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ATIVIDADE DA ADENOSINA DESAMINASE, CONCENTRAÇÃO DE NUCLEOTIDEOS E NUCLEOSIDEO DE ADENINA EM RATOS INFECTADOS COM *Trypanosoma evansi*

TESE DE DOUTORADO

Aleksandro Schafer da Silva

Santa Maria, RS, Brasil

2011

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Aleksandro Schafer da Silva

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: Sonia Terezinha dos Anjos Lopes

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Elaborada por
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como requisito parcial para obtenção do grau de
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RESUMO

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

ATIVIDADE DA ADENOSINA DESAMINASE, CONCENTRAÇÃO DE NUCLEOTIDEOS E NUCLEOSIDEO DE ADENINA EM RATOS INFECTADOS COM *Trypanosoma evansi*

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Santa Maria, 09 de dezembro de 2011

O sistema purinérgico é conhecido por ser uma via de sinalização importante em diversos tecidos. Entre os componentes desse sistema destacamos a adenosina, um modulador do sistema nervoso central, circulatório e imunológico. A concentração de adenosina no hospedeiro é controlada pela enzima adenosina deaminase (ADA), presentes em tecidos, células e fluidos. Em virtude disso, os objetivos deste estudo foram (1) determinar a atividade da ADA no *Trypanosoma evansi*; (2) avaliar a atividade da ADA no soro, eritrócitos, linfócitos e encéfalo e (3) determinar a concentração de nucleotídeos e nucleosideos no soro e córtex cerebral de ratos infectados com *T. evansi*. Para um primeiro estudo foram infectados dois camundongos com *T. evansi*. Quando estes animais apresentavam elevada parasitemia ($\pm 10^8$ parasito/µL) foi realizada a coleta de sangue e separação dos flagelados por coluna de DEAE-celulose, a fim realização dos ensaios enzimáticos no parasito. Atividade da ADA nas formas trypomastigotas de *T. evansi* foi determinada por espectofotometria. Em um segundo estudo foi utilizado 39 ratos, divididos em três grupos: grupo A e B (infectado) e grupo C (C1 e C2/controle). Amostras de sangue e encéfalo foram colhidas nos dias 4 pós-infecção (PI) (grupos A e C1) e 20 PI (grupos B e C2). A partir do sangue total colhido com anticoagulante foram separados os linfócitos e eritrócitos para mensuração da atividade da ADA, já o soro foi obtido de amostras de sangue armazenadas em tubos sem anticoagulante. O encéfalo foi separado em cerebelo, córtex cerebral, hipocampo e estriado para avaliar a atividade da ADA em cada estrutura. Então, observou-se redução da atividade de ADA no soro e eritrócitos em ratos infectados com *T. evansi* em comparação com não-infectados ($P < 0,05$). A atividade de ADA em linfócitos estava diminuída no dia 4 PI e aumentou no dia 20 PI. Não houve diferença da ADA no cerebelo. No córtex cerebral, no hipocampo e estriado ocorreu redução da atividade da ADA nos dia 4 e 20 PI, respectivamente. Em todas as estruturas do encéfalo foi detectada a presença do parasito por PCR. Em um terceiro estudo foram utilizados 24 ratos, sendo 12 controles negativos e outros 12 infectados com *T. evansi*. Nos dias 4 (n=6 por grupo) e 20 (n=6 por grupo) foram realizadas as coletas de sangue para obtenção do soro e amostras do córtex cerebral para mensuração dos níveis de ATP, ADP, AMP e adenosina. Neste estudo, foi constatado aumento das concentrações de ATP, AMP e adenosina no encéfalo e soro de ratos infectados com *T. evansi* nos dois períodos avaliados, com exceção dos níveis de adenosina que reduziram no dia 4 PI. Não houve alteração na concentração de ADP. Portanto, na infecção por *T. evansi* os componentes do sistema purinérgico pode ser alterados, podendo estar envolvido na resposta imunológica, na anemia e nos sinais neurológicos.

Palavras-chave: *Trypanosoma evansi*, ratos, adenosina, adenosina deaminase.

ABSTRACT

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

ACTIVITY OF ADENOSINE DEAMINASE, CONCENTRATION OF ADENINE NUCLEOTIDES AND NUCLEOSIDE IN RATS INFECTED WITH *Trypanosoma evansi*

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Santa Maria, 09 December 2011

The purinergic system is known to be an important signaling pathway in different tissues. Among the components of this system have adenosine, a modulator of central nervous, circulatory and immune systems. The concentration of adenosine in the host is controlled by the enzyme adenosine deaminase (ADA), present in tissues, cells and fluids. As a result, the objectives of this study were (1) to determine the ADA activity in *Trypanosoma evansi*, (2) evaluate the activity of ADA in serum, erythrocytes, lymphocytes and brain of infected rats, and (3) determine the concentration of nucleotides and nucleosides in serum and cerebral cortex of rats infected with *T. evansi*. In the first study two mice were infected with *T. evansi*. When these animals showed high parasitemia ($\pm 10^8$ parasites/uL) was performed with blood collection and separation of trypomastigotes by DEAE-cellulose column for performing the assays. Spectrometry was performed by the biochemical detection of ADA in the form trypomastigotes of *T. evansi*. In a second study, we used 39 rats divided into three groups: group A and B (infected) and group C (C1 and C2 – control group) Samples of blood and brain samples were collected on day 4 PI (A and C1) and 20 PI (B and C2). From the blood (with anticoagulant) were separated lymphocytes and erythrocytes for measurement of ADA activity, since the serum was obtained from blood samples stored in tubes without anticoagulant. The brain was separated into cerebellum, cerebral cortex, hippocampus and striatum to evaluate the ADA activity in each structure. Decrease of ADA activity in serum and erythrocytes in rats infected with *T. evansi* when compared not-infected ($P<0.05$). ADA activity in lymphocytes was decreased at day 4 PI and increased in day 20 PI. There was no difference in ADA activity in the cerebellum. In the cerebral cortex caused a reduction of ADA activity on days 4 and 20 PI. Decrease of ADA activity in hippocampus and striatum in the day 4 and day 20 PI, respectively. In a third study, 24 rats were used, 12 used as a negative control and 12 infected with *T. evansi*. On day 4 ($n = 6$ per group) and 20 PI ($n = 6$ per group) were performed to obtain blood samples of serum and cerebral cortex for analysis. The samples were prepared for quantification of ATP, ADP, AMP and adenosine. This study found increased concentrations of ATP, AMP and adenosine in the brain and serum of rats infected with *T. evansi* in both periods, except that the levels of adenosine decreased on day 4 PI. The ADP concentration did not change in this study. Therefore, the infection by *T. evansi* purinergic system components can be changed, may be involved in immune response, in anemia and neurological signs.

Keywords: *Trypanosoma evansi*, rats, adenosine, adenosine deaminase.

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1 INTRODUÇÃO

O *Trypanosoma evansi* é um protozoário digenético da seção salivaria, agente etiológico da doença conhecida como “Mal das Cadeiras” ou “Surra” em equinos (SILVA et al., 2002; HERRERA et al., 2004). Apresenta ampla distribuição geográfica, podendo ocorrer na África, Ásia, América Central e do Sul. Comumente é observado parasitando diversas espécies de animais domésticos e silvestres (SILVA et al., 2002). Os humanos eram considerados refratários à infecção por *T. evansi* (KUBIAK; MOLFI, 1954), entretanto Joshi et al. (2005) relataram o primeiro caso de infecção pelo parasito em um fazendeiro na Índia e posteriormente uma investigação sorológica e parasitológica identificou 410 pessoas positivas para *T. evansi* em populações de vilarejos na Índia (SHEGOKAR et al., 2006).

Os tripomastigotas presentes nos vasos sanguíneos de vertebrados são adquiridos por insetos durante a ingestão de sangue contaminado, sendo a transmissão atribuída principalmente aos tabanídeos (*Tabanus* sp., *Chrysops* sp. e *Hematopota* sp.). Há também a possibilidade de transmissão por morcegos hematófagos (HOARE, 1972). A doença causada por este protozoário é caracterizada por rápida perda de peso, graus variáveis de anemia, febre intermitente, edema dos membros pélvicos e das partes baixas do corpo e fraqueza progressiva (HERRERA et al., 2004; RODRIGUES et al., 2005).

Algumas pesquisas têm mostrado que ratos são altamente suscetíveis à tripanosomose, mostrando alterações bioquímicas, hematológicas e patológicas associadas a sinais clínicos como ataxia, tremores e coma terminal em animais não tratados (MENEZES et al., 2004; WOLKMER et al., 2009). Em um estudo recente, nosso grupo de pesquisa concluiu que ratos são um ótimo modelo experimental para estudar *T. evansi*, pois foi observado que os ratos infectados agudamente e cronicamente podem manifestar sinais neurológicos e problemas locomotores como paralisia de membros pélvicos com lesões histológicas (Da Silva et al. *in press*) semelhantes aos equinos, principais animais afetados naturalmente. A patogenia das alterações clínicas não está completamente esclarecida e como o sistema purinérgico é responsavel por várias funções vitais dos mamíferos consideramos oportuno inverstigar esse sistema na infecção por *T. evansi* em ratos.

O sistema purinérgico é conhecido por ser uma via de sinalização importante em diversos tecidos, desencadeando múltiplos efeitos celulares relacionados à neuromodulação, as resposta imune e inflamatória, dor, agregação plaquetária, vasodilatação mediada pelo

endotélio, proliferação e morte celular. Fazem parte desse sistema os nucleotídeos (ATP, ADP e AMP) e nucleosídeo (adenosina) extracelulares, receptores para os nucleotídeos (P2X e P2Y) e nucleosideos (A_1, A_{2a}, A_{2b}, A_3) extracelulares e ectoenzimas (NTPDase, 5'-nucleotidase e adenosina deaminase), responsáveis pela regulação dos níveis dessas moléculas (FRANCO et al., 1997; YEGUTKIN, 2008).

A adenosina age como um modulador do sistema nervoso central (SNC) em mamíferos, regulando o metabolismo das células e desencadeando uma série de efeitos fisiológicos que participam na apoptose, na necrose e na proliferação celular. Em condições patológicas, a adenosina desempenha um papel protetor, modulando a liberação de neurotransmissores e também atuando como um regulador endógeno da imunidade inata, a defesa do hospedeiro de lesão tecidual excessiva associada à inflamação (FRANCO et al., 1997; YEGUTKIN, 2008). A concentração de adenosina extracelular é regulada pela atividade de um pequeno grupo de enzimas importantes, incluindo a adenosina desaminase (ADA, EC 3.5.4.4), que catalisa a conversão da adenosina em inosina. Altos níveis dessa enzima são encontrados no sistema linfóide e SNC, podendo também ser encontrada em menor quantidade nos eritrócitos. Conforme a literatura, a ADA desempenha um papel importante na função dos linfócitos e é essencial para o crescimento normal, a diferenciação e a proliferação de linfócitos T (FRANCO et al., 1997; YEGUTKIN, 2008).

A atividade da ADA pode ser um marcador sensível na infecção e ser utilizada para o acompanhamento do curso da doença. A atividade da ADA mostra-se elevada no soro de pacientes com tuberculose, theileriose, malária e leishmaniose visceral (OZCAN et al., 1997; MELO et al., 2000; KHAMBU et al., 2007; ALTUG et al., 2008), porém a atividade dessa importante enzima não foi investigada nas tripanossomoses, o que justifica este estudo. Portanto, os objetivos destes experimentos foram: (1) determinar bioquimicamente a atividade da enzima ADA no *T. evansi*; (2) investigar a atividade da ADA no soro, eritrócitos, linfócitos e encéfalo de ratos infectados experimentalmente com *T. evansi*; (3) mensurar a concentração de nucleotídeos e nucleosídeo da adenina no soro e córtex cerebral em ratos infectados experimentalmente com *T. evansi.*

2 CAPÍTULO I
REVISÃO DE LITERATURA

2.1 – *Trypanosoma evansi*

Os tripanossomas são micro-organismos pertencentes ao reino Protozoa, filo Euglenozoa, subfilo Sarcomastigophora, superclasse Mastigophora, classe Zoomastigophora, ordem Cinetoplastida, família Trypanosomatidae, gênero *Trypanosoma*. Os tripanossomas podem ser distribuídos em duas seções: Salivaria, aqueles transmitidos por picadas de vetores biológicos e Stercoraria, pela contaminação da pele ou das mucosas do hospedeiro (HOARE, 1972; SILVA et al., 2002). O gênero de *Trypanosoma* da seção salivaria são altamente patogênicos para pessoas e animais domésticos e estão distribuídos em quatro subgêneros: *Trypanozoon* (*T. brucei*, *T. evansi*, *T. equiperdum*), *Nannomonas* (*T. congolense*, *T. simiae*), *Duttonella* (*T. vivax*) e *Pycnomonas* (*T. suis*) (CONNOR; VAN DEN BOSSCHE, 2004).

O *Trypanosoma evansi* (*T. evansi*) foi o primeiro tripanossoma patogênico descoberto em 1880 por Griffith Evans, que encontrou organismos móveis no sangue de cavalos e camelos doentes (MAUDLIN et al., 2004). É o agente etiológico da doença secularmente conhecida como “mal das cadeiras” ou “surra” em equinos com ocorrência na África, Índia, Malásia, Indonésia, China, Rússia, Filipinas, América Central e do Sul (LEVINE, 1973; SILVA et al., 2002). Este protozoário teve sua origem no continente africano e foi introduzido nas Américas pelos primeiros colonizadores europeus. Desde então, tem causado numerosos surtos em equinos, resultando em morte e elevados prejuízos aos pecuaristas (SILVA et al., 2002). Surtos ou casos isolados de tripanossomose têm sido relatados, há vários anos, em diversas regiões brasileiras (FRANKE et al., 1994; SILVA et al., 1995; HERRERA et al., 2004). Na região sul do país, onde até 2005 não havia registro de ocorrência desse flagelado, o número de casos tem aumentado gradativamente anos após ano (COLPO et al., 2005; CONRADO et al., 2005; RODRIGUES et al., 2005; FRANCISCATO et al., 2007; ZANETTE et al., 2008).

O *T. evansi* tem origem africana como mencionado anteriormente, e trabalhos indicam que ele surgiu a partir da perda do DNA mitocondrial, ou cinetoplasto, do *Trypanosoma brucei*, causador da “doença do sono” em humanos. O cinetoplasto (kDNA) é uma rede de

DNA circular com replicação independente adicional ao DNA nuclear. Ele é composto por maxicírculos e minicírculos, que complementarmente expressam o DNA mitocondrial e RNA ribossômico. Os maxicírculos expressam proteínas que geralmente são componentes de complexos respiratórios, mas para que essa expressão ocorra, são necessárias certas inserções ou deleções que são comandadas por RNAs guias (gRNAs) que são produtos da transcrição dos minicírculos (Liu et al., 2005). No entanto, este protozoário flagelado é geralmente monomórfico, tendo um pequeno cinetoplasto subterminal. Porém, existem formas acinetoplásticas em que o DNA cinetoplástico circular é ausente. Estes exemplares são encontradas em cepas silvestres como resultados de mutação ou após tratamento com tripanocidas (aceturato de diminazeno). Formas acinetoplásticas também são relatadas após longo tempo em cultura *in vitro* e criopreservação (ZWEYGARTH et al., 1990). As cepas brasileiras são comprovadamente acinetoplásticas (VENTURA et al., 2000). As formas encontradas na corrente sanguínea são basicamente lancetadas e o corpo é alongado e achatado. Um flagelo livre está sempre presente. Há uma membrana ondulante bem desenvolvida e a extremidade posterior pode ser arredondada ou afilada (Figura 1). Seu tamanho varia de 15 a 33 µm, com média de 24 µm (HOARE, 1972).

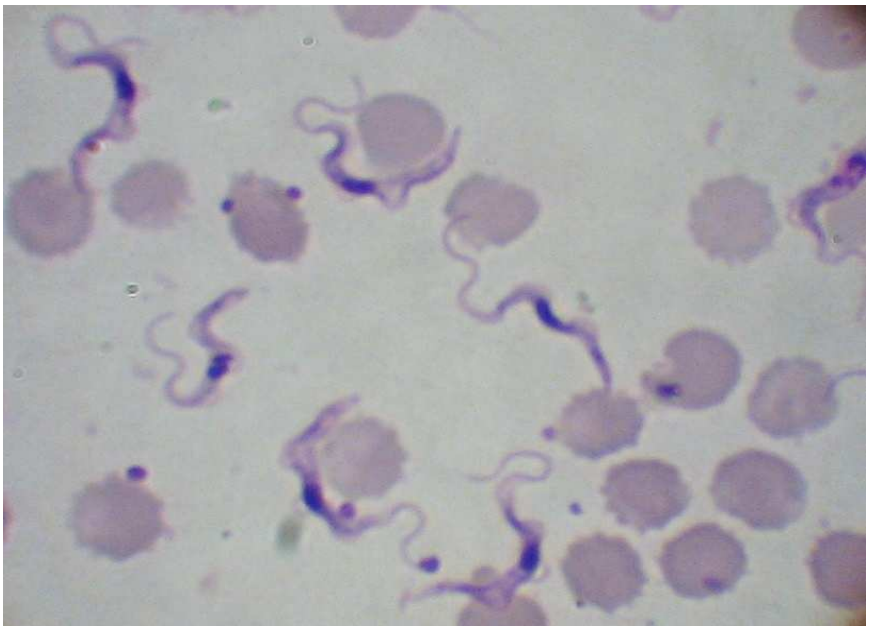

Figura 1 – Formas tripomastigotas de *T. evansi* em esfregaço sanguíneo de ratos infectados experimentalmente.

Nos últimos anos, uma grande variedade de pesquisas bioquímicas e moleculares têm sido empregadas no campo da tripanossomose, tais como a identificação molecular e análise filogenética dos tripanossomas (AMER et al., 2011) e a regulação da concentração de cálcio que é extremamente essencial para a vida destes parasitos (DOCAMPO; MORENO, 1996). Também recentemente foi realizada a detecção da atividade de enzimas como a acetilcolinesterase em compartimentos subcelulares (MIJARES et al., 2011) e a cisteína proteinases em *T. evansi* (YADAV et al., 2011), uma das proteinases liberadas por tripanossomas vivos e mortos que acarretam imunossupressão no hospedeiro infectado, contribuindo imensamente na patogênese da doença. O gene de uma selenoproteína exclusiva de tripanossomatídeos, a *selTRYP*, foi amplificado do cDNA e parcialmente seqüenciado de *T. evansi*, portanto os autores sugerem que este parasito é capazes de utilizar selênio para a formação de selenoproteínas, capaz de proteger o protozoário dos radicas livres produzidos pelo hospedeiro (TAVARES et al., 2011). Em *Trypanosoma brucei*, os autores demonstraram que a atividade de transporte de purinas é regulada pela captação de nucleosídeos e, em alguns casos, de hipoxantina (SANCHEZ et al., 2002). A existência de dois sistemas de transporte distintos de adenosina em *T. evansi* já foi bem documentada, os quais são necessários para manter as funções vitais do flagelado (SUSWAM et al., 2001; SUSWAM et al., 2003). Estas novas descobertas podem auxiliar à elucidar a patogênese do *T. evansi*, assim como os mecanismos utilizados pelo parasito para sobreviver no hospedeiro.

O *T. evansi* causa a tripanossomose em 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). Em 2005, foi relatado o primeiro caso de infecção humana em um fazendeiro na Índia (JOSHI et al., 2005; SHEGAKAR et al., 2006).

A infecção por *T. evansi* em humanos não é comum, pois os mesmos possuem em seu plasma sanguíneo uma apolipoproteína ligada a lipoproteínas de alta densidade que é considerada um fator tripanolítico, chamado apolipoproteína L-1 (APOL1). A APOL1 entra no protozoário por endocitose e promove a formação de poros na membrana lisossomal, induzindo o rompimento destes compartimentos e a morte celular (VANHAME et al., 2003). Um dos tripanossomatídeos Africanos que causa a “doença do sono” em humanos (*T. brucei rhodesiense*) expressa uma proteína que confere resistência a APOL1, conhecida como proteína associada à resistência ao soro (SRA) (XONG et al., 1998). O *T. evansi* é

normalmente susceptível ao plasma humano, como demonstrado por Hawking (1978) e também por Otto et al. (2010) para um isolado brasileiro. Uma análise molecular do gene da APOL1 do paciente indiano demonstrou uma rara mutação nos dois alelos, que levava à formação de dois *stop codons* no meio da fase aberta de leitura do gene, impedindo então a expressão da APOL1 funcional neste paciente, o que provavelmente foi determinante para o desenvolvimento da infecção (VANHOLLEBEKE et al., 2006).

Tripomastigota é a forma dos tripanossomas presentes nos vasos sanguíneos de vertebrados, que são disseminados por insetos hematófagos durante o repasto sanguíneo (SILVA et al., 2002). Como a transmissão é mecânica, não há o desenvolvimento do hematozoário em nenhum órgão do vetor, e quanto menor a diferença de tempo entre os repastos sanguíneos, maiores são as possibilidades de passagem do parasita para um novo hospedeiro (HOARE, 1972). Os principais vetores pertencem aos gêneros *Tabanus* sp. (mutucas), porém insetos dos gêneros *Stomoxys* sp, *Haematopota* sp. e *Lyperosia* sp. podem transmitir o parasita (SILVA et al., 2002). Na América Central e do Sul o morcego hematófago *Desmodus rotundus* é considerado um vetor importante, uma vez que os tripomastigotas multiplicam-se na corrente circulatória destes animais, os quais podem permanecer infectados por até um mês, atuando como vetor e também como hospedeiro do protozoário (HOARE, 1972). Ainda, existe a possibilidade de transmissão oral em carnívoros que se alimentam da carcaça de animais infectados (RAMIREZ et al., 1979). A via oral pode ser importante na dispersão de infecção de *T. evansi* em cachorros, quatis e capivaras, que podem ser infectados em consequência das brigas frequentes entre animais infectados e não infectados. Além disso, espécies gregárias como coatis e capivaras têm um comportamento agressivo facilitando a transmissão oral do protozoário entre eles, e mantendo a infecção no grupo social, já que a forma crônica da doença causada por *T. evansi* foi identificada em capivaras (*Hydrochaeris hydrochaeris*) e quatis (*Nasua nasua*), possíveis reservatórios do agente. Os cães e ruminantes também podem atuar como reservatórios do *T. evansi* quando o curso da doença for crônico (HERRERA et al., 2004).

A patogenicidade dos tripanossomas no hospedeiro varia de acordo com a cepa do *Trypanosoma* sp., a espécie do hospedeiro, fatores não específicos afetando o animal (outras doenças, estresse, etc.) e condições epizootiológicas locais (HOARE, 1972). Os *T. evansi* se reproduzem por fissão binária longitudinal quando estão no sangue de seu hospedeiro (BRUN et al., 1998). Esta multiplicação inicia-se no local da picada, na pele, invadindo a corrente sanguínea e o sistema linfático do hospedeiro, levando a picos de febre e induzindo a uma resposta inflamatória (CONNOR; VAN DEN BOSSCHE, 2004).

Os tripanossomatídeos africanos da seção salivaria, a qual pertence o *T. evansi*, possuem um interessante mecanismo para evadir as defesas do hospedeiro: as glicoproteínas variáveis de superfície, ou *variant surface glycoproteins* (VSGs). Toda a superfície do protozoário (aproximadamente 95%) é recoberta por VSGs, que possuem a propriedade de se alterar, "enganando" o sistema imune humoral do hospedeiro (PAYS et al., 2004). O genoma desses tripanossomatídeos possui centenas de genes que codificam para diferentes VSGs, e apenas um é expresso por vez. As VSGs são traduzidas com um domínio N- terminal que é variável e um domínio C-terminal que é altamente conservado e possui uma sequência para âncoras de GPI (glicofosfatidilinositol) que as sustentam na superfície do parasito. Quando os protozoários mudam sua cobertura de VSGs ocorrem os picos de parasitemia, observados na forma crônica da doença (CARRINGTON et al., 1991).

Em infecções naturais e experimentais, observou-se que a tripanossomose por *T. evansi* pode apresentar-se com 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. 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, ocorre o agravamento dos sinais clínicos e 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).

A principal alteração hematológica identificada em animais com tripanossomose é a anemia acentuada (CONNOR; VAN DEN BOSSCHE, 2004). A doença é marcada pela diminuição no valor de hematócrito, na concentração de hemoglobina e no número de eritrócitos totais. As alterações eritrocitárias podem incluir microesferócitos, acantócitos, dacriócitos, micrócitos, vacuolização eritrocitária, policromasia, poiquilocitose, adesão eritrocitária e eritrofagocitose (ANOSA; KANEKO, 1983; SILVA et al., 1995; CONRADO et al. 2005). Conforme a literatura, o principal mecanismo responsável pela anemia seria a liberação de hemolisinas e enzimas pelos tripanossomas, que induziram lesões diretamente na membrana dos eritrócitos, aumentando a fragilidade dos mesmos. A adesão do complexo antígeno-anticorpo às membranas eritrocitárias e dos componentes do complemento aos

eritrócitos também contribui para anemia, pois promove a eritrofagocitose (CONNOR; VAN DEN BOSSCHE, 2004). Shehu et al. (2006) relataram que a anemia ocorre em consequência da atividade da neuraminidase, a qual tornaria os glóbulos vermelhos mais propensos à fagocitose pelo sistema reticuloendotelial. Recentemente, a anemia também foi atribuída à peroxidação lipídica, pois o aumento de radicais livres acarreta danos à membrana eritrocitária (WOLKMER et al., 2009).

Os principais componentes da resposta imune à infecção por *T. evansi* em camundongos foram estudados por Baral et al. (2007) e Paim et al. (2011a). Segundo os autores, o fator de necrose tumoral (TNF), que é importante na infecção de outros tripanossomatídeos, não influencia na parasitemia ou tempo de sobrevivência dos animais. O interferon-gama (IFN- γ) também não influenciou a parasitemia e o tempo de sobrevivência, mas os animais sem o gene do IFNγ$^{-/-}$ apresentaram maior chance de desenvolver anemia. Durante a infecção, outras citocinas que são ativadas tais como a interleucina 1 e 6 (PAIM et al., 2011). Baral et al. (2007) concluíram que o óxido nítrico, produzido pelo hospedeiro mediante a ação de IFNγ tem efeito supressivo nas células T do hospedeiro, mas esse efeito não influencia na parasitemia e tempo de sobrevivência dos camundongos. Estes autores também observaram o papel da IgM no controle da infecção por *T. evansi*. Os animais foram capazes de controlar a infecção em seu início, onde haviam altos níveis de IgM e baixos níveis de IgG. A queda dos níveis de IgM e aumento de IgG coincidiu com a perda do controle da infecção. Os camundongos deficientes em IgM também não foram capazes de controlar o primeiro pico de parasitemia. Para confirmar esta teoria, camundongos deficientes em IgM foram tratados, antes da infecção, com IgM e IgG purificados de animais infectados, e apenas os que receberam IgM foram capazes de controlar a infecção, demonstrando assim o papel fundamental da IgM na resposta à tripanossomose por *T. evansi*.

O diagnóstico presuntivo desta doença em equinos pode ser feito a partir dos sinais clínicos, que são bastante característicos nesta espécie. Entretanto, o diagnóstico definitivo somente poderá ser estabelecido através de exames laboratoriais, como a identificação dos tripomastigotas em esfregaço de sangue corado, podendo-se também visualizar as formas móveis em uma gota de sangue fresco entre lâmina e lamínula ao microscópio de luz e inoculação em animais susceptíveis (KUBIAK; MOLFI, 1954). Segundo Tourantier (1993), a técnica do micro-hematócrito é a mais adequada para diagnóstico em termos de praticidade, custo e sensibilidade. A técnica de reação em cadeia da polimerase (PCR) é de grande sensibilidade (VENTURA et al., 2000).

O aceturato de diminazeno é o produto mais comumente usado no controle da

tripanossomose dos animais domésticos, pois apresenta maior índice terapêutico que as outras drogas na maioria das espécies domésticas. Tem atividade contra tripanossomas que são resistentes a outros medicamentos e apresenta baixa incidência de resistência (PEREGRINE; MAMMAM, 1993). Em um estudo recente, uma nova terapia com aceturato de diminazeno apresentou sucesso de 85,7% na cura de gatos infectados com *T. evansi* (DA SILVA et al., 2009). Outro produto de eficácia curativa para *T. evansi* é o suramim, fármaco este utilizado no humano infectado com o parasito (JOSHI et al., 2006). No entanto, este fármaco tem uma limitação para animais devido ao elevado custo do tratamento. Em virtude disso, terapias alternativas com plasma humano (OTTO et al., 2010) devem ser testadas para serem utilizadas em casos de resistência do protozoário aos quimioterápicos.

Estudos recentes mostraram que um produto análogo da purina, 3-desoxiadenosina (*cordycepin*), foi eficaz na cura da infecção por *T. brucei* em camundongos, tanto na fase aguda e crônica (com envolvimento do sistema nervoso central) da doença (ROTTENBERG et al., 2005; VODNALA et al., 2008). Segundo esses autores, a eficácia do tratamento está relacionado com a proteção do *cordycepin* contra a enzima adenosina desaminase (ADA), que é responsável pela desaminação do análogo da adenosina. Portanto, o protocolo de tratamento exige a combinação de cordycepin com um inibidor da ADA, como *deoxycoformycin*. *Cordycepin*, quando protegido contra desaminação, também possui atividade biológica contra tripanossomas (ROTTENBERG et al., 2005; VODNALA et al., 2008). Os nucleosídeos do parasito são alvos de uma via metabólica que torna os tripanosomas vulneráveis, de uma forma que outras drogas disponíveis não fazem (ROTTENBERG et al., 2005). O metabolismo das purinas em tripanossomas e outros parasitas representa uma vulnerabilidade específica, pois tripanossomas, como outros protozoários, não podem participar na síntese de novas purinas quando o *cordycepin* liga-se aos recepetores específicos das purinas e portanto esta incapacidade de tripanossomas em sintetizar novas purinas tem sido explorado como um alvo terapêutico na tripanossomose (ROTTENBERG et al., 2005; VODNALA et al., 2008). Foi constatada susceptibilidade de *T. evansi* ao *cordycepin in vitro* (100%) e uma eficácia curativa de 42,5% em ratos infectados, quando administrado combinado ao *cordycepin* (2 mg/kg) com EHNA hydrochloride (2 mg/kg), pela via intraperitonial (DA SILVA et al., 2011b).

Pesquisas tem mostrado que ratos são altamente suscetíveis à tripanossomose, mostrando alterações bioquímicas, hematológicas e patológicas associadas à sinais clínicos como ataxia, tremores e coma terminal em animais não tratados (MENEZES et al., 2004; WOLKMER et al., 2009). Em estudo recente, concluiu-se que os ratos são um ótimo modelo

experimental para estudar *T. evansi*, pois foi observado que ratos infectados agudamente e cronicamente podem manifestar sinais neurológicos e problemas locomotores, como paralisia de membros pélvicos com lesões histológicas (DA SILVA et al., in press) semelhantes aos equinos, principais animais afetados naturalmente. Nestes mesmos animais, foi constatado, uma redução de atividade da enzima Ca^{2+} ATPase associada à peroxidação lipídica em músculos do membro pélvico de ratos infectados com *T. evansi*, fato este que dificulta a saída de cálcio das células e consequentemente leva à lesão celular (TONIN et al., 2011). O estresse oxidativo também já foi relatando em roedores parasitados por *T. evansi* (OMER et al., 2007; WOLKMER et al., 2009) e associado à patogenia da anemia nesta doença.

Os sinais neurológicos e a resposta inflamatória de ratos infectados com *T. evansi* foram correlacionados com as alterações no sistema colinérgico, mais especificamente às enzimas acetilcolinesterase e butirilcolinesterase, que são responsáveis pela regulação da aceticolina, um importante neurotransmissor e modulador imunológico (DA SILVA et al., 2011a; 2011b). Com base nestes resultados, os ratos Wistar foram considerados um bom modelo experimental para estudos de tripanossomose por *T. evansi*, e avaliação de sua influência sobre o sistema purinérgico.

2.2 - Sistema purinérgico

O sistema purinérgico é conhecido por ser uma via de sinalização importante em diversos tecidos, desencadeando múltiplos efeitos celulares. É considerado um sistema primitivo, envolvido em muitos mecanismos neurais e não-neurais e em eventos de curta e longa duração, incluindo a resposta imune e a inflamatória, a dor, a agregação plaquetária, a vasodilatação mediada pelo endotélio, a proliferação e a morte celular (BURNSTOCK, 2004).

Três componentes principais fazem parte do sistema purinérgico: nucleotídeos e nucleosídeos extracelulares, seus receptores (Figura 2) e ectoenzimas (Figura 3) responsáveis pela regulação de níveis destas moléculas (YEGUTKIN, 2008). Os nucleosídeos (inosina e adenosina) são moléculas resultantes da união de uma base púrica ou pirimídica com uma pentose (ATKINSON et al., 2006). Os nucleotídeos de adenina como ATP, ADP e AMP são considerados importantes moléculas sinalizadoras em tecidos (YEGUTKIN, 2008).

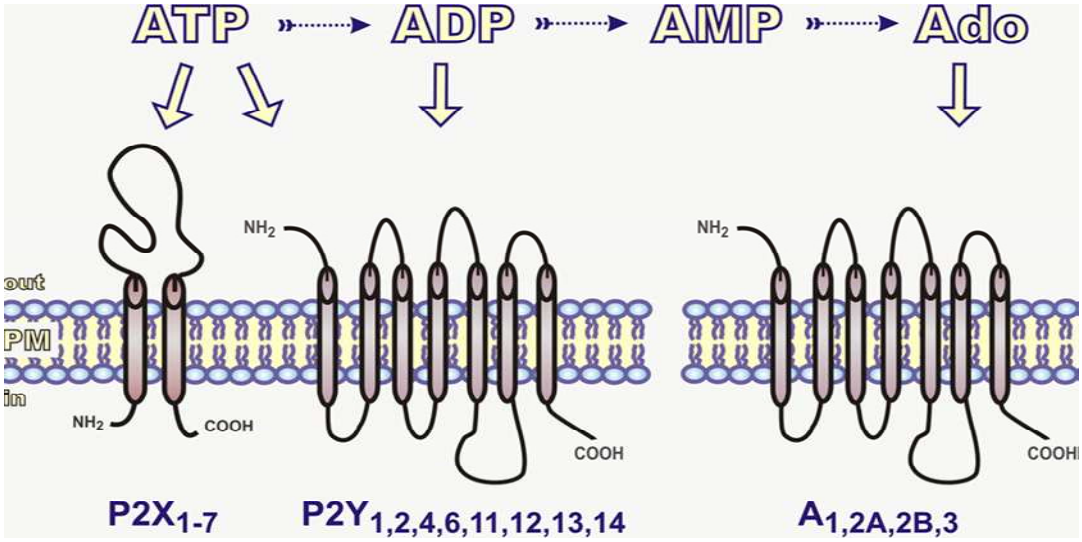

Figura 2 – Tipos de receptores para nucleotídeos e nucleosídeo de adenina (Fonte: Yegutkin 2008).

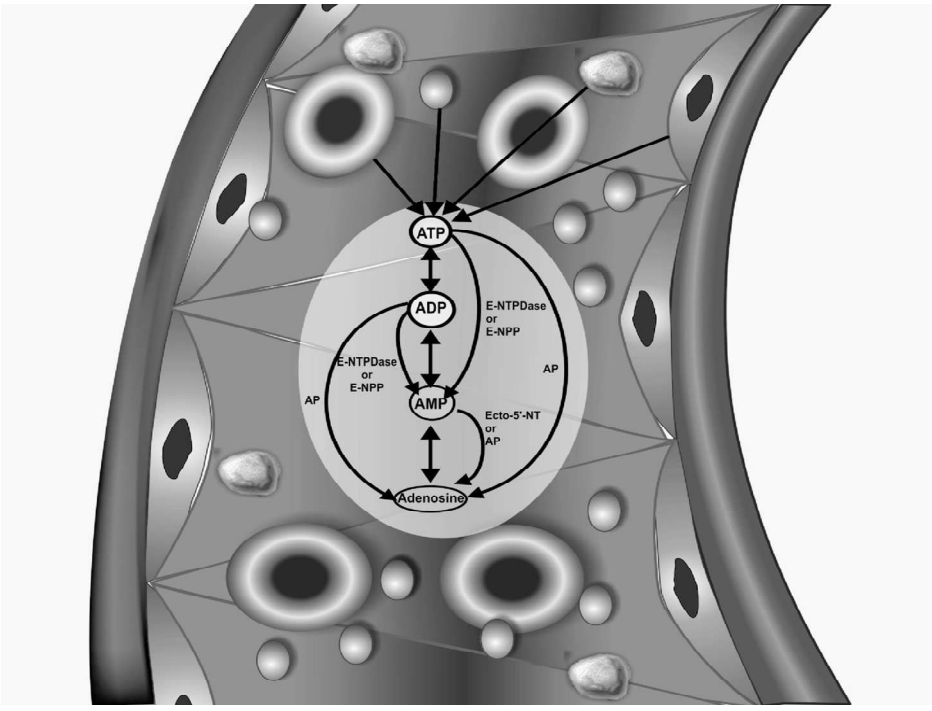

Figura 3 – Enzimas envolvidas na degradação extracelular de nucleotídeos e nucleosídeo de adenina (Fonte: Schetinger et al., 2007).

Estudos têm demonstrado que os nucleotídeos e nucleosídeos da adenina regulam processos relacionados à tromborregulação, modulam a resposta imune e sinalizam vias

crucias para o desenvolvimento e funcionamento do sistema nervoso (BURNSTOCK, 2002). No sistema vascular estas moléculas participam nas funções cardíacas em respostas vasomotoras e atividade plaquetária, sendo o ADP o principal agonista envolvido no recrutamento e agregação das plaquetas (ATKINSON et al., 2006). Já o ATP, em altas concentração, e a adenosina pode atuar inibindo a agregação plaquetária e modulando o tônus vascular (SOSLAU; YOUNGPRAPAKORN, 1997; ANFOSSI et al., 2002). ATP e a adenosina também participam na ativação ou inibição do sistema imunológico (Figure 4). Dependendo da concentração, o ATP tem funções pró-inflamatórias, pois é responsável pela estimulação e a proliferação de linfócitos, células envolvidas na liberação de citocinas (BOURS et al., 2006). Enquanto isso, a adenosina apresenta-se como uma molécula antiinflamatória (GESSI et al., 2007).

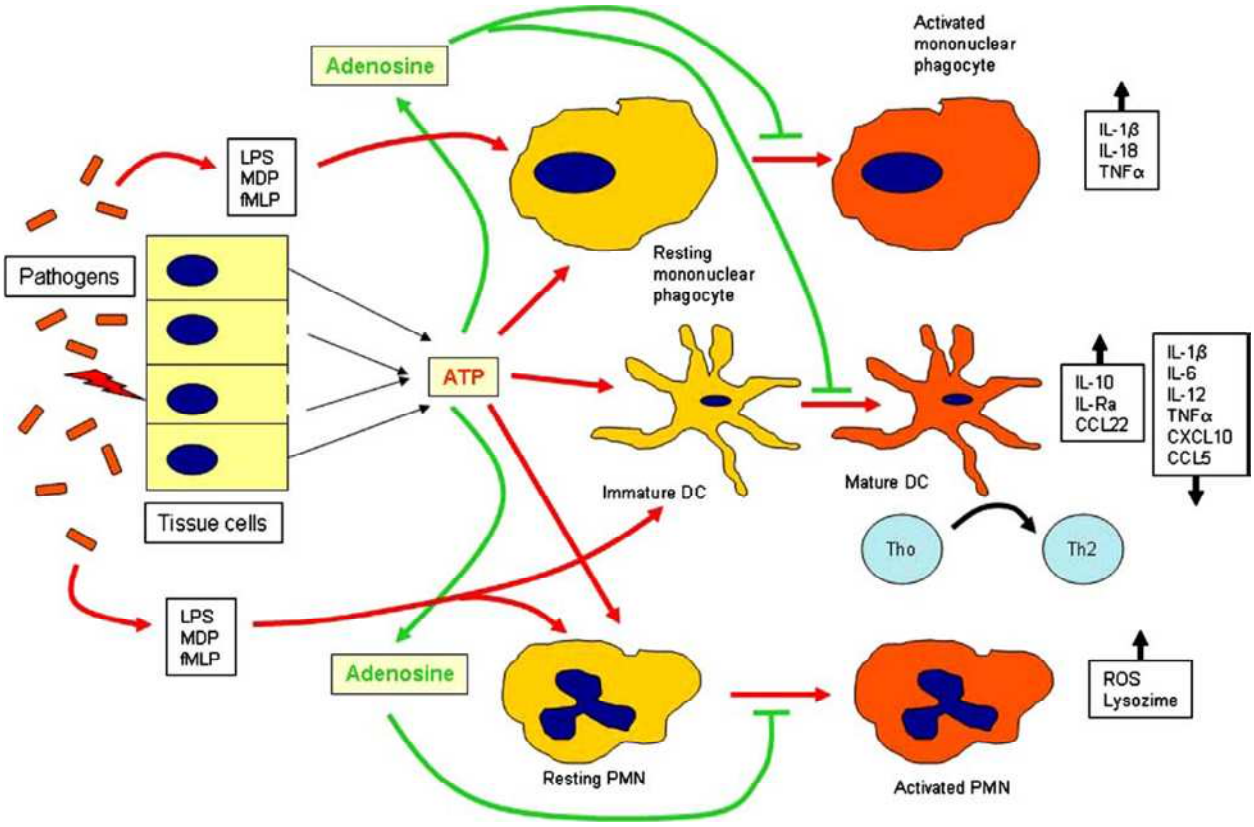

Figure 4 – Relação entre o sistema imunológico e purinérgico durante a resposta inflamatória frente a um patógeno (Fonte: Bours et al., 2006)

Todas as funções dos nucleotídeos e nucleosídeo de adenina são mediadas por receptores purinérgicos presentes na superfície de diferentes tipos de células (YEGUTKIN,

2008). Para nucleotídeos existem dois grupos de receptores (P2X e P2Y), sendo o P2X um receptor acoplado a canais iônicos e P2Y acoplado à proteína G (Figura 2). Os receptores para adenosina incluem quatro tipos (A_1, A_{2a}, A_{2b}, A_3), os quais são proteínas transmembrana acopladas à proteína G (YEGUTKIN, 2008).

O controle dos níveis extracelulares de nucleotídeos e nucleosídeo de adenina são realizados por enzimas ancoradas na membrana celular ou meio intersticial. Dentre estas enzimas destacamos as ecto-nucleosídeo trifosfato difosfohidrolase (E-NTPDase), ecto-nucleotídeo pirofosfatase (E-NPPs), 5'-nucleotidase e adenosina desaminase (ADA) (YEGUTKIN, 2008). Estas enzimas atuam em conjunto, formando uma cadeia enzimática que tem início com a ação da E-NTPDase e da E-NPP as quais hidrolisam o ATP e ADP, formando o AMP, que em seguida é hidrolisado pela 5'-nucleotidase formando adenosina. Finalmente, a adenosina é desaminada pela ADA em inosina (YEGUTKIN, 2008).

2.3 Adenosina e adenosina desaminase (ADA)

A adenosina, um importante componente do sistema purinérgico e age como um modulador do SNC (Figure 5). Em mamíferos, regula o metabolismo das células e desencadeia uma série de efeitos fisiológicos que participam na apoptose, necrose e proliferação celular. Em condições patológicas, a adenosina desempenha um papel protetor, modulando a liberação de neurotransmissores e atuando como um regulador endógeno da imunidade inata, a defesa do hospedeiro de lesão tecidual excessiva associada à inflamação (RATHBONE et al., 1999; HASKO; CRONSTEIN, 2004; SITKOVSKY; OHTA, 2005; BURNSTOCK, 2006; DESROSIERS et al., 2007).

A concentração de adenosina extracelular é regulada pela atividade de um pequeno grupo de enzimas importantes, incluindo a adenosina desaminase (ADA, EC 3.5.4.4 – Figure 6), que catalisa a conversão da adenosina em inosina, seu metabólito inativo. Altos níveis desta enzima são encontrados no sistema linfóide (linfonodos, baço e timo), podendo também ser encontrada, mas em menor quantidade, nos eritrócitos (CRISTALLI et al., 2001; SABOURY et al., 2003). A ADA foi detectada na superfície de muitos tipos celulares, incluindo sinaptossomas cerebrais. A expressão de atividade desta enzima é heterogênea em tecidos periféricos e no SNC. A atividade da ADA apresenta uma grande variação em áreas

cerebrais de acordo com as vias purinérgicas (GEIGER et al., 1986; FRANCO et al., 1986; 1997). Estudos têm demonstrado que a ADA desempenha um papel importante na função dos linfócitos e é essencial para a diferenciação e a proliferação de linfócitos T (FRANCO et al., 1997; CODERO et al., 2001). Na superfície das células hematopoiéticas, pode atuar na maturação de células vermelhas (ARAN et al., 1991). A deficiência de ADA pode contribuir para condições patológicas (ALDRICH et al., 2000).

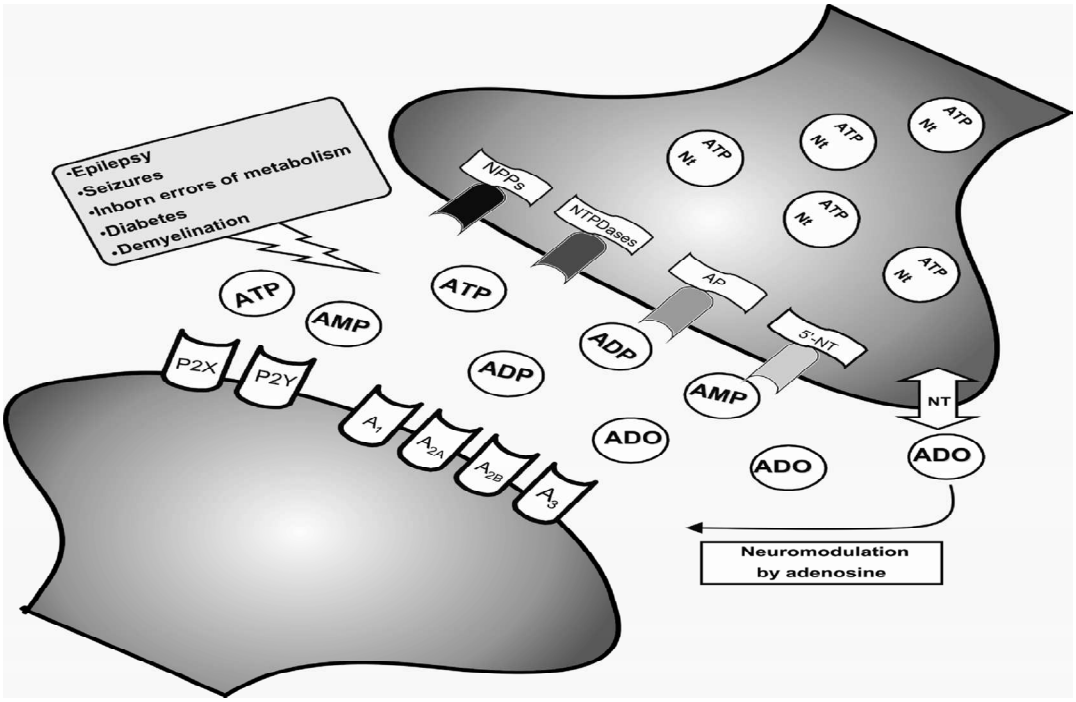

Figura 5 – Os nucleotídeos e nucleosído da adenina têm participação intensa no SNC, atuando como neurotransmissor (ATP) e neuromoduladores (ADA) em condiçoes fisiológicas e/ou patológicas. (Fonte: Schetinger et al., 2007).

Como mencionado anteriormente, a ADA é amplamente distribuída nos tecidos dos animais vertebrados e divide-se em duas isoformas ADA1 e ADA2. Os tecidos contêm predominantemente ADA1. Já a ADA2 é o principal componente do soro e é um suposto estimulador de células-T (FRANCO et al., 1997; BURNSTOCK, 2006).

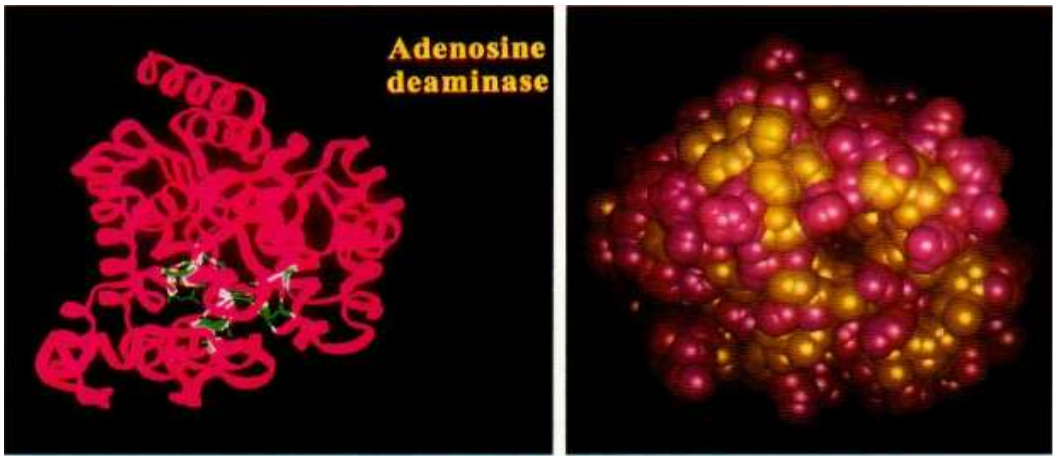

Figura 6 – Estrutura tridimensional da ADA. As imagens são formadas a partir de dados relatados por Wilson et. al. (1991). A imagem a direita apresenta o sítio ativo no centro da estrutura, e as cadeias laterais polares e não polares estão representadas em rosa e amarelo respectivamente (FRANCO et al., 1998).

A ADA1 é uma proteína monômera com uma massa molecular de aproximadamente 40 kDa. A localização da ADA_1 é principalmente citosólica, sendo encontrada em todo o organismo e também na superfície de macrófagos, linfócitos B e em alguns linfócitos T. Esta pode estar combinada com uma glicoproteína dimérica não específica (CD26) de aproximadamente 200 kDa, designada proteína combinante (cp) (TSUBOI et al., 1995). O complexo ADA-proteína combinante constitui uma ecto-ADA, a qual é responsável pelo controle dos níveis de adenosina extracelulares (SAURA et al., 1996; FRANCO et al., 1997). Estudos envolvendo a sinalização mediada pela adenosina no SNC demonstraram que além da interação com CD26, a ADA1 pode atuar como uma ecto-enzima ancorada aos receptores de adenosina (A1 e A2b), mediando os processos de sinalização deste nucleosídeo neuromodulador (CIRUELA et al., 1996; ROMANOWSKLA et al., 2007).

A ADA1 e a ADA2 apresentam diferenças, tanto estruturais quanto cinéticas. A massa molecular da ADA2 é de aproximadamente 100 kDa e representa uma menor parte da atividade da ADA em tecidos, sendo abundante no plasma (IWAKI-EGAWA et al., 2004). A fonte celular e a função da ADA2 plasmática ainda não estão completamente esclarecidas (KOBAYASHI et al., 1993), porém dados recentes têm sugerido que ela pode ser secretada por monócitos ativados em processos inflamatórios (IWAKI-EGAWA et al., 2006).

A atividade da ADA pode ser um marcador sensível na infecção e ser utilizada para o acompanhamento do curso na mesma. A atividade da ADA mostra-se elevada no soro de pacientes com tuberculose, theileriose, malária e leishmaniose visceral (OZCAN et al., 1997;

MELO et al., 2000; KHAMBU et al., 2007; ALTUG et al., 2008). Apesar da vasta literatura sobre as alterações induzidas no SNC pelo *T. brucei* em humanos “doença do sono” e animal “Nagana” (MAULDIN et al., 2004), o conhecimento das alterações causadas por *T. evansi* no SNC dos animais são limitada aos estudos histopatológicos. Portanto, nos propomos a avaliar o sistema purinérgico na infecção por *T. evansi*, utilizando como modelo experimental ratos Wistar.

3 - CAPÍTULO II

ARTIGOS & MANUSCRITO

Os resultados desta tese são apresentados na forma de três artigos e um manuscrito, com sua formatação de acordo com as orientações das revistas ao quais foram submetidos:

3.1 – ARTIGO I

Biochemical detection of adenosine deaminase in *Trypanosoma evansi*

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Experimental Parasitology

Artigo publicado na Revista “Experimental Parasitology”

(ANEXO I)

Biochemical detection of adenosine deaminase in *Trypanosoma evansi*

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Biochemical detection of adenosine deaminase in *Trypanosoma evansi*

Abstract

Biochemical and molecular research on parasites has increased considerably in trypanosomes in the recent years. Many of them have the purpose of identify areas, proteins and structures of the parasite which are vulnerable and could be used in therapy against the protozoan. Based on this hypothesis this study aimed to detect biochemically the enzyme adenosine deaminase (ADA) in *Trypanosoma evansi,* and to adapt an assay to the measurement of its activity in trypomastigotes. Firstly, the parasites were separated from the blood of mice experimentally infected with a DEAE-cellulose column. The ADA activity in trypomastigotes was evaluated at concentrations of 0.1, 0.2, 0.5, 0.6 and 0.8 mg of protein by spectrophotometry. ADA activity was observed in the parasites at all concentrations tested and its activity was proportional to the concentration of protein, ranging between 0.64 and 2.24 U/L in the lowest and highest concentration of protein, respectively. Therefore, it is possible to detect biochemically ADA in *T. evansi*, an enzyme that may be associated with vital functions of the parasite, similar to what occurs in mammals. This knowledge may be useful in the association of the chemotherapic treatment with specific inhibitors of the enzyme, in future studies.

Keywords: Trypomastigotes, ADA, adenosine, inosine.

1. Introduction

Flagellates of genus *Trypanosoma* are ubiquitous parasites and infect a wide range of vertebrate hosts, resulting in immeasurable economic losses (Dobson et al., 2009). *Trypanosoma evansi* is the most widely distributed of the pathogenic African animal trypanosomes, affecting domestic livestock and wildlife in Asia, Africa and Latin America (Luckins and Dwinger, 2004). The parasite is transmitted mechanically by hematophagous flies such as *Tabanus* and *Stomoxys* spp. and/or vampire bats (Hoare, 1972). The main affected animals are horses, camels and dogs, but a large number of species may be parasitized. The animals showed typical clinical signs such as anemia, weight loss and locomotive disturbance (Hoare, 1972; Maudlin et al., 2004).

T. evansi is classified as monomorphic and is represented by trypomastigotes found in the bloodstream in the lanced shape, elongated body and flat. The parasite presents free flagellum, undulating and well developed membrane, the sub-terminal portion kinetoplast or marginal body and a core (Hoare, 1972; Maudlin et al., 2004). In the recent years, a wide variety of biochemical and molecular researches have been developed in the field of trypanosomosis, such as molecular identification and phylogenetic analysis of parasites (Amer et al., 2011), regulation of calcium concentration (Docampo and Moreno, 1996) and the detection of enzymes such as acetylcholinesterase in *T. evansi* (Mijares et al., 2011). In *Trypanosoma brucei*, authors demonstrated that purine transport activities are differentially regulated in the lifecycle stages of parasite, and mediate uptake of purine nucleosides and in some cases the nucleobase, hypoxanthine (Sanchez et al., 2002). Other researchers reported the existence of 2 distinct adenosine transport systems in *T. evansi* (Suswam et al., 2001; Suswam et al., 2003). According with the authors, this fact is related with the resistance to the melaminophenyl arsenical drug. These tools help to elucidate the relationships among

different species and subspecies and their potency of virulence and pathogenesis (Morrison et al., 2007).

Recently, our research group reported alterations in the activity of the enzyme adenosine deaminase (ADA: EC 3.5.4.4) in serum, cells (lymphocytes and erythrocytes) and brain of rats infected with *T. evansi* (Da Silva et al., 2011a, Da Silva et al., 2011b). ADA is considered to be a key enzyme in the purine metabolism, catalyzing the irreversible deamination of adenosine and deoxyadenosine to inosine and deoxyinosine, respectively, and closely regulating extracellular adenosine and inosine concentrations in mammals (Franco et al., 1997). Furthermore, adenosine is a CNS modulator, regulates cell metabolism and triggers a variety of physiological effects participating in apoptosis, necrosis, cell proliferation, and modulating the release of the neurotransmitters and tropic factors (Hasko and Cronstein, 2004; Sitkovsky and Ohta, 2005; Desrosiers et al., 2007).

Adenosine and inosine has already been restored in *T. evansi* and is involved in various vital functions of the parasite (Suswam et al., 2001; Suswam et al., 2003). In this study we aimed to investigate the presence of ADA in *T. evansi* as well as to adapt a technique to measure the activity of ADA in the parasite.

2. Material and Methods

For this experiment, it was utilized a strain of *T. evansi* obtained from a naturally infected dog (Colpo et al., 2005). Isolates were kept in liquid nitrogen according to the methodology described by Silva et al. (2003). The procedure was approved by the Animal Welfare Committee of Federal University de Santa Maria (UFSM), number 23081.012513/2009-52.

The trypomastigotes were obtained by inoculation of two mice with 0.2 mL of cryopreserved blood (Silva et al., 2003) containing 10^6 parasites per microliter,

intraperitoneally. Subsequently, the parasitemia was estimated daily by microscopic examination of smears. Each slide was mounted with blood collected from the tail vein, stained by the panoptic method, and visualized by optical microscopy. After the 5^{th} day of infection the parasitemia was over 100 trypanosomes per microscopic field (1000x). At this time the animals were anesthetized with isoflurane for blood collection, stored in tubes with anticoagulant (EDTA 10%).

The volume of 3 mL collected from both mice was eluted with PBS buffer containing 1% glucose (PSG - 1v/v). Thereafter, the separation of trypomastigotes forms by chromatography was performed on a Poly-Prep® column (Bio-Rad Laboratories, Hercules, USA) using the DEAE-cellulose resin, according to the technique described by Tavares et al. (2011). The number of parasites purified was measured by counting in a Neubauer chamber. In order to concentrate the number of parasites as a pellet, the purified samples (2 mL of PSG + parasite) were centrifuged for 30 minutes (14,000 g at 4 C). After the *T. evansi* ($1x10^9$ trypomastigotes per mL) was obtained it was stored in microtubes and kept frozen at -20 ºC until analyses.

On the day of analysis, the pelleted trypanosomes were resuspended with 50 mmol/L per mM phosphate buffer, pH 6.5. Then the protein concentration of the trypomastigotes was measured by the method of Peterson (1977) with bovine serum albumin used as a standard. The concentration of proteins in the parasite was expressed in mg mL^{-1}.

To measure the ADA activity in the parasites it was adapted the technique used to evaluate the ADA activity in lymphocytes. ADA activity was measured spectrophotometrically in trypomastigotes forms of *T. evansi* by the method of Guisti (1974) modified. The reaction was started by the addition of the substrate (adenosine) to a final concentration of 21 mmol/L and incubations were carried out for 1h at 37 °C. The reaction was stopped by adding 106 mmol/L/0.16 mmol/L phenol-nitroprusside solution. The reaction

mixtures were immediately mixed to 125 mmol/L/11 mmol/L alkalinehypochlorite (sodium hypochlorite) and vortexed. Ammonium sulphate 75 umol/L was used as ammonium standard. The ammonia concentration is directly proportional to the absorption of indophenol at 650 nm. The specific activity is reported as U/L. The estimation was performed out in triplicate and the mean was used for calculation.

3. Results and discussion

In this study, the pelleted trypanosomes eluted with phosphate buffer showed a protein concentration of 0.86 mg mL^{-1}. The ADA activity was assessed at concentrations of 0, 0.1, 0.2, 0.5, 0.6 and 0.8 mg mL^{-1}. In this study it was detected biochemically the presence of ADA enzyme in *T. evansi*. In the lowest and highest concentrations of proteins tested, the ADA activity was between 0.64 and 2.24 U/L, respectively. Therefore, the ADA activity increased in proportionately with the concentration of protein used (Fig. 1).

Studies have reported changes in adenosine transport in parasites and ADA activity in mammals associated with infections by *T. brucei*, *T. evansi*, *Trypanosoma vivax*, *Leishmania donovani* and *Leishmania infantum* (Okochi et al., 1983; De Koning and Jarvis, 1999; Suswam et al., 2003; Tripathi et al., 2008; Da Silva et al., 2011a,b). However, there is still a great complexity in the purine transport in trypanosomes. There is the necessity for more detailed biochemical characterizations of purine transporters in order to provide useful information for improved drug delivery, and then to achieve a better understanding of drug resistance phenotypes associated with purine transporters (Suswam et al., 2003). As a result, this study aimed to investigate the presence of ADA in *T. evansi*, an enzyme important for many vital functions in mammals and possibly for the parasites. In the genome of *T. brucei* it was identified ADA, which showed similarity among these trypanosomes.

The biochemical tests showed ADA activity in *T. evansi*. Probably, in the parasite this enzyme is responsible for the regulation of adenosine concentration and consequently inosine, as occurs in mammals (Franco et al., 1997). In future studies, we aim to investigate the ADA presence within the parasite using immune markers, as well as the purification and molecular characterization of the ADA of *T. evansi* in order to find differences with its counterpart in vertebrates, which could allow us to propose this enzyme as a potential target for chemotherapy.

Recent studies showed that the treatment with the adenosine analogue called cordycepin (3'-deoxyadenosine) when protected by an inhibitor of ADA was effective in the curative treatment of mice infected with *T. brucei* (Rottenberg et al., 2005; Vodnala et al., 2009). The curative effect is obtained because cordycepin binds to receptors, binding site for nucleosides obtained from the host to vital functions of the parasite. In contrast to most mammalian cells, trypanosomes cannot synthesize purines *de novo*. Instead, they depend on the salvage pathway of nucleosides from the body fluids of the host (Hammond and Gutteridge, 1984). When used only ADA inhibitor in the treatment of *T. brucei* in mice, researchers did not observe curative action and the animals died as a consequence of the disease (Rottenberg et al., 2005). With the discovery of ADA in the parasite, it becomes interesting to test *in vitro* the action of ADA inhibitors on *T. evansi*, to assess whether the inhibitor could have some direct harmful effects on the protozoan, by a mechanism that interferes with vital functions and causes the death of flagellates. This hypothesis will be the subject of a forthcoming study, together with the characterization of the enzyme in the parasite, as previously described.

Based on these results, we can conclude that *T. evansi* has the enzyme adenosine deaminase, which probably regulates the concentration of adenosine and inosine in the besieged, as it occurs in mammals. The technique provided demonstrated to be adequate to

detect biochemically ADA activity in the parasite. So, this is the first step for the time to come we can test specific inhibitors of this enzyme in infected animals.

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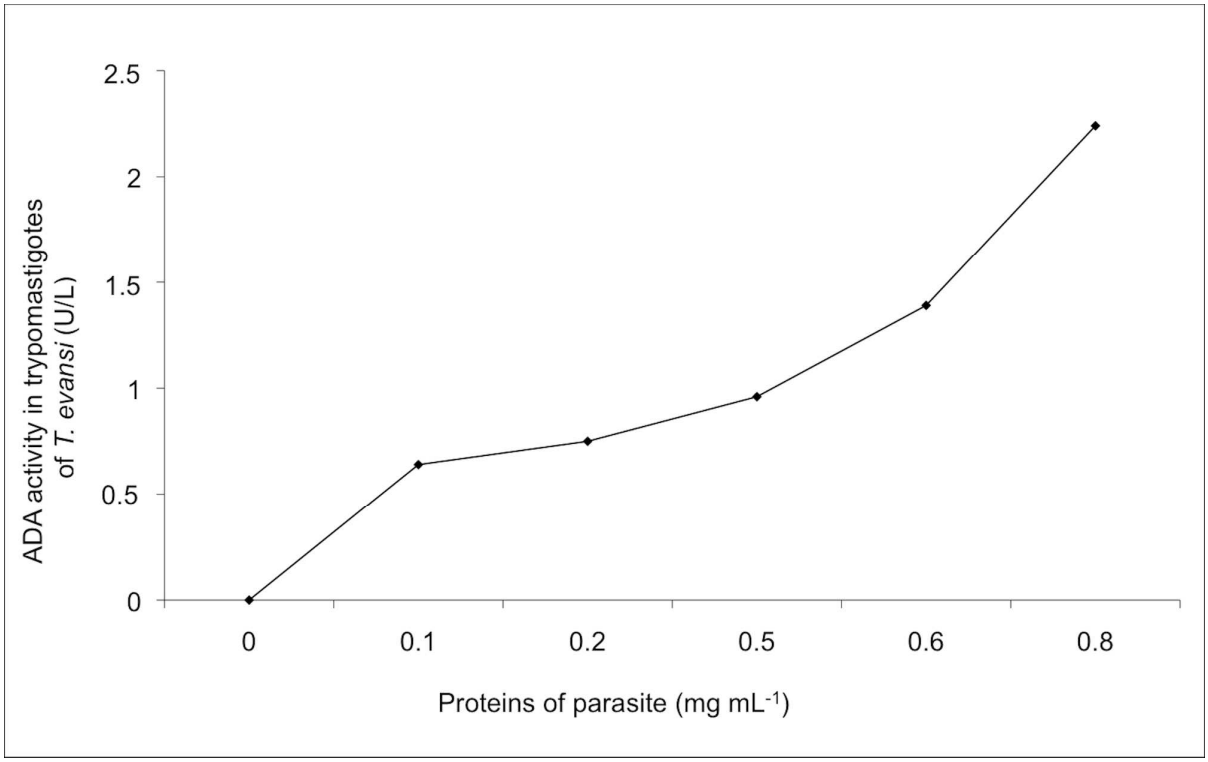

Fig. 1: Biochemical detection of adenosine deaminase activity in trypomastigotes forms of *T. evansi*. Relationship between ADA activity and protein concentration of the parasite.

3.2 – ARTIGO II

Activity of the enzyme adenosine deaminase in serum, erythrocytes and lymphocytes of rats infected with *Trypanosoma evansi*

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(ANEXO II)

Activity of the enzyme adenosine deaminase in serum, erythrocytes and lymphocytes of rats infected with *Trypanosoma evansi*

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Activity of the enzyme adenosine deaminase in serum, erythrocytes and lymphocytes of rats infected with *Trypanosoma evansi*

Abstract

In *Trypanosoma evansi* infections are commonly observed changes in hemogram and the enzyme adenosine deaminase (ADA) plays important roles in the production and differentiation of blood cells. Thus, this study aim was to evaluate the activity of ADA in serum, erythrocytes and lymphocytes of rats infected with *T. evansi* compared not-infected. Were used 30 adult rats divided into three uniform groups. The animals in groups A and B were infected with 2 x 10^6 trypomastigotes/rats, intraperitoneally. Rodents from group C (control group), were not-infected. Blood collection was performed on days 4 and 20 post-infection (PI) in order to get an acute and other chronic infection stage of disease. The blood collected was used to assess the activity of ADA. In the blood, reduced hematocrit and increased lymphocytes were correlated with ADA activity in erythrocytes and lymphocytes. We observed reduction of ADA activity in serum and erythrocytes in rats infected with *T. evansi* compared to not-infected ($P<0.05$). ADA activity in lymphocytes was decreased in 4 days, when the parasitemia was high and increased after 20 days, when the number of circulating parasites was low. In conclusion, our results showed that the ADA activity was altered in serum, lymphocytes and erythrocytes of rats in experimental infection by *T. evansi*, concomitantly with hematological parameters.

Keywords: trypanosomosis, ADA, anemia, lymphocytosis, rats.

1. Introduction

Trypanosoma evansi is a digenetic flagellate implicated in the infection of a large number of domestic and wild animals, such as equines, canines, felines, rabbits, capybaras, ring-tailed coatis, bovines and buffaloes (Dávila and Silva 2000; Herrera *et al.* 2004; Tarello 2005; Da Silva *et al.* 2008) and humans (Joshi *et al.* 2005). This protozoan is the agent of trypanosomosis, a disease with broad distribution in Africa, Asia, and Latin America (Lun and Desser 1995). The trypomastigotes present in blood vessels of vertebrate hosts are transmitted by blood-sucking insects during feeding. The insect vectors are most commonly tabanide species (*Tabanus* sp., *Chrysops* sp., and *Hematopota* sp.) and vampire bats (Hoare 1972).

Two features of the disease were reported in Brazil: the acute syndrome, responsible for the death of equines and non-treated canines, and the chronic syndrome, which affects many wild animals as *Hydrochaeris hydrochaeris* and *Nasua nasua* (Herrera *et al.* 2004). The acute form is characterized by intermittent fever, subcutaneous widespread edema, progressive anemia and blindness. Clinical signs disappear during the subacute phase and the trypanosomosis may often go undiagnosed during clinical examination. Accurate diagnosis is only possible during the chronic stage of the disease, where clinical signs are more evident and the animal's condition is more severely affected (Silva *et al.* 2002).

Adenosine deaminase (ADA: EC 3.5.4.4) is considered to be a key enzyme in purine metabolism, catalyzing the irreversible deamination of adenosine and deoxyadenosine to inosine and deoxyinosine, respectively, closely regulating extracellular adenosine concentrations (Franco *et al.* 1997). Adenosine deaminase activity has been detected on the surface of hematopoietic cells (Aran *et al.* 1991). Researchers described a family in which there is a dominantly inherited form of hemolytic anemia associated with a notable increase of

ADA activity in erythrocytes but with normal ADA levels in other blood cells, including lymphocytes (Valentine *et al.* 1977).

ADA is present in all cell types, but high ADA activity is present in the thymus, lymphoid tissues and peripheric lymphocytes. It has been demonstrated that this enzyme plays an important role in lymphocyte function and is essential for the normal growth, differentiation and proliferation of T lymphocytes (Franco *et al.* 1997; Codero *et al.* 2001). The observation that ADA deficiency leads to severe combined immunodeficiency syndrome points to the physiological importance of controlling extracellular adenosine levels in the immune system (Aldrich *et al.* 2000).

Anemia by *T. evansi* is often described and it is characterized by decreased values of erythrocytes, hemoglobin and hematocrit. However, its causes are not completely understood (Silva *et al.* 1995; Aquino *et al.* 2002). In infections by this protozoan, leukocyte changes are described as neutropenia, neutrophilia, monocytosis, lymphopenia, lymphocytosis (Silva *et al.* 1995; Marques *et al.* 2000; Wolkmer *et al.* 2009).

Considering the functions of ADA in leukocyte and hematopoietic system, this study aimed to evaluate the activity of this enzyme in serum, erythrocytes and lymphocytes of rats infected with *T. evansi*.

2. Material and methods

A total of 30 adult rats, males, with a mean age of 90 days and weighing in average 300 (±29) grams were used in this study. The animals were kept in cages with 10 animals each in a room experiment with temperature and humidity controlled (25ºC; 70%). They were fed with commercial ration and water *ad libitum*. All animals received a formulation containing pyrantel pamoate, praziquantel and fenbendazole and were submitted to a period of

15 days of adaptation. All animals were apparently healthy when the experimental period begun (day 0).

These rats were divided into three groups of 10 animals each. The rats in groups A and B were inoculated intraperitoneally (Day 1) with a strain of *T. evansi* that had been obtained from a naturally infected dog (Colpo *et al.* 2005) and had been kept in liquid nitrogen. The number of inoculated flagellates was estimated by using a Neubauer chamber (Wolkmer *et al.* 2007). This study aimed to evaluate the acute and chronic disease in rat, so the infectious dose used for groups A and B was 2 x 10^6 trypomastigotes/animal in fresh blood and blood cryopreserved, respectively (Da Silva *et al.* 2009a). The collection of samples from animals in group A was performed on day 4 post-infection (PI) and group B was on day 20 PI. Group C (negative control) consisted of healthy rats, not infected by *T. evansi*, but received a physiological solution by the same way. Group C was divided into two groups (C1 and C2) and the material was collected on day 4 and 20 PI in order to compare with the infected groups (A and B). Parasitemia was estimated daily by microscopic examination of smears. Each slide was mounted with blood collected from the tail vein, stained by the panoptic method, and visualized at a magnification of 1000x.

The animals were anesthetized in a chamber with isoflurane for collection of blood by cardiac puncture (8mL). The storage of the samples was considered accordingly to the analysis. Thus, part of the material collected was allocated in tubes containing anticoagulant for separation of lymphocytes (4mL), separation of erythrocytes (2mL) and analysis of hemogram (1mL). The volume of 1mL was stored in a tube without anticoagulant to obtain serum.

Erythrocytes count, hematocrit (Ht), hemoglobin concentration (Hb), mean corpuscular volume (MCV), mean corpuscular hemoglobin concentration (MCHC) and total leukocytes were evaluated. Smears were mounted and stained by the panoptic method. The

determination of microhematocrit was performed according to the technique described by Feldman *et al.* (2000), and blood smears were stained with panoptic method to perform differential leukocyte. Erythrocytes count and hemoglobin concentration were determined using an electronic counter.

Erythrocytes were obtained from whole blood with EDTA, according to the technique described by Hostetter and Johnson (1989). Erythrocytes were resuspended to hematocrit in Hepes-buffered at 10%. As the erythrocytes, lymphocytes were also obtained from whole blood with EDTA by gradient separation using Ficoll-Histopaque™ plus, according to the technique described by Böyum (1968). The samples stored in tubes without anticoagulant was centrifuged for 10 minutes, and the serum was obtained. The ADA activity was measured immediately after obtaining the erythrocytes, lymphocytes and serum.

ADA activity was measured spectrophotometrically in serum, lymphocytes and erythrocytes by the method of Giusti and Gakis (1971). The reaction was started by addition of the substrate (adenosine) to a final concentration of 21 mmol/l and incubations were 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 sulphate of 75 umol/l was used as ammonium standard. The ammonia concentration is directly proportional to the absorption of indophenol at 650 nm. The specific activity is reported as U/L in serum and lymphocytes and U/g of Hb in erythrocytes.

The data were submitted to one-way analysis of variance (ANOVA) followed by the Tukey's test ($P<0.05$). The effect of ADA in erythrocytes and lymphocytes on hematocrit and lymphocytes was analyzed by linear regression, respectively. The analyses were performed using SAS statistical package (SAS Institute, Cary, NC, USA) with a significance level of 5% ($P <0.05$).

The procedure was approved by the Animal Welfare Committee of Federal University de Santa Maria (UFSM), number 23081.012513/2009-52, in accordance to Brazilian laws and ethical principles published by the Colégio Brasileiro de Experimentação Animal (COBEA).

3. Results

Examination of peripheral smear blood showed that the pre patent period in rats experimentally infected had variation between 24 and 72 hours. The peak of parasitemia at day 4 PI in group A (63 trypanosomes per microscopic field at 1000 x magnification) and irregular waves of parasitemia were observed in group B, ranging from zero to three trypomastigotes per microscopic field (Fig. 1). Seven (7/10) rats in group A showed up apathetic, slow movements, disorientation and gasping on day 4 PI. In group B, the animals did not show any clinical change, as well as the not-infected rats (group C).

Hematological changes was observed in the infected rats as the decrease in hematocrit (Fig. 3a), erythrocyte count and hemoglobin content (Table 1 - $P<0.05$). The mean of MCV and the mean of MCHC did not differ among groups, characterizing a normocytic–normochromic anemia (Table 1). Simultaneously with hematological changes, the rats of group A showed leukocytosis (Table 1) and lymphocytosis (Fig. 4a), as compared to group B and C.

The ADA activity in serum was reduced in the groups infected with *T. evansi* compared to not-infected rats ($P<0.001$ – Fig. 2). In erythrocytes, the ADA activity was reduced on day 4 and 20 (Figure 3b), but was more pronounced in 20 days when the parasitemia was low (Fig. 1). In the chronic phase it was observed a positive correlation (r^2: 0.82) between the ADA activity in erythrocytes and the hematocrit values ($P<0.001$ – Fig. 3d).

In lymphocytes, the activity of ADA was reduced in the acute phase (Day 4), when the parasitemia was high, but showed an increase of ADA activity in 20 days (Fig. 4b), when the parasitemia was low (Fig. 1) and the number of lymphocytes was normal (Fig. 4-a). In acute phase it was observed negative correlation (r^2: -0.60) between the ADA activity in lymphocytes and total number of lymphocytes ($P<0.05$).

4. Discussion

Many studies have reported changes in adenosine transport and ADA activity associated with infections by *Trypanosoma brucei, Trypanosoma evansi, Trypanosoma vivax, Leishmania donovani* and *Leishmania infantum* (Okochi *et al.* 1983; De Koning and Jarvis 1999; Suswam *et al.* 2003; Tripathi *et al.* 2008). However, a study that correlates the acute and chronic effect of experimental infection with *T. evansi* in the ADA activity in the serum, erythrocytes and lymphocytes of rats has not been found in the literature.

It has been observed an increased ADA activity in serum samples, erythrocytes, leukocytes and plasma hemoglobin concentrations with vivax malaria as compare to control group (Ozcan *et al.* 1997). Authors described significantly increased ADA activity in visceral leishmaniasis patients compared to healthy controls (Khambu *et al.* 2007). Researchers showed that intraperitoneal injection of the adenosine analogue cordycepin (3´-deoxyadenosine) for treatment of the encephalitic stage of human African trypanosomosis, together with an adenosine deaminase inhibitor (coformycin or deoxycoformycin), cures *T. brucei* infection in mice (Rottenberg *et al.* 2005). Therefore, we see how ADA activity may be associated with trypanosome infection, as in this study with *T. evansi* that rat had reduced enzyme activity in blood cells and serum.

In the acute phase of this study we observed a reduction of ADA activity in serum, erythrocytes and lymphocytes of rats infected with *T. evansi* compared to healthy rats. At this

stage, rats had four days of infection and high parasitemia (Fig. 1). The reduction in ADA activity would have caused an increase in the extracellular concentrations of adenosine, which would be converted to inosine. Adenosine acts as a sensor and provides information to the immune system about the tissue damage or acute inflammatory changes occurring in the vicinity of the immune system (Kumar and Sharma 2009). The reduction in ADA activity in lymphocytes, would lead to interaction of adenosine with adenosine receptors that exist in many cell types, with possible anti-inflammatory effects, among them the inhibition of Th1 immune response. In acute infection caused by *T. cruzi* there is a predominance of Th1 and cellular response with production of interferon-γ (Kumar and Tarleton 2001). Therefore, inhibition of this response by the action of extracellular adenosine in purinergic receptors could be a compensatory effect, attenuating inflammation and tissue damage. The treatment of macrophages with interferon-γ up regulates the expression of the adenosine receptor, A_{2B}, and the activation of A_{2B} receptors is involved with the deactivation of macrophages, possibly through an increase of cAMP (Xaus *et al.* 1999). This reinforces the concept of anti-inflammatory action of adenosine as a way to preserve cells and tissues.

The ADA may be expressed as an ectoenzyme on the surface of lymphocytes. In the serum there is another isoenzyme, ADA_2, which has a low affinity for the substrate (Muraoka *et al.* 1990). Thus, it will only increase its activity in higher concentrations of substrate, unlike the lymphocyte isoform. The concentrations of extracellular adenosine in this study seems to be sufficient for activation of the enzyme in serum, assuming that they could be binding on the adenosine receptors in blood cells. Regarding the ADA activity in erythrocytes, reduction of enzyme activity seems to follow the decrease in total erythrocytes, which could be verified in future by observing the expression of ectoenzymes the surface of red blood cells.

Researchers mentioned that red blood cells are relatively well supplied with ADA and circulating damaged erythrocytes release significant amounts of ADA, a process that may

predispose to vasoocclusive events (Muraoka *et al.* 1990). Based on this information, we hypothesized that ADA activity was increased in serum and/or plasma, in consequence of the decrease in red blood cells due to a hemolytic process, a cause of anemia in trypanosomosis (Jackson *et al.* 1996). However, this was not observed in our study. Therefore, despite the positive correlation between hematocrit and activity levels in erythrocytes, further studies are necessary to clear the role of ADA in anemia caused by *T. evansi* in rats.

In addition, in the chronic phase our experiment showed that the parasitemia had stabilized and fluctuated between 0 and 3 parasites per field (1000x). At the same time we find that the ADA activity in serum and erythrocytes was reduced in rats with trypanosomosis, and in lymphocytes ADA activity was increased when compared to not-infected. The low parasitemia suggests that the compensatory effect of an anti-inflammatory like adenosine is no longer necessary. Thus, increased ADA activity in lymphocytes, reducing the extracellular concentrations of adenosine and favoring an inflammatory response that would be sufficient to contain the spread of the parasite without major tissue damage. The low concentration of extracellular adenosine also prevents activation of isoforms present in erythrocytes and serum, probably due to a low affinity for the substrate.

Researches observed that ADA activity in serum of patients with idiopathic Parkinson's disease has been recently found significantly higher than in normal controls, suggesting that high serum ADA activity may be involved in the pathogenesis of Parkinson's disease through peripheral T-lymphocyte activation (Chiba *et al.* 1995). Increase in serum ADA activities in patients with cutaneous leishmaniasis (Ozcan *et al.* 1998), change in activity of this enzyme may also be related to pathogenesis of the parasite, as well as in Parkinson's disease. Rather, this study showed reduced ADA activity in serum of rats infected with *T. evansi*, different from what occurred in infection by *Leishmania* sp. (Ozcan *et al.* 1998; Khambu *et al.* 2007).

An important aspect to be discussed is that *T. evansi* caused an acute infection in the rats, but can also be chronic for some rodents. These protozoa are Salivarian trypanosomes which are usually more virulent and pathogenic than Stercorarian trypanosomes (Menezes *et al.* 2004). The detection of parasitemia in rats (24 h) occurred earlier than previously reported in experimentally infected rats (Queiroz *et al.* 2000; Al-Mohammed 2006; Omer *et al.* 2007), probably due to the high pathogenicity of this strain. Although authors reported that *R. norvegicus* is a suitable model for the study of the parasitemic wave of *T. evansi* (Queiroz *et al.* 2000), the typical undulating course of parasitemia was observed in the group B of this experiment. According to Da Silva *et al*. (2009a) the longevity of rats may be related to the type of inoculum used. When they are made of successive infections in rats, there is an acute phase with high parasitemia and death of animals within 5 days PI. Now, when the inoculum used was cryopreserved in liquid nitrogen the longevity of rodents can increase considerably, resulting in a chronic phase. In rats the disease was characterized by high levels of parasitemia along with clinical signals of apathy, weakness, ataxia and severe anemia (Wolkmer *et al.* 2009), similar to what occurred in the acute phase of this experiment.

Previous studies from our laboratory showed that in the infection with *T. evansi* hematological changes are commonly related to other factors, but major changes depend on the degree of parasitemia and period of infection (Wolkmer *et al.* 2007; Da Silva *et al.* 2009b). Our research group has already found in the erythrocytes a decreased activity of acetylcholinesterase in cats (Da Silva *et al.* 2010) and an increased lipid peroxidation in rats (Wolkmer *et al.* 2009) infected with *T. evansi*. It is important to state that these alterations might be related to pathogenesis of the disease or just a consequence of anemia according to researchers, because adenosine is related to maturation of erythrocytes (Franco *et al.* 1990; Jackson *et al.* 1996). This question also persists for the decreased ADA activity in erythrocytes in this study.

Based on these results, we conclude that the parasitism by *T. evansi* alters the activity of ADA in serum, erythrocytes and lymphocytes of rats experimentally infected, suggesting that trypanosomiasis can interfere with a purinergic signaling. Other studies should be performed to verify the expression of ectoenzymes in the surface of red blood cells and lymphocytes in trypanosomiasis, in order to understand the relationship of the ADA with anemia and lymphocytosis in this disease.

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Table 1: Means and standard deviation of the hematological parameters of rats experimentally infected with *T. evansi*.

Parameters	Group*		
	A: Infected (Day 4)	B: Infected (Day 20)	C: Not-infected
Total erythrocytes ($x10^6/\mu l$)	6.15^b (±0.46)	6.34^b (±0.65)	7.0^a (±0.24)
Hemoglobin (g/dl)	11.8^b (±0.46)	12.5^b (±0.46)	14.0^a (±0.46)
MCV (fl)	62.6^a (±2.10)	62.4^a (±1.74)	61.0^a (±2.40)
MCHC (%)	30.8^a (±0.90)	32.6^a (±1.10)	31.3^a (±0.80)
Total leukocytes ($x10^3/\mu l$)	12.71^a (±3.10)	5.27^b (±1.2)	5.75^b (±0.9)

* Means in the same line followed by different letters are statistically different among them by Tukey's test at 5% probability.

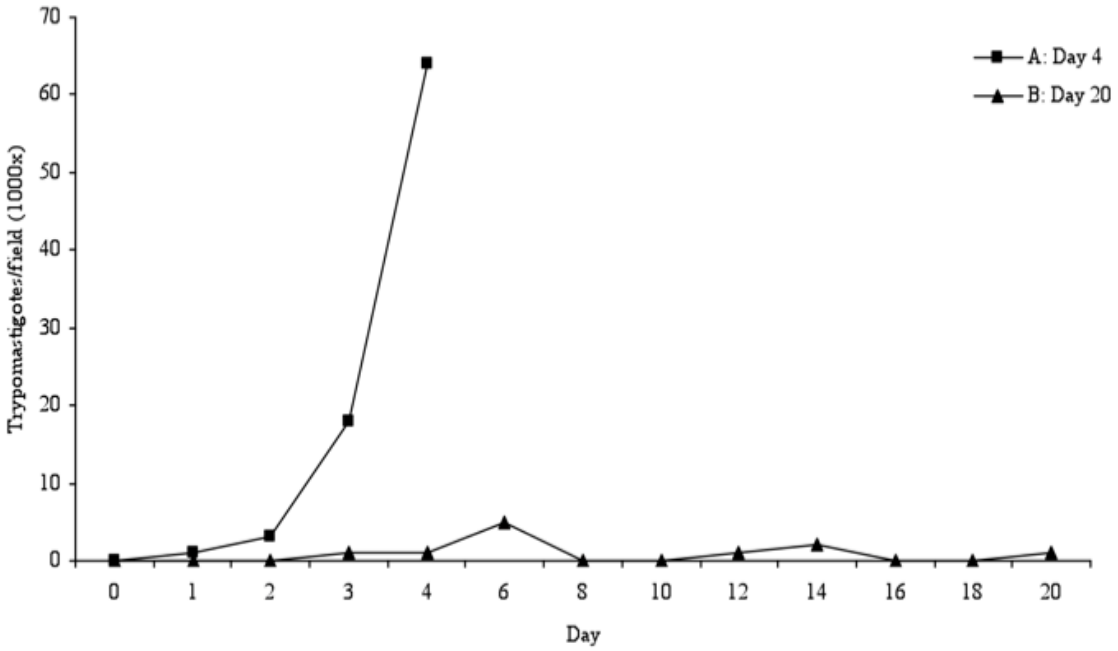

Fig. 1: Parasitemia of *T. evansi* in infected rats at day 20 post-inoculation. The collection of material was performed in groups A and B on day 4 and 20 post-infection when high and low parasitemia, respectively.

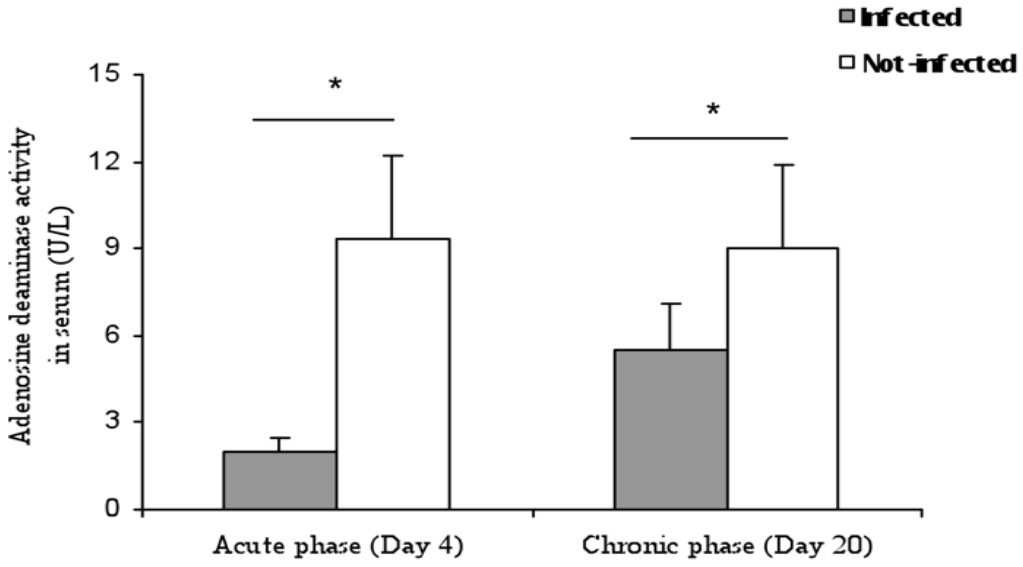

Fig. 2: Means and standard deviation of the adenosine deaminase activity in serum of rats infected whit *Trypanosoma evansi* (Day 4 and 20 PI) compared to not-infected (n=10). (*p<0.05)

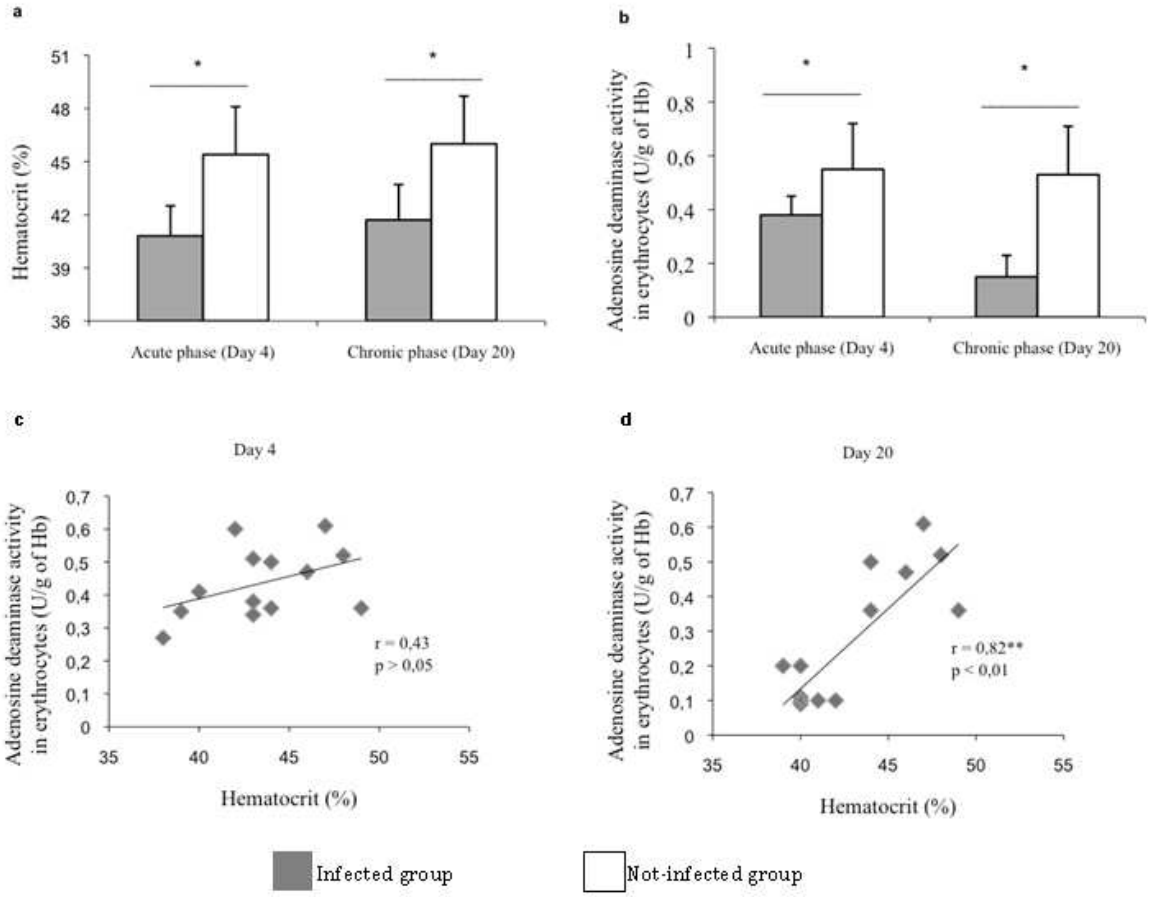

Fig. 3: Means and standard deviation of the hematocrit (a) and adenosine deaminase activity in erythrocytes (b) of rats infected whit *Trypanosoma evansi* (Day 4 and 20 PI) compared not-infected (n=10). Linear regression analysis of individual infected rat hematocrit with adenosine deaminase activity in erythrocytes of the acute phase (c) and chronic phase (d). (*p<0.05)

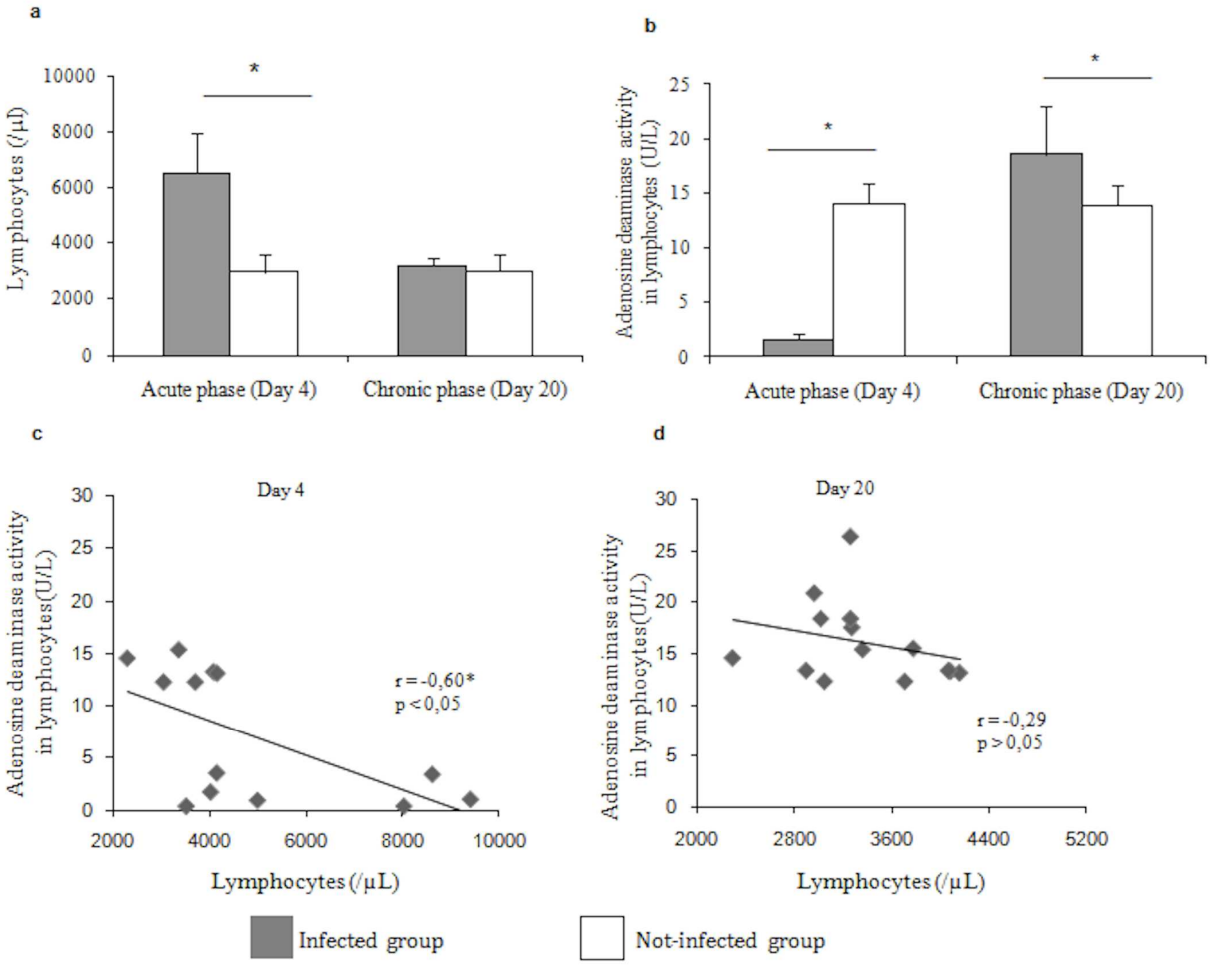

Fig. 4: Means and standard deviation of the lymphocytes (a) and adenosine deaminase activity in lymphocytes (b) of rats infected with *Trypanosoma evansi* (Day 4 and 20 PI) compared to not-infected (n=10). Linear regression analysis of individual infected rat number of lymphocytes with adenosine deaminase activity in lymphocytes of the acute phase (c) and chronic phase 20 (d). (*p<0.05)

3.3 – ARTIGO III

***Trypanosoma evansi*: Adenosine deaminase activity in the brain of infected rats**

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(ANEXO III)

***Trypanosoma evansi*: Adenosine deaminase activity in the brain of infected rats**

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Trypanosoma evansi: Adenosine deaminase activity in the brain of infected rats

Abstract

The study was undertaken to evaluate changes in the activity of adenosine deaminase (ADA) in brains of rats infected by *Trypanosoma evansi*. Each rat was intraperitoneally infected with 10^6 trypomastigotes either suspended in fresh (Group A; n=13) and cryopreserved blood (Group B; n=13). Thirteen animals were used as control (Group C). ADA activity was estimated in the cerebellum, cerebral cortex, striatum and hippocampus. No differences ($P > 0.05$) in ADA activity were observed in the cerebellum between infected and non-infected animals. Significant ($P < 0.05$) reductions in ADA activity occurred in cerebral cortex in acutely (day 4 post-infection; PI) and chronically (day 20 PI) infected rats. ADA activity was significantly ($P < 0.05$) decreased in the hippocampus in acutely infected rats, but significantly ($P < 0.05$) increased in the chronically infected rats. Significant ($P < 0.05$) reductions in ADA activity occurred in the striatum of chronically infected rats. Parasites could be found in peripheral blood and brain tissue through microscopic examination and PCR assay, respectively, in acutely and chronically infected rats. The reduction of ADA activity in the brain was associated with high levels of parasitemia and anemia in acute infections. Alterations in ADA activity of the brain in *T. evansi*-infected rats may have implications for pathogenesis of the disease.

Keywords: *Trypanosoma evansi*, adenosine deaminase activity, adenosine, brain, rats.

1. Introduction

Trypanosoma evansi and *T. brucei* are closely phylogenetically related trypanosomes of African origin (Brun et al., 1998; Lai et al., 2008). *T. evansi* is the only mechanically

transmitted by biting flies and displays the broadest host range and geographical distribution among all pathogenic trypanosomes, infecting domestic and wild mammals in Africa, Asia and Latin America (Brun et al., 1998; Ventura et al., 2002; Herrera et al., 2005).

The infection caused by *T. evansi* in horses develops in two stages, the early, or haemolymphatic stage, when the parasites multiply and spread in the blood and lymph nodes, followed by the late or encephalitic stage, when the parasites cross the blood–brain barrier to invade the central nervous system (CNS). *T. evansi* causes a devastating horse disease, called ‘‘mal de cadeiras’’ or “surra”, characterized by anemia, immunosuppression, emaciation, severe neurological signs, motor incoordination, paralysis of hind limbs and death of untreated animals (Rodrigues et al., 2009; Berlin et al., 2009).

Clinical signs of neurological disorders are reported in horses, camels, buffaloes, cattle, deer and cats infected by *T. evansi* (Tuntasuvan et al., 1997; Tuntasuvan et al., 2000; Rodrigues et al., 2005; Berlin et al., 2009; Da Silva et al., 2010). Brain lesions were reported in bovines and equines (Tuntasuvan et al., 1997; Rodrigues et al., 2009). Rats are highly susceptible to the disease, showing hematological, biochemical and pathological changes associated with ataxia, tremors and terminal coma of untreated animals (Menezes et al., 2004; Wolkmer et al., 2009). Human infection by *T. evansi* was reported for the first time in 2005 in an Indian farmer that showed signs of sensory deficit, disorientation and violent behavior (Joshi et al., 2005).

Adenosine acts as a CNS modulator in mammals, regulates cell metabolism and triggers a variety of physiological effects participating in apoptosis, necrosis and cell proliferation. Under pathological conditions, adenosine plays a protective role by modulating the release of the neurotransmitters and tropic factors. Adenosine also acts as an endogenous regulator of innate immunity, protecting the host from excessive tissue injury associated with

strong inflammation (Rathbone et al., 1999; Beraudi et al., 2003; Hasko and Cronstein, 2004; Sitkovsky and Ohta, 2005; Burnstock, 2006; Desrosiers et al., 2007).

The concentration of extracellular adenosine is regulated by the activity of a small group of important enzymes including adenosine deaminase (ADA; EC 3.5.4.4), which catalyses the conversion of the adenosine into its inactive metabolite inosine. ADA activity is widely distributed in tissues and fluids from vertebrate animals in isoforms of ADA1 and ADA2. Tissue extracts contain predominantly ADA1, which is supposed to be derived mainly from injured tissues. ADA2 is found in serum and derived from stimulated T-cells. ADA has been detected on the surface of many cell types, including brain synaptosomes. A heterogeneous expression of ADA activity can be found among peripheral tissues and even within the CNS, where high activities of ADA were reported in discrete and diverse brain areas (Geiger et al., 1986; Franco et al., 1986, 1997).

ADA activities may be sensitive markers for infection severity and for monitoring the course of infections. The activity of ADA was elevated in the serum of hosts with tuberculosis, theileriosis, malaria and visceral leishmaniasis (Ozcan et al., 1997; Melo et al., 2000; Khambu et al., 2007; Altug et al., 2008). No study has demonstrated a relationship of *T. evansi* infection with ADA activity in the CNS. Thus, the purpose of the present investigation was to determine whether *T. evansi* infection induces changes in ADA activity in the brain tissues of adult rats.

2. Material and methods

2.1. Experimental animals

Thirty nine adult outbreed male rats (mean age of 90 days) weighing 300 ± 18 g were maintaining in cages in a room with controlled temperature (25ºC) and humidity (70%). They were fed (commercial ration) and water *ad libitum*. Before the experiment, they were

treated with pyrantel pamoate, praziquantel and fenbendazole, and submitted to an adaptation period of 15 days. The procedure was approved by the Animal Welfare Committee of Universidade Federal de Santa Maria (UFSM), number 23081.012513/2009-52, in accordance to Brazilian laws and ethical principles of the Colégio Brasileiro de Experimentação Animal (COBEA).

2.2. Experimental design and trypanosome infection

The rats were divided in three groups of 13 animals each. Animals in groups A and B were inoculated intraperitoneally (day 0) with a strain of *T. evansi* that had been obtained from a naturally infected dog (Colpo et al., 2005) and had been maintaining in liquid nitrogen. The infective dose (estimated using a hemocytometer) for each animal was 10^6 trypomastigotes in either fresh (Group A; 0.1 ml) and cryopreserved blood (Group B; 0.2 ml) in order to elicit acute and chronic infections, respectively (Da Silva et al., 2009). The collection of blood samples and brains from animals in group A was performed at day 4 post-infection (PI) while samples for group B were collected at day 20 PI. Group C consisted of 13 healthy non-infected control rats. This group was divided into groups C1 and C2 and blood samples and brains were collected on days 4 (C1) and 20 (C2) PI for comparison with the infected groups A and B.

2.3. Estimation of parasitemia

The presence and degree of parasitemia were determined daily for each animal by blood film examination. A drop of blood was collected from the tail and placed on a slide, and a thin blood smear was prepared manually (Da Silva et al., 2006). The blood films were Romanovsky stained and then examined under a microscope, counting 10 fields at 1000x magnification.

2.4. Collection of samples

The animals were anesthetized in a chamber with isoflurane before collection of blood by cardiac puncture. Thereafter, animals were decapitated following recommendations of the Ethics Committee and brains of 10 rats from each group were carefully removed avoiding contamination with peripheral blood, and dissected to separate cerebellum, cerebral cortex, striatum and hippocampus. Each part of the brain was weighed, homogenized in 10 volumes of 50 mmol/l per mM phosphate buffer (pH 7.0) and centrifuged for 30 min at 14,000 × g at 4 ^{o}C. The supernatant was then collected as described by Bellé et al. (2009).

2.5. Hematologic Parameters

Erythrocyte count, hemoglobin concentration and total leukocyte count were determined using an electronic counter (CC-550-Celm, São Paulo, Brazil).

2.6. ADA activity in brain

ADA activities were estimated spectrophotometrically (Hitachi U-2800A - spectrophotometer) by the method of Giusti (1974), which is based on the direct measurement of the formation of ammonia produced when the enzyme acts on adenosine. Brain homogenate of cerebral cortex (15mg/mL protein), cerebellum (10mg/mL protein), striatum (3mg/mL protein) or hippocampus (3mg/mL protein) was added to 21mM of adenosine in 50 mM sodium phosphate buffer (pH 6.5) and incubated at 37 °C for 1 h. The reaction was stopped by adding 1.5 ml of 106/0.16 mM phenol–nitroprusside solution, which was immediately mixed with 1.5 ml of 125/11 mM alkaline-hypochlorite (sodium hypochlorite). The ammonia released would react with alkaline hypochlorite and phenol in the presence of a catalyst-sodium nitroprusside to produce indophenol (a blue color) and the concentration of

ammonia is directly proportional to the absorbance of indophenol read at 620 nm. Ammonium sulphate of 75µM was used as ammonium standard.

Protein concentration of the brain homogenate was measured by the method of Peterson (1977) with bovine serum albumin used as a standard. The value of ADA activity in the brain tissue was expressed as U/mg of protein. The estimation was performed out in triplicate and the mean was used for calculation.

2.7. DNA extraction and PCR detection of *T. evansi* in brains of rats

Three rats from each infected group (A and B) and controls (C1 and C2) were randomly selected to investigate the presence of *T. evansi* DNA in their brains by PCR. For this assay, cerebellum, cerebral cortex, striatum and hippocampus, removed using one sterile blade for each structure from each animal, was individually transferred to sterile tubes containing 0.5mL ethanol.

For preparation of DNA templates, a small section (0.4 x 0.4 mm) of each brain were removed, transferred to sterile tubes and washed three times (5 min. each) in bi-distillated water under shaker. Then, the tissues were cut in small segments, incubated with lysis buffer (1% SDS, 100 mM EDTA pH 8.0, 20 mM Tris-HCl, pH 8.0, and 350 mg/ml of proteinase K), at 37ºC for 18 h, centrifuged at 14,000 *g* for 5 min, and DNA purified using Wizard Purification Systems (Promega, USA). Purified DNA samples were used as templates for PCR amplifications of a spliced leader gene sequence using primers and reaction conditions previously described (Ventura et al., 2002). Amplified DNA fragments were resolved in 2% agarose gel, stained with ethidium bromide and visualized under U.V. light.

2.8. Statistical analysis

The data were summarized means and standard deviations analyzed by ANOVA followed by the Tukey's post-test ($P < 0.05$).

3. Results

3.1. Parasitemia, hematological parameters and clinical signs

Examination of the peripheral blood smears showed a prepatent period between 1-3 days PI. No difference in prepatent period between group A and B. The peak of parasitemia occurred on day 4 PI in group A (63 trypomastigotes per microscopic field), and irregular waves of parasitemia (0-3 trypomastigotes per microscopic field) were observed in group B (Figure 1). Decreased ($P < 0.05$) levels of erythrocyte count and hemoglobin were observed in rats of groups A and B, when compared to group C. Animals from group A showed a significant ($P < 0.05$) increase in the number of total leukocytes (Figure 2). Seven (7/10) rats of group A presented apathy, lethargy, disorientation and gasping at day 4 PI. Animals from group B did not show any clinical sign.

3.2. ADA activity in brain

No difference in ADA activity was detected in the cerebellum between infected and non-infected animals. A significant ($P < 0.05$) decrease occurred in cerebral cortex of acutely and chronically infected animals. In acutely infected rats, the activity was significantly ($P < 0.05$) reduced in hippocampus, but no alteration was observed in striatum. However, in chronically infected rats, ADA activity increased significantly ($P < 0.05$) in the hippocampus with a concomitant reduction ($P < 0.05$) in the striatum (Figure 3).

3.3. Detection of *T. evansi* in brain of rats using PCR assay

The PCR assays detected *T. evansi* DNA in brain parts of acutely and chronically infected rats. Tissue samples of control animals were all negative for the parasite. The PCR did not allow for parasite count and the intensity of amplified band could not be quantified.

4. Discussion

Variations in ADA activity occurred in brains of rats during infection by *T. evansi*, with respect to components of the brain (cerebral cortex, striatum and hippocampus) and severity of the disease (acute or chronic infection). Acutely infected animals with high levels of parasitemia showed neurological disturbances, but chronically infected ones with low parasitemia had no neurological signs.

The reduction in ADA activity in some brain regions (cerebral cortex, striatum and hippocampus) may have increased of adenosine levels in the brain. Adenosine plays an important regulatory role in neuronal activity and has neuroprotective actions in P1 purinoreceptor-mediated pathological conditions (Cunha and Ribeiro, 2000; Cunha, 2001). In addition, reduction in ADA activity could also contribute to limit inflammation and subsequent cellular damage (Abbracchio and Ceruti, 2007). Adenosine protects host cells from excessive tissue injury associated with strong inflammation, existing evidence that elevated level of this nucleoside potently down-regulates the activation of lymphocytes during inflammation, playing a regulatory role on dendritic cell immune responses (Desrosiers et al., 2007). Increased ADA in chronic infected animals may increase the severity of the lesion, because a decrease in brain adenosine can lead to damage of brain tissue.

Our evidence of different ADA activities among the regions of the brain corroborated with spatial activity of the enzyme, which correlates with mRNA expressions (Mackiewicz et al., 2000). Thus, ADA activity may play an important role in the mechanisms that control regional concentrations of adenosine in the brain, and the differences observed are likely to

have important physiological consequences. In experimentally infected horses, the severity of encephalomyelitis varied in different parts of the brain (Lemos et al., 2008).

Changes in ADA activities were associated with the presence of parasites in peripheral blood and brain, anemia and neurological signs. These data suggest that the presence of parasites may be primarily responsible for the reduced ADA activity in the brains of *T. evansi*-acutely infected rats (highly parasitemic). Based on our previous studies, rats that develop the acute infection invariable develop severe hematological and neurological disorders and died (Wolkmer et al., 2009). The neurological disturbances in *T. evansi*-infected hosts could be related to changes in ADA activity in the brains especially in the cerebral cortex and hippocampus (Mesulam et al., 2002).

In contrast to most mammalian cells, trypanosomatids are unable to engage in *de novo* purine synthesis and depend on the salvage pathway of nucleosides from their mammalian hosts. Studies have been done to identify targets for purine pathway inhibitors of *Leishmania* spp., *T. brucei, T. vivax* and *T. evansi* (Ogbunude and Ikediobi, 1983; De Koning et al., 1999; Suswam et al., 2003; Witola et al., 2004; Carter et al., 2008). Although some enzymes of purine salvage were detected in the bloodstream forms of *T. brucei*, *T. congolense* and *T. vivax*, homogenates of these trypanosomes apparently lacked adenosine deaminase (Ogbunude and Ikediobi, 1983). *T. brucei* and *T. cruzi* genomes include genes encoding putative ADA-like enzymes, but to date these enzymes have not been expressed nor was their function analyzed, as well as not disclosed by the first broad proteomic analysis of *T. evansi* (Roy et al., 2010).

In conclusion, *T. evansi* infection resulted in either the reduction or increase in the ADA activity in brain of rat. The alterations in ADA activity in the brain of infected rats may have implications for pathogenesis and neurological signs of the disease.

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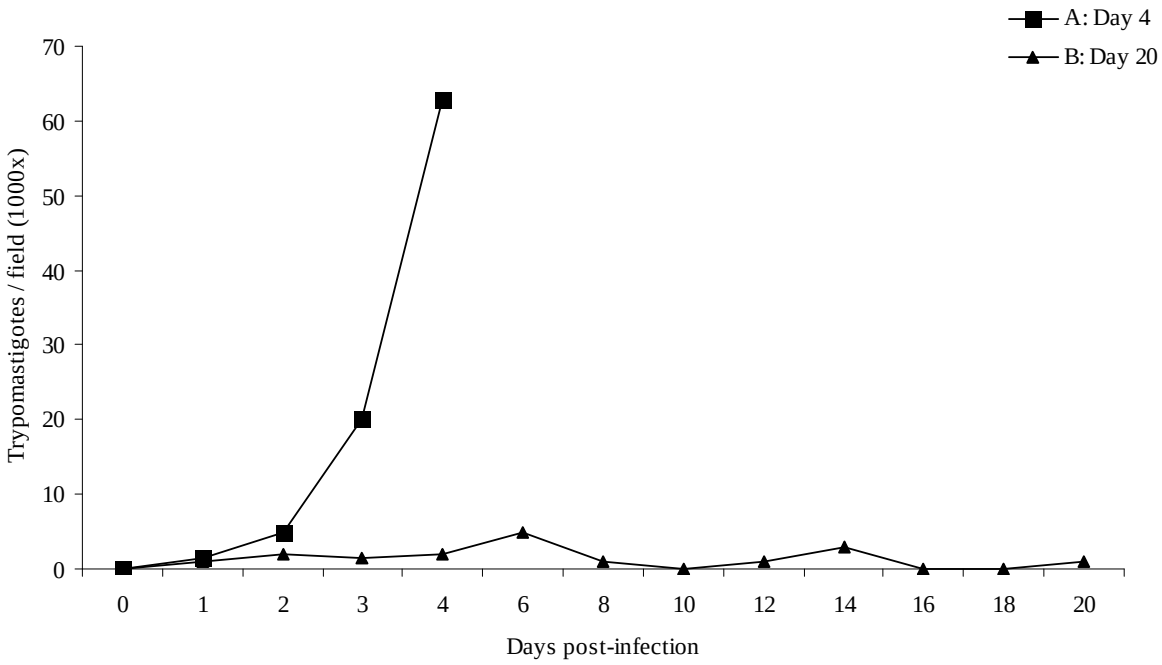

Figure 1: Parasitemia of *T. evansi*-infected rats with acute or chronic infections.

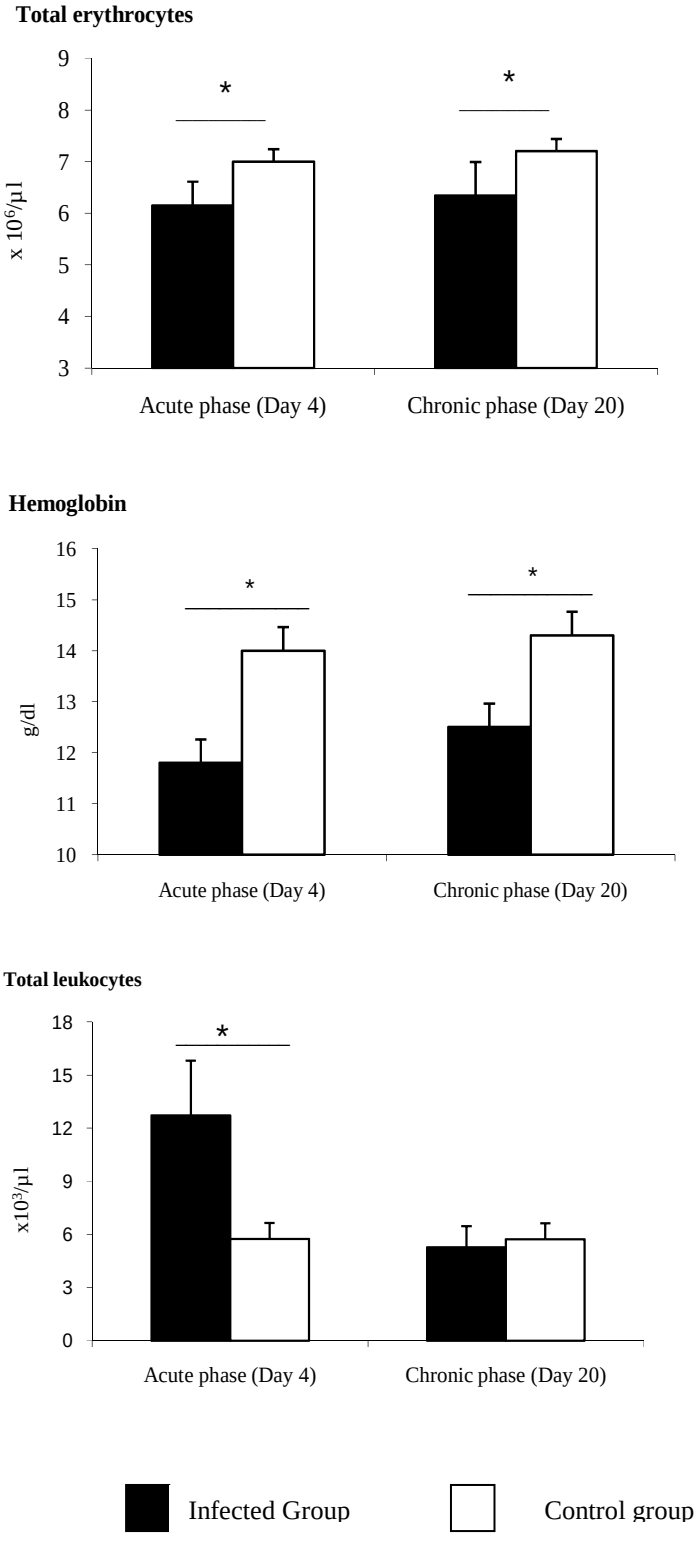

Figure 2: Total erythrocytes, hemoglobin concentration and total leukocytes of *T. evansi*- infected (days 4 and 20 post-infection) and non-infected rats.

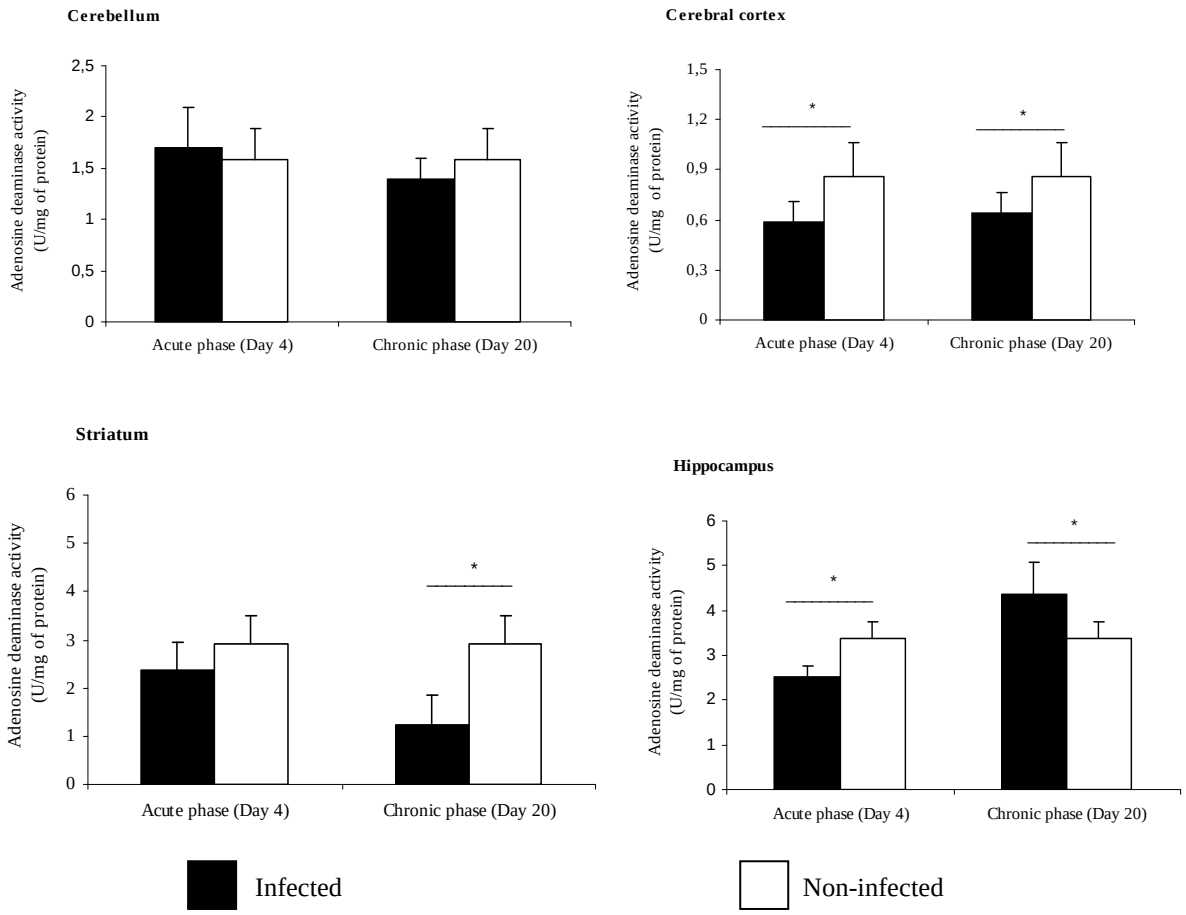

Figure 3: Adenosine deaminase activity in cerebellum, cerebral cortex, striatum and hippocampus of *T. evansi*-infected (days 4 and 20 post-infection) and non-infected rats.

3.4 – MANUSCRITO I

Influence of *Trypanosoma evansi* in adenine nucleotides and nucleoside concentration in serum and cerebral cortex of infected rats

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Influence of *Trypanosoma evansi* in adenine nucleotides and nucleoside concentration in serum and cerebral cortex of infected rats

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Abstract

This study aimed to evaluate the adenine nucleotides and nucleoside concentration in serum and cerebral cortex of rats infected with *Trypanosma evansi*. Each rat was intraperitoneally infected with $1x10^6$ trypomastigotes suspended in cryopreserved blood (Group A; n=18). Twelve animals were used as controls (Group B). The infected animals were monitored daily by blood smears. At days 4 and 20 post-infection (PI) it was collected serum and cerebral cortex to measure the levels of ATP, ADP, AMP and adenosine by high performance liquid chromatography (HPLC). In serum there was a significant ($P<0.05$) increase in the ATP, AMP and adenosine concentrations at days 4 and 20 PI in infected rats when compared to not-infected. Furthermore, in the cerebral cortex it was observed a significant ($P<0.05$) increase in the concentrations of ATP, AMP and decreased adenosine levels at day 4 PI. At day 20 PI it was only observed an increase in the AMP and adenosine concentrations in brain of infected rats when compared to not-infected. It was not observed any difference in ADP concentration in serum and brain at days 4 and 20 PI. No change was observed histologically in the cerebral cortex of infected animals. The results allow us to conclude that infection with *T. evansi* in rats causes an increase in the concentrations of ATP, AMP and adenosine in serum and cerebral cortex. These alterations occurred as a result of *T. evansi* pathogenesis which involves neurotransmission, neuromodulation and immune response impairment, as discussed in the manuscript.

Keywords: trypanosomosis, ATP, ADP, AMP, adenosine.

1. Introduction

Trypanosoma evansi is a digenetic flagellate implicated in the infection of a large number of domestic and wild animals (Dávila and Silva, 2000; Silva et al., 2002; Herrera et al., 2004) and rarely humans (Joshi et al., 2005). This parasite is the agent of trypanosomosis, a disease with broad distribution in Africa, Asia, and Latin America (Lun and Desser, 1995) and recently in Europe (Gutierrez et al., 2010). The trypomastigotes present in blood vessels of vertebrate hosts are transmitted by blood-sucking insects during feeding (Hoare, 1972).

T. evansi causes a devastating mammals disease characterized by anemia, thrombocytopenia, immunosuppression, emaciation, severe neurological signs, motor incoordination, paralysis of hind limbs and death of untreated animals (Silva *et al.* 2002; Berlin et al., 2009; Rodrigues et al., 2009). Rats (Menezes et al., 2004; Wolkmer et al., 2009; Tochetto et al. 2010) and horses (Berlin et al. 2009; Rodrigues et al. 2009) are highly susceptible to this disease, showing hematological, biochemical and pathological changes associated with ataxia, tremors, terminal coma and paralysis of hind limbs when untreated. According to the literature, locomotor clinical signs can be associated with inflammatory infiltrates, meningoencephalitis, edema, necrosis and demyelination in equines (Lemos et al., 2008; Rodrigues et al., 2009). In a recent study of our research group it has been suggested that changes in the activity of purinergic system (Da Silva et al., 2011a; 2011b; Oliveira et al., 2011a; 2011b) and cholinergic system enzymes (Da Silva et al., 2011c) could be involved in the pathogenesis of trypanosomiasis and exacerbate the clinical signs, hematological and inflammatory responses caused by *T. evansi*.

The purinergic system (consisting of enzymes, nucleotides, nucleosides and receptors) is involved in the regulation of several vital functions of mammals (Gödeche, 2008). The nucleotides ATP, ADP, AMP and the nucleoside adenosine are secreted by hematological and endothelial cells and used as mediators able to modulate the inflammation process, vascular

thrombosis, muscle contraction, neurotransmission and pain (Ralevic and Burnstock, 1998; Sitkovsky and Ohta, 2005; Sneddon et al., 1999; Burnstock, 2006; Desrosiers et al., 2007). The adenosine also acts as a central nervous system (CNS) modulator in mammals, regulates cell metabolism and triggers a variety of physiological effects participating in apoptosis, necrosis and cell proliferation (Rathbone et al., 1999). NTPDase family (ecto-diphosphohydrolase, apyrase or CD39) are responsible by hydrolyze ATP and ADP into AMP, while 5'-nucleotidase hydrolyses AMP to adenosine (Zimmermann, 1996). The adenosine deaminase (ADA) is responsible for the irreversible deamination of adenosine to inosine, closely regulating extracellular adenosine concentrations (Franco et al., 1997).

In a previous study by our research group it was found an increased activity of NTPDase and 5'nuclotidase (Oliveira et al., 2011b) and a decreased ADA activity in the cerebral cortex of rats infected with *T. evansi* (Da Silva et al., 2011b). Reduction in serum ADA activity was also detected (Da Silva et al., 2011a). Considering the functions of nucleotides and nucleosides for CNS and hematological cells, as well as the clinical alterations observed in trypanosomosis, this study aimed to evaluate the adenine nucleotides and nucleoside concentration in serum and cerebral cortex of rats infected with *T. evansi*, to confirm the results of enzymatic activity presented in other articles of the research group.

2. Material and methods

2.1. Experimental animals

Thirty outbreed male rats (mean age of 90 days) weighing 284 ± 12 g were kept in cages in an experimental room with controlled temperature (25ºC) and humidity (70%). The food (commercial ration) and water were disposed *ad libitum*. Previous to the experiment, animals were submitted to an adaptation period of 15 days. The procedure was approved by

the Animal Welfare Committee of Universidade Federal de Santa Maria (UFSM), number 52/2009.

2.2. Experimental design and trypanosome infection

Rats were divided into two groups as follows: group A was constituted by 18 Wistar rats inoculated with *T. evansi* strain and group B formed by 12 animals used as negative controls. A strain of *T. evansi* obtained from a naturally infected dog was used (Colpo et al., 2005), kept in liquid nitrogen at the laboratory. At day zero the parasites were thawed and the number of trypanosomes per mL was determined using a hemocytometer under microscope (Wolkmer et al., 2007). The animals from group A were inoculated intraperitoneally with cryopreserved blood (0.2 mL) containing $1x10^6$ trypomastigotes per animal. The control animals received 0.2 mL of sterile saline (0.9% NaCl) by the same route.

Both groups were divided into two subgroups each, organized according to the time of infection and degree of parasitemia. Two subgroups defined as controls (B4 and B20), composed by six non-inoculated animals each, and the infected subgroups (A4 and A20), inoculated with *T. evansi*, and set by six animals each. The animals were weighed at days 0, 4 and 20 PI.

2.3. Estimation of parasitemia

The presence and degree of parasitemia were determined daily for each animal by blood film examination. A drop of blood was collected from the tail, placed on a slide and a thin blood smear was prepared manually (Da Silva et al., 2006). The blood films were Romanovsky stained and then examined under a microscope, counting 10 fields at 1000x of magnification.

2.4. Collection of samples

The animals were anesthetized in a chamber with isoflurane for collection of blood by cardiac puncture (3mL) at days 4 (A4 and B4) and 20 (A20 and B20) post-infection. The

material collected was allocated in tubes without anticoagulant to obtain serum. Thereafter, animals were euthanized following recommendations of the Ethics Committee. The brains were carefully removed avoiding contamination with peripheral blood, and cerebral cortex was dissected. A portion of the cerebral cortex was used for biochemical analysis and a histopathological study.

2.5. Samples preparation

2.5.1. Serum

ATP and its breakdown products were extracted according to Furstenau *et al.* (2008). The denaturation of sample proteins was performed using 0.6mol/L perchloric acid. All samples were then centrifuged (14000×g for 10min) and the supernatants were neutralized with 4.0 N KOH and clarified with a second centrifugation (14000×g for 15 min) (Furstenau et al., 2008).

2.5.2. Cerebral cortex

ATP and its breakdown products were extracted according to Ryder (1985). Briefly, differents amounts of cortex were weighted and homogenized with 0.6 M perchloric acid at 0°C for 1 min with an Ultra-turrax homogenizer (model T 18, IKA® Works Inc., Wilmington, Del., U.S.A.). The homogenate was centrifuged at 2000×*g* for 10 min, and the supernatant was immediately neutralized to pH 6.5 to 6.8 with 1M potassium hydroxide.

2.6. Analysis of purines levels in serum and brain by high performance liquid chromatography (HPLC)

High performance liquid chromatography (HPLC) was performed with a Shimadzu (Kyoto, Japan) equipment composed of a model LC-20AT reciprocating pumps, a model DGU-20A5 degasser, a diode array detector (DAD) model SPD-M20A, auto-sampler (SIL-20A) and model CBM-20A integrator, operated by software LC Solution 1.22 SP1. Separation was achieved with a Phenomenex Synergi 4µ Fusion RP-80A column (150 x 4.60

mm, 4 µm) with precolumn, using 0.04 M potassium dihydrogen orthophosphate (KH_2PO_4) and 0.06 M dipotassium hydrogen orthophosphate (K_2HPO_4) as mobile phase A and acetonitrile as mobile phase B. A gradient elution was used according to the specifications of Scherer *et al.* (2005), at a flow rate of 0.7 mL/min. Mobile phases were filtered through a 0.45 µm Millipore filter prior to analysis, and all the reagent utilized were of HPLC grade. Purines in the samples (ATP, ADP, AMP and adenosine) were identified by their retention times and DAD spectrum (in the range 200-400 nm), and quantified by comparison of the peak's area with standards. The results ATP, ADP, AMP and adenosine in serum were expressed by nmol per L; and in brain were expressed by nmol per g of tissue.

2.7. Histopathology

Histopathologically, it was investigated a possible damage to the central nervous system of rats infected with *T. evansi*. From sagittal sections with an interval of 3 mm in region was a mounted slide of cerebral cortex. Slides were stained with hematoxylin and eosin.

2.8. Statistical analysis

The data were presented as means and standard deviations analyzed by student t-test ($P < 0.05$).

3. Results

3.1. Parasitemia and clinical course of infection

T. evansi could be detected in the blood of all infected rats from 24 to 48 h after inoculation. Parasitemia levels increased progressively in most animals until day 4 PI, when the first peak of parasitemia was observed (mean of 47 trypanosomes/field). In this first peak of parasitemia, six infected rats maintained a progressive quantitative increase in blood parasites and died between days 5–6 PI with high parasitemia (more than 200

trypanosomes/field). After day 5 PI, the remaining rats from subgroups A20 showed a reduction in parasitemia, which oscillated from 0 to 2 parasites/field until day 20 PI. The six rats which died were not used for nucleotides and nucleosides quantification.

At the time of sample collection, parasitemia of rats showed an average of 59 ± 9.7 trypanosomes/filed at day 4 PI (A4) and 1.6 ± 0.7 trypanosomes/field at day 20 PI (A20). Animals from subgroup A4 showed weight loss (mean 287.6g to 274.9g), disorientation and prostration. Rats from subgroup A20 also showed weight loss (282.1g to 277.3g). The animals from control group remained clinically healthy during the experimental period.

3.2. Purines levels in serum

At day 4 PI it was observed a significant ($P<0.05$) increase in ATP (40%), AMP (113%) and adenosine (54%) concentrations in serum of infected rats when compared to not-infected. Similarly, at day 20 PI it was also observed an increased ATP (80%), AMP (61%) and adenosine (481%) concentrations in serum of infected rats when compared to not-infected (Figure 1). Concentration of ADP in serum of rats was not altered between groups ($P>0.05$; Figure 1b).

3.3. Purines levels in cerebral cortex

At day 4 PI it was observed a significant ($P<0.05$) increase in both ATP (48%) and AMP (44%) concentrations, while adenosine level was decreased (29%) in cerebral cortex of infected rats when compared to not-infected (Figure 2). At day 20 PI it was observed an increase in AMP (33%) and adenosine (36%) concentrations in cerebral cortex of infected rats when compared to not-infected (Figure 2). Regarding the ATP levels, no difference was observed between groups in day 20 PI ($P>0.05$). Concentrations of ADP in cerebral cortex had no variation between groups ($P>0.05$; Figure 2b).

3.4. Histology

In subgroups A4 and A20 were not observed histological changes that give evidence to damage in the cerebral cortex, as well as in group B.

4. Discussion

Increased NTPDase activity for the substrates ATP and ADP was observed in the brain and reduced in platelets of infected rats with *T. evansi* at day 5 PI (Oliveira et al., 2011a; 2011b). In this study, we found an increased ATP concentration in serum and cerebral cortex, unlike the levels of ADP which did not change between groups. The increase in ATP level may be related to the inflammatory response and neurotoxicity, once it is an important neutransmitter (Edwards et al., 1992; Agresti et al., 2005). According with Oliveira et al. (2011b) the increased enzymatic activity may be associated with the elevated release of ATP, which promotes an increase in the levels of intracellular calcium mediated by P2X receptors, and this event could represent a significant damage to the cells (Edwards *et al.* 1992). Then the increase in ATP level may cause the neurological alterations observed in infected rats (Wolkmer et al., 2009; Oliveira et al., 2011b), because ATP could lead to excitotoxicity by excitatory neurotransmitters release, such as glutamate (Lima et al., 2007). At day 15 PI, Oliveira et al., (2011b) observed that a decrease in NTPDase activity may have a compensatory effect in order to increase the concentrations of neurotransmitter (ATP) in the brain of rats infected with *T. evansi*, but this was not confirmed in this study with 20 days PI, because ATP level was not changed in the brain, unlike the serum concentration of the nucleotide which was significantly increased.

In this study, no change was observed in ADP levels in serum and cerebral cortex, although there were changes in enzymatic activity in rats infected with *T. evansi*, as well as by increasing the NTPDase activity in the brain in day 5 PI and platelets in day 15 PI (Oliveira et al., 2011a; 2011b). At 5 days PI there was a decrease in NTPDase activity in platelets

(Oliveira et al., 2011a). The ADP nucleotide, which is primarily released by platelets (Lee et al., 1998), is mainly related to thrombocytopenia and platelet aggregation (Lunkes *et al.* 2004). However, in *T. evansi* infection occurred a severe decrease in platelets count (Oliveira et al., 2011a), which could lead to reduction in ADP concentration. However, this was not verified in this study, since the concentration of ADP was normal, probably due to an increased release of this nucleotide by platelets, as a compensatory effect of coagulation disorders.

The AMP concentration in both serum and brain increased significantly in rats infected with *T. evansi* at days 4 and 20 PI, because the activation in the enzymatic cascade for hydrolysis of ATP and ADP to AMP was identified by increased ectonucleotidases activity (Oliveira et al., 2011a; 2011b). A previous study of our research group presented this hypothesis, since there was an increased 5'-nucleotidase activity and consequently increased AMP hydrolysis to adenosine, as observed in this study. At day 4 PI, despite the increased activity of 5'-nucleotidase in the brain as previously described by Oliveira et al. (2011b) and high concentration of AMP observed in this study, it was observed a decreased adenosine level in cerebral cortex, probably due the increased requirement of this nucleoside during infection, once adenosine is an important neuromodulador. Another hypothesis for the reduction of adenosine at day 4 PI would be high parasitemia and severe deamination of adenosine to inosine by ADA present in *T. evansi* according to Da Silva et al. (2011d).

In recent studies it was observed a reduction in ADA activity in serum, erythrocytes, lymphocytes and cerebral cortex of rats infected with *T. evansi* compared to healthy rats after 4 days PI (Da Silva et al., 2011a; 2011b). According to the study aforementioned, the reduction in ADA activity would have caused an increase in the extracellular concentrations of adenosine, which would be converted to inosine. In this study it was confirmed that indeed there was an increase in the concentration of adenosine in serum as suggested

by Da Silva et al. (2011a), but in cerebral cortex there was a reduction in adenosine levels. According with literature, the increase of adenosine acts as a sensor and provides information to the immune system about tissue injuries or acute inflammatory changes occurring in the vicinity of the immune system (Kumar and Sharma, 2009). The interaction of adenosine with adenosine receptors may promote anti-inflammatory effects, because it causes the inhibition of Th1 immune response attenuating inflammation and tissue damage (Xaus et al., 1999).

Adenosine plays an important regulatory role in neuronal activity and has neuroprotective actions in P1 purinoreceptor-mediated pathological conditions (Cunha and Ribeiro, 2000; Cunha, 2001). Therefore, the reduction in cerebral cortex adenosine levels after 4 days PI could be the cause of neurological disorders observed in rats infected with *T. evansi* (Wolkmer et al., 2009; Tochetto et al., 2010; Da Silva et al., 2011b), once histological lesions in brain are not observed in infected rats (Oliveira et al., 2011b), reconfirmed in this study. At day 20 PI, the concentration of adenosine increased in serum and brain, probably in the inflammatory response against the parasite and compensatory effects, because as mentioned previously adenosine may inhibit the immune response and reduce cell and tissue damage caused by inflammation.

The results allow us to conclude that infection with *T. evansi* in rats causes an increase in the concentrations of ATP, AMP and adenosine in serum and cerebral cortex. This increase in nucleotides and nucleosides levels associated with increased activities of ectonuclotidases and ADA cause the activation of enzyme cascade with hydrolysis of ATP and ADP to AMP; and AMP to adenosine. This nucleoside acts as an anti-inflammatory and neuromodulator signaling molecule, in addition to other actions already mentioned in this manuscript. The data show that increasing the activity of NTPDase and 5'nucleotidase consequently increase the concentrations of ATP and AMP, and the reduction of ADA activity was designed to increase the concentration of adenosine in accordance with literature.

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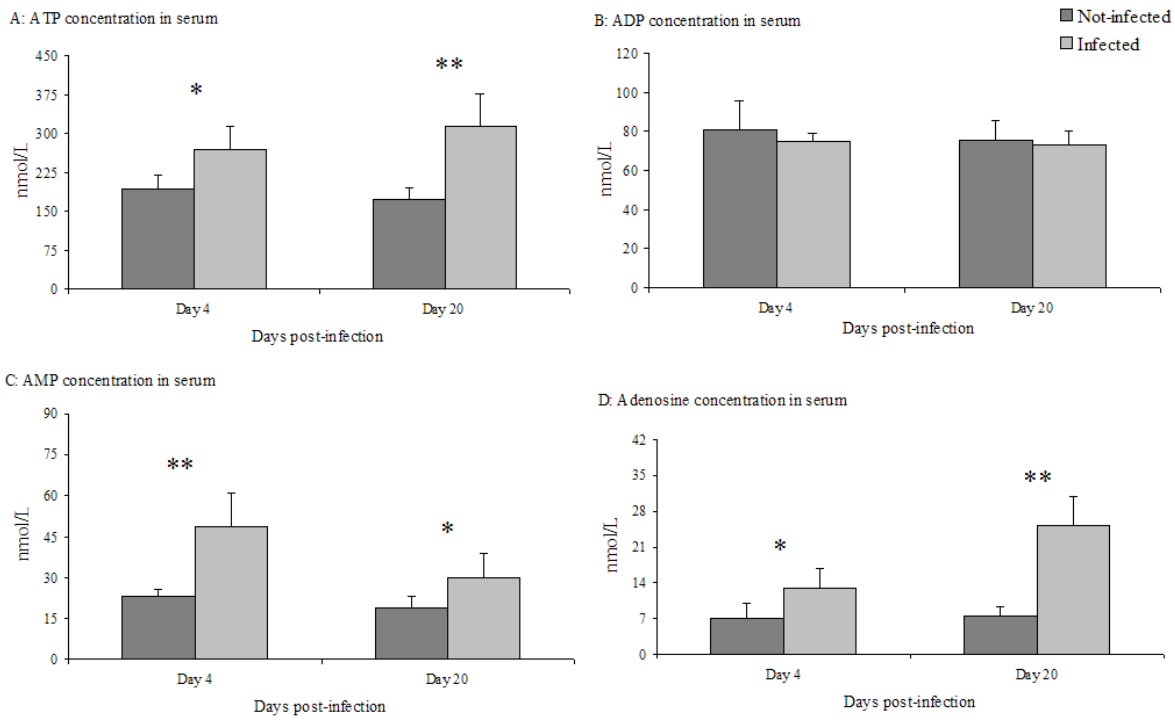

Fig. 1: Concentration of ATP (A), ADP (B), AMP (C) and adenosine (D) in serum of rats infected with *Trypanosoma evansi* (Day 4 and 20 PI) compared to not-infected (n=6). (*$p<0.05$; **$p<0.01$)

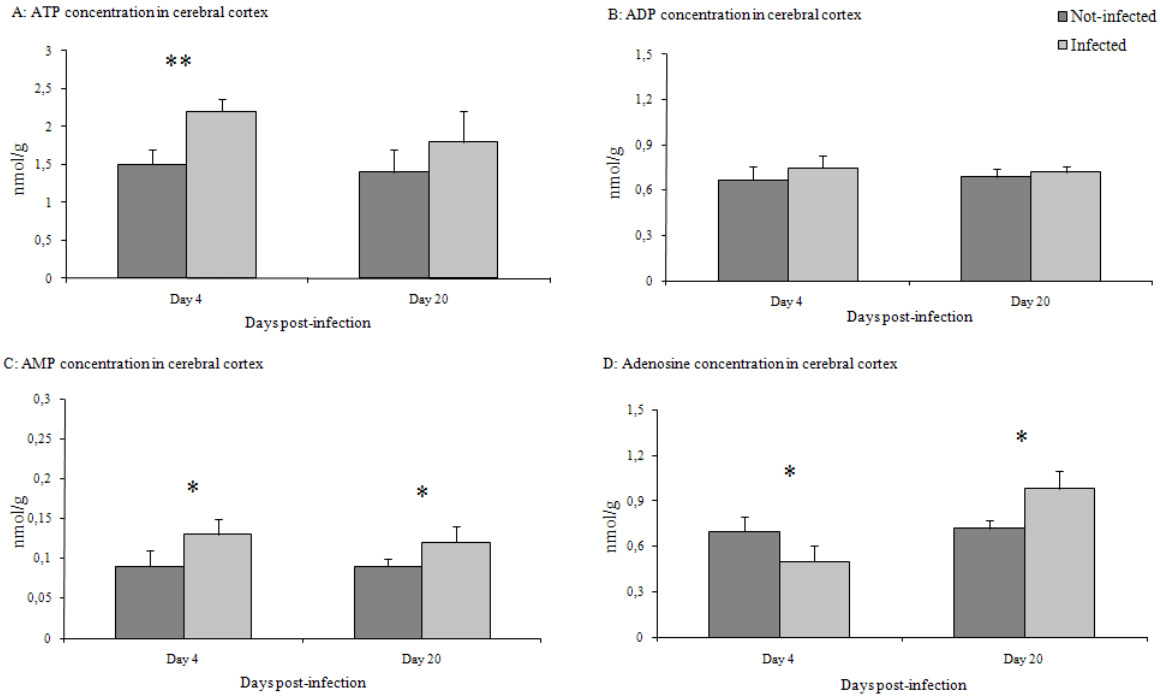

Fig. 2: Concentration of ATP (A), ADP (B), AMP (C) and adenosine (D) levels in cerebral cortex of rats infected with *Trypanosoma evansi* (Day 4 and 20 PI) compared to not-infected (n=6). (*p<0.05; **p<0.01).

4 DISCUSSÃO

Nos últimos anos nosso grupo de pesquisa tem descrito diferentes casos de infecção natural por *T. evansi* em bovinos, equinos e cães (DA SILVA et al., 2007; 2008; ZANETTE et al., 2008) e investigado, experimentalmente a patogenia da doença em gatos (DA SILVA et al., 2010) e, em ratos, como relatado nesta tese (Artigos: A-II e A-III; manuscrito: M-I). Ratos infectados com *T. evansi* desenvolvem anemia, trombocitopenia, sinais neurológicos e paralisia de membros pélvicos (DA SILVA et al., 2010; TOCHETTO et al., 2010), similar ao que ocorre em equinos (SILVA et al., 1995).

Na patogenia da anemia por *T. evansi*, discute-se ter causas multifatoriais. Wolkmer et al. (2009) concluiu que a peroxidação lipídica, causava a fragilidade da membrana eritrocitária, levando à lise das hemácias. Em um estudo recente, Paim et al. (2011a) observou que a infecção pelo flagelado causa um aumento significativo das citocinas pró-inflamatórias no soro, e esta resposta imunológica afetaria a produção de células vermelhas. Conforme os autores, o aumento das citocinas poderia contribuir em 24% para a anemia observada na tripanossomose em ratos. A redução de acetilcolinesterase no sangue também pode contribuir para a anemia (WOLKMER et al., 2010), pois esta enzima desempenha funções importantes na superfície do eritrócito.

Neste estudo, foi envestigado o envolvimento do sistema purinérgico na anemia, para isso foi mensurado a atividade da ADA no eritrócito. A alteração na atividade da ADA pode estar envolvida em caso de anemia hemolítica (VALENTINE et al., 1977). A redução na atividade da ADA em eritrócitos foi relacionada à diminuição de hematócrito (Artigo II). Para confirmar esta relação, seria necessário analisar a expressão da ectoenzima na superfície das células vermelhas do sangue. As hemáceas são relativamente bem supridas de ADA, portanto estas células quando danificadas liberam quantidades significativas de ADA (MURAOKA et al., 1990). Com base nessas informações, a hipótese seria que a atividade da ADA aumenta no soro e/ou plasma, como consequência da diminuição dos glóbulos vermelhos devido a um processo hemolítico (JACKSON et al., 1996). No entanto, isso não foi observado no Artigo II, já que a atividade da ADA estava reduzida no soro. Embora, a correlação positiva entre hematócrito e atividade da ADA em eritrócitos nada é conclusivo, portanto, mais estudos são necessários para confirmar o papel da ADA na anemia causada por *T. evansi* em ratos.

Distúrbios de coagulação são comumente observados na tripanossomose por *T. evansi*.

O envolvimento do sistema purinérgico na hemostasia já está bem documentado, pois são as plaquetas as grandes responsáveis pela secreção de nucleotídeos que desempenham funções relacionadas à neurotransmissão (ATP), ativação da agregação plaquetária (ADP) e inibição da agregação (adenosina). Em um estudo específico em plaquetas, Oliveira et al. (2011a) observou que a hidrólise de ATP, ADP e AMP e desaminação da adenosina foram alterados nas células de ratos infectados com *T. evansi*. Segundo o autor, durante o período de infecção, a diminuição da atividade enzimática pode estar relacionada à trombocitopenia. Alterações nas atividades dessas enzimas podem implicar na fisiopatologia da tripanossomose.

A infecção por *T. evansi* estimula a resposta imunológica, levando ao aumento de imunoglogulinas (GRESSLER et al., 2010), citocinas (PAIM et al., 2011a) e proteínas de fase aguda (COSTA et al., 2010). No hemograma, observou-se um aumento no número de leucócitos totais em decorrência da linfocitose. Em um estudo recente conduzido pelo nosso grupo de pesquisa (DA SILVA et al, 2011a,b), constatou-se que durante a fase aguda da doença ocorreu um aumento da atividade da enzima AChE nos linfócitos, o que levaria, consequentemente a um aumento na hidrólise de ACh, que tem ação anti-inflamatória, por inibir a produção de mediadores inflamatórios, reduzindo os danos celulares e teciduais durante a infecção. Neste estudo, verificamos que a ADA nos linfócitos desempenha função similar, pois ocorreu redução na atividade da ADA a fim de aumentar as concentrações extracelulares de adenosina, nucleosídeo que tem ação anti-inflamatória como discutido detalhadamente no Artigo II. Conforme a literatura, a redução da atividade de ADA em linfócitos levaria a interação da adenosina com receptores purinérgicos que existem em muitos tipos de células, levando a efeitos anti-inflamatórios, entre eles a inibição da resposta imune Th1. Na infecção aguda causada por *T. cruzi*, há uma predominância de Th1 e resposta celular com produção de interferon-γ (KUMAR; TARLETON, 2001), assim como na infecção por *T. evansi* relatada recentemente (PAIM et al., 2011a). Portanto, a inibição dessa resposta pela ação da adenosina extracelular em receptores purinérgicos poderia atenuar a inflamação e os danos teciduais.

Os distúrbios neurológicos são relatados em cavalos, camelos, búfalos, bovinos, veados e gatos infectados por *T. evansi* (TUNTASUVAN et al., 1997; 2000; RODRIGUES et al., 2005; BERLIN et al., 2009; DA SILVA et al., 2010). As lesões cerebrais foram relatadas em bovinos e equinos (TUNTASUVAN et al., 1997; RODRIGUES et al., 2005). Ratos infectados com o parasito e apresentando sinais neurológicos na fase aguda da doença não apresentaram lesão histológica no SNC (OLIVEIRA et al., 2011a). Ao contrário, na fase crônica os ratos apresentaram paralisia de membros pélvicos. Histologicamente, estes animais

apresentaram infiltrado inflamatório no encéfalo e lesões nos músculos pélvicos como miosite, degeneração valeriana, atrofia das fibras e infiltrados inflamatórios (DA SILVA et al., in press). Com base nestas informações, pode-se sugerir que os sinais clínicos observados na fase aguda são causados por alterações nas concentrações de neurotransmissores (ATP e ACh) e neuromodulador (adenosina) em animais infectados com *T. evansi*, como apresentado no Artigo III. Outro elemento importante na neurotransmissão é o óxido nítrico, que em ratos infectados com *T. evansi* encontrou-se elevado em diferentes regiões do encéfalo (PAIM et al., 2011b). Conforme, a literatura, o excesso de óxido nítrico no SNC pode levar a citotoxidade, causando lesões histológicas em células nervosas, o que não foi verificado no manuscrito I.

Como mencionado anteriormente, ratos são altamente suscetíveis à tripanossomose, apresentando alterações hematológicas, bioquímicas e patológicas associadas com ataxia, tremores e coma terminal de animais não tratados (MENEZES et al., 2004; WOLKMER et al., 2009). A infecção humana por *T. evansi* foi relatada pela primeira vez em 2005, em um agricultor indiano que mostrou alterações comportamentais, tais como: desorientação, ataxia e déficits sensoriais (JOSHI et al., 2005). Relacionando estas alterações com nosso estudo (Artigo III), verificamos que a redução da atividade da ADA em algumas regiões do cérebro (córtex cerebral, estriado e hipocampo) poderia ter aumentado os níveis de adenosina, porém isso não ocorreu. Nos ratos com sinais neurológicos, na fase aguda, observamos redução na concentração de adenosina (Manuscrito I). Então, como a adenosina desempenha um importante papel de regulador da atividade neuronal e tem ações neuroprotetoras em P1 purinoreceptor mediada por condições patológicas (CUNHA; RIBEIRO, 2000; CUNHA, 2001), sua deficiência poderia causar os distúrbios neurológicos já observados e relatados em infecções por este parasito.

A enzima ADA é amplamente distribuída em tecidos e fluídos de mamíferos em duas isoformas, ADA1 e ADA2. Em células teciduais, principalmente sistema nervoso predomina a isoforma ADA1 e a isoforma de ADA2 é encontrado no soro e na superfície de células sanguíneas. Uma expressão heterogênea da atividade da ADA pode ser encontrada entre as células periféricas e, até mesmo, dentro das distintas regiões do SNC em uma mesma condição patológica (FRANCO et al, 1986, 1997). Nestes estudos (Artigos II e III), foi observada heterogenidade na atividade da ADA no soro, linfócitos, eritrócitos, plaquetas que predomina ADA2 e regiões do encéfalo que predomina ADA1. Estas diferenças na atividade enzimática podem ser explicadas pelas duas isoformas da ADA, e também, pela diferentes funções que a ADA desempenha nas células e tecidos.

No Artigo I tivemos como objetivo de investigar a presença de ADA em *T. evansi*, uma enzima importante para muitas funções vitais nos mamíferos e, possivelmente para o parasito também. Esta enzima já havia sido detectada no genoma de *T. brucei*, assim como foi detectado no *T. evansi* por técnicas bioquímicas. A ideia de investigar a presença desta enzima no parasito surgiu no momento que foi detectado o *T. evansi* no encéfalo dos ratos infectados, pela técnica de PCR (Artigo III). Como as análises bioquímicas são realizadas a partir de homogenizado da estrutura cerebral, possivelmente a ADA do parasito poderia ser detectada no teste bioquímico juntamente com a ADA do hospedeiro. No entanto, na maioria das estruturas dos animais infectados foi verificado redução da atividade da ADA.

Em um dos nossos estudos foi verificado um aumento da atividade da NTPDase e 5'-nucleotidase (OLIVEIRA et al., 2011a) e uma redução na atividade da ADA no córtex cerebral de ratos infectados com *T. evansi* (Artigo III); e redução e/ou aumento da atividade da ADA no soro, eritrócitos e linfócitos (Artigo II). Considerando que estas enzimas são responsáveis pela regulação da concencentração de nucleotídeos e nucleosídeos de adenina no SNC e em células hematológicas, outro estudo conduzido pelo nosso grupo de pesquisa teve a finalidade de mensurar os níveis de ATP, ADP, AMP e adenosina no soro e encéfalo de ratos infectados com o parasito (Manuscrito I).

Conforme mencionado anteriormente, a atividade da enzima NTPDase aumentou para os substratos ATP e ADP no cérebro (OLIVEIRA et al., 2011a) e reduziu em plaquetas (OLIVEIRA et al., 2011b) de ratos infectados com *T. evansi* no dia 5 PI. No manuscrito I, foi observado um aumento na concentração de ATP no córtex cerebral e no soro, ao contrário dos níveis de ADP que não alterou entre os grupos. O aumento do ATP pode estar relacionado com a resposta inflamatória e neurotoxicidade, devido ao fato de ser um importante neutransmissor (EDWARDS et al., 1992; AGRESTI et al., 2005). O aumento da atividade enzimática pode estar associado à elevada liberação de ATP, que promove um aumento nos níveis de cálcio intracelular mediada por receptores P2X, e esse evento poderia representar um prejuízo significativo para as células (EDWARDS et al., 1992). Então, o aumento no nível de ATP pode causar as alterações neurológicas observadas em ratos infectados (WOLKMER et al., 2009; OLIVEIRA et al., 2011a), porque o ATP pode levar a excitotoxicidade por liberação de neurotransmissores excitatórios, como o glutamato (LIMA et al., 2007). No dia 15 PI, pesquisadores observaram uma diminuição na atividade da NTPDase explicada como um efeito compensatório (OLIVEIRA et al., 2011a), a fim de aumentar a concentração do neurotransmissor (ATP) no cérebro de ratos infectados com *T. evansi*. Esse fato não foi confirmado com 20 dias PI (Manuscrito I), porque os níveis de ATP não diferiram no cérebro,

ao contrário da concentração sérica do nucleotídeo que foi significativamente elevada.

Nenhuma mudança foi observada nos níveis de ADP no córtex cerebral e soro (Manuscrito I), embora haja alteração na atividade enzimática dos ratos infectados com *T. evansi*, como o aumento da atividade da NTPDase no encéfalo (OLIVEIRA et al., 2011a) e plaquetas (OLIVEIRA et al., 2011b) no dia 5 e 15 PI, respectivamente. Já no dia 5 PI, houve uma diminuição na atividade da NTPDase em plaquetas (OLIVEIRA et al., 2011b). O ADP está relacionada principalmente à trombocitopenia e agregação plaquetária (LUNKER et al., 2004), sendo que o ADP é secretado principalmente pelas plaquetas (LEE et al., 1998). No entanto, na infecção por *T. evansi* ocorreu severa redução de plaquetas (OLIVEIRA et al., 2011b), o que poderia levar à redução na concentração de ADP. Porém verificou-se que a concentração de ADP foi similar ao grupo controle, provavelmente devido a um aumento da secreção destes nucleotídeos por plaquetas, como resposta aos distúrbios de coagulação.

A concentração de AMP no soro e no cérebro aumentou significativamente em ratos infectados com *T. evansi* no dia 4 e 20 PI (Manuscrito I), isso poderia ser explicado pela ativação da cascata enzimática na hidrólise de ATP e ADP para AMP, já que houve um aumento na atividade das ectonucleotidases (OLIVEIRA et al., 2011a,b). Na sequência da cascata, o aumento na atividade da enzima 5'-nucleotidase, gera consequentemente um aumentou na hidrólise de AMP para adenosina, como observado no Manuscrito I. No dia 4 PI, apesar do aumento da atividade da enzima 5'-nucleotidase no córtex cerebral previamente descrito por Oliveira et al. (2010a) e alta concentração de AMP descrita no Manuscrito I, observamos que a redução dos níveis de adenosina no córtex cerebral, provavelmente ocorreu devido a uma maior exigência deste nucleosídeo durante a infecção, já que a adenosina é um neuromodulador importante. Outra hipótese para a redução da adenosina no dia 4 PI, seria a elevada parasitemia, a qual proporcionaria uma maior degradação de adenosina em inosina pela ADA presentes no *T. evansi*, uma enzima que foi detectada neste estudo (Artigo I).

No Manucrito II foi relatada uma redução na atividade da ADA no soro, eritrócitos, linfócitos e córtex cerebral de ratos infectados com *T. evansi* em comparação com ratos saudáveis no dia 4 PI. Segundo o estudo, a redução da atividade da ADA ocorreu devido ao aumento na concentração extracelular de adenosina, a qual seria convertido em inosina. No Manuscrito I foi confirmado que realmente houve um aumento na concentração de adenosina no soro como sugerido no Artigo II. Já no córtex cerebral, houve uma redução nos níveis de adenosina (Manuncrito I). Conforme a literatura, o aumento de adenosina pode ser um sensor, fornecendo informações para o sistema imunológico sobre o dano tecidual ou alterações inflamatórias agudas (KUMAR; SHARMA, 2009). A interação da adenosina a receptores de

adenosina pode ter um efeito anti-inflamatório, levando à inibição da resposta imune mediada por Th1, e reduzindo o processo inflamatório e os danos teciduais (XAUS et al., 1999).

Como já mencionado, a adenosina desempenha um importante papel regulador na atividade neuronal. Portanto, a redução dos níveis de adenosina no córtex cerebral no dia 4 PI (Manuscrito I) poderia ser a causa dos distúrbios neurológicos observados em ratos infectados com *T. evansi* (WOLKMER et al., 2009; TOCHETTO et al., 2010; OLIVEIRA et al., 2011a), já que lesões histológicas no cérebro não são observados em ratos infectados (OLIVEIRA et al., 2011a). No dia 20 PI, a concentração de adenosina no cérebro e soro aumentou, provavelmente devido à adenosina ser capaz de inibir a resposta imune, e assim reduzir dano celular e tecidual devido ao processo inflamatório.

5 CONCLUSÃO

Neste estudo concluiu-se que o *Trypanosoma evansi* apresenta em sua composição química a enzima ADA, que deve ser responsável pela desaminação de adenosina em inosina no parasito, similar ao que ocorre nos mamíferos;

O estudo com ratos infectados, experimentalmente, com *T. evansi* permitiu elaborar algumas conclusões relacionas ao sistema purinérgico, descritas a seguir: (1) a redução na atividade ADA nos eritrócitos pode estar relacionada à patogenia da anemia nas tripanossomoses; (2) a redução da atividade da ADA no soro, eritrócitos e linfócitos ocorreu com a finalidade de aumentar as concentrações de adenosina extracelular, que tem caráter anti-inflamatório a fim de minimizar o processo inflamatório e danos teciduais causado pela infecção.

Na análise dos nucleotídeos e nucleosídeo também possibilitou fazer conclusões como: (1) o aumento de ATP e redução de adenosina no córtex cerebral pode ser responsável pelos sinais neurológicos observados na fase aguda da doença; (2) a redução da atividade da ADA no encéfalo ocorreu para aumentar as concentrações de adenosina, fundamental para a neuromudulação durante o parasitismo.

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ANEXOS

ANEXO I – Artigo I

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Research Brief

Biochemical detection of adenosine deaminase in *Trypanosoma evansi*

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ABSTRACT

Biochemical and molecular research on parasites has increased considerably in trypanosomes in the recent years. Many of them have the purpose of identify areas, proteins and structures of the parasite which are vulnerable and could be used in therapy against the protozoan. Based on this hypothesis this study aimed to detect biochemically the enzyme adenosine deaminase (ADA) in *Trypanosoma evansi*, and to adapt an assay to the measurement of its activity in trypomastigotes. Firstly, the parasites were separated from the blood of mice experimentally infected with a DEAE-cellulose column. The ADA activity in trypomastigotes was evaluated at concentrations of 0.1, 0.2, 0.5, 0.6 and 0.8 mg of protein by spectrophotometry. ADA activity was observed in the parasites at all concentrations tested and its activity was proportional to the concentration of protein, ranging between 0.64 and 2.24 U/L in the lowest and highest concentration of protein, respectively. Therefore, it is possible to detect biochemically ADA in *T. evansi*, an enzyme that may be associated with vital functions of the parasite, similar to what occurs in mammals. This knowledge may be useful in the association of the chemotherapic treatment with specific inhibitors of the enzyme, in future studies.

1. Introduction

Flagellates of genus *Trypanosoma* are ubiquitous parasites and infect a wide range of vertebrate hosts, resulting in immeasurable economic losses (Dobson et al., 2009). *Trypanosoma evansi* is the most widely distributed of the pathogenic African animal trypanosomes, affecting domestic livestock and wildlife in Asia, Africa and Latin America (Luckins and Dwinger, 2004). The parasite is transmitted mechanically by hematophagous flies such as *Tabanus* and *Stomoxys* spp. and/or vampire bats (Hoare, 1972). The main affected animals are horses, camels and dogs, but a large number of species may be parasitized. The animals showed typical clinical signs such as anemia, weight loss and locomotive disturbance (Hoare, 1972; Maudlin et al., 2004).

T. evansi is classified as monomorphic and is represented by trypomastigotes found in the bloodstream in the lanced shape, elongated body and flat. The parasite presents free flagellum, undulating and well developed membrane, the sub-terminal portion kinetoplast or marginal body and a core (Hoare, 1972; Maudlin et al., 2004). In the recent years, a wide variety of biochemical and molecular researches have been developed in the field of trypanosomosis, such as molecular identification and phylogenetic analysis of parasites (Amer et al., 2011), regulation of calcium concentration (Docampo and Moreno, 1996) and the detection of enzymes such as acetylcholinesterase in *T. evansi* (Mijares et al., 2011). In *Trypanosoma brucei*, authors demonstrated that purine transport activities are differentially regulated in the lifecycle stages of parasite, and mediate uptake of purine nucleosides and in some cases the nucleobase, hypoxanthine (Sanchez et al., 2002). Other researchers reported the existence of two distinct adenosine transport systems in *T. evansi* (Suswam et al., 2001, 2003). According with the authors, this fact is related with the resistance to the melaminophenyl arsenical drug. These tools help to elucidate the relationships among different species and subspecies and their potency of virulence and pathogenesis (Morrison et al., 2007).

Recently, our research group reported alterations in the activity of the enzyme adenosine deaminase (ADA: EC 3.5.4.4) in serum, cells (lymphocytes and erythrocytes) and brain of rats infected with *T. evansi* (Da Silva et al., 2011a,b). ADA is considered to be a key enzyme in the purine metabolism, catalyzing the irreversible deamination of adenosine and deoxyadenosine to inosine and deoxyinosine, respectively, and closely regulating extracellular

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adenosine and inosine concentrations in mammals (Franco et al., 1997). Furthermore, adenosine is a CNS modulator, regulates cell metabolism and triggers a variety of physiological effects participating in apoptosis, necrosis, cell proliferation, and modulating the release of the neurotransmitters and tropic factors (Hasko and Cronstein, 2004; Sitkovsky and Ohta, 2005; Desrosiers et al., 2007).

Adenosine and inosine has already been restored in *T. evansi* and is involved in various vital functions of the parasite (Suswam et al., 2001, 2003). In this study we aimed to investigate the presence of ADA in *T. evansi* as well as to adapt a technique to measure the activity of ADA in the parasite.

2. Material and methods

For this experiment, it was utilized a strain of *T. evansi* obtained from a naturally infected dog (Colpo et al., 2005). Isolates were kept in liquid nitrogen according to the methodology described by Silva et al. (2003). The procedure was approved by the Animal Welfare Committee of Federal University de Santa Maria (UFSM), number 23081.012513/2009-52.

The trypomastigotes were obtained by inoculation of two mice with 0.2 mL of cryopreserved blood (Silva et al., 2003) containing 10^6 parasites per microliter, intraperitoneally. Subsequently, the parasitemia was estimated daily by microscopic examination of smears. Each slide was mounted with blood collected from the tail vein, stained by the panoptic method, and visualized by optical microscopy. After the 5th day of infection the parasitemia was over 100 trypanosomes per microscopic field (1000×). At this time the animals were anesthetized with isoflurane for blood collection, stored in tubes with anticoagulant (EDTA 10%).

The volume of 3 mL collected from both mice was eluted with PBS buffer containing 1% glucose (PSG – 1v/v). Thereafter, the separation of trypomastigotes forms by chromatography was performed on a Poly-Prep® column (Bio-Rad Laboratories, Hercules, USA) using the DEAE-cellulose resin, according to the technique described by Tavares et al. (2011). The number of parasites purified was measured by counting in a Neubauer chamber. In order to concentrate the number of parasites as a pellet, the purified samples (2 mL of PSG + parasite) were centrifuged for 30 min (14,000*g* at 4 °C). After the *T. evansi* (1×10^9 trypomastigotes per mL) was obtained it was stored in microtubes and kept frozen at −20 °C until analyses.

On the day of analysis, the pelleted trypanosomes were resuspended with 50 mmol/L per mM phosphate buffer, pH 6.5. Then the protein concentration of the trypomastigotes was measured by the method of Peterson (1977) with bovine serum albumin used as a standard. The concentration of proteins in the parasite was expressed in mg mL^{-1}.

To measure the ADA activity in the parasites it was adapted the technique used to evaluate the ADA activity in lymphocytes. ADA activity was measured spectrophotometrically in trypomastigotes forms of *T. evansi* by the method of Giusti (1974) modified. The reaction was started by the addition of the substrate (adenosine) to a final concentration of 21 mmol/L and incubations were carried out for 1 h at 37 °C. The reaction was stopped by adding 106/0.16 mmol/L phenol–nitroprusside solution. The reaction mixtures were immediately mixed to 125/11 mmol/L alkalinehypochlorite (sodium hypochlorite) and vortexed. Ammonium sulfate 75 µmol/L was used as ammonium standard. The ammonia concentration is directly proportional to the absorption of indophenol at 650 nm. The specific activity is reported as U/L. The estimation was performed out in triplicate and the mean was used for calculation.

3. Results and discussion

In this study, the pelleted trypanosomes eluted with phosphate buffer showed a protein concentration of 0.86 mg mL^{-1}. The ADA activity was assessed at concentrations of 0, 0.1, 0.2, 0.5, 0.6 and 0.8 mg mL^{-1}. In this study it was detected biochemically the presence of ADA enzyme in *T. evansi*. In the lowest and highest concentrations of proteins tested, the ADA activity was between 0.64 and 2.24 U/L, respectively. Therefore, the ADA activity increased in proportionately with the concentration of protein used (Fig. 1).

Studies have reported changes in adenosine transport in parasites and ADA activity in mammals associated with infections by *T. brucei*, *T. evansi*, *Trypanosoma vivax*, *Leishmania donovani* and *Leishmania infantum* (Okochi et al., 1983; De Koning and Jarvis, 1999; Suswam et al., 2003; Tripathi et al., 2008; Da Silva et al., 2011a,b). However, there is still a great complexity in the purine transport in trypanosomes. There is the necessity for more detailed biochemical characterizations of purine transporters in order to provide useful information for improved drug delivery, and then to achieve a better understanding of drug resistance phenotypes associated with purine transporters (Suswam et al., 2003). As a result, this study aimed to investigate the presence of ADA in *T. evansi*, an enzyme important for many vital functions in mammals and possibly for the parasites. In the genome of *T. brucei* it was identified ADA, which showed similarity among these trypanosomes.

The biochemical tests showed ADA activity in *T. evansi*. Probably, in the parasite this enzyme is responsible for the regulation of adenosine concentration and consequently inosine, as occurs in mammals (Franco et al., 1997). In future studies, we aim to investigate the ADA presence within the parasite using immune markers, as well as the purification and molecular characterization of the ADA of *T. evansi* in order to find differences with its counterpart in vertebrates, which could allow us to propose this enzyme as a potential target for chemotherapy.

Recent studies showed that the treatment with the adenosine analogue called cordycepin (3′-deoxyadenosine) when protected by an inhibitor of ADA was effective in the curative treatment of mice infected with *T. brucei* (Rottenberg et al., 2005; Vodnala et al., 2009). The curative effect is obtained because cordycepin binds to receptors, binding site for nucleosides obtained from the host to vital functions of the parasite. In contrast to most mammalian cells, trypanosomes cannot synthesize purines *de novo*. Instead, they depend on the salvage pathway of nucleosides from the body fluids of the host (Hammond and Gutteridge, 1984). When used only ADA inhibitor in the treatment of *T. brucei* in mice, researchers did not observe curative action and the animals died as a consequence of the disease (Rottenberg et al., 2005). With the discovery of ADA in the parasite, it becomes interesting to test

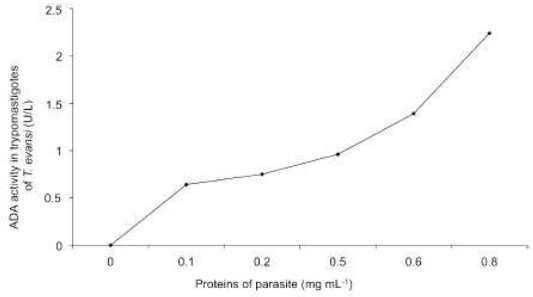

Fig. 1. Biochemical detection of adenosine deaminase activity in trypomastigotes forms of *T. evansi*. Relationship between ADA activity and protein concentration of the parasite.

in vitro the action of ADA inhibitors on *T. evansi*, to assess whether the inhibitor could have some direct harmful effects on the protozoan, by a mechanism that interferes with vital functions and causes the death of flagellates. This hypothesis will be the subject of a forthcoming study, together with the characterization of the enzyme in the parasite, as previously described.

Based on these results, we can conclude that *T. evansi* has the enzyme adenosine deaminase, which probably regulates the concentration of adenosine and inosine in the besieged, as it occurs in mammals. The technique provided demonstrated to be adequate to detect biochemically ADA activity in the parasite. So, this is the first step for the time to come we can test specific inhibitors of this enzyme in infected animals.

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ANEXO II - Artigo II

Activity of the enzyme adenosine deaminase in serum, erythrocytes and lymphocytes of rats infected with *Trypanosoma evansi*

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SUMMARY

In *Trypanosoma evansi* infections changes in the haemogram are commonly observed, and the enzyme adenosine deaminase (ADA) plays an important role in the production and differentiation of blood cells. Thus, the aim of this study was to evaluate the activity of ADA in serum, erythrocytes and lymphocytes of rats infected with *T. evansi* compared to non-infected rats. Thirty adult rats were used, divided into 3 uniform groups. The animals in groups A and B were infected intraperitoneally with 2×10^6 trypomastigotes/rat. Rodents from group C (control group), were not-infected. Blood collection was performed on days 4 and 20 post-infection (p.i.) in order to obtain acute and chronic infection stages of disease. The blood was used to assess the activity of ADA. In the blood, reduced haematocrit and increased lymphocytes were correlated with ADA activity in erythrocytes and lymphocytes. We observed reduction of ADA activity in serum and erythrocytes in rats infected with *T. evansi* compared to non-infected rats ($P<0{\cdot}05$). ADA activity in lymphocytes was decreased after 4 days, when the parasitaemia was high and increased after 20 days, when the number of circulating parasites was low. In conclusion, our results showed that the ADA activity was altered in serum, lymphocytes and erythrocytes of rats, concomitantly with haematological parameters, in experimental infection by *T. evansi*.

Key words: trypanosomosis, ADA, anaemia, lymphocytosis, rats.

INTRODUCTION

Trypanosoma evansi is a digenetic flagellate implicated in the infection of a large number of domestic and wild animals, such as equines, canines, felines, rabbits, capybaras, ring-tailed coatis, bovines and buffaloes (Dávila and Silva, 2000; Herrera *et al*. 2004; Tarello, 2005; Da Silva *et al*. 2008) and humans (Joshi *et al*. 2005). This protozoan is the agent of trypanosomosis, a disease with broad distribution in Africa, Asia, and Latin America (Lun and Desser, 1995). The trypomastigotes present in blood vessels of vertebrate hosts are transmitted by blood-sucking insects during feeding. The insect vectors are most commonly tabanide species (*Tabanus* sp., *Chrysops* sp., and *Hematopota* sp.) and vampire bats (Hoare, 1972).

Two features of the disease were reported in Brazil: the acute syndrome, responsible for the death of equines and non-treated canines, and the chronic syndrome, which affects many wild animals such as *Hydrochaeris hydrochaeris* and *Nasua nasua* (Herrera *et al*. 2004). The acute form is characterized by intermittent fever, subcutaneous widespread oedema, progressive anaemia and blindness. Clinical signs disappear during the subacute phase and the trypanosomosis may often go undiagnosed during clinical examination. Accurate diagnosis is only possible during the chronic stage of the disease, where clinical signs are more evident and the animal's condition is more severely affected (Silva *et al*. 2002).

Adenosine deaminase (ADA: EC 3.5.4.4) is considered to be a key enzyme in purine metabolism, catalysing the irreversible deamination of adenosine and deoxyadenosine to inosine and deoxyinosine, respectively and closely regulating extracellular adenosine concentrations (Franco *et al*. 1997). Adenosine deaminase activity has been detected on the surface of haematopoietic cells (Aran *et al*. 1991). Researchers have described a family in which there is a dominantly inherited form of haemolytic anaemia associated with a notable increase of ADA activity in erythrocytes but with normal ADA levels in other blood cells, including lymphocytes (Valentine *et al*. 1977).

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ADA is present in all cell types, but high activity is present in the thymus, lymphoid tissues and peripheric lymphocytes. It has been demonstrated that this enzyme plays an important role in lymphocyte function and is essential for the normal growth, differentiation and proliferation of T lymphocytes (Franco *et al*. 1997; Codero *et al*. 2001). The observation that ADA deficiency leads to severe combined immunodeficiency syndrome points to the physiological importance of controlling extracellular adenosine levels in the immune system (Aldrich *et al*. 2000).

Anaemia caused by *T. evansi* is often described and is characterized by decreased levels of erythrocytes, haemoglobin and haematocrit. However, its causes are not completely understood (Silva *et al*. 1995; Aquino *et al*. 2002). In infections by this protozoan, leukocyte changes are described as neutropenia, neutrophilia, monocytosis, lymphopenia, lymphocytosis (Silva *et al*. 1995; Marques *et al*. 2000; Wolkmer *et al*. 2009).

Considering the functions of ADA in leukocytes and the haematopoietic system, this study aimed to evaluate the activity of this enzyme in serum, erythrocytes and lymphocytes of rats infected with *T. evansi*.

MATERIALS AND METHODS

A total of 30 adult rats, males, with a mean age of 90 days and weighing on average 300 (±29) grams were used in this study. The animals were kept in cages with 10 animals each, in a room experiment with controlled temperature and humidity (25 °C; 70%). They were fed with commercial ration and water *ad libitum*. All animals received a formulation containing pyrantel pamoate, praziquantel and fenbendazole and were submitted to a period of 15 days of adaptation. All animals were apparently healthy when the experimental period began (day 0).

These rats were divided into 3 groups of 10 animals each. The rats in groups A and B were inoculated intraperitoneally (Day 1) with a strain of *T. evansi* that had been obtained from a naturally infected dog (Colpo *et al*. 2005) and had been kept in liquid nitrogen. The number of inoculated flagellates was estimated by using a Neubauer chamber (Wolkmer *et al*. 2007). This study aimed to evaluate the acute and chronic disease in the rat, so the infectious dose used for groups A and B was 2×10^6 trypomastigotes/animal in fresh blood and cryopreserved blood, respectively (Da Silva *et al*. 2009*a*). The collection of samples from animals in group A was performed on day 4 post-infection (p.i.) and group B was on day 20 p.i. Group C (negative control) consisted of healthy rats, not infected by *T. evansi*, but received a physiological solution administered in the same way. Group C was divided into 2 groups (C1 and C2) and the material was collected on day 4 and day 20 p.i. in order to compare with the infected groups (A and B). Parasitaemia was estimated daily by microscopic examination of smears. Each slide was mounted with blood collected from the tail vein, stained by the panoptic method, and visualized at a magnification of 1000×.

The animals were anaesthetized in a chamber with isoflurane for collection of blood by cardiac puncture (8 ml). The storage of the samples was considered according to the analysis. Thus, part of the material collected was allocated in tubes containing anticoagulant for separation of lymphocytes (4 ml), separation of erythrocytes (2 ml) and analysis of haemogram (1 ml). The volume of 1 ml was stored in a tube without anticoagulant to obtain serum.

Erythrocyte count, haematocrit (Ht), haemoglobin concentration (Hb), mean corpuscular volume (MCV), mean corpuscular haemoglobin concentration (MCHC) and total leukocytes were evaluated. Smears were mounted and stained by the panoptic method. The determination of microhematocrit was performed according to the technique described by Feldman *et al*. (2000), and blood smears were stained with the panoptic method to perform differential leukocyte counts. Erythrocyte counts and haemoglobin concentrations were determined using an electronic counter.

Erythrocytes were obtained from whole blood with EDTA, according to the technique described by Hostetter and Johnson (1989). Erythrocytes were resuspended to haematocrit in Hepes-buffer at 10%. Lymphocytes were also obtained from whole blood with EDTA by gradient separation using Ficoll-Histopaque™ plus, according to the technique described by Böyum (1968). The samples stored in tubes without anticoagulant were centrifuged for 10 min, and the serum was obtained. The ADA activity was measured immediately after obtaining the erythrocytes, lymphocytes and serum.

ADA activity was measured spectrophotometrically in serum, lymphocytes and erythrocytes by the method of Giusti and Gakis (1971). The reaction was started by addition of the substrate (adenosine) to a final concentration of 21 mmol/l and incubations were 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 sulphate at a concentration of 75 μmol/l was used as ammonium standard. The ammonia concentration is directly proportional to the absorption of indophenol at 650 nm. The specific activity is reported as U/L in serum and lymphocytes and U/g of Hb in erythrocytes.

The data were submitted to one-way analysis of variance (ANOVA) followed by the Tukey's test ($P<0{\cdot}05$). The effect of ADA in erythrocytes and lymphocytes on haematocrit and lymphocytes was

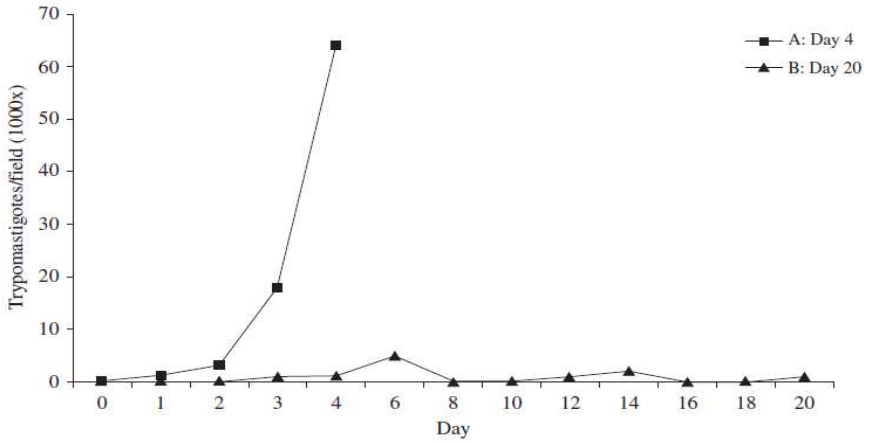

Fig. 1. Parasitaemia of *Trypanosoma evansi* in infected rats at day 20 post-inoculation. The collection of material was performed in groups A and B on day 4 and day 20 post-infection at high and low parasitaemia, respectively.

analysed by linear regression, respectively. The analyses were performed using SAS statistical package (SAS Institute, Cary, NC, USA) with a significance level of 5% ($P<0{\cdot}05$).

The procedure was approved by the Animal Welfare Committee of Federal University de Santa Maria (UFSM), number 23081.012513/2009-52, in accordance to Brazilian laws and ethical principles published by the Colégio Brasileiro de Experimentação Animal (COBEA).

RESULTS

Examination of peripheral smear blood showed that the pre-patent period in experimentally infected rats varied between 24 and 72 h. The peak of parasitaemia at day 4 p.i. in group A (63 trypanosomes per microscopic field at 1000 × magnification) and irregular waves of parasitaemia were observed in group B, ranging from 0 to 3 trypomastigotes per microscopic field (Fig. 1). Seven (7/10) rats in group A demonstrated apathetic slow movements, disorientation and gasping on day 4 p.i. In group B, the animals did not show any clinical change, neither did the non-infected rats (group C).

Haematological changes were observed in the infected rats as a decrease in haematocrit (Fig. 3a), erythrocyte count and haemoglobin content (Table 1 – $P<0{\cdot}05$). The mean MCV and the mean MCHC did not differ among groups, characterizing a normocytic–normochromic anemia (Table 1). Simultaneously with haematological changes, the rats of group A showed leukocytosis (Table 1) and lymphocytosis (Fig. 4a), as compared to groups B and C.

The ADA activity in serum was reduced in the groups infected with *T. evansi* compared to the non-infected rats ($P<0{\cdot}001$ – Fig. 2). In erythrocytes, the ADA activity was reduced on day 4 and day 20 (Fig. 3b), but was more pronounced after 20 days

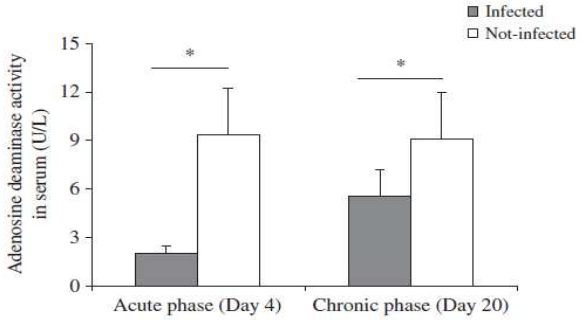

Fig. 2. Means and standard deviation of the adenosine deaminase activity in serum of rats infected with *Trypanosoma evansi* (day 4 and day 20 p.i.) compared to non-infected rats ($n=10$). (*$P<0{\cdot}05$).

when the parasitaemia was low (Fig. 1). In the chronic phase a positive correlation (r^2: 0·82) was observed between the ADA activity in erythrocytes and the haematocrit values ($P<0{\cdot}001$ – Fig. 3d).

In lymphocytes, the activity of ADA was reduced in the acute phase (day 4), when the parasitaemia was high, but showed an increase of ADA activity after 20 days (Fig. 4b), when the parasitaemia was low (Fig. 1) and the number of lymphocytes was normal (Fig. 4-a). In the acute phase a negative correlation (r^2: −0·60) was observed between the ADA activity in lymphocytes and total number of lymphocytes ($P<0{\cdot}05$).

DISCUSSION

Many studies have reported changes in adenosine transport and ADA activity associated with infections by *Trypanosoma brucei*, *T. evansi*, *T. vivax*, *Leishmania donovani* and *L. infantum* (Okochi *et al.* 1983; De Koning and Jarvis, 1999; Suswam *et al.*

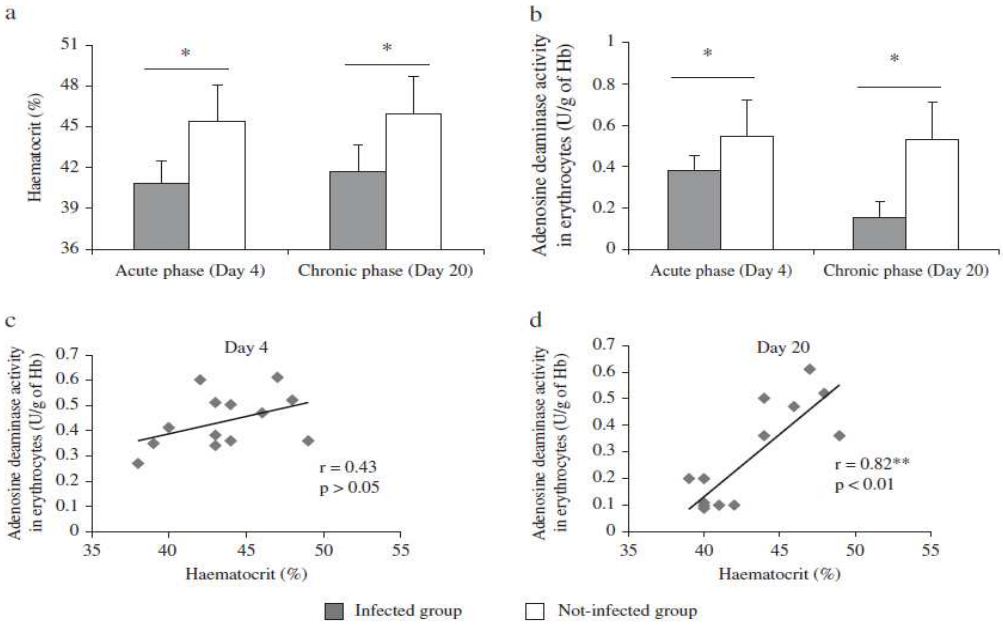

Fig. 3. Means and standard deviation of the haematocrit (a) and adenosine deaminase activity in erythrocytes (b) of rats infected with *Trypanosoma evansi* (day 4 and day 20 p.i.) compared to non-infected ($n = 10$). Linear regression analysis of individual infected rat haematocrit with adenosine deaminase activity in erythrocytes during the acute phase (c) and chronic phase (d). (*$P < 0{\cdot}05$.)

2003; Tripathi *et al*. 2008). However, a study that correlates the acute and chronic effect of experimental infection with *T. evansi* in the ADA activity in the serum, erythrocytes and lymphocytes of rats has not been found in the literature.

An increased ADA activity has been observed in serum samples, erythrocytes, leukocytes and in plasma haemoglobin concentrations with vivax malaria as compare to the control group (Ozcan *et al*. 1997). Authors described significantly increased ADA activity in visceral leishmaniasis patients compared to healthy controls (Khambu *et al*. 2007). Researchers showed that intraperitoneal injection of the adenosine analogue cordycepin (3´-deoxy-adenosine) for treatment of the encephalitic stage of human African trypanosomosis, together with an adenosine deaminase inhibitor (coformycin or deoxycoformycin), cures *T. brucei* infection in mice (Rottenberg *et al*. 2005). In this study we demonstrate that ADA activity may be associated with trypanosome infection, since the *T. evansi*-infected rat had reduced enzyme activity both in blood cells and in serum.

In the acute phase of this study we observed a reduction of ADA activity in serum, erythrocytes and lymphocytes of rats infected with *T. evansi* compared to healthy rats. At this stage, rats had 4 days of infection and high parasitaemia. The reduction in ADA activity would have caused an increase in the extracellular concentrations of adenosine, which would then be converted to inosine. Adenosine acts as a sensor and provides information to the immune system regarding the tissue damage or acute inflammatory changes occurring in the vicinity of the immune system (Kumar and Sharma, 2009). The reduction in ADA activity in lymphocytes would lead to interaction of adenosine with adenosine receptors that exist in many cell types, with possible anti-inflammatory effects, among them the inhibition of the Th1 immune response. In acute infection caused by *T. cruzi* there is a predominance of Th1 and cellular response with production of interferon-γ (Kumar and Tarleton, 2001). Therefore, inhibition of this response by the action of extracellular adenosine in purinergic receptors could be a compensatory effect, attenuating inflammation and tissue damage. The treatment of macrophages with interferon-γ upregulates the expression of the adenosine receptor, A_{2B}, and the activation of A_{2B} receptors is involved with the deactivation of macrophages, possibly through an increase of cAMP (Xaus *et al*. 1999). This reinforces the concept of the anti-inflammatory action of adenosine as a way to preserve cells and tissues.

The ADA may be expressed as an ectoenzyme on the surface of lymphocytes. In the serum there is another isoenzyme, ADA_2, which has a low affinity for the substrate (Muraoka *et al*. 1990). Thus, it will only increase its activity in higher concentrations of substrate, unlike the lymphocyte isoform. The concentration of extracellular adenosine in this study seems to be sufficient for activation of the

Table 1. Means and standard deviation of the haematological parameters of rats experimentally infected with *Trypanosoma evansi*

Parameters	Group*		
	A: Infected (Day 4)	B: Infected (Day 20)	C: Not-infected
Total erythrocytes ($\times 10^6/\mu$l)	$6{\cdot}15^b$ ($\pm 0{\cdot}46$)	$6{\cdot}34^b$ ($\pm 0{\cdot}65$)	$7{\cdot}0^a$ ($\pm 0{\cdot}24$)
Haemoglobin (g/dl)	$11{\cdot}8^b$ ($\pm 0{\cdot}46$)	$12{\cdot}5^b$ ($\pm 0{\cdot}46$)	$14{\cdot}0^a$ ($\pm 0{\cdot}46$)
MCV (fl)	$62{\cdot}6^a$ ($\pm 2{\cdot}10$)	$62{\cdot}4^a$ ($\pm 1{\cdot}74$)	$61{\cdot}0^a$ ($\pm 2{\cdot}40$)
MCHC (%)	$30{\cdot}8^a$ ($\pm 0{\cdot}90$)	$32{\cdot}6^a$ ($\pm 1{\cdot}10$)	$31{\cdot}3^a$ ($\pm 0{\cdot}80$)
Total leukocytes ($\times 10^3/\mu$l)	$12{\cdot}71^a$ ($\pm 3{\cdot}10$)	$5{\cdot}27^b$ ($\pm 1{\cdot}2$)	$5{\cdot}75^b$ ($\pm 0{\cdot}9$)

* Means in the same line followed by different letters are statistically different among them by Tukey's test at 5% probability.

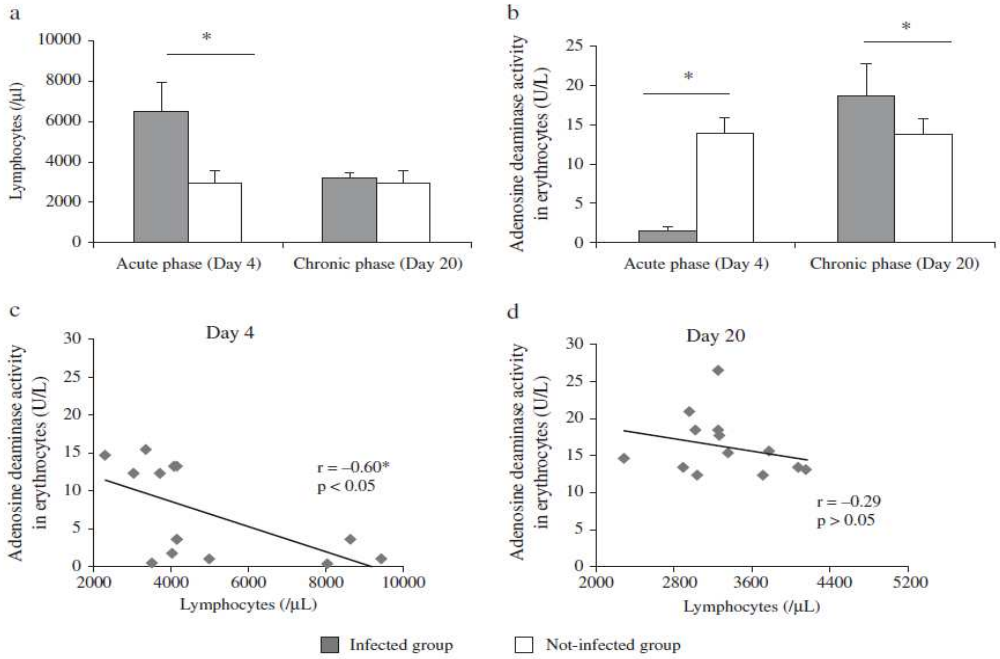

Fig. 4. Means and standard deviation of the lymphocytes (a) and adenosine deaminase activity in lymphocytes (b) of rats infected with *Trypanosoma evansi* (day 4 and day 20 p.i.) compared to non-infected ($n=10$). Linear regression analysis of individual infected rat number of lymphocytes with adenosine deaminase activity in lymphocytes of the acute phase (c) and chronic phase day 20 (d). (*$P<0{\cdot}05$.)

enzyme in serum, assuming that it could be binding on the adenosine receptors in blood cells. Regarding the ADA activity in erythrocytes, reduction of enzyme activity seems to follow the decrease in total erythrocytes, which could be verified in future studies by observing the expression of ectoenzymes on the surface of red blood cells.

Researchers mentioned that red blood cells are relatively well supplied with ADA and circulating damaged erythrocytes release significant amounts of ADA, a process that may predispose to vaso-occlusive events (Muraoka *et al*. 1990). Based on this information, we hypothesized that ADA activity was increased in serum and/or plasma, as a consequence of the decrease in red blood cells due to a haemolytic process, causing anaemia in trypanosomosis (Jackson *et al*. 1996). However, this was not observed in our study. Therefore, despite the positive correlation between haematocrit and activity levels in erythrocytes, further studies are necessary to confirm the role of ADA in anaemia caused by *T. evansi* in rats.

In addition, our experiment showed that in the chronic phase the parasitaemia had stabilized and fluctuated between 0 and 3 parasites per field (1000×). At the same time we found that the ADA activity in serum and erythrocytes was reduced in rats

with trypanosomosis, and in lymphocytes ADA activity was increased when compared to non-infected rats. The low parasitaemia suggests that the compensatory effect of an anti-inflammatory like adenosine is no longer necessary. Thus, increased ADA activity in lymphocytes, reduces the extracellular concentrations of adenosine, promoting an inflammatory response that would be sufficient to contain the spread of the parasite without major tissue damage. The low concentration of extracellular adenosine also prevents activation of the isoforms present in erythrocytes and serum, probably due to a low affinity for the substrate.

Other workers recently observed that ADA activity in the serum of patients with idiopathic Parkinson's disease was significantly higher than in normal controls, suggesting that high serum ADA activity may be involved in the pathogenesis of Parkinson's disease via peripheral T-lymphocyte activation (Chiba *et al*. 1995). An increase in serum ADA activity in patients with cutaneous leishmaniasis (Ozcan *et al*. 1998), and a change in activity of this enzyme may also be related to pathogenesis of the parasite, also in Parkinson's disease. In contrast, this study showed reduced ADA activity in the serum of rats infected with *T. evansi*, compared with infection by *Leishmania* sp. (Ozcan *et al*. 1998; Khambu *et al*. 2007).

An important aspect to be discussed is that *T. evansi* caused an acute infection in rats, in contrast to a chronic infection in some rodents. These protozoa are Salivarian trypanosomes which are usually more virulent and pathogenic than Stercorarian trypanosomes (Menezes *et al*. 2004). The detection of parasitaemia in rats (24 h) occurred earlier than previously reported in experimentally infected rats (Queiroz *et al*. 2000; Al-Mohammed, 2006; Omer *et al*. 2007), probably due to the high pathogenicity of this strain. Although authors reported that *R. norvegicus* is a suitable model for the study of the parasitaemic wave of *T. evansi* (Queiroz *et al*. 2000), the typical undulating course of parasitaemia was observed in group B of this experiment. According to Da Silva *et al*. (2009*a*) the longevity of rats may be related to the type of inoculum used. When they are made by successive infections in rats, there is an acute phase with high parasitaemia and death of animals within 5 days p.i. Now, when the inoculum used was cryopreserved in liquid nitrogen the longevity of rodents could increase considerably, resulting in a chronic phase. In rats the disease was characterized by high levels of parasitaemia along with clinical signals of apathy, weakness, ataxia and severe anaemia (Wolkmer *et al*. 2009), similar to what occurred in the acute phase of this experiment.

Previous studies from our laboratory showed that in the infection with *T. evansi* haematological changes are commonly related to other factors, but major changes depend on the degree of parasitaemia and period of infection (Wolkmer *et al*. 2007; Da Silva *et al*. 2009*b*). Our research group has already found decreased activity of acetylcholinesterase in erythrocytes in cats (Da Silva *et al*. 2010) and an increased lipid peroxidation in rats (Wolkmer *et al*. 2009) infected with *T. evansi*. It is important to state that these alterations might be related to pathogenesis of the disease or may just be a consequence of anaemia, because adenosine is related to maturation of erythrocytes (Franco *et al*. 1990; Jackson *et al*. 1996). This question also persists for the decreased ADA activity in erythrocytes found in this study.

Based on these results, we conclude that parasitism by *T. evansi* alters the activity of ADA in serum, erythrocytes and lymphocytes of experimentally infected rats, suggesting that trypanosomiasis can interfere with purinergic signalling. Other studies need to be performed to verify the expression of ectoenzymes on the surface of red blood cells and lymphocytes in trypanosomiasis, in order to understand the relationship of the ADA with anaemia and lymphocytosis in this disease.

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ANEXO III – Artigo III

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Trypanosoma evansi: Adenosine deaminase activity in the brain of infected rats

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ABSTRACT

The study was undertaken to evaluate changes in the activity of adenosine deaminase (ADA) in brains of rats infected by *Trypanosoma evansi*. Each rat was intraperitoneally infected with 10^6 trypomastigotes either suspended in fresh (group A; $n = 13$) and cryopreserved blood (group B; $n = 13$). Thirteen animals were used as control (group C). ADA activity was estimated in the cerebellum, cerebral cortex, striatum and hippocampus. No differences ($P > 0.05$) in ADA activity were observed in the cerebellum between infected and non-infected animals. Significant ($P < 0.05$) reductions in ADA activity occurred in cerebral cortex in acutely (day 4 post-infection; PI) and chronically (day 20 PI) infected rats. ADA activity was significantly ($P < 0.05$) decreased in the hippocampus in acutely infected rats, but significantly ($P < 0.05$) increased in the chronically infected rats. Significant ($P < 0.05$) reductions in ADA activity occurred in the striatum of chronically infected rats. Parasites could be found in peripheral blood and brain tissue through microscopic examination and PCR assay, respectively, in acutely and chronically infected rats. The reduction of ADA activity in the brain was associated with high levels of parasitemia and anemia in acute infections. Alterations in ADA activity of the brain in *T. evansi*-infected rats may have implications for pathogenesis of the disease.

1. Introduction

Trypanosoma evansi and *Trypanosoma brucei* are closely phylogenetically related trypanosomes of African origin (Brun et al., 1998; Lai et al., 2008). *T. evansi* is the only mechanically transmitted by biting flies and displays the broadest host range and geographical distribution among all pathogenic trypanosomes, infecting domestic and wild mammals in Africa, Asia and Latin America (Brun et al., 1998; Ventura et al., 2002; Herrera et al., 2005).

The infection caused by *T. evansi* in horses develops in two stages, the early, or haemolymphatic stage, when the parasites multiply and spread in the blood and lymph nodes, followed by the late or encephalitic stage, when the parasites cross the blood–brain barrier to invade the central nervous system (CNS). *T. evansi* causes a devastating horse disease, called "mal de cadeiras" or "surra", characterized by anemia, immunosuppression, emaciation, severe neurological signs, motor incoordination, paralysis of hind limbs and death of untreated animals (Rodrigues et al., 2009; Berlin et al., 2009).

Clinical signs of neurological disorders are reported in horses, camels, buffaloes, cattle, deer and cats infected by *T. evansi* (Tuntasuvan et al., 1997, 2000; Rodrigues et al., 2005; Berlin et al., 2009; Da Silva et al., 2010). Brain lesions were reported in bovines and equines (Tuntasuvan et al., 1997; Rodrigues et al., 2009). Rats are highly susceptible to the disease, showing hematological, biochemical and pathological changes associated with ataxia, tremors and terminal coma of untreated animals (Menezes et al., 2004; Wolkmer et al., 2009). Human infection by *T. evansi* was reported for the first time in 2005 in an Indian farmer that showed signs of sensory deficit, disorientation and violent behavior (Joshi et al., 2005).

Adenosine acts as a CNS modulator in mammals, regulates cell metabolism and triggers a variety of physiological effects participating in apoptosis, necrosis and cell proliferation. Under pathological conditions, adenosine plays a protective role by modulating the release of the neurotransmitters and tropic factors. Adenosine also acts as an endogenous regulator of innate immunity, protecting the host from excessive tissue injury associated with strong inflammation (Rathbone et al., 1999; Beraudi et al., 2003; Hasko and Cronstein, 2004; Sitkovsky and Ohta, 2005; Burnstock, 2006; Desrosiers et al., 2007).

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The concentration of extracellular adenosine is regulated by the activity of a small group of important enzymes including adenosine deaminase (ADA; EC 3.5.4.4), which catalyses the conversion of the adenosine into its inactive metabolite inosine. ADA activity is widely distributed in tissues and fluids from vertebrate animals in isoforms of ADA1 and ADA2. Tissue extracts contain predominantly ADA1, which is supposed to be derived mainly from injured tissues. ADA2 is found in serum and derived from stimulated T-cells. ADA has been detected on the surface of many cell types, including brain synaptosomes. A heterogeneous expression of ADA activity can be found among peripheral tissues and even within the CNS, where high activities of ADA were reported in discrete and diverse brain areas (Greiger and Nagy, 1986; Franco et al., 1986, 1997).

ADA activities may be sensitive markers for infection severity and for monitoring the course of infections. The activity of ADA was elevated in the serum of hosts with tuberculosis, theileriosis, malaria and visceral leishmaniasis (Ozcan et al., 1997; Melo et al., 2000; Khambu et al., 2007; Altug et al., 2008). No study has demonstrated a relationship of *T. evansi* infection with ADA activity in the CNS. Thus, the purpose of the present investigation was to determine whether *T. evansi* infection induces changes in ADA activity in the brain tissues of adult rats.

2. Material and methods

2.1. Experimental animals

Thirty nine adult outbreed male rats (mean age of 90 days) weighing 300 ± 18 g were maintaining in cages in a room with controlled temperature (25 °C) and humidity (70%). They were fed (commercial ration) and water *ad libitum*. Before the experiment, they were treated with pyrantel pamoate, praziquantel and fenbendazole, and submitted to an adaptation period of 15 days. The procedure was approved by the Animal Welfare Committee of Universidade Federal de Santa Maria (UFSM), Number 23081.012513/2009-52, in accordance to Brazilian laws and ethical principles of the Colégio Brasileiro de Experimentação Animal (COBEA).

2.2. Experimental design and trypanosome infection

The rats were divided in three groups of 13 animals each. Animals in groups A and B were inoculated intraperitoneally (day 0) with a strain of *T. evansi* that had been obtained from a naturally infected dog (Colpo et al., 2005) and had been maintaining in liquid nitrogen. The infective dose (estimated using a hemocytometer) for each animal was 10^6 trypomastigotes in either fresh (group A; 0.1 ml) and cryopreserved blood (group B; 0.2 ml) in order to elicit acute and chronic infections, respectively (Da Silva et al., 2009). The collection of blood samples and brains from animals in group A was performed at day 4 post-infection (PI) while samples for group B were collected at day 20 PI. Group C consisted of 13 healthy non-infected control rats. This group was divided into groups C1 and C2 and blood samples and brains were collected on days 4 (C1) and 20 (C2) PI for comparison with the infected groups A and B.

2.3. Estimation of parasitemia

The presence and degree of parasitemia were determined daily for each animal by blood film examination. A drop of blood was collected from the tail and placed on a slide, and a thin blood smear was prepared manually (Da Silva et al., 2006). The blood films were Romanovsky stained and then examined under a microscope, counting 10 fields at 1000× magnification.

2.4. Collection of samples

The animals were anesthetized in a chamber with isoflurane before collection of blood by cardiac puncture. Thereafter, animals were decapitated following recommendations of the Ethics Committee and brains of 10 rats from each group were carefully removed avoiding contamination with peripheral blood, and dissected to separate cerebellum, cerebral cortex, striatum and hippocampus. Each part of the brain was weighed, homogenized in 10 volumes of 50 mmol/l per mM phosphate buffer (pH 7.0) and centrifuged for 30 min at 14,000*g* at 4 °C. The supernatant was then collected as described by Bellé et al. (2009).

2.5. Hematologic parameters

Erythrocyte count, hemoglobin concentration and total leukocyte count were determined using an electronic counter (CC-550-Celm, São Paulo, Brazil).

2.6. ADA activity in brain

ADA activities were estimated spectrophotometrically (Hitachi U-2800A – spectrophotometer) by the method of Giusti (1974), which is based on the direct measurement of the formation of ammonia produced when the enzyme acts on adenosine. The volumes of 25 μl of the brain homogenates (cerebral cortex, cerebellum, striatum and hippocampus) were 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 106/0.16 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 would react with alkaline-hypochlorite and phenol in the presence of a catalyst-sodium nitroprusside to produce indophenol (a blue color) and the concentration of ammonia is directly proportional to the absorbance of indophenol read at 620 nm. Ammonium sulphate of 75 μM was used as ammonium standard.

Protein concentration of the brain homogenate was measured by the method of Peterson (1977) with bovine serum albumin used as a standard. The value of ADA activity in the brain tissue was expressed as U/mg of protein. The estimation was performed out in triplicate and the mean was used for calculation.

2.7. DNA extraction and PCR detection of T. evansi in brains of rats

Three rats from each infected group (A and B) and controls (C1 and C2) were randomly selected to investigate the presence of *T. evansi* DNA in their brains by PCR. For this assay, cerebellum, cerebral cortex, striatum and hippocampus, removed using one sterile blade for each structure from each animal, was individually transferred to sterile tubes containing 0.5 ml ethanol.

For preparation of DNA templates, a small section (0.4 × 0.4 mm) of each brain were removed, transferred to sterile tubes and washed three times (5 min each) in bi-distillated water under shaker. Then, the tissues were cut in small segments, incubated with lysis buffer (1% SDS, 100 mM EDTA pH 8.0, 20 mM Tris–HCl, pH 8.0, and 350 mg/ml of proteinase K), at 37 °C for 18 h, centrifuged at 14,000*g* for 5 min, and DNA purified using Wizard Purification Systems (Promega, USA). Purified DNA samples were used as templates for PCR amplifications of a spliced leader gene sequence using primers and reaction conditions previously described (Ventura et al., 2002). Amplified DNA fragments were resolved in 2% agarose gel, stained with ethidium bromide and visualized under UV light.

2.8. Statistical analysis

The data were summarized means and standard deviations analyzed by ANOVA followed by the Tukey's post-test ($P < 0.05$).

3. Results

3.1. Parasitemia, hematological parameters and clinical signs

Examination of the peripheral blood smears showed a prepatent period between 1 and 3 days PI. No difference in prepatent period between groups A and B. The peak of parasitemia occurred on day 4 PI in group A (63 trypomastigotes per microscopic field), and irregular waves of parasitemia (0–3 trypomastigotes per microscopic field) were observed in group B (Fig. 1). Decreased ($P < 0.05$) levels of erythrocyte count and hemoglobin were observed in rats of groups A and B, when compared to group C. Animals from group A showed a significant ($P < 0.05$) increase in the number of total leukocytes (Fig. 2). Seven (7/10) rats of group A presented apathy, lethargy, disorientation and gasping at day 4 PI. Animals from group B did not show any clinical sign.

3.2. ADA activity in brain

No difference in ADA activity was detected in the cerebellum between infected and non-infected animals. A significant ($P < 0.05$) decrease occurred in cerebral cortex of acutely and chronically infected animals. In acutely infected rats, the activity was significantly ($P < 0.05$) reduced in hippocampus, but no alteration was observed in striatum. However, in chronically infected rats, ADA activity increased significantly ($P < 0.05$) in the hippocampus with a concomitant reduction ($P < 0.05$) in the striatum (Fig. 3).

3.3. Detection of T. evansi in brain of rats using PCR assay

The PCR assays detected *T. evansi* DNA in brain parts of acutely and chronically infected rats. Tissue samples of control animals were all negative for the parasite. The PCR did not allow for parasite count and the intensity of amplified band could not be quantified.

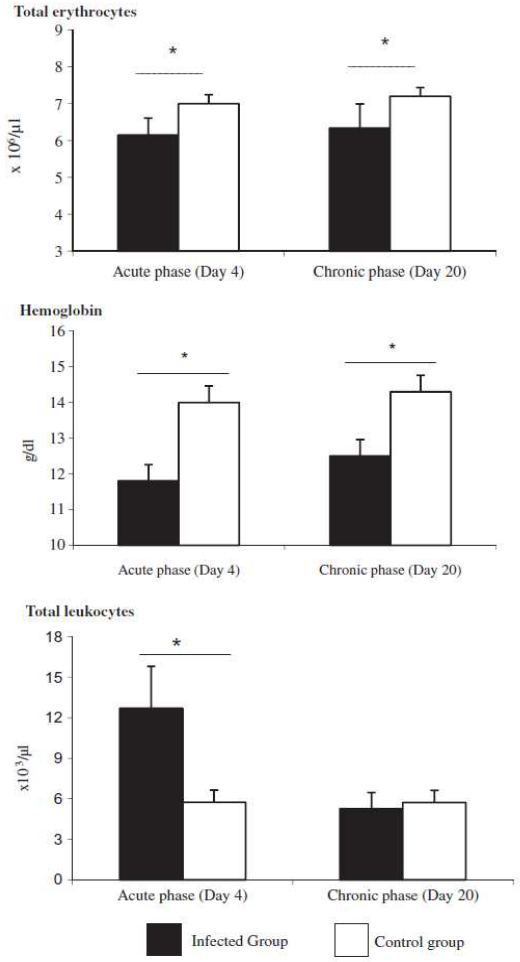

Fig. 2. Total erythrocytes, hemoglobin concentration and total leukocytes of *T. evansi*-infected (days 4 and 20 post-infection) and non-infected rats.

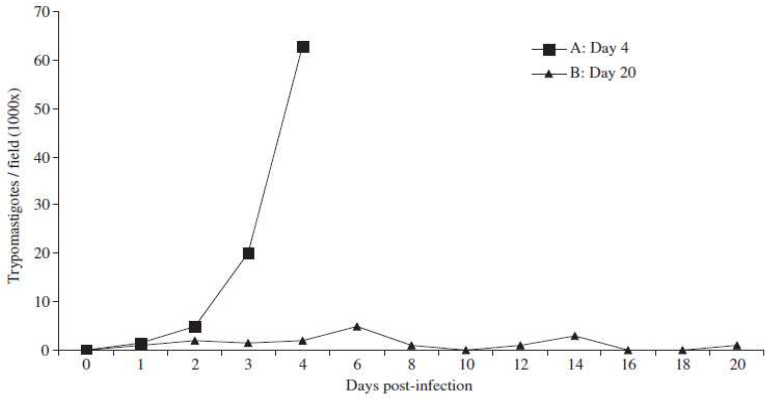

Fig. 1. Parasitemia of *T. evansi*-infected rats with acute or chronic infections.

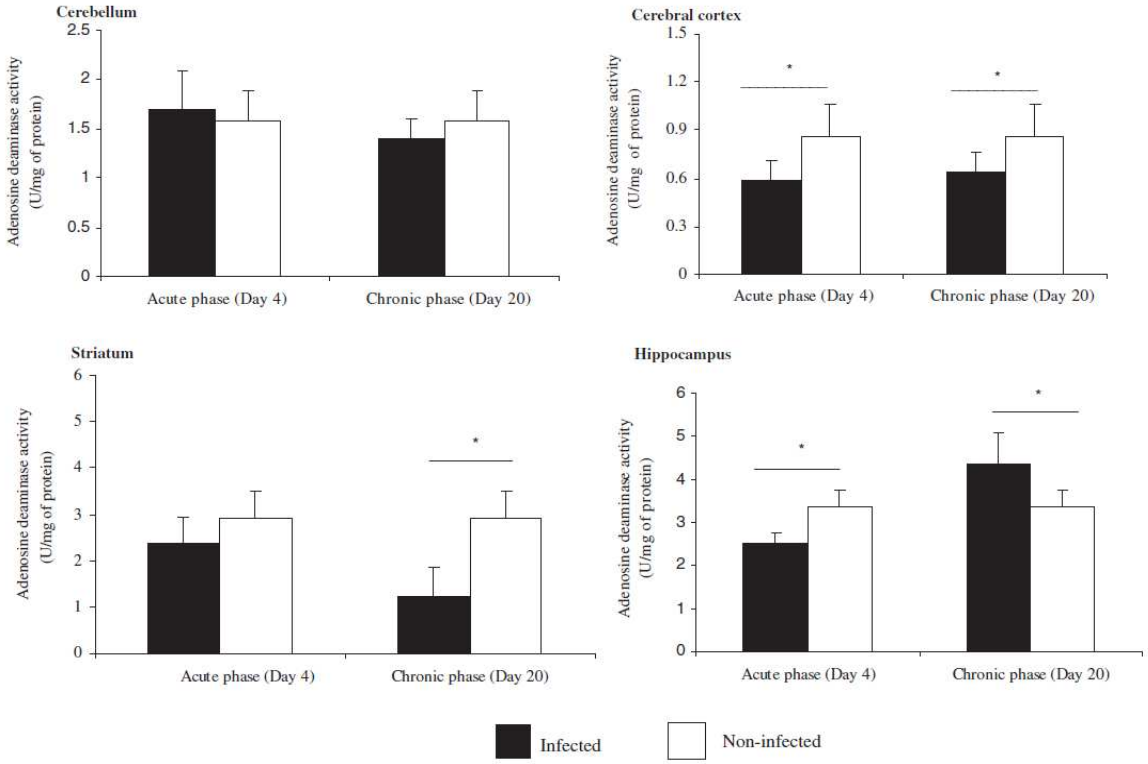

Fig. 3. Adenosine deaminase activity in cerebellum, cerebral cortex, striatum and hippocampus of *T. evansi*-infected (days 4 and 20 post-infection) and non-infected rats.

4. Discussion

Variations in ADA activity occurred in brains of rats during infection by *T. evansi*, with respect to components of the brain (cerebral cortex, striatum and hippocampus) and severity of the disease (acute or chronic infection). Acutely infected animals with high levels of parasitemia showed neurological disturbances, but chronically infected ones with low parasitemia had no neurological signs.

The reduction in ADA activity in some brain regions (cerebral cortex, striatum and hippocampus) may have increased of adenosine levels in the brain. Adenosine plays an important regulatory role in neuronal activity and has neuroprotective actions in P1 purinoreceptor-mediated pathological conditions (Cunha and Ribeiro, 2000; Cunha, 2001). In addition, reduction in ADA activity could also contribute to limit inflammation and subsequent cellular damage (Abbracchio and Ceruti, 2007). Adenosine protects host cells from excessive tissue injury associated with strong inflammation, existing evidence that elevated level of this nucleoside potently down-regulates the activation of lymphocytes during inflammation, playing a regulatory role on dendritic cell immune responses (Desrosiers et al., 2007). Increased ADA in chronic infected animals may increase the severity of the lesion, because a decrease in brain adenosine can lead to damage of brain tissue.

Our evidence of different ADA activities among the regions of the brain corroborated with spatial activity of the enzyme, which correlates with mRNA expressions (Mackiewicz et al., 2000). Thus, ADA activity may play an important role in the mechanisms that control regional concentrations of adenosine in the brain, and the differences observed are likely to have important physiological consequences. In experimentally infected horses, the severity of encephalomyelitis varied in different parts of the brain (Lemos et al., 2008).

Changes in ADA activities were associated with the presence of parasites in peripheral blood and brain, anemia and neurological signs. These data suggest that the presence of parasites may be primarily responsible for the reduced ADA activity in the brains of *T. evansi*-acutely infected rats (highly parasitemic). Based on our previous studies, rats that develop the acute infection invariable develop severe hematological and neurological disorders and died (Wolkmer et al., 2009). The neurological disturbances in *T. evansi*-infected hosts could be related to changes in ADA activity in the brains especially in the cerebral cortex and hippocampus (Mesulam et al., 2002).

In contrast to most mammalian cells, trypanosomatids are unable to engage in *de novo* purine synthesis and depend on the salvage pathway of nucleosides from their mammalian hosts. Studies have been done to identify targets for purine pathway inhibitors of *Leishmania* spp., *T. brucei*, *Trypanosoma vivax* and *T. evansi* (Ogbunude and Ikediobi, 1983; De Koning and Jarvis, 1999; Suswam et al., 2003; Witola et al., 2004; Carter et al., 2008). Although some enzymes of purine salvage were detected in the bloodstream forms of *T. brucei*, *Trypanosoma congolense* and *T. vivax*, homogenates of these trypanosomes apparently lacked adenosine deaminase (Ogbunude and Ikediobi, 1983). *T. brucei* and *T. cruzi* genomes include genes encoding putative ADA-like enzymes, but to date these enzymes have not been expressed nor was their function analyzed, as well as not disclosed by the first broad proteomic analysis of *T. evansi* (Roy et al., 2010).

In conclusion, *T. evansi* infection resulted in either the reduction or increase in the ADA activity in brain of rat. The alterations in

ADA activity in the brain of infected rats may have implications for pathogenesis and neurological signs of the disease.

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