR, ou seja, quando a concentração deste produto final estiver aumentada ele agirá como inibidor da RNR e consequentemente diminuirá a velocidade de produção da sua própria via. Caso o produto comece a ser consumido e consequentemente sua concentração diminua, ele vai deixar de inibir a via.

Naturalmente o organismo do hospedeiro produz a adenosina e a desoxiadenosina, as quais são vitais aos tripanossomas. Sinteticamente produz-se a cordicepina, um análogo da adenosina sem a hidroxila no carbono 3’ (LENNON, SUHADOLNIK, 1976). E a pentostatina, um análogo da desoxiadenosina com uma base modificada. Devido a grande semelhança entre essas moléculas, algumas enzimas de reconhecimento da adenosina e da desoxiadenosina acabam confundindo-as (TSAI, et al., 2010) (Figura 3). A cordicepina, por ser um análogo da adenosina, é confundida pela adenosina kinase e é fosforilada a AMP, ADP e ATP e assim inserida na molécula de RNA. Após ser inserida, a síntese irá parar, pois a cordicepina não possui a hidroxila exatamente no carbono 3’ onde o grupo fosfato se ligaria (SIEV, et al., 1969). A penstostatina, por sua vez, é um análogo da desoxiadenosina. Consequentemente as enzimas que fosforilam a desoxiadeanosina e as enzimas que inserem o dATP na síntese do DNA se confundirão e irão inserir a pentostatina. Como a molécula da pentostatina possui uma base diferente da base adenina encontrada na desoxiadenosina, haverá uma falha na geração da dupla fita de DNA, pois é exatamente nesta base que as ligações moleculares da dupla fita são formadas. Além disso, a pentostatina é um inibidor irreversível da ADA, ou seja ela interfere na reação química, interrompendo as reações

enzimáticas. Sua consequência é diminuir a concentração efetiva da enzima (SPIERS, 1987). O bloqueio da ADA leva ao acumulo e proteção contra a degradação da cordicepina, fazendo com que ela desenvolva a sua ação. Consequentemente ao bloqueio da degradação da adenosina, há o acumulo de ADP o que resulta na regulação alostérica da RNR bloqueando ou diminuindo a velocidade desta via (ULLMAN et al., 1978).

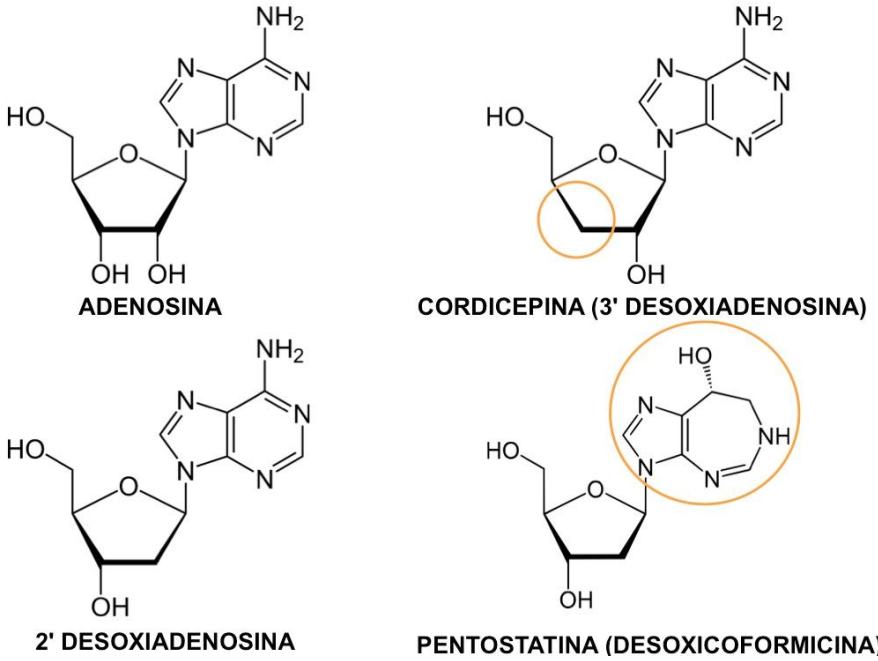

Figura 3 – Moléculas de adenosina e desoxiadenosina com seus respectivos análogos, cordicepina e pentostatina. Fonte: Arquivo pessoal

Devido a alta afinidade dessas drogas pelos receptores do parasita, P1 e P2, e baixa afinidade pelos receptores do hospedeiro, além da grande concentração de substratos competidores para os transportadores do hospedeiro, sugere-se que os análogos da adenosina não causem interferência na síntese do DNA e RNA do hospedeiro (ROTTENBERG et al., 2005).

Assim, o objetivo deste estudo foi testar a susceptibilidade do *T. evansi* ao tratamento com cordicepina associado à deoxicoformicina (pentostatina) em camundongos infectados experimentalmente e a verificação dos parâmetros hematológicos, bioquímicos, histológicos, atividade da ADA e os marcadores de estresse oxidativo e toxicidade celular nesses animais, além de determinar uma dose ótima para o tratamento da tripanossomose.

3 ARTIGO I

Cordycepin (3'-deoxyadenosine) pentostatin (deoxycoformycin) combination treatment of mice experimentally infected with *Trypanosoma evansi*

Publicado na **PARASITOLOGY**

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SUMMARY

The aim of this study was to evaluate the anti-trypanosomal effect of treatment with 3′-deoxyadenosine (cordycepin) combined with deoxycoformycin (pentostatin: inhibitor of the enzyme adenosine deaminase) *in vitro* by using mice experimentally infected with *Trypanosoma evansi*. *In vitro*, a dose-dependent trypanocidal effect of cordycepin was observed against the parasite. In the *in vivo* trials, the two drugs were used individually and in combination of different doses. The drugs when used individually had no curative effect on infected mice. However, the combination of cordycepin (2 mg kg^{-1}) and pentostatin (2 mg kg^{-1}) was 100% effective in the *T. evansi*-infected groups. There was an increase in levels of some biochemical parameters, especially on liver enzymes, which were accompanied by histological lesions in the liver and kidneys. Based on these results we conclude that treatment using the combination of 3′-deoxyadenosine with deoxycoformycin has a curative effect on mice infected with *T. evansi*. However, the therapeutic protocol tested led to liver and kidney damage, manifested by hepatotoxicity and nephrotoxicity.

Key words: trypanosomiasis, adenosine, adenosine deaminase inhibitor.

INTRODUCTION

Trypanosoma evansi is the aetiological agent of the disease known as ‘Mal das Cadeiras’ or ‘Surra’ in horses. However, this parasite has also been reported to affect domestic and wild mammals (Maudlin et al. 2004) and, in rare cases, humans (Powar et al. 2006). *Trypanosoma evansi* is the most widely distributed of the pathogenic African animal trypanosomes, affecting animals in Asia, Africa and Latin America and resulting in immeasurable economic losses (Luckins and Dwinger, 2004; Dobson et al. 2009). It is

mechanically transmitted by haematophagous flies such as *Tabanus* spp. and *Stomoxys* spp. and/or vampire bats (Hoare, 1972). Infected animals showed typical clinical signs such as anaemia, oedema, weight loss and locomotor disturbance (Hoare, 1972; Maudlin et al. 2004).

Therapy for animal trypanosomiasis relies on the use of diminazene aceturate, which is effective for treatment of the disease in cattle, buffalo, sheep, pigs and camels (Peregrine and Mamman, 1993; Sirivan et al. 1994). However, a single dose is not effective for horses, mules and dogs (Tuntasuvan et al. 2003; Colpo et al. 2005), resulting in lack of efficacy of these drugs (Tuntasuvan et al. 2003; Da Silva et al. 2008). In addition, the drugs used to treat *T. evansi* are considered hepatotoxic and nephrotoxic (Spinosa et al. 1999), and their prolonged use can cause worsening of the disease. Thus, it is important to investigate alternative therapies to improve the success of the treatment using new drugs, drug combinations and other components that could increase the curative efficacy. Accordingly, some studies have emerged suggesting new options of treatment for trypanosomiasis.

A recent study showed low therapeutic efficacy of the combination of cordycepin (3-deoxyadenosine) with the inhibitor of adenosine deaminase (ADA_1) in mice infected with *T. evansi* (Da Silva et al. 2011a). However, studies have shown efficacy of treatment with cordycepin in *T. brucei* infection in mice (Rottenberg et al. 2005; Vodnala et al. 2008, 2009). The efficacy of the treatment is related to the protection of cordycepin against the enzyme adenosine deaminase (ADA), which is responsible for the deamination of the analogue adenosine (Rottenberg et al. 2005; Vodnala et al. 2008, 2009). However, this protocol requires the combination of cordycepin with an inhibitor of ADA_1 and ADA_2 known as deoxycoformycin (Rottenberg et al. 2005), a different inhibitor from that used in the study with *T. evansi*.

The trypanocidal potential of cordycepin was noticed in experiments performed in the 1970s (Williamson, 1972; Williamson and Macadam, 1976). However, the administration of cordycepin did not result in a complete cure of infection (Aiyedun et al. 1973; Da Silva et al. 2011a). Cordycepin targets a vulnerable pathway in the trypanosomal metabolic economy in a way that is not targeted by currently available drugs (Rottenberg et al. 2005). Accordingly, the purine metabolism in trypanosomes and in other parasites presents a particular vulnerability because these parasites cannot engage in a new purine synthesis (James and Born, 1980; Hammond and Gutteride, 1984; Hassan and Coombs, 1988). Rather, they depend on the salvage pathway of nucleosides from the body fluids of the host (Agarwal et al. 1975). The inability of trypanosomes to engage in *de novo* purine synthesis has been exploited as a

therapeutic target (Vodnala et al. 2009). Based on the aforementioned, we designed an experiment to evaluate the susceptibility of *T. evansi* in mice to treatment with a combination of 3′-deoxyadenosine and deoxycoformycin.

MATERIALS AND METHODS

Reagents

3′-deoxyadenosine (Cordycepin) was purchased from Sigma. Deoxycoformycin (Pentostatin; Tocris) was used as an inhibitor of ADA. Unless otherwise indicated, all reagents were diluted in PBS, aliquoted and stored at -20 °C until further use. The medium components used, except the antibiotics were purchased from Sigma.

Trypanosoma evansi isolate

Trypanosoma evansi was originally isolated from a naturally infected dog (Colpo et al. 2005). Two rats (R_1 and R_2) were intraperitoneally infected with blood cryopreserved in liquid nitrogen. The strain was later reactivated to obtain a large quantity of bloodstream forms of the parasite for subsequent infection of mice that formed the experimental groups and also for use in *in vitro* tests.

In vitro tests

Culture medium. The cultivation for *T. evansi* was adapted from the method reported by Baltz (1985). To prepare the culture medium, minimum essential medium (MEM) without glutamine (0.376 g), glutamine (0.016 g), sodium bicarbonate (0.088 g), glucose (0.04 g), HEPES free acid (0.238 g), nonessential amino acid solution (200 µL), penicillin (1596 U mL^{-1}) and estreptomicin (100 µg mL^{-1}) were used. The components were dissolved and homogenized in 30 mL of water, after adjustment of the pH to 7.1 with NaOH. The volume of the solution was then raised to 42mL with sterile distilled water at an osmolarity of 0.30. Later, the culture medium was sterilized by filtration at 0.22 µm and stored in a refrigerator. On the day of testing, 10 mL were separated into a Falcon tube to which were added 1 µl mL^{-1} of 50 mM hypoxanthine (dissolved in 0.1 M NaOH) and 2 µl mL^{-1} of 1.2 mM 2-mercaptoethanol. Subsequently, the complete culture medium was equilibrated in a CO_2 incubator for 2h (37°C and 5% CO_2).

Acquisition of trypanosomes - For the *in vitro* tests, the trypomastigotes were obtained from

rat No. 1 (R_1); when it showed high parasitaemia (10^7 trypanosomes μL^{-1}), it was anaesthetized under isoflurane anaesthesia to aid blood collection by intracardiac puncture. The samples were stored in EDTA tubes at 13°C. For separation of trypanosomes, 200 µl of blood were diluted in complete culture medium (200 µL), stored and centrifuged at 400g for 10min. The supernatant was removed and the trypanosomes were counted in a Neubauer chamber.

***In vitro* assay -** The culture medium containing the parasites was mixed on microtitre plates (270µL) followed by addition of cordycepin at concentrations of 0.5, 1.0, 5.0 or 10 mg mL^{-1}. The same concentrations of pentostatin were used in the tests that utilized a combination of cordycepin and pentostatin. For the control group, the highest concentration of PBS (diluent) was used. At 1, 3, 6 and 12 h after the onset of the experiment, counting of live parasites was performed in a Neubauer chamber. The tests were carried out in duplicate.

***In vivo* trials**

Animals. A total of 49 female mice (mean age of 60 days) weighing approximately 25 ± 21 g were kept in cages (7 animals each), in a room with controlled temperature and humidity (25°C; 70%). The animals were fed with commercial ration and received water ad libitum. All animals were submitted to an adaptation period of 10 days before the beginning of the experiment.

Experimental mice groups and trypanosomal infection. The mice were divided into 7 groups (A, B, C, D, E, F and G) of 7 animals each. Group A was not infected with the parasite and, thus, served as a negative control. Mice in groups B to G were infected intraperitoneally with 1.4×10^6 trypanosomes in 0.1 mL of blood from rat N°. 2 (R_2).

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

Experimental design. Group A mice were the negative control and group B mice (infected with *T. evansi* and untreated) were the positive control. Mice in groups C to G were treated intraperitoneally at 24h intervals for 3 days post-inoculation (p.i.), as follows: group C was treated with cordycepin (3′-deoxyadenosine) (2 mg kg^{-1}); group D was treated with pentostatin (deoxycoformycin) (1 mg kg^{-1}); group E was treated with pentostatin (2 $mgkg^{-1}$);

group F was treated with the combination of cordycepin (2 mg kg^{-1}) and pentostatin (1 mg kg^{-1}); and group G was treated with the combination of cordycepin (2 mg kg^{-1}) and pentostatin (2 mg kg^{-1}). The effect of the treatment on the group was evaluated based on the parasitaemia levels, longevity, mortality of the mice and molecular analysis for *T. evansi* in infected mice that survived after treatment and thereby verify the trypanocidal effectiveness of the therapeutic protocol.

Collection of samples. On day 40 of the experiment, the survivor animals (in groups A, F and G) were anaesthetized with isoflurane in an anaesthetic chamber for collection of blood by cardiac puncture. The blood samples were collected in tubes without anticoagulant to obtain the serum. Thereafter, mice were euthanized in accordance with the recommendations of the Ethics Committee. The brains, livers and kidneys of the animals were removed for histological analysis.

Hepatic and renal function. The blood samples were centrifuged at 3500g for 10min to obtain serum. Liver function (alanine transaminase - ALT and alkaline phosphatase - ALP) and renal function (creatinine) were evaluated.

Histopathology. Evidence of drug-induced histopathological damage to the brains, livers and kidneys of *T. evansi*-infected mice treated with the drugs was investigated to detect the toxicity of the treatment. Samples of the organs were fixed in buffered formalin (10%), embedded in paraffin, sectioned at a thickness of 6 μm and stained with haematoxylin/eosin (H&E). The sections were qualitatively evaluated for differences in microarchitecture, organization and the presence of inflammatory cells. Measurements were made of the nuclear areas of hepatocytes and kidney corpuscles.

Molecular diagnosis. The brain and blood samples collected with EDTA (as anticoagulant) were preserved in ethanol for DNA extraction and posterior analysis using *T. evansi*-specific PCR (Ventura et al. 2002).

Statistical analysis

Data were submitted to analysis of variance (ANOVA) followed by Duncan test. The histological results were analysed by Student's t-test. Results were considered significant when $P<0.05$.

RESULTS

In vitro tests

The results showed a dose-dependent trypanocidal effect of cordycepin (Fig. 1A), pentostatin (Fig. 1B), and cordycepin and pentostatin combination (Fig. 1C) on *T. evansi* trypomastigotes. At 1 h post-treatment, a reduction of 48% and 55% in the levels of live trypomastigotes was observed at cordycepin concentrations of 5 and 10 $mgmL^{-1}$, respectively, when compared with the control group. The combination of cordycepin and pentostatin potentiated the trypanocidal effect. At 1h after the beginning of the experiment there was a reduction of 69% (5 mg mL^{-1}) and 81% (10 mg mL^{-1}) in the number of live trypanosomes when compared with the control. At 3, 6 and 12 h post-treatment, a significant decrease ($P < 0.001$) in the levels of live parasites was observed at all the concentrations used of cordycepin and the combination of cordycepin and pentostatin. No live parasites were detected at 6 h of incubation of the drug at concentrations of 10 mg mL^{-1} and 12 h of treatments with cordycepin eliminated the parasite, unlike in the control group (Fig. 1A). Tests using pentostatin showed no significant reduction in the number of live trypomastigotes in most groups, except in those treated with concentrations of 5 $mgmL^{-1}$ (3, 6 and 12h) and 10 $mgmL^{-1}$ (1, 3, 6 and 12 h).

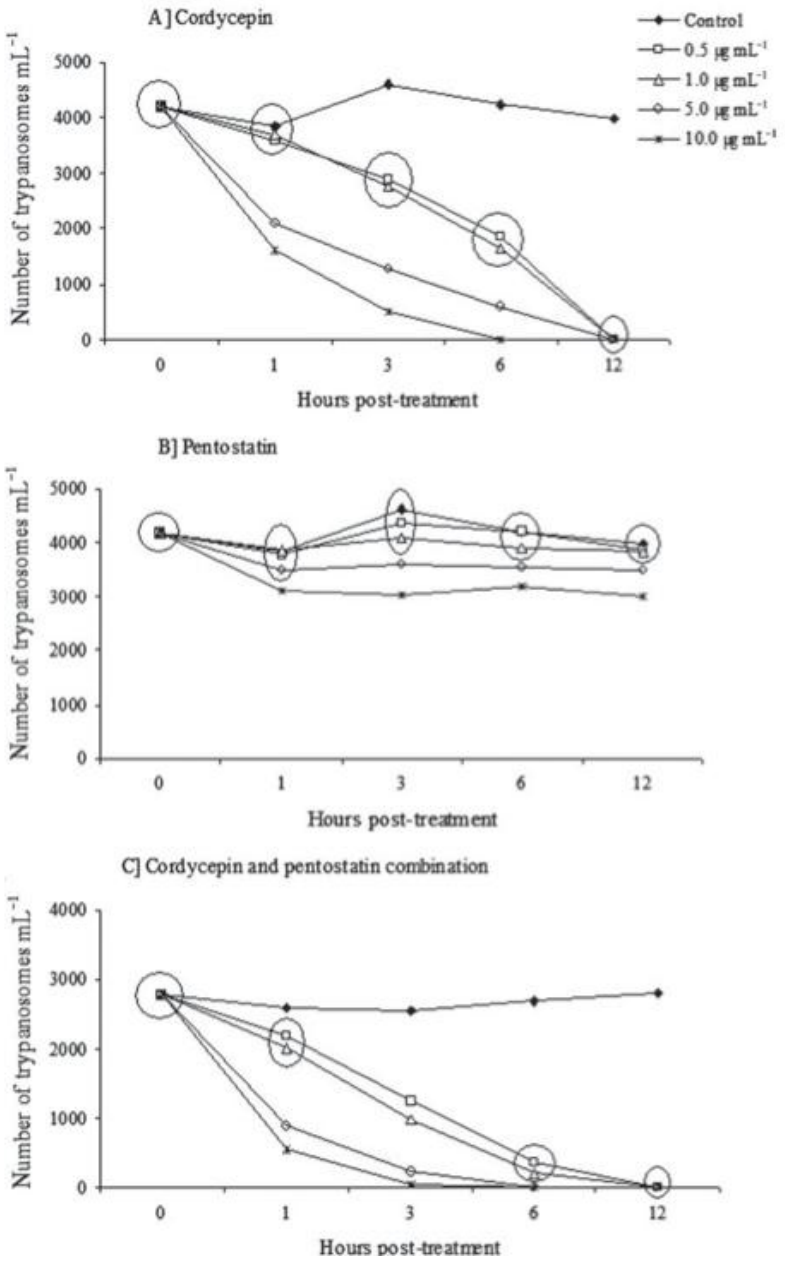

Fig. 1 – Dose–response effect of cordycepin (A), pentostatin (B) and cordycepin and pentostatin combination (C) on the viability of *Trypanosoma evansi in vitro* compared with the control group. Means followed by the same letters within a column (circles) are not statistically different among themselves at 5% probability by Tukey's test.

In vivo tests

Data of the *T. evansi*-infected groups are presented in Table 1. Parasitaemia in mice of groups B, C, D and E increased with time until death of the mice (Fig. 2). One mouse in group F presented with positive blood smears at day 21 p.i., while the other mice in the same group remained negative (Fig. 2). Mice in group G showed no parasitaemia, with longevity of 40 days p.i. (Table 1).

Table 1 – Mean and standard deviation of the pre-patent period, longevity, mortality and success of therapy using treatment with cordycepin (3′-deoxyadenosine) combined with pentostatin (deoxycoformycin) in mice experimentally infected with *Trypanosoma evansi*.

Groups n=7	Treatment	Prepatent period (days)	Longevity (days)	Mortality (No. dead/No. in group)	*Therapeutic success (%)
A	Negative control: non-infected	-	40.0^{a} (±0.0)	0/6	-
B	Positive control: Infected and untreated	4.86^{ab} (±0.38)	8.86^{c} (±0.38)	6/6	0.0
C	Treated with 2mg kg^{-1} of cordycepin	7.1^{a} (±1.51)	12.1^{b} (±3.08)	6/6	0.0
D	Treated with 1 mg kg^{-1} of pentostatin	4.1^{ab} (±1.21)	8.14^{c} (±0.90)	6/6	0.0
E	Treated with 2 mg kg^{-1} of pentostatin	3.5^{b} (±0.82)	8.0^{c} (±0.0)	6/6	0.0
F	Cordycepin (2mg $kg^{-}1$) + pentostatin (1mg kg^{-1})	37.6^{bc} (±6.7)	38.1^{a} (±5.67)	1/6	83.3
G	Cordycepin (2mg $kg^{-}1$) + pentostatin (2mg kg^{-1})	0.0^{c} (±0.0)	40.0^{a} (±0.0)	0/6	100.0

Means followed by same letters in the same column do not differ significantly in the test of Duncan. * Considered a therapeutic success for drug-treated mice that survived for 40 days and remained negative for the parasite by examination of their blood smears and PCR.

The mice that died during the experiment showed parasitaemia above 100 trypomastigotes per microscopic field (1000× magnification). In groups B, C, D and E all mice died, while in group F only 1 mouse died. Death did not occur in mice in groups A and G within 40 days post-treatment.

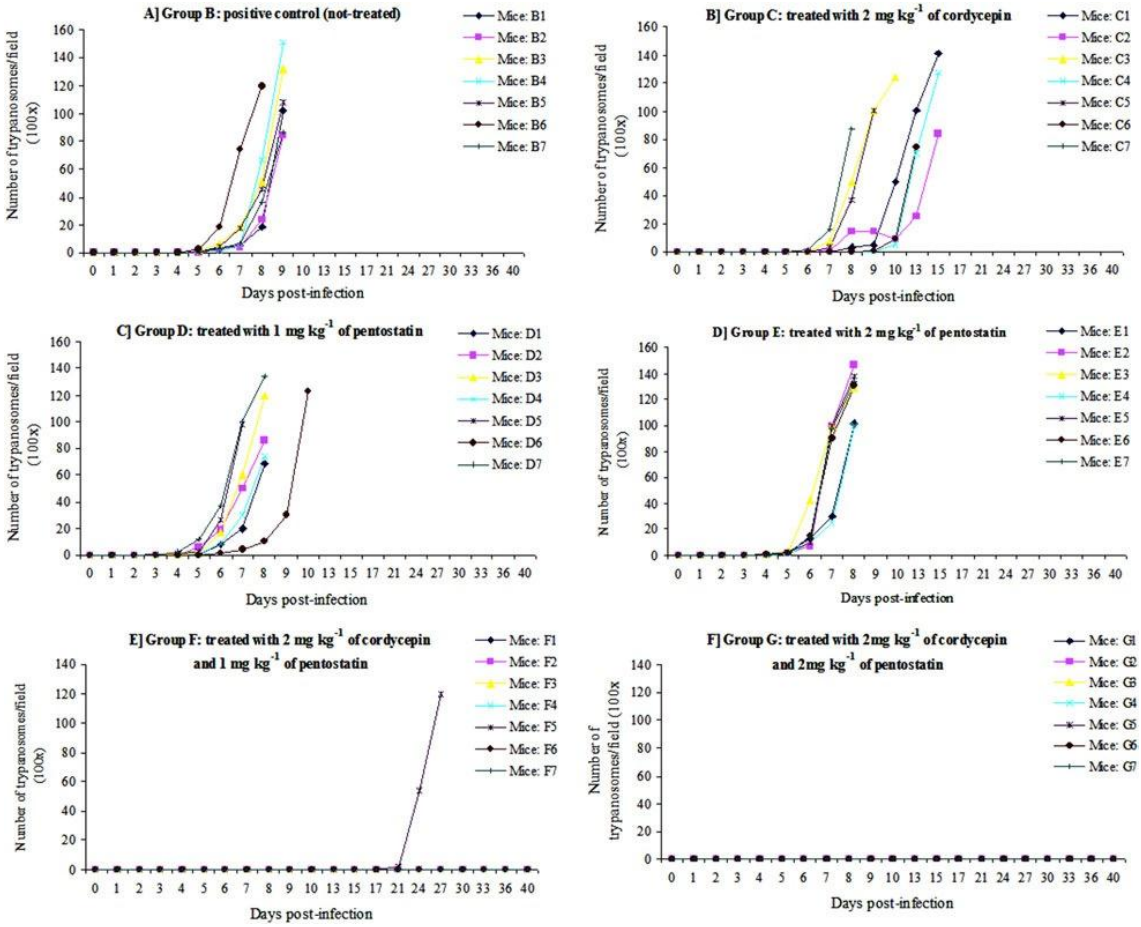

Fig. 2 – Parasitaemia of *Trypanosoma evansi*-infected mice at day 40 post-inoculation (p.i.) in groups B to G. The treatment started at day 1 p.i. (3 doses at 24-h intervals).

Hepatic and renal function. Results of the biochemical analyses of samples obtained from mice in groups A, F and G performed at the end of the experiment (day 40 p.i.) are presented in Fig. 3. A significant increase in the levels of alanine aminotransferase was found in the sera of mice treated with the combination of the two drugs, groups F and G (Fig. 3A). In the same groups a similar increase in alkaline phosphatase levels was observed (Fig. 3B). Serum creatinine levels in the mice did not show significant change compared with the control group (Fig. 3C).

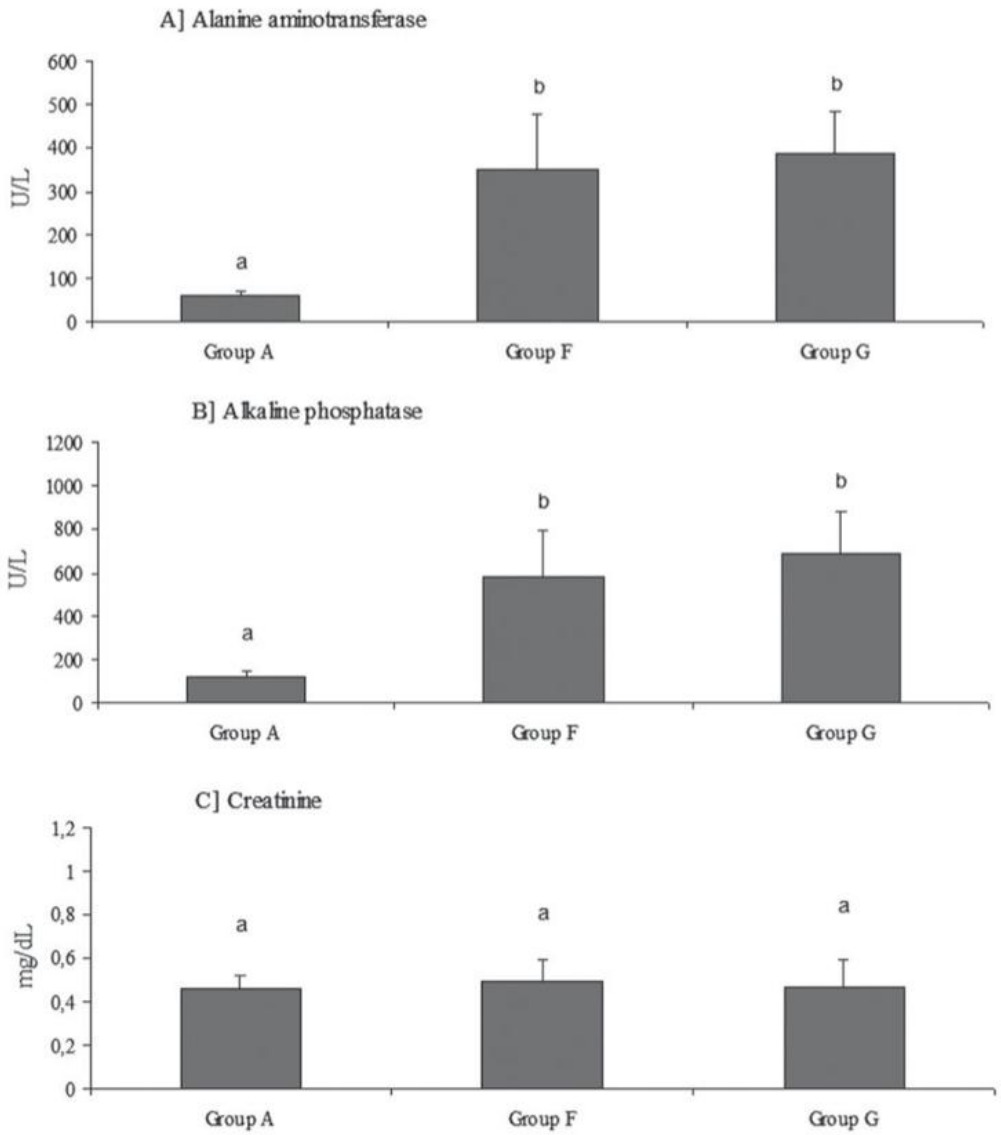

Fig. 3 – Alanine aminotransferase, alkaline phosphatase, and creatinine in mice infected with *Trypanosoma evansi* treated with cordycepin combined with pentostatin (group F and G) compared with those not infected (group A).

Histopathology. The liver indicated strong nuclear and cytoplasmic tumefaction and necrotic degeneration. Furthermore, intense congestion of the sinusoids by leukocytes was observed. Thus, the microarchitecture of the organ was lost. There were statistically significant differences (P=0.0001) between the nuclear areas of the hepatocyte of the control and infected animals (Fig. 4). In the renal tissue, a significant increase in the glomerular area (P=0.041) of the infected animals was observed (Fig. 5A). No changes were observed in the brain tissues of animals studied and treated with cordycepin and pentostatin combination (Fig. 5B).

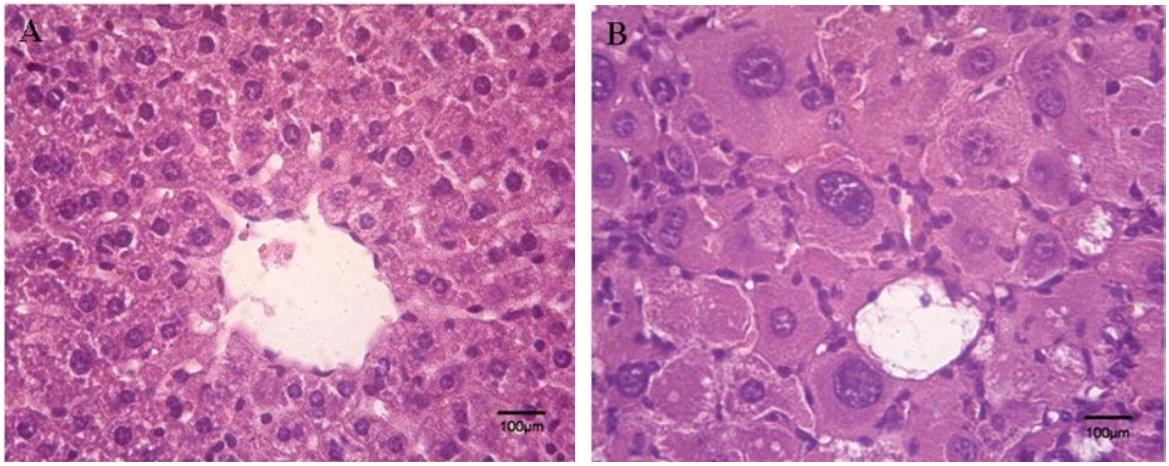

Fig. 4 – Histological section of liver of healthy mice (A). Photomicrographs of liver sections of infected mice with *Trypanosoma evansi* and treated with the cordycepin and pentostatin combination showing a strong nuclear and cytoplasmic tumefaction and necrotic degeneration in the hepatocytes (B), haematoxylin-eosin stain.

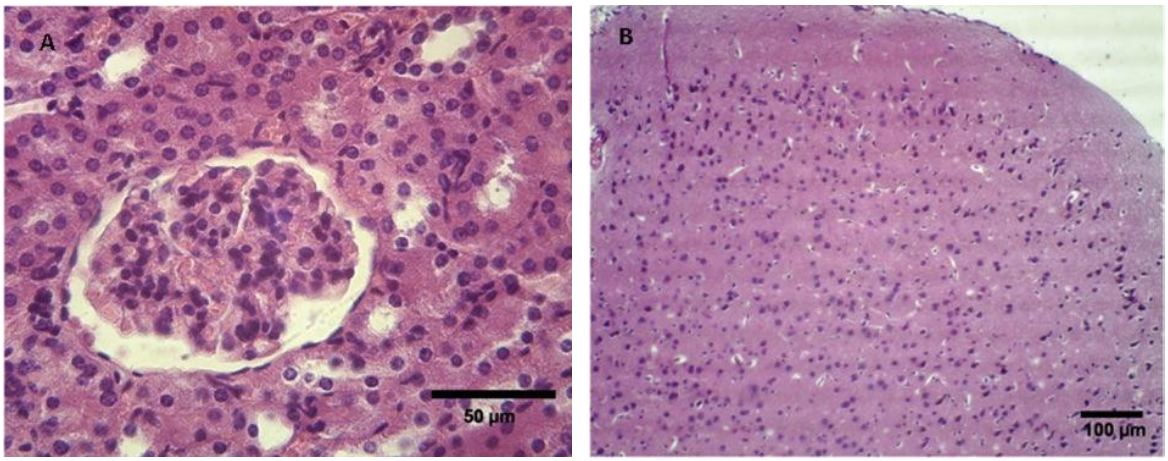

Fig. 5 – Mice infected with *Trypanosoma evansi* and treated with the cordycepin and pentostatin combination. Photomicrographs showing the renal tissue that showed a significant increase in glomerular area (A), and cortical region of the brain without histological alterations, despite parasite infection and treatment (B).

PCR. The molecular analysis was performed to confirm the effectiveness of the therapeutic protocol used, since parasitaemia was not detected in the survivor animals in groups F and G. Specific PCR assays from blood and brain of these animals were negative for the presence of *T. evansi*. Therefore, treatment was 83.3 and 100% effective for groups F and G, respectively (Table 1).

Committee on ethics and animal welfare

The experimental procedures used in this study were approved by the Animal Welfare Committee of Universidade Federal de Santa Maria (UFSM), under number 026/2012.

DISCUSSION

In this study, a dose-dependent effect of cordycepin against *T. evansi* was observed *in vitro* in contrast to the relative susceptibility expressed *in vitro* to the same drug by *T. congolense*, *T. vivax*, *T. brucei rhodesiense*, *T. evansi*, *T. brucei brucei*, *T. cruzi* and *Leishmania* sp. in previous studies (Williamson, 1972; Aiyedun et al. 1973; Williamson and Macadam, 1976; Maser et al. 2001; Rottenberg et al. 2005; Vodnala et al. 2009; Da Silva et al. 2011a). The susceptibility of *T. evansi* to this adenosine analogue is likely to be related to the parasite's inability to engage in de novo purine synthesis (James and Born, 1980; Hammond and Gutteridge, 1984; Hassan and Coombs, 1988). Our study showed that the ADA inhibitor exerts trypanocidal activity, *in vitro*. This effect was, however, seen only in the first evaluation post-treatment at 1h, which can be explained by the short half-life of the inhibitor (McConnell et al. 1978; Major et al. 1981). Deoxycoformycin can be unstable in acid and neutral media but relatively stable in the vicinity of pH 9 (Bzowska et al. 1985). However, in the *in vitro* tests, the culture medium was pH 7.1, which may have rendered the molecule pentostatin unstable over time and thereby reduced its trypanocidal action. But, the reduction in the number of parasites in the first hour of study suggests that the inhibitor (pentostatin) acts on the ADA present in *T. evansi* (Da Silva et al. 2011b) and thus kills the parasite. The use of inhibitors, such as anticholinesterase, explains the mechanism of action of some pesticides such as organophosphates (Santos et al. 2007).

Pentostatin is an ADA inhibitor that can prevent degradation of cordycepin, and thus a combination of the two drugs has been used for the treatment of certain malignant tumours in humans, such as leukaemia and melanoma (Adamson et al. 1977). This combination has also been effective against *T. brucei* (Rottenberg et al. 2005; Vodnala et al. 2009); in this study, we observed that the highest dose used proved 100% effective against *T. evansi*. However, cordycepin when used alone was not curative *in vivo*, but rather increased the longevity of the treated animals. This was probably because of the rapid deamination of cordycepin (3′-deoxyadenosine) to 3′-deoxyinosine, which can then be inactivated by the trypanosomal enzymes inosine and deoxyinosine hydrolases (Rottenberg et al. 2005). Based on the *in vitro* results, we believe that the addition of pentostatin prevents the degradation of the adenosine

analogue, and thus the combination of the two drugs produced a brief trypanocidal action. However, when cordycepin was used alone, it had no apparent effect on the parasitaemia and in the longevity of mice.

The anti-trypanosomal activity of cordycepin against *T. brucei* was noted to be enhanced by the addition of the ADA inhibitor EHNA (Williamson and Scott-Finnigan, 1978). However, in contrast, this combination failed to induce a curative effect in 42.5% of mice infected with *T. evansi* (Da Silva et al. 2011a). The combination of adenosine analogue 3′-deoxyadenosine and coformycin or deoxycoformycin produced a curative effect in *T. brucei* infection in mice (Rottenberg et al. 2005) as was similarly observed in the *T. evansi*-infected mice in our study. The difference in curative efficacy may be related to specificity of the ADA inhibitor, i.e. the inhibitor EHNA is specific for the isoform ADA_1, since the other two inhibitors (coformycin or deoxycoformycin) are capable of inhibiting both ADA_1 and ADA_2. It should be remembered that tissue extracts contain predominantly ADA_1, which is supposed to be derived mainly from injured tissues. ADA_2 is found in serum and derived from stimulated T cells (Greiger and Nagy, 1986; Franco et al. 1997).

The administration of cordycepin and coformycin/deoxycoformycin eliminated parasites in the brain parenchyma when administered after *T. brucei brucei* penetration into the brain (Rottenberg et al. 2005). This is a highly desirable outcome, because the difficulty of chemotherapy in *T. evansi* infections has been attributed to survival of trypanosomes in cerebrospinal fluid as trypanocidal drugs do not cross the blood–brain barrier or do so in insufficient doses to cure infection (Jennings et al. 1977; Spinosa et al. 1999). Therefore, treatment with cordycepin can be a viable alternative for animals of high economic or sentimental value due to the high costs of cordycepin and ADA inhibitors in Brazil.

Apparently the mice of this study showed no clinical signs suggestive of intoxication. However, in the biochemical tests an increase in liver enzymes associated with histological lesions in the liver of animals in groups F and G was observed. The treatment also caused low intensity, histological renal lesions without impairment of the renal function given that the creatinine levels remained within normal limits. Thus, any application of the promising treatment results observed from our studies should be made cautiously to avoid untoward effects. Effects of toxicity of cordycepin in combination with the ADA inhibitor 2-deoxycoformycin in beagle dogs were also observed as well as when only the inhibitor was used. But the dogs which received only cordycepin showed no drug-related toxicities (Rodman et al. 1997). In a future study, we plan to investigate whether toxicity may be related

to the ADA inhibitor.

Data from this study did not detect resistance of the parasite to cordycepin combined with the inhibitor except in 1 of the 7 mice of group F in which infection relapsed (and which died later). However, it has been reported that *T. b. brucei* developed resistance to cordycepin upon prolonged culture with low doses of the compound; such resistant parasites showed diminished virulence and reduced growth *in vivo* (Vodnala et al. 2009). In other studies, the cordycepin-resistant parasites showed a genetic defect in TbAT1 and surprisingly without cross resistance to other trypanocidal drugs (Maser et al. 1999; Koning et al. 2004). Therefore, cordycepin combined with an ADA inhibitor may be an optimal treatment option when there is resistance to other anti-trypanosomal drugs. Results from our studies suggest curative efficacy of a combination of 3′-deoxyadenosine and deoxycoformycin in mice experimentally infected with *T. evansi*. Note that *in vitro* and *in vivo* the cordycepin and pentostatin combination potentiated the trypanocidal effect against *T. evansi*. Future studies are necessary to evaluate the effects of the treatment on haematological and biochemical parameters, enzymatic purinergic system, as well as to verify if pentostatin has the ability to inhibit the ADA of *T. evansi.*

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4 ARTIGO II

Influence of treatment with 3'-deoxyadenosine associated deoxycoformycin on hematological parameters and activity of adenosine deaminase in infected mice with *Trypanosoma evansi*

Publicado na **EXPERIMENTAL PARASITOLOGY**

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Abstract

This study aimed to verify the effect of 3'-deoxyadenosine and deoxycoformycin on hematologic parameters and adenosine deaminase (ADA) activity in plasma and brain of mice infected with *Trypanosoma evansi*. Seventy animals were divided into seven groups, which were divided into two subgroups each for sampling on days 4 and 8 post-infection (PI). The groups were composed of three uninfected groups (A–C), namely, not-treated (A), treated with 3'-deoxyadenosine (B), and treated with deoxycoformycin (C) and four infected groups, mice with *T. evansi* (D–G), namely, not-treated (D), treated with 3'-deoxyadenosine (E), treated with deoxycoformycin (F), and treated with a combination 3'-deoxyadenosine and deoxycoformycin (G). Hematological parameters and ADA activity were evaluated in plasma and brain. Animals in groups B and C exhibited a reduction in the levels of plasma total protein compared group A. Animals in groups D and F showed changes in the hematological parameters. The ADA activity significantly reduced in the animals of groups C, D, F and G. Mice in the group E presented increased ADA activity in plasma. Therefore, we conclude that the treatment interferes significantly in the hematologic parameters in mice infected with *T. evansi*. On the other hand, when the ADA inhibitor was used we observed a significant decrease in the values of hematocrit, total erythrocytes, and hemoglobin concentration. The deoxycoformycin was able to inhibit the ADA activity of parasite thus it may be one of the mechanisms of efficacy of this treatment.

1. Introduction

The *Trypanosoma evansi* is the etiologic agent of trypanosomosis, a disease with broad distribution in Africa, Asia, and Latin America (Lun and Desser, 1995), which may result in immeasurable economic losses (Luckins and Dwinger, 2004 and Dobson et al., 2009). The parasite is transmitted mechanically by blood-sucking insects during feeding such as *Tabanus* spp. and *Stomoxys* spp. and/or vampire bats (Hoare, 1972). A large number of species may be parasitized by *T. evansi* including horses (in which the disease is called "Mal das cadeiras" or "Surra"), camels, dogs, and in rare cases, humans (Powar et al., 2006). Trypanosome-infected animals present clinical signs such as fever, poor body condition, weakness, subcutaneous edema, petechiae of the serous membranes, and instability of the hind limbs (Silva et al., 1995). An accurate diagnosis of this disease is possible during its acute and chronic stage, but chronic clinical signs are more evident and the animal condition is more severely affected (Silva et al., 2002).

In Brazil, the therapy for trypanosomosis relies on the use of diminazene aceturate, which is effective for the treatment in cattle, buffalo, sheep, pigs and camels (Peregrine and Mamman, 1993 and Sirivan et al., 1994). However, when only one dose is used the treatment is not effective for horses, mules or dogs (Tuntasuvan et al., 2003 and Colpo et al., 2005), which results in lack of efficacy of this drug as a consequence (Tuntasuvan et al., 2003 and Da Silva et al., 2008). Thus, it is important to investigate alternatives to improve the success of the treatment using new drugs, anti-protozoa associations, and other components that could increase the curative efficacy as occurred when diminazene aceturate is associated with selenium in the therapy of infected rats with *T. evansi* (Tonin et al., 2011). Based on this idea, some studies have emerged suggesting new options for the treatment of trypanosomosis.

The effective treatment in the cure of infected mice with *Trypanosoma brucei* was observed when an analogue product of purine, 3'-deoxyadenosine was used (Rottenberg et al., 2005, Vodnala et al., 2008 and Vodnala et al., 2009). The efficacy of the treatment is related to the protection of 3-deoxyadenosine against the enzyme adenosine deaminase (ADA), which is responsible for the deamination of this adenosine analogue (Rodrigues et al., 2005, Vodnala et al., 2008 and Vodnala et al., 2009). However, the administration of 3'-deoxyadenosine alone did not result in a complete cure from the infection (Aiyedun et al., 1973 and Da Silva et al., 2011d). Therefore, this treatment requires the combination of 3'-deoxyadenosine with an inhibitor of ADA_1 and ADA_2, known as deoxycoformycin (Rottenberg et al., 2005).

In addition to the treatment of trypanosomosis, 3'-deoxyadenosine combinations with

deoxycoformycin has been used for the treatment of certain malignant tumors in humans, e.g., leukemia and melanoma (Adamson et al., 1977). This adenosine analogue can perform similar functions to adenosine that is present in all tissues of mammals, demonstrating important functions related to cell signaling, neuroprotection, thromboregulation, and immune processes (Burnstock, 2006 and Desrosiers et al., 2007). In addition, adenosine has an anti-inflammatory action playing a central role in inflammation and immunomodulation (Di Virgilio et al., 1998 and Luttikhuizen et al., 2004). The concentration of extracellular adenosine is regulated by the ADA activity, which is considered an enzyme in the purine metabolism, catalyzing the irreversible deamination of adenosine and deoxyadenosine to inosine and deoxyinosine, respectively (Franco et al., 1997). The observation that ADA deficiency leads to a severe combined immunodeficiency syndrome points to the physiological importance of controlling extracellular adenosine levels (Aldrich et al., 2000).

A recent study showed that the combination of 3'-deoxyadenosine with deoxycoformycin was effective in treating mice infected with *T. evansi*, but the cured animals showed histological lesions in their liver and kidneys (Dalla Rosa et al., 2013). Thus, this study has as objective to verify the effect of 3′-deoxyadenosine and deoxycoformycin on hematologic parameters and ADA activity in plasma and brain of mice infected with *T. evansi* and to check the effect of deoxycoformycin on the ADA activity of parasite.

2. Materials and methods

2.1. Drugs

3′-Deoxyadenosine (Cordycepin®) was obtained from Sigma Chemical Co (St. Louis, MO, USA). Deoxycoformycin (Pentostatin®) was obtained from Tocris Bioscience (Minneapolis, MN, USA). Unless otherwise indicated, all reagents were diluted in PBS, aliquoted and stored at -20°C until further use.

2.2. *T. evansi* isolate

To reactivate the isolated and obtain a large amount of blood parasites for the subsequent infection of mice that formed the experimental groups, in this study, two Wistar rats (R_1 and R_2) were intraperitoneally infected with blood cryopreserved in liquid nitrogen containing 1.3×10^6 trypanosomes.

2.3. Animal groups and *T. evansi* infection

Seventy adult female mice with a mean age of 60 days and weighing average

23.2 ± 1.9 g were used in this study. The animals were housed in cages, ten in each cage, in a room with controlled temperature and humidity (25°C; 70%) on a 12 h light/dark cycle with free access to food and water. All animals were submitted to an adaptation period of 15 days before the beginning of the experimental period.

Mice were divided into seven groups (A, B, C, D, E, F and G) with 10 animals each. The groups A, B and C were formed by uninfected animals with the parasite. Animals of groups D–G were intraperitoneally infected with 0.1 mL of blood from rat (R_1) containing 1.1×10^6 trypanosomes (Day 0). Subsequently, the parasitemia was estimated daily by microscopic examination of smears. Each slide was mounted with blood collected from the tail vein, Romanowsky stain, and visualized at a magnification of 1000×.

2.4. Experimental design

Group A was composed of uninfected and untreated animals (negative control), group B consisted of uninfected and treated with 1 mg/kg/day of 3′-deoxyadenosine and the animals of group C were uninfected and treated with 1 mg/kg/day of deoxycoformycin. Group D was composed of infected and untreated animals (positive control). Animals of groups E, F and G were infected with *T. evansi* and treated with 1 mg/kg/day of 3′-deoxyadenosine, 1 mg/kg/day of deoxycoformycin and treated with combination 1 mg/kg/day of 3′-deoxyadenosine with 1 mg/kg/day of deoxycoformycin, respectively. The treatment (groups B, C, E, F and G) began 2 h after infection by the parasite and lasted for 3 days, i.e., three doses at intervals of 24 h by intraperitoneal.

2.5. Collection of samples

The groups (n = 10) were divided into two subgroups (n = 5) according to the date of sample collection (day 4 post-infection – A1, B1, C1, D1, E1, F1 and G1; day 8 post-infection – A2, B2, C2, D2, E2, F2 and G2). On days 4 and 8 post-infection (PI), the animals were anesthetized in a chamber with isoflurane for collection of blood by cardiac puncture. The material collected was allocated in tubes with anticoagulant (ethylene-diaminetetraacetic acid-EDTA) to evaluate the influence of treatment on hematologic parameters and ADA activity in plasma. Thereafter, animals were decapitated following recommendations of the Ethics Committee and the brain was removed to evaluate the influence of the treatment on the ADA activity of this tissue.

2.6. Hematological parameters

Erythrocyte count, hematocrit, hemoglobin concentration, total leukocytes, and plasma total protein were evaluated. Erythrocytes and total leukocytes were performed using an automated cell counter Mindray BC-2800 Auto Hematology Veterinary Blood Analyzer. The hematocrit was obtained by centrifugation using a microcentrifuge (Sigma) at 19,720g/5 min according to Thrall et al. (2004).

2.7. ADA activity

2.7.1. ADA activity in plasma

ADA activity was measured spectrophotometrically in plasma by the method of Giusti and Galanti (1984). The reaction was started by the addition of the substrate (adenosine) to a final concentration of 21 mmol/L and incubation carried out for 1 h at 37 °C. The reaction was stopped by adding 106 mmol/L/0.16 mmol/L phenol–nitroprusside/mL solution. The reaction mixtures were immediately mixed to 125 mmol/L/11 mmol/L alkalinehypochlorite (sodium hypochlorite) and vortexed. Ammonium sulfate 75 μM was used as ammonium standard. The ammonia concentration was directly proportional to the absorption of indophenol at 620 nm. The specific activity is reported as U/L in plasma.

2.7.2. ADA activity in brain

First, the brain was weighed, homogenized in 10 volume of 50 mmol/L per mM phosphate buffer (pH 6.5), and centrifuged for 30 min at 14,000g at 4 °C. The supernatant was then collected as described by Bellé et al. (2009). ADA activities were estimated spectrophotometrically by the method of Giusti and Galanti (1984), which is based on the direct measurement of the formation of ammonia produced when the enzyme acts on adenosine. The volume of 25 μL of the brain homogenates was used. The enzymatic reaction was started by addition of 500 μL of 21 mM adenosine as substrate. The reaction was stopped by adding 1.5 mL of 106.2/0.16 mM phenol–nitroprusside to the reaction mixture, which was immediately mixed with 1.5 mL of 125/11 mM alkaline-hypochlorite (sodium hypochlorite). The ammonia released reacted with alkaline-hypochlorite and phenol in the presence of a catalyst-sodium nitroprusside to produce indophenol (a blue color) and the concentration of ammonia was directly proportional to the absorbance of indophenol read at 620 nm. Ammonium sulphate of 75 μM was used as ammonium standard. For the calculations of ADA activity, the protein concentration of the brain homogenate was measured by the method of Bradford (1976) with bovine serum albumin used as a standard. The value of ADA activity in

the brain tissue was expressed as U/mg of protein.

2.8. Influence of deoxycoformycin on ADA activity of *T. evansi*

First, trypomastigotes of *T. evansi* in the blood of a rat (R_2) were separated with the aid of column diethylaminoethyl cellulose (DEAE-C) according to the technique described by Tavares et al. (2011). In pellet trypanosomes we evaluated ADA activity according to the technique by Giusti and Galanti (1984), adapted and published by Da Silva et al., 2011a, Da Silva et al., 2011b, Da Silva et al., 2011c and Da Silva et al., 2011d. For the assays *in vitro*, the deoxycoformycin was used at concentrations of 0, 25 pM, 1 nM and 10 nM. The ADA activity in the trypanosomes was expressed in U/L.

2.9. Statistic analysis

Data were submitted to one-way analysis of variance followed by the Student's t test ($P < 0.05$). Values were represented as mean ± standard deviation. All samples were processed in triplicate.

3. Results

3.1. Parasitemia

Animals in group D (positive control) showed increased parasitemia, being observed an average of 17 trypanosomes/field on day 4 PI (subgroup D1) and 113 trypanosomes/field on day 8 PI (subgroup D2). Similar results were observed in mice in group F (treated with deoxycoformycin), i.e., 11 and 87 trypanosomes/field on days 4 and 8 PI, respectively. In the rodent groups E and G, the presence of trypanosomes in blood smears on day 8 of the experiment was not observed. After the treatment, no clinical sign showing intoxication of animals was observed.

3.2. Hematological parameters

Animals in groups B and C (uninfected and treated) showed no variation in the values of hematocrit, total erythrocytes, hemoglobin, and total leukocytes when compared to group A (uninfected and untreated). On the other hand, the levels of plasma total protein decreased ($P < 0.05$) in animals treated with 3′-deoxyadenosine (group B) and deoxycoformycin (group C) when compared to group A on day 4 PI (Table 1).

A decrease ($P < 0.05$) in the values of hematocrit, total erythrocytes, and hemoglobin concentration was observed in the infected and untreated mice (group D) and infected and

treated mice with deoxycoformycin (group F) on day 8 PI (Table 1). The number of total leukocytes was significantly ($P < 0.05$) increased in mice infected and untreated (group D) on days 4 and 8 PI when compared to the other groups, with the exception of group E and group F which had a similar increase of leukocytes only on day 8 PI. The group G treated with the combination 3′-deoxyadenosine and deoxycoformycin did not show significant variation ($P > 0.05$) in hematologic parameter when compared to group A (Table 1).

3.3. ADA activity in plasma and brain

The results of ADA activity of animals in this experiment are shown in Table 2. The animals in group B (uninfected and treated with 3′-deoxyadenosine) showed no significant ($P > 0.05$) variation in ADA activity in plasma and brain when compared to group A. The treatment with deoxycoformycin in group C was able to significantly ($P < 0.05$) reduce the ADA activity in plasma and brain on days 4 and 8 PI when compared to group A. Animals infected with *T. evansi* and untreated (group D) showed reduction ($P < 0.05$) in ADA activity in plasma only on day 8 PI compared to negative control. Animals in group E (infected and treated with 3′-deoxyadenosine) showed an increase in ADA activity in plasma on day 8 PI ($P < 0.05$). In both groups F and G, the ADA activity in plasma and brain of mice had a significant ($P < 0.05$) reduction compared to the negative control group.

Table 1 – Mean and standard deviation of hematologic parameters (hematocrit, total erythrocytes, hemoglobin concentration, plasma total protein levels and total leukocytes) in mice uninfected/infected with *Trypanosoma evansi* and untreated/treated with 3′-deoxyadenosine and deoxycoformycin. Blood samples were analyzed on days 4 and 8 post-infection.

Parameters	Day	Group A	Group B	Group C	Group D	Group E	Group F	Group G
Hematocrit (%)	04	45.8^{a} (±2.4)	44.2^{a} (±2.5)	45.5^{a} (±2.6)	45.1^{a} (±1.5)	47.0^{a} (±1.5)	47.5^{a} (±2.0)	45.0^{a} (±2.3)
	08	44.7^{ab} (±2.6)	45.3^{ab} (±1.5)	46.3^{a} (±2.5)	38.0^{c} (±1.0)	44.0^{ab} (±1.5)	37.3^{c} (±1.5)	49.5^{a} (±3.5)
Erythrocytes (x10^{6}µL)	04	6.7^{a} (±1.0)	8.0^{a} (±0.1)	8.2^{a} (±0.5)	8.4^{a} (±0.4)	8.1^{a} (±0.3)	8.4^{a} (±0.3)	7.5^{a} (±0.4)
	08	7.9^{ab} (±0.9)	8.1^{abc} (±0.1)	8.3^{ab} (±0.5)	6.7^{c} (±0.2)	7.5^{bc} (±0.5)	6.9^{c} (±0.4)	8.8^{a} (±0.6)
Hemoglobin (g/dL)	04	12.0^{a} (±2.0)	12.7^{a} (±0.5)	12.8^{a} (±0.7)	13.1^{a} (±0.6)	13.2^{a} (±0.2)	13.0^{a} (±0.7)	12.3^{a} (±0.8)
	08	12.0^{ab} (±2.1)	12.6^{ab} (±0.6)	12.6^{ab} (±0.7)	10.3^{c} (±0.6)	12.1^{ab} (±0.7)	10.8^{c} (±0.7)	13.4^{a} (±1.1)
Plasma total protein (g/dL)	04	7.5^{a} (±0.3)	6.8^{c} (±0.3)	6.9^{bc} (±0.2)	7.9^{a} (±0.1)	7.8^{a} (±0.2)	7.5^{ab} (±0.3)	7.4^{abc} (±0.2)
	08	7.6^{abc} (±0.1)	7.0^{c} (±0.0)	6.9^{c} (±0.2)	7.4^{bc} (±0.1)	8.0^{ab} (±0.2)	8.4^{a} (±0.7)	7.2^{c} (±0.3)
Leukocytes (x10^{3}/µL)	04	4.2^{b} (±1.7)	5.4^{ab} (±1.3)	4.8^{b} (±2.1)	8.0^{a} (±2.1)	4.8^{b} (±2.3)	4.6^{b} (±1.1)	3.6^{b} (±1.3)
	08	4.6^{b} (±2.1)	4.8^{b} (±0.6)	3.9^{b} (±1.4)	8.9^{a} (±1.0)	8.5^{a} (±0.7)	10.9^{a} (±3.5)	5.8^{ab} (±1.4)

Same letters in the same row are not statistically different from each other, the significance level of 5% in Student test. Group A: uninfected and untreated animals, Group B: uninfected and treated with 3'-deoxyadenosine, Group C: uninfected and treated with deoxycoformycin, Group D: infected and untreated, Group E: infected and treated with 3'-deoxyadenosine, Group F: infected and treated with deoxycoformycin, and group G: infected and treated with combination 3'-deoxyadenosine and deoxycoformycin.

Table 2 – Mean and standard deviation of the activity of adenosine deaminase (ADA) in plasma and brain of mice uninfected/infected with *Trypanosoma evansi* and untreated/treated with 3′-deoxyadenosine and deoxycoformycin. Blood samples were analyzed on days 4 and 8 post-infection.

Groups	Mean and standard deviation of ADA activity in plasma (U/L) and brain (U/mg of protein)			
	Day 4 post-infection		Day 8 post-infection	
	Plasma	Brain	Plasma	Brain
Group A	2.53±0.31 [bc]	14.52±3.20 [a]	2.75±0.47 [b]	13.68±3.53 [a]
Group B	3.35±0.34 [ab]	11.32±1.21 [abc]	3.13±0.22 [b]	10.93±0.90 [a]
Group C	1.40±0.14 [de]	8.36±0.54 [cd]	1.33±0.08 [cd]	1.63±0.85 [b]
Group D	2.03±0.39 [cd]	12.50±1.48 [ab]	1.70±0.10 [c]	12.52±0.50 [a]
Group E	3.71±1.00 [a]	11.06±2.65 [abc]	5.07±0.7 [a]	11.07±0.97 [a]
Group F	0.48±0.22 [e]	3.74±0.87 [e]	0.48±0.10 [d]	4.24±0.10 [b]
Group G	0.67±0.23 [e]	5.20±0.84 [de]	0.66±0.30 [d]	4.96±0.62 [b]

Mean followed by the same letter in the same column does not differ statistically itself, the probability of 5% ($P<0.05$) in Student test. Group A: uninfected and untreated animals, Group B: uninfected and treated with 3'-deoxyadenosine, Group C: uninfected and tretaed with deoxycoformycin, Group D: infected and untreated, Group E: infected and treated with 3'-deoxyadenosine, Group F: infected and treated with deoxycoformycin, and group G: infected and treated with combination 3'-deoxyadenosine and deoxycoformycin.

3.4. Effect of deoxycoformycin on the ADA activity of *T. evansi*

The concentration of proteins in the trypanosome pellet chosen for the tests was 0.6 mg mL^{-1}, being this concentration used for the tests with inhibitor. The results of ADA activity in the parasite and deoxycoformycin effects on the activity of this enzyme are shown in Fig. 1. A dose-dependent inhibition of ADA activity in the parasite was observed when the inhibitor deoxycoformycin was used.

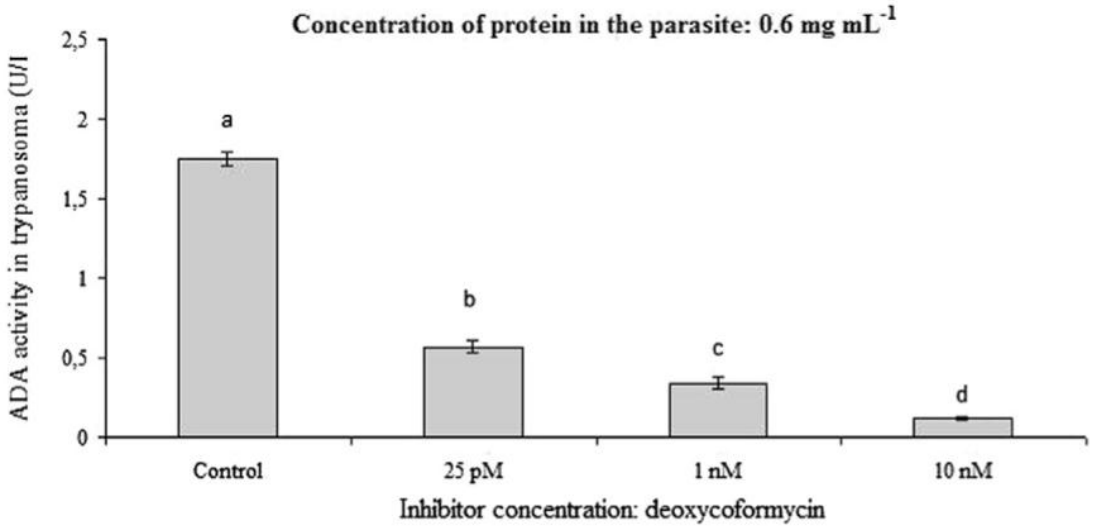

Fig. 1 – Activity of adenosine deaminase (ADA) at a concentration of 0.6 mg mL^{-1} of pellet *Trypanosoma evansi* and tests with deoxycoformycin in different concentrations. Different letters in the column determine that there is a significant difference between treatments ($P < 0.05$).

4. Discussion

In previous studies, our research group has found that the 3′-deoxyadenosine associated with ADA inhibitors such as EHNA hydrochloride (Da Silva et al., 2011d) and deoxycoformycin (Dalla Rosa et al., 2013) can be 42.5% and 100% effective in treating infected mice with *T. evansi*, respectively. In both experiments mentioned above, when only the ADA inhibitors were tested, no curative effect was observed because parasitemia increased progressively. When 3′-deoxyadenosine was used, the elimination of the parasite from the blood for some time occurred, but with recurrent parasitemia. Similar results were observed in this study.

The present study was made due to histological lesions observed in the liver and kidney of mice infected with *T. evansi* and treated with the combination 3′-deoxyadenosine and deoxycoformycin (Dalla Rosa et al., 2013). Therefore, we aimed to check if the treatment could influence the hematological parameters and ADA activity in plasma and also in the brain, where it performs vital functions in mammals related to neuromodulation. However, Dalla Rosa et al. (2013) found no histological lesions in the brain of treated animals. The toxic effect of treatment was attributed to the ADA inhibitor (deoxycoformycin) alone or associated with 3′-deoxyadenosine in a study with dogs, being observed in the animal clinical signs of gastrointestinal toxicity, and bone marrow injury causing lymphopenia, thrombocytopenia, and depletion of hematopoietic cells (Rodman et al., 1997). However,

apparently the same did not occur in this study with the dose used.

Infections by *T. evansi* commonly cause anemia and leukocytosis in domestic animals (Rodrigues et al., 2005 and Wolkmer et al., 2009). This finding was also visualized in mice in this study, which showed the parasite circulating during the experiment (groups D and F). The individual treatment with 3′-deoxyadenosine and with deoxycoformycin had no effect on hematology parameters, a fact observed in healthy animals and treated with inhibitors of ADA (groups B and C). In a first moment of the study, a reduction in the levels of plasma total protein (day 4 PI) was observed in healthy mice treated with 3′-deoxyadenosine and deoxycoformycin (groups B and C) when compared to other groups. This finding may suggest that drugs may tax and/or injure the liver, which is the main organ involved in protein synthesis, as it has been observed in a previous study (Dalla Rosa et al., 2013). This did not occur when the same treatment was used in infected animals which can be explained because the infection with *T. evansi* stimulates the increase of protein as evidenced by Costa et al. (2010) in cats experimentally infected with the parasite.

The ADA activity in plasma and brain of rodents infected with *T. evansi* reduced significantly (Da Silva et al., 2011a and Da Silva et al., 2011b), which occurred in this study in group D formed by infected and untreated animals. Both drugs used in our experimental protocol caused at some point alteration in ADA activity in plasma and/or brain. Inhibition of ADA activity drew attention for groups that were treated with deoxycoformycin which had a large decrease of ADA activity in plasma and brain on days 4 and 8 PI. This result was expected on day 4 PI, since the last dose of the drug was administered on day 3 PI. However, on day 8 PI, 5 days after the last dose of the drug, the inhibition was not expected. This is because the ADA inhibitors have a relatively short half-life (McConnell et al., 1978). According to the researchers, the administration of deoxycoformycin in dogs, rats and humans has rapid uptake and clearance of tissues, followed by its rapid elimination through the urine, mainly in an unmetabolized form (Borondy et al., 1977, McConnell et al., 1978 and Major et al., 1981). In rats, more than 90% of the administered dose was recovered in the urine within 2 h after intraperitoneal injection (McConnell et al., 1978), confirming that the half life of the drug is short. The same did not occur in our study. The ADA activity was inhibited and remained inhibited compared to healthy animals. Therefore, despite the fact the curative treatment shows efficacy in the treatment for trypanosomosis, it can interfere with other physiological functions that the ADA has participation of the host.

ADA is widely distributed in tissues of vertebrate animals and divided into two isoforms: ADA_1 and ADA_2. The deoxycoformycin has the ability to inhibit both isoforms

(Franco et al., 1986). As mentioned in the introduction, this enzyme plays different roles in mammalian and its deficiency may contribute to some disorders. This reduction in ADA activity may have caused an increase in the concentration of adenosine, which develops a neuroprotective and modulator role regarding the tissue damage and impaired metabolism (Pearson et al., 2003). This may cause an imbalance in the levels of nucleosides in the central nervous system (CNS), reduce deamination to inosine and thereby interfere the sequence of enzymatic cascade. In addition to interfering with the CNS, ADA deficiency could interfere with the immune response and hematopoiesis, where this enzyme has important roles (Aran et al., 1991, Xaus et al., 1999 and Kumar and Sharma, 2009).

ADA inhibitors available in the pharmaceutical industry presents a number of drawbacks for clinical use such as problems in their pharmacokinetics and/or severe toxic effects and high cost (Cavalcante, 2010). The deoxycoformycin promotes intracellular accumulation of adenosine nucleotides and deoxyadenosine, blocking DNA synthesis by inhibiting ribonucleotide reductase, and deoxyadenosine inactive to S-adenosyl homocysteine hydrolase, promoting the accumulation of S-adenosyl cysteine, which is toxic to lymphocytes (Chabner et al., 2005). The results of this study are worrisome because despite the fact that therapeutic protocol is effective in controlling trypanosomosis, it causes interference in the purinergic system.

The activity of ADA was recently detected in *T. evansi* (Da Silva et al., 2011c). Based on this information, this study suggests that the deoxycoformycin is capable of inhibiting ADA present in parasite. It is worth investigating whether this inhibition may lead to parasite death and if this enzyme plays a vital role as it does in mammals. In *in vitro* tests a reduction was visualized in the number of parasites living deoxycoformycin only when administered within the first hour of experiment (Dalla Rosa et al., 2013). Therefore, we believe that the association of the ADA inhibitor 3′-deoxyadenosine enhances the trypanocidal activity *in vivo*.

Based on these results, we conclude that the treatment with 3′-deoxyadenosine and deoxycoformycin in the doses used caused no alterations in hematocrit, number of erythrocytes, and hemoglobin concentration in mice infected with *T. evansi*. The number of leukocyte differs between groups, but this variation seems to be associated with animals that had parasitemia by *T. evansi*. The plasma total protein levels can be reduced as a consequence of this treatment. The activity of ADA suffers strong inhibition in plasma and brain of animals treated with deoxycoformycin. Therefore, the therapeutic protocol influences the activity of ADA and this may cause disturbances and side effects. As ADA and adenosine have

participation in the immune response, we conclude that this system could be harmed by the treatment used in the current study. Consequently, more research must be carried out before recommending this treatment for trypanosomosis. Another conclusion drawn from this experiment was that the deoxycoformycin is able to inhibit the ADA activity in *T. evansi*, which may be related to the mechanism of trypanocidal action of drug.

Committee on ethics and animal welfare

The procedure was approved by the Animal Welfare Committee of Universidade Federal de Santa Maria, number 026/2012.

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5 ARTIGO III

3'deoxyadenosine and deoxycoformycin for the treatment of *Trypanosoma evansi* infection: An effective and nontoxic dose

Submetido para a **PATHOLOGY - RESEARCH AND PRACTICE**

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SUMMARY

The aim of this study was to evaluate the therapeutic efficacy and viability of using 3′deoxyadenosine (Cordycepin - adenosine analogue) combined with deoxycoformycin (Pentostatin - an adenosine deaminase inhibitor) in mice infected with *Trypanosoma evansi*. We show that the combination of Cordycepin (2.0 mg kg^{-1}) and Pentostatin (0.2, 0.5, 1.0, 2.0 mg kg^{1}) is effective in the clearance of *T. evansi*, although at higher concentrations (Cordycepin 2mg kg^{-1} and Pentostatin 2mg kg^{-1}), some toxicity was observed in the liver and kidney. Since the Cordycepin 2.0 mg kg^{-1} and Pentostatin 0.2 mg kg^{-1} combination was effective and had low toxicity, we recommend this as a therapeutic option. Therefore, we conclude that when combined, Cordyceptin and Pentostatin are able to effectively eliminate *T. evansi*.

Key words: adenosine, trypanosomiasis, toxicity

1. INTRODUCTION

Trypanosoma evansi is a flagellated protozoan that infects a range of hosts, including a devastating disease in equines, and is the most prevalent pathogenic trypanosome throughout the tropical and subtropical areas of the world [1, 2]. Transmission occurs via bloodsucking flies (*Tabanus* spp. and *Stomoxys* spp.), and occasionally vampire bats in Latin America [3]. Clinically, infection is characterized by rapid weight loss, various degrees of anemia, intermittent fever, edema of the hind limbs, progressive weakness and locomotor

disturbance, eventually leading to death [4, 5]. To date, several drugs have been used for the treatment of *T. evansi* infection, yet their efficacy and toxicity, particularly towards the kidneys and liver, have proven to be problematic. [6, 7, 8]. Moreover, in some instances drug resistance has been reported [9]. Thus, it is important to investigate alternative therapies for the treatment of *T. evansi*.

Importantly, one characteristic of trypanosomes is their inability to engage in *de novo* purine synthesis [10, 11]. In particular, their dependence on the nucleoside salvage pathway from the body fluids of their hosts [12] has been exploited as a therapeutic target [13]. Our recent studies suggest that a combination of 3′deoxyadenosine (Cordycepin) and deoxycoformycin (Pentostatin) in mice experimentally infected with *T. evansi* is curative [14], and has a beneficial role in treating hematological parameters and the activity of ecto-adenosine deaminase (E-ADA) in the host [15]. Mechanistically, as an adenosine analogue, Cordycepin most likely replaces the ribose of adenosine and consequently interrupts nucleolar RNA synthesis [16, 17, 18]. On the other hand, Pentostatin is a deoxyadenosine analog and a potent inhibitor of E-ADA, an enzyme of purine metabolism [19]. Blockade of E-ADA leads to the accumulation of cytotoxic de-oxyadenosine triphosphate metabolites in the cell, inhibition of DNA synthesis and eventual cell death [20, 21]. Moreover, when Pentostatin is used in combination with Cordycepin, deamination is protected, leading to another biological activity against other trypanosomatids [22, 13].

The high affinity of the trypanocidal for the parasite transporter, P1 and P2, the low affinity for the mammalian transporters, and the low abundance of competing substrates for the trypanosome transporters probably underlie the toxicity of Cordycepin and Pentostatin to trypanosomes [23]. Yet, although we previously showed that Cordyceptin and Pentostatin did lead to some liver and kidney damage [14], we re-visited the therapeutic potential of these drugs to determine whether an optimal dose could be found that was both nontoxic to the host yet had the ability to clear *T. evansi* in experimentally infected mice.

2. MATERIALS AND METHODS

2.1. Reagents

3′-deoxyadenosine (Cordycepin) was acquired from Sigma – Aldrich, EUA. Deoxycoformycin (Pentostatin; Tocris) was used as an inhibitor of E-ADA. Unless otherwise indicated, all reagents were diluted in PBS pH 7, aliquoted and stored at -20°C until further use.

2.2. *Trypanosoma evansi* isolate

T. evansi was originally isolated from a naturally infected dog [24]. One rat (R_1) was intraperitoneally (I.P.) infected with blood that had been cryopreserved containing *T. evansi* in order to obtain a large enough quantity of bloodstream forms of the parasite for subsequent infection of mice that formed the experimental groups.

2.3. Animals

A total of 120 mice, BALB/c female (mean age of 60 days) weighing approximately 25±2g were used. All animals were kept in cages (303 x 193 x 126 mm; n=10/cage), in a room with controlled temperature and humidity (25°C; 70%) and a light–dark illumination cycle of 12/12 h. Cages and bedding were changed three times a week. Tap water and food were provided *ad libitum*. All animals were submitted to an adaptation period of 10 days before the beginning of the experiment. The experimental procedures used in this study were approved by the Animal Welfare Committee of UFSM, under number 026/2012.

2.4. Infections

Mice were divided into 6 groups (A, B, C, D, E and F; n=20/group). Group A served as a negative control (uninfected and untreated). Mice in Group B served as a positive control and were infected with $1x10^6$ trypanosomes in 0.1 mL of blood from rat (R_1). Mice in Groups C through F were infected similar to Group B mice but also treated, 24 hs after infection, for three days post-infection (PI) with a constant dose of Cordyceptin (2mg kg^{-1}) and different doses of Pentostatin, respectively: 0.2mg kg^{-1} (Group C), 0.5mg kg^{-1} (Group D), 1.0 mg kg^{-1} (Group E), 2.0 mg kg^{-1} (Group F).

2.5. Parasitemia evaluation

Parasitemia was estimated daily by microscopic examination of blood smears from mice. Each slide was prepared from tail vein mouse blood, stained by the Romanowsky method and visualized at a magnification of 1000x.

2.6. Collection of samples

On day 4 (n=10/group) and 18 (n=10/group) post-infection, mice were anesthetized with isofluorane in an anesthetic chamber for the collection of blood by cardiac puncture. Due to the small sample volume, animals from each group were evaluated in each period, i.e., the blood samples were collected in tubes without anticoagulant to obtain the serum (5 mice) and

in tubes with EDTA to obtain the plasma (5 mice). Thereafter, mice were euthanized in accordance with the recommendations of the Ethics Committee and livers, spleens and kidneys were removed to assess cell viability of the organs and determine toxicity after treatment.

2.7. Cell viability Assay - MTT Assay

For the culture of splenocytes and hepatocytes, we used a modified method [25]. Briefly, the spleens and livers were surgically removed and immediately transferred to *Petri* dishes containing culture medium (RPMI 1640-GIBCO® enriched with 10% fetal calf serum, 1% Penicillin/Streptomicin, and 1% Amphotericin B). The organs were mashed, passed through a strainer, and centrifuged for 5 minutes at 1000 rpm. Subsequently, the supernatant was resuspended in an erythrocyte lysis buffer (8.26g NH_4Cl, 1g $KHCO_3$ and 0.037g EDTA) for 2 minutes. After one more wash in culture medium, cells were re-suspended in complete media and seeded at a concentration of 1.5×10^5 cells [25].

Thiazolyl Blue Tetrazolium Bromide (MTT) was then added to each well at a concentration of 5 mg ml^{-1}. The plate was homogenized (150 rpm per 5 minutes) and maintained in a CO_2 incubator (5%) at 37ºC for 4 hours after which the supernatant was removed and we added 150 µL of DMSO per well. The plate was then centrifuged and read at 570 nm.

2.8. Cytotoxicity Assay - PicoGreen Assay

PicoGreen® was diluted to a working solution in TE buffer (10 mM Tris-HCL and 1 mM EDTA). Due to the reagent's light sensitivity, we used solid black 96-well microplates. Before use, plates were soaked in 1M NaOH for a day, washed with autoclaved distilled water and dried in an incubator at 37˚C. Next, both plasma and PicoGreen® reagent (10 µL each) or PicoGreen® alone were added to wells in a 100 µL total volume (brought up with TE buffer). Plates were kept at room temperature for 5 minutes, and then read in a spectroflourimeter. PicoGreen® stained samples were excited at 485 nm and emission at 520 nm was recorded. All samples were processed in triplicate.

2.9. Measurement of oxidative/antioxidant imbalances

In order to identify oxidative imbalance, certain antioxidants and oxidative variables were evaluated, including AOPP (advanced oxidation protein products), TOS (total oxidant status), FRAP (ferric reducing ability of plasma) and TAC (total antioxidant capacity). All

measurements were performed on a Cobas MIRA® (Roche Diagnostics, Basel, Switzerland) automated analyzer. AOPP protein oxidation levels in liver tissue were evaluated by the method described in [26].

TOS in liver tissue was also determined using a novel automated measurement method [27]. In this method, oxidants present in the sample oxidize the ferrous ion-o-dianisidine complex to ferric ion. Abundant glycerol molecules in the reaction medium enhance the oxidation reaction. The ferric ion forms a colored complex with xylenol orange in an acidic medium. The color intensity, which can be measured spectrophotometrically, is related to the total amount of oxidant molecules in the sample. The assay is calibrated with hydrogen peroxide and results are expressed in terms of μmol H_2O_2 Equivalents/g protein.

The total antioxidant potential of liver samples was determined using a FRAP assay [28] FRAP assay measures the change in absorbance at 593 nm owing to the formation of a blue colored Fe^{2+} tripyridyltriazine compound from colorless oxidized Fe^{3+} form by the action of electron donating antioxidants. Result was expressed in μmol L^{-1}.

TAC was determined in liver using a novel automated method [29] involving the production of hydroxyl radical. The antioxidant effect of the sample against the potent free radical reactions initiated by the hydroxyl radicals produced is measured. Results were expressed as μmol Trolox Equivalents/g protein.

2.10. Hepatic and renal function

The serum activities of alanine aminotransferase (ALT), aspartate aminotransferase (AST) and concentrations of creatinine were measured using commercial kits (Bioclin®, Belo Horizonte, MG, Brazil). All measurements were performed on a Cobas MIRA® automated analyzer (Roche Diagnostics, Basel, Switzerland).

2.11. Histopathology.

Samples of the livers and kidneys of *T. evansi* infected mice treated or not treated with Cordycepin and Pentostatin were fixed in buffered formalin (10%), embedded in paraffin, sectioned at 5μm and stained with haematoxylin/eosin (H&E). The sections were qualitatively evaluated for differences in microarchitecture, organization and the presence of inflammatory cells.

2.12. Effectiveness of treatment

Treatment outcome was based on parasitaemia levels and molecular analysis for *T.*

evansi in infected mice that survived after treatment. Blood and brain samples were collected and DNA extracted for *T. evansi*-specific PCR [30]. Treatment was considered effective when clinical signs were not observed, parasitemia levels were reduced to zero and any signs of toxic damage to livers and kidneys was detected.

2.13. Statistical analysis

Initially, the raw data distribution was tested for normality of variance by Shapiro-Wilk test, and it was found that the data had a normal distribution. Then, data were submitted to analysis of variance (ANOVA) followed by Duncan test. Results were considered significant when $P<0.05$.

3. RESULTS

3.1. Administration Cordycepin and Pentostatin eliminates *T. evansi* burden *in vivo*

To determine whether treatment with Cordycepin and Pentostatin improved clearance of *T. evansi*, we conducted a dose-dependent time course with the two drugs *in vivo*. Mice were spilt into six groups, with Group A serving as a negative control (uninfected) and Group B a positive control (infected but not treated.) Groups B through F were infected with *T. evansi* and three days post-infection, treated with a constant dose of Cordycetin (2 mg kg^{-1}) and increasing concentrations of Pentostatin (Group C: 0.2 mg kg^{-1}; Group D: 0.5 mg kg^{-1}; Group E: 1 mg kg^{-1}; Group F: 2 mg kg^{-1}). As shown in Figure 1, examination of peripheral blood smears showed a pre-patent period between 24 and 48 hours in the infected and untreated mice. Groups C through F presented low parasitemia on day one post-infection. Importantly however, as time post-infection increased, mice treated with Cordycepin and Pentostatin showed a decrease in parasitemia, with the highest doses of the drugs (Group F) not demonstrating any evidence of trypanosomes after day one post-infection (Table 1 and Figure 1). Furthermore, molecular analysis was performed to confirm the effectiveness of the therapeutic protocol used. Animals treated with Cordycepin and Pentostatin at every dose showed a negative blood smear and were negative for the presence of *T. evansi* by PCR from the blood and brains of mice (Table 1). Therefore, these results suggest that Cordycepin and Pentostatin can completely clear *T. evansi* infection *in vivo.*

Table 1 – Mean and standard deviation of the pre-patent period, longevity, mortality and success of therapy with Cordycepin (3'-deoxyadenosine) combined with Pentostatin (deoxycoformycin) in mice experimentally infected with *Trypanosoma evansi*.

Groups n=6	Treatment	Pre-patent period (days)	Longevity (days)	Mortality (n° dead / n° in group)	*Therapeutic success (%)
A	Negative control Non-infected	-	18	0/10	-
B	Positive control Infected and untreated	1.1 (±0.3)	5.4 (±0.7)	10/10	0
C	Infected and treated 2mg/kg^{-1} cordycepin + 0.2mg/kg^{-1} pentostatin	0	18	0/10	100
D	Infected and treated 2mg/kg^{-1} cordycepin + 0.5mg/kg^{-1} pentostatin	0	18	0/10	100
E	Infected and treated 2mg/kg^{-1} cordycepin + 1mg/kg^{-1} pentostatin	0	18	0/10	100
F	Infected and treated 2mg/kg^{-1} cordycepin + 2mg/kg^{-1} pentostatin	0	18	0/10	100

*Considered a therapeutic success for drug-treated mice that survived for 18 days and remained negative for the parasite by examination of their blood smears and by PCR.)

3.2. Effect of Cordycepin and Pentostatin treatment on renal and hepatic toxicity

To determine whether Pentostatin and Cordycepin led to increased cellular toxicity and mitochondrial damage, the MTT assay for cell viability was used. This assay is based on the conversion of MTT (yellow color) into formazan crystals (blue-purple color) by living cells, which determines mitochondrial activity [31]. For most cell populations the total mitochondrial activity is related to the number of viable cells. Thus, it is possible to establish parameters for cytotoxicity and the cell proliferation rate by this assay [32]. As shown in Figure 2A, Cordycepin and Pentostatin produced a decrease in the percentage of cell survival in the liver in a dose dependent manner. However, even at the highest concentrations, cell survival was better than untreated mice (Figure 2A, compare Groups B to F). Within spleen, we detected increased levels of cell survival relative to uninfected controls in all but the highest drug dose (Group F), which showed minimal deviation from our negative control mice.

To further characterize whether Pentostatin and Cordycepin causes cellular toxicity,

we employed the use of PicoGreen. PicoGreen® dye is an ultrasensitive fluorescent reagent that allows quantification of dsDNA in the solution and can detect minute concentrations of DNA, up to 25 pg mL^{-1} [33]. PicoGreen® binds double-stranded DNA and forms a highly fluorescent complex. Thus, PicoGreen® is used to follow DNA denaturation due to a decreasing fluorimetric signal intensity, corresponding to the production of ssDNA and mononucleotide content, which can be indicative of DNA damage and possible cell death [34]. At lower doses of Pentostatin, we observed decreased levels of dsDNA relative to uninfected controls, indicative of cell survival (Figure 2C, Groups C and D). At higher doses (Groups E and F), we observed greater levels of dsDNA relative to uninfected Group A mice. Thus, taken together, these results suggest that Cordycepin and Pentostatin treatment causes minimal to moderate cytoxicity in a dose dependent fashion.

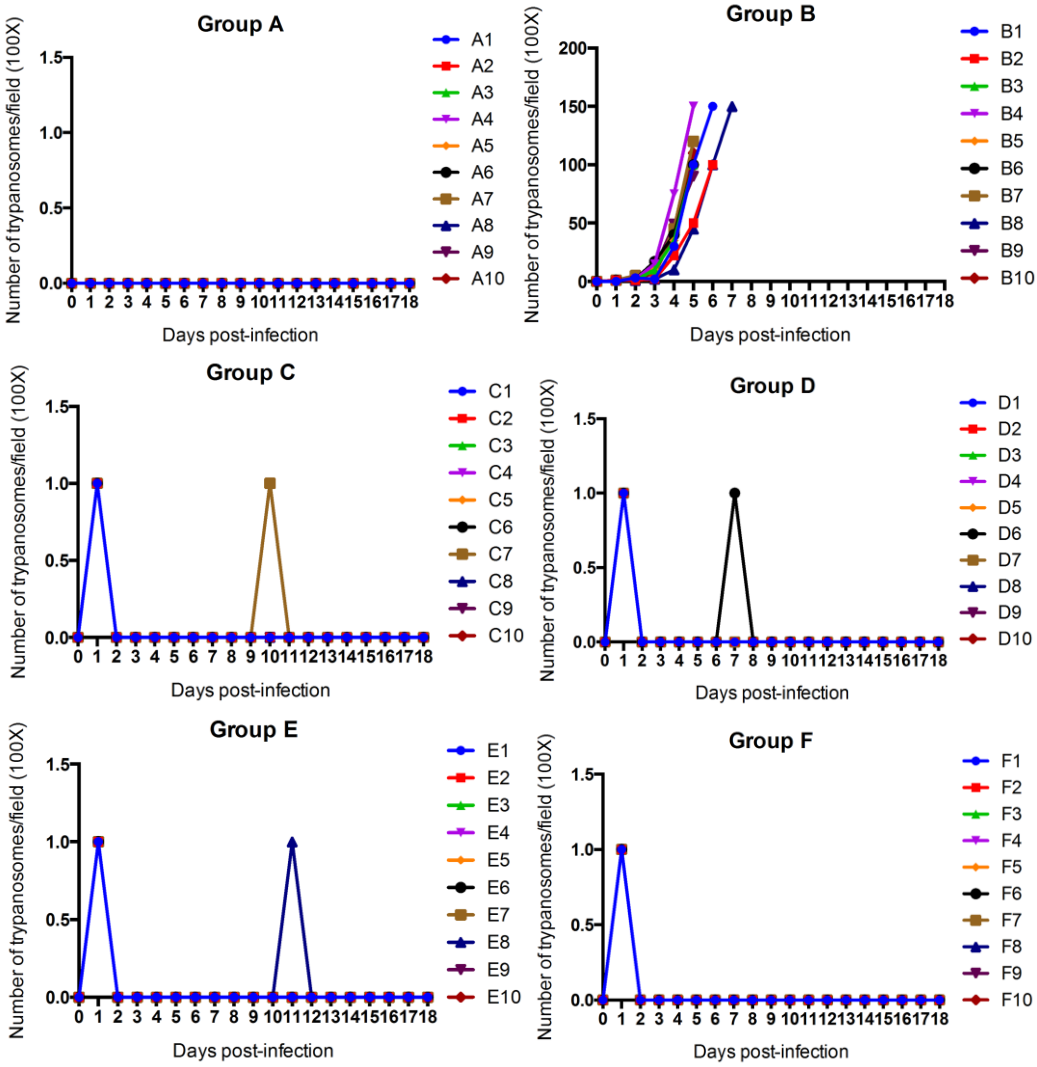

Fig 1 – Parasitemia of *Trypanosoma evansi*-infected mice day 18 post-infection. The different treatments started in Groups C through F at day 1 post-infection (3 doses at 24 h intervals). Groups: A- Negative control B- Positive control C- 2mg/kg^{-1} cordycepin + 0.2mg/kg^{-1} pentostatin D- 2mg/kg^{-1} cordycepin + 0.5mg/kg^{-1} pentostatin E- 2mg/kg^{-1} cordycepin + 1mg/kg^{-1} pentostatin F- 2mg/kg^{-1} cordycepin + 2mg/kg^{-1} pentostatin.

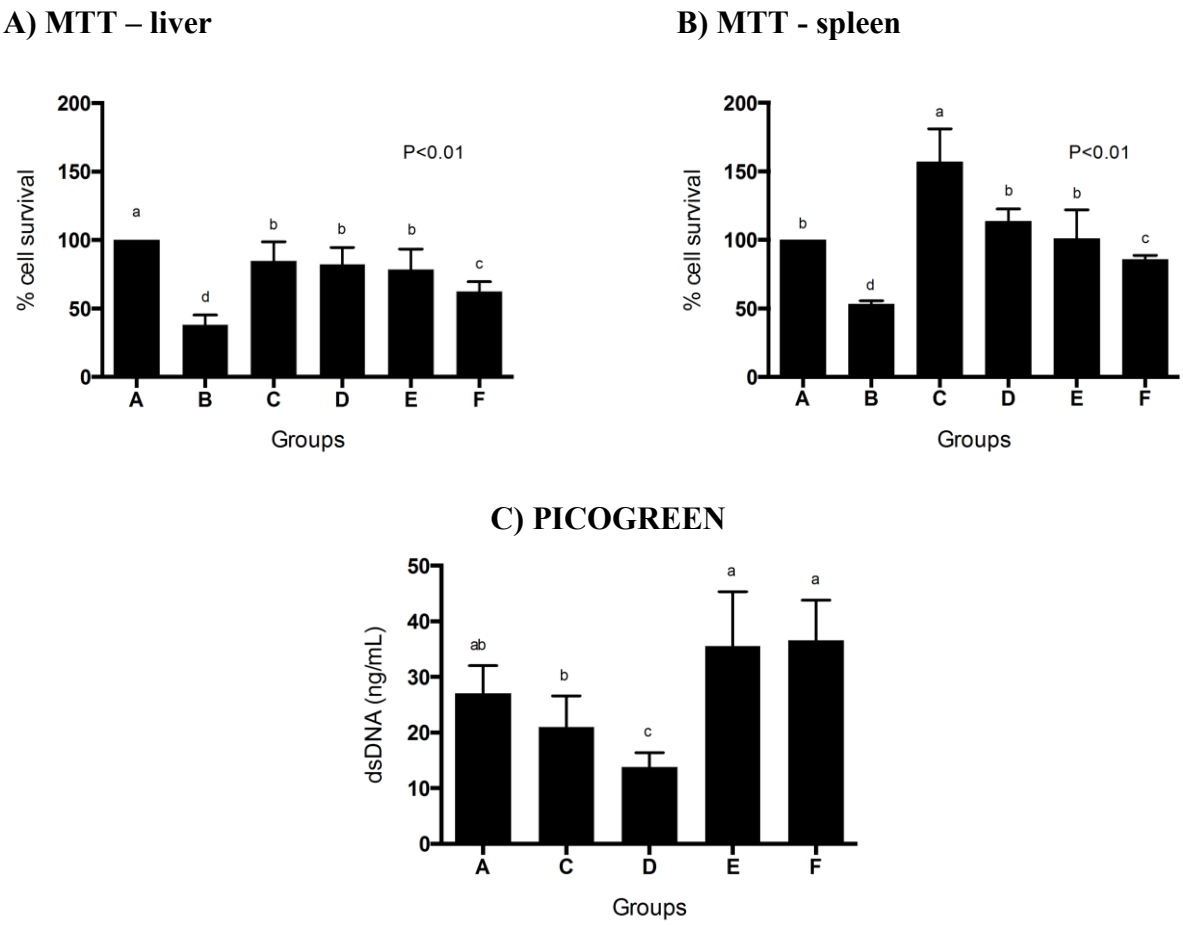

Fig 2 – Effect of Cordycepin (3'deoxyadenosine) and Pentostatin (Deoxycoformicin) treatment on cell survival percentage based in MTT Assay using liver (A) and spleen (B), day 4 post-infection. And based in Picogreen® Assay using plasma (C), day 18 post-infection. Groups: A- Negative control B- Positive control C- 2mg/kg^{-1} cordycepin + 0.2mg/kg^{-1} pentostatin D- 2mg/kg^{-1} cordycepin + 0.5mg/kg^{-1} pentostatin E- 2mg/kg^{-1} cordycepin + 1mg/kg^{-1} pentostatin F- 2mg/kg^{-1} cordycepin + 2mg/kg^{-1} pentostatin.

3.3. Mice treated with Cordyceptin and Pentostatin display variable oxidative stress metabolite levels

Next, we decided to analyze levels of oxidative stress metabolites to determine whether they were being regulated by Cordyceptin and Pentostatin. The study did not reveal variations in liver AOPP levels on either day 4 or 18 PI compared with the control group (Figure 3A). An increase of oxidative stress in the liver of mice (increased TOS) occurred on day 4 PI in Group F ($P<0.01$), but on day 18 PI both Groups E and F showed increased levels ($P<0.05$) (Figure 3B).

There was a significant decrease in FRAP levels on day 4 PI in Groups E and F ($p<0.01$) when compared to Group A (control) (Figure 3C). Also, there was a significant

increase in antioxidant defense (increased TAC) day 4 PI from the liver of animals from groups D, E and F (P<0.01) compared to A. Finally, at day 18 PI, only Groups E and F showed increased levels (P<0.01) (Figure 3D).

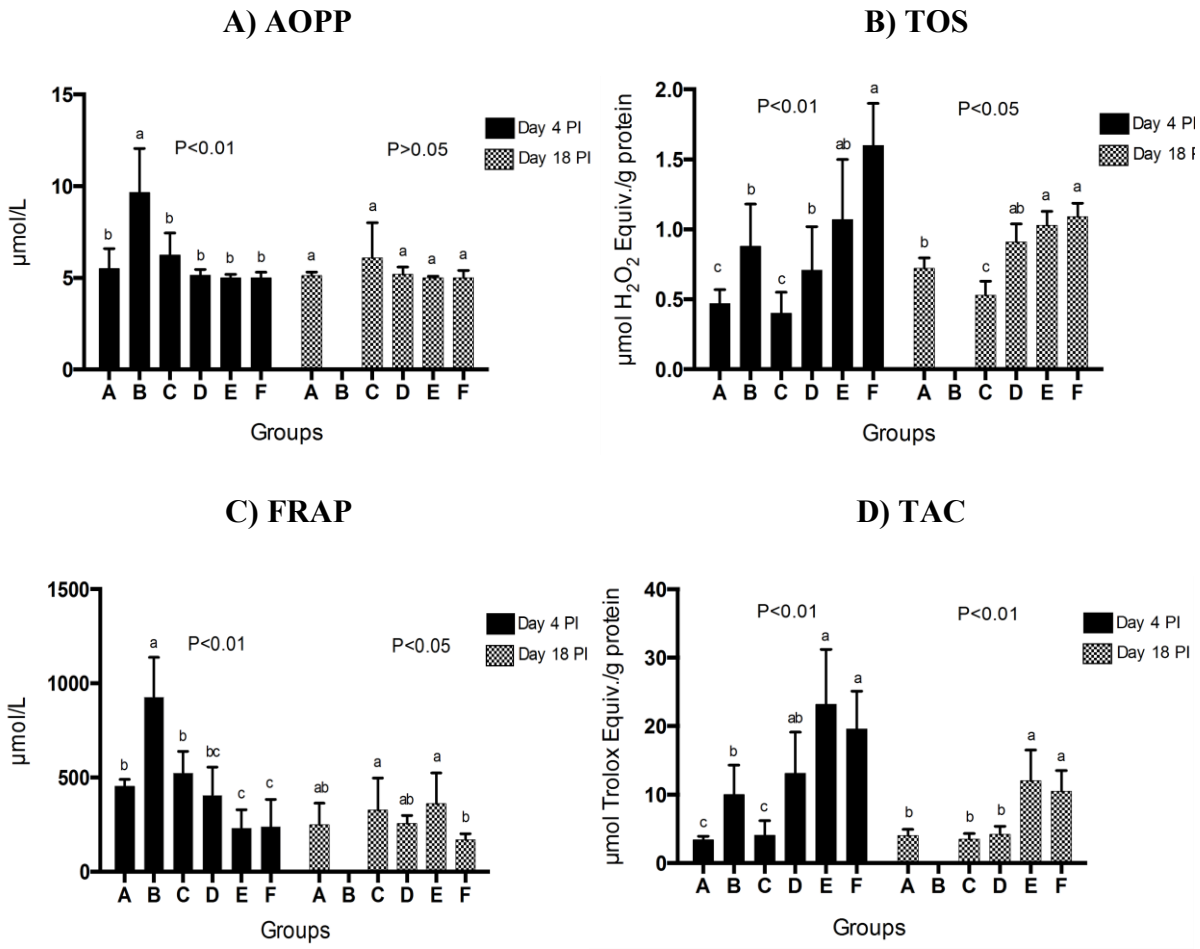

Fig 3 – Effect of Cordycepin (3'deoxyadenosine) and Pentostatin (Deoxycoformicin) treatment on the oxidative imbalance. AOPP - advanced oxidation protein produtcs, TOS - total oxidant status, FRAP - ferric reducing ability of plasma and TAC - total antioxidant capacity. Groups: A- Negative control B- Positive control C- 2mg/kg-1 cordycepin + 0.2mg/kg-1 pentostatin D- 2mg/kg-1 cordycepin + 0.5mg/kg-1 pentostatin E- 2mg/kg-1 cordycepin + 1mg/kg-1 pentostatin F- 2mg/kg-1 cordycepin + 2mg/kg-1 pentostatin.

3.4. Hepatic and renal function, and pathology is minimally perturbed by low doses of Cordycepin and Pentostatin treatment

Figure 4 depicts the results of alanine aminotransferase (ALT), aspartate aminotransferase (AST) and creatinine levels obtained from all mouse groups at day 4 and 18 PI. A significant increase in the levels of ALT was found in the sera of mice treated with the

combination of the two drugs (groups D, E and F when compared with group A - Figure 4A). A similar increase in the AST levels was observed at day 4 (groups D, E and F) and 18 PI (groups C, D, E and F) compared with the control group (Figure 4B). Serum creatinine levels in the treated mice did not show significant changes compared to the control group, but there were increases in Group B untreated mice (positive control). Therefore, to further determine whether these increases in biochemical metabolites resulted in histopathology, we analyzed histological sections of mice treated with Cordycepin and Pentostatin. Group A (uninfected) animals did not show histological alterations in liver (data not shown). Two animals from Group C (treated with the lowest dose) showed mild multifocal inflammatory infiltrates (Figure 5A), due to probably the *T. evansi* infection and not primarily related with the treatment. However, the liver from infected and treated mice (Groups D, E and F) indicated progressive histologic lesions reaching severe multifocal hepatocyte, hydropic degeneration and hypertrophy of hepatocytes (Figure 5B). Lastly, the kidneys from Groups A, C, D and E did not show histologic lesions (Figure 6A). Group B (positive control) and Group F (highest dose of drugs) showed a moderate diffuse hypercellularity of the glomerulus (Figure 6B). Therefore, these results suggest that renal and hepatocytic pathology do occur at higher concentrations of Cordycepin and Pentostatin treatment, but at lower doses this pathology is largely absent.

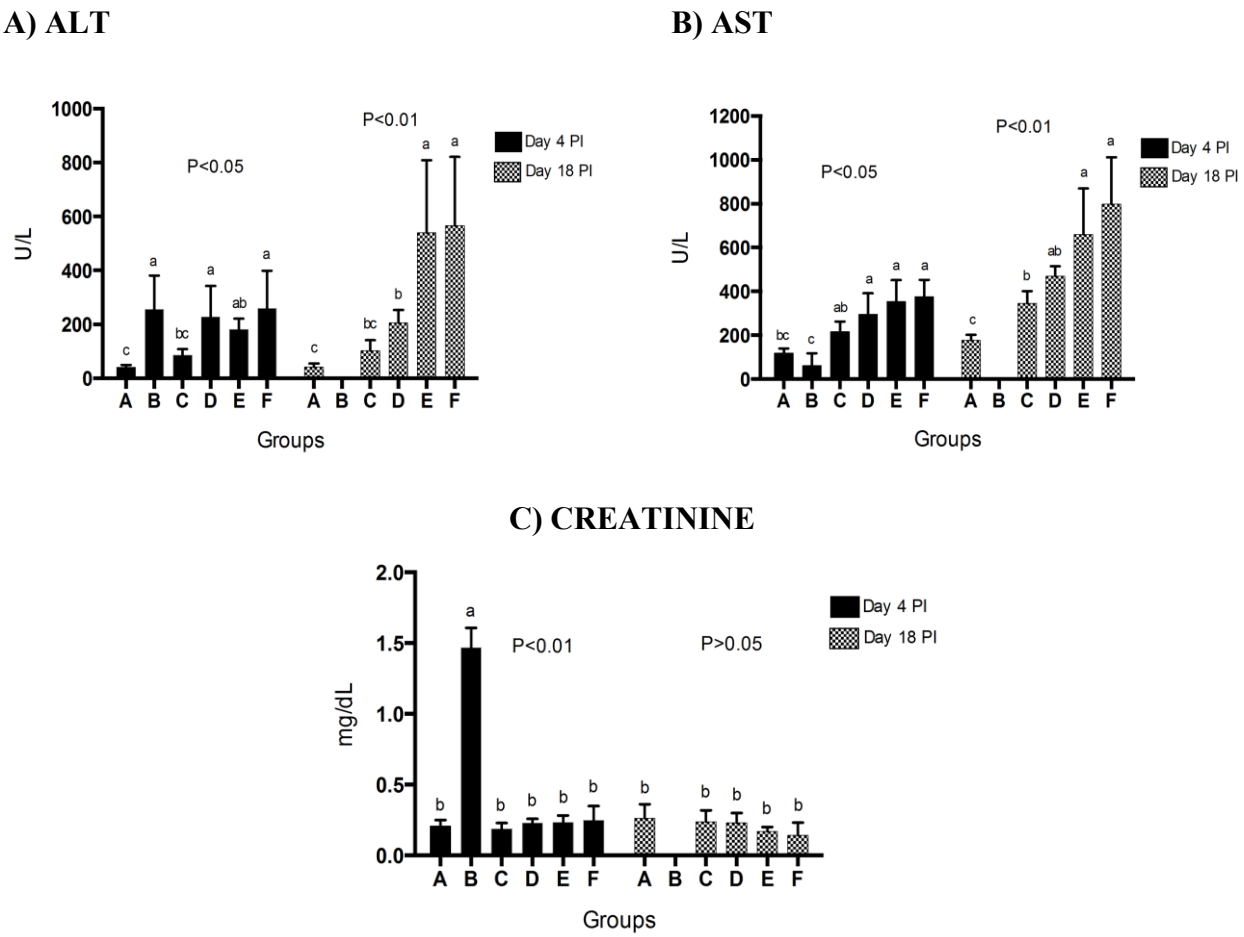

Fig 4 – Alanine aminotransferase (ALT), aspartate aminotransferase (AST) and creatinine in mice infected with *Trypanosoma evansi* and treated with different doses of Cordycepin and Pentostatin combination. Groups: A- Negative control B- Positive control C- 2mg/kg-1 cordycepin + 0.2mg/kg-1 pentostatin D- 2mg/kg-1 cordycepin + 0.5mg/kg-1 pentostatin E- 2mg/kg-1 cordycepin + 1mg/kg-1 pentostatin F- 2mg/kg-1 cordycepin + 2mg/kg-1 pentostatin

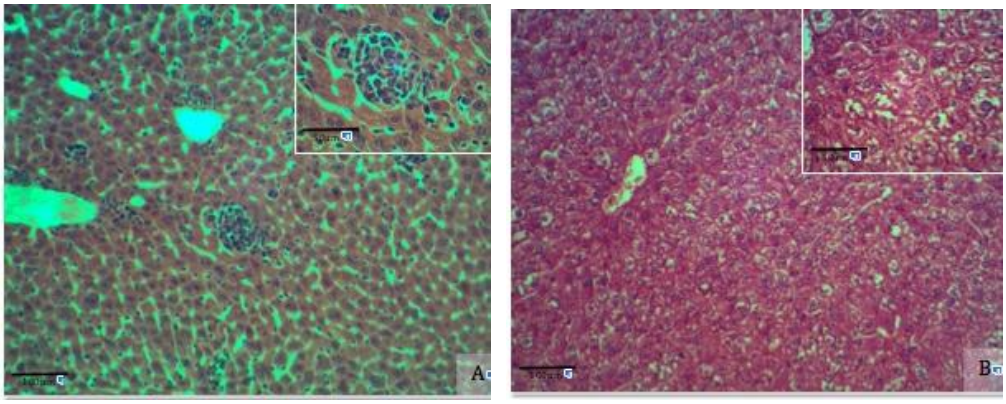

Fig 5 – (A) Histological section of liver of mice treated with the lowest dose (2 mg kg^{-1} Cordycepin associated with 0.2 mg kg^{-1} Pentostatin - Group C) showing mild multifocal lymphocytic inflammatory infiltrate (square inside). (B) Histological section of liver of mice treated with the highest dose (2 mg kg^{-1} Cordycepin associated with 2 mg kg^{-1} Pentostatin - Group F) exhibiting moderate diffuse hydropic degeneration in the hepatocytes (square inside). Haematoxylin-eosin stain.

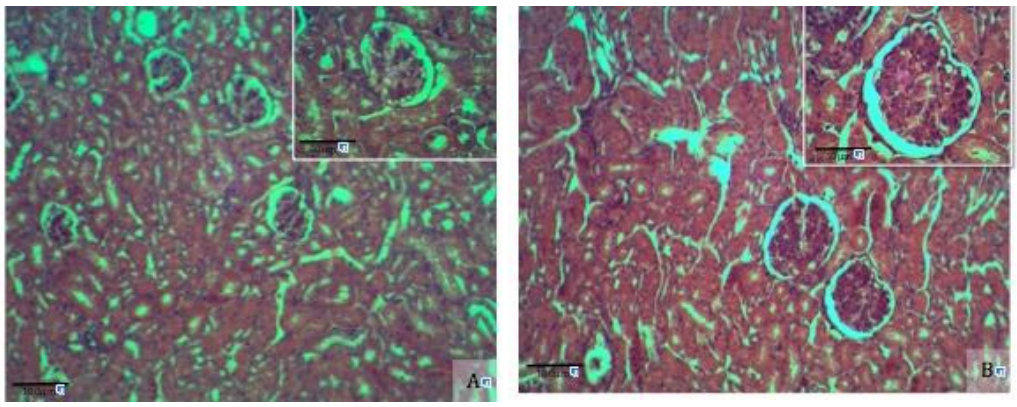

Fig 6 – (A) Photomicrographs showed the renal tissue of mice treated with the lowest dose (2 mg kg^{-1} Cordycepin associated with 0.2 mg kg^{-1} Pentostatin - Group C) without histological alterations. (B) Histological section of liver of mice treated with the highest dose (2 mg kg^{-1} Cordycepin associated with 2 mg kg^{-1} Pentostatin - Group F) demonstrating moderate diffuse hypercellularity of the glomerulus (square inside). Haematoxylin-eosin stain.

4. DISCUSSION

We describe a preclinical evaluation of the chemotherapeutic efficacy of Cordycepin and Pentostatin for early infection with *T. evansi*. Briefly, these drugs were selected based on previous studies from our research group. We show that the magnitude of trypanosome growth in infected mice was restricted by the supply of purine analogues as well as an inhibitor of purine synthesis. The effect of these drugs on infected mice was clearly evident in

all groups treated. In particular, mice infected with the highest doses of drugs (Group F) only showed detectable parasitemia 24 hours PI.

Kinetically, previous research has shown that these drugs are rapidly distributed in the tissues of mice with a concomitant decrease in parasitemia [35, 36]. Administration of coformycin and deoxycoformycin eliminated parasites in the brain parenchyma when administered after *T. brucei brucei* penetration into the brain [22]. Our results suggest that the purine nucleoside analogues can penetrate the blood-brain barrier blocking parasitemia recurrence. In the current study, we found that the lowest dose of Pentostatin (0.2 mg kg^{-1}), when associated with Cordycepin (2.0 mg kg^{-1}) was 100% effective and non-toxic to mice, thus achieving our main objective: to define an optimal therapeutic dose.

Our study also showed that low concentrations of Cordycepin and Pentostatin drugs are protective in splenocytes. In hepatocytes, the MTT assay showed that all *T. evansi* infected mice treated with Cordycepin and Pentostatin had decreased cell survival levels when compared with our control group, however, this reduction was very small. Indeed, many compounds have the ability to alter cellular signaling pathways and the cell cycle, thereby promoting resistance to apoptosis, inhibiting DNA repair, altering methylation processes and increasing oxidative stress disorders [37]. Particularly, within beagle dogs Cordycepin and Pentostatin toxicity has already ben observed, most likely due to the formation of higher levels of active Cordycepin metabolites [38]. Similar to the MTT assay, the Picogreen® assay confirmed the toxic effects of high doses of Cordycepin and Pentostatin. This class of agents affects the structural integrity of DNA, generally after incorporation during replication or DNA excision repair synthesis, leading to stalled replication forks and chain termination [39].

Our biochemical tests demonstrated an increase in liver enzymes, particularly at day 18 PI, that were associated with the lesions observed in the histological sections. Moreover, Group F treated mice (highest dose of drugs) caused low intensity histological renal lesions. However, this lead to no impairment of renal function given that creatinine levels were normal. Therefore, these results confirm data found in our previous work [14]. Moreover, other studies have found that Cordycepin and Pentostatin levels were higher in liver, kidney and spleen than in lung, heart, pancreas, thymus and brain [35].

Oxidative stress arises from an imbalance between oxidant and antioxidant compounds that generate specific metabolites, which can be identified and quantified. This process leads to the oxidation of biomolecules with the consequent loss of biological function. Detection can be analyzed by an increase of TOS levels in liver [40]. Therefore, we examined four markers of oxidative stress in the liver and found that the highest dose groups (E and F)

showed a decrease in FRAP (reducing "antioxidant power"), which most likely lead to a protective effect in the body by increasing antioxidant levels.

Alternatively, there are results indicating that Cordycepin potentially affects the regulation of the immune system and may be the reason (or part of the reason) for the elimination of parasites within experimentally infected *T. evansi* mice [41]. Future studies would be needed to assess whether this is true. In any case, Group E and F mice displayed histological lesions, altered liver enzymes levels and variation within oxidative parameters, leading us to conclude that these doses should not be used therapeutically. However, we recommend that a dose of 2.0 mg kg^{-1} Cordycepin and 0.5 mg kg^{-1} Pentostatin (Group D mice) is effective for the treatment of *T. evansi* infection but with some caution to avoid potential intoxication. In conclusion, our main objective of this study was achieved, and therefore we can conclude that the lowest combination of Cordycepin (2.0 mg kg^{-1}) and Pentostatin (0.2 mg kg^{-1}) can be used in the treatment of mice experimentally infected with *T. evansi*. When tested, this dose was effective in the elimination of parasites and was nontoxic to animals however we do not know whether even smaller doses of Pentostatin could be effective, and therefore future studies could be performed.

Conflict of interest

The authors have declared that no conflict of interests exists between the funders.

Acknowledgements

We are grateful to reviewers for their comments and suggestions. This work was supported by Brazilian Government - Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (Capes).

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6 CONSIDERAÇÕES FINAIS

Os resultados desta tese, apresentados na forma de três artigos publicados ou submetidos em revistas internacionais e representativas na área de Parasitologia Veterinária, permitem concluir que:

A cordicepina e pentostatina quando utilizadas isoladamente nas doses testadas não mostraram efeito curativo em camundongos infectados com *T. evansi*. Porém, quando combinadas apresentaram-se 100% de eficácia.

Grupos tratados com as maiores doses de pentostatina apresentaram aumento nos níveis de alguns parâmetros bioquímicos, especialmente sobre as enzimas hepáticas, as quais foram acompanhadas por lesões histológicas, além de demonstrarem toxicidade celular aumentada.

A atividade da enzima adenosine deaminase sofre uma forte inibição no plasma e no cérebro de animais tratados com pentostatina isolada e/ou associada a cordicepina, indicando possivelmente alterações na resposta imune, o que pode ser também responsável pelos efeitos colaterais.

E finalmente, após avaliar o potencial terapêutico e a toxicidade de novas doses da combinação cordicepina e pentostatina, recomendamos 2,0 mg kg^{-1} de cordicepina e 0,2 mg kg^{-1} de pentostatina para o tratamento de roedores infectados experimentalmente por *Trypanosoma evansi*. Essa dose obteve 100% de cura, sem apresentar toxicidade aos animais.

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