UNIVERSIDADE FEDERAL DO RIO GRANDE DO SUL
FACULDADE DE VETERINÁRIA
PROGRAMA DE PÓS-GRADUAÇÃO EM CIÊNCIAS VETERINÁRIAS

TESE DE DOUTORADO

MARIA ISABEL DE AZEVEDO

INFECÇÃO EXPERIMENTAL POR *Cryptococcus neoformans*: INFLUÊNCIA DA DOENÇA SOBRE OS SISTEMAS PURINÉRGICO E COLINÉRGICO

PORTO ALEGRE

2016

UNIVERSIDADE FEDERAL DO RIO GRANDE DO SUL
FACULDADE DE VETERINÁRIA
PROGRAMA DE PÓS-GRADUAÇÃO EM CIÊNCIAS VETERINÁRIAS

TESE DE DOUTORADO

MARIA ISABEL DE AZEVEDO

INFECÇÃO EXPERIMENTAL POR *Cryptococcus neoformans*: INFLUÊNCIA DA DOENÇA SOBRE OS SISTEMAS PURINÉRGICO E COLINÉRGICO

Tese apresentada como requisito parcial para obtenção do grau de Doutor em Ciências Veterinárias, junto à Faculdade de Medicina Veterinária da Universidade Federal do Rio Grande do Sul.
Subárea: Microbiologia
Especialidade: Micologia
Orientador: **PROF. DR. LAERTE FERREIRO**
Coorientador: **PROF. DR. JANIO MORAIS SANTURIO**

PORTO ALEGRE
2016

UNIVERSIDADE FEDERAL DO RIO GRANDE DO SUL
FACULDADE DE VETERINÁRIA
PROGRAMA DE PÓS-GRADUAÇÃO EM MEDICINA VETERINÁRIA

A comissão examinadora, abaixo assinada
Aprova a tese de Doutorado

INFECÇÃO EXPERIMENTAL POR *Cryptoccocus neoformans*: INFLUÊNCIA DA DOENÇA SOBRE OS SISTEMAS PURINÉRGICO E COLINÉRGICO

elaborada por
Maria Isabel de Azevedo

Como requisito parcial para obtenção do grau de
Doutor em Ciências Veterinárias

COMISSÃO EXAMINADORA

Laerte Ferreiro, Dr.
(Presidente/Orientador)

Flávio de Mattos de Oliveira, Dr. UFSM

Gustavo Wissmann Neto, Dr. HCPA-Porto Alegre

Régis Adriel Zanette, Dr. UFRGS

CIP - Catalogação na Publicação

DE AZEVEDO, MARIA ISABEL
INFECÇÃO EXPERIMENTAL POR Cryptococcus neoformans: INFLUÊNCIA DA DOENÇA SOBRE OS SISTEMAS PURINÉRGICO E COLINÉRGICO / MARIA ISABEL DE AZEVEDO. -- 2016.
96 f.

Orientador: LAERTE FERREIRO.
Coorientador: JANIO MORAIS SANTURIO.

Tese (Doutorado) -- Universidade Federal do Rio Grande do Sul, Faculdade de Veterinária, Programa de Pós-Graduação em Ciências Veterinárias, Porto Alegre, BR-RS, 2016.

1. Cryptococcus neoformans. 2. Infecção Experimental. 3. Sistema purinérgico. 4. Sistema colinérgico. 5. Enzimas. I. FERREIRO, LAERTE, orient. II. MORAIS SANTURIO, JANIO, coorient. III. Título.

Elaborada pelo Sistema de Geração Automática de Ficha Catalográfica da UFRGS com os dados fornecidos pelo(a) autor(a).

"A mente que se abre a uma nova ideia,
jamais voltará ao seu estado original"
Albert Einstein.

Dedico este trabalho a minha tia Dalva Maria de Azevedo (*in memorian*) pelo carinho, amor e ensinamentos.

AGRADECIMENTOS

- À Deus por sempre guiar meus passos;
- À minha família, meu pai Dario Luiz Pereira de Azevedo, minha mãe Vera Marlene de Azevedo, meu irmão Maurício Cristiano de Azevedo, minha irmã Simone Maria de Azevedo e meu sobrinho Gustavo de Azevedo, sem o amor e apoio de vocês eu não teria chegado até aqui. Amos vocês!!!
- Ao meu namorado Thiago Guilherme Schwanz, pela paciência, pelo amor e carinho, pelo aprendizado e principalmente pelo companheirismo.
- Ao meu orientador Prof. Dr. Laerte Ferreiro, pela oportunidade, amizade, confiança e incentivo;
- Ao meu co-orientador, Prof. Dr. Janio Morais Santurio, agradeço não somente pelo doutorado, mas também pelos 11 anos e meio de orientação e amizade. Faltam palavras para expressar a minha gratidão;
- Ao Prof. Dr. Sydney Hartz Alves, pela sua amizade, apoio e disposição;
- Ao Prof. Dr. Aleksandro Schaefer da Silva, pela ajuda e orientação;
- Às professoras Daniela Pereira e Sonia Botton pela amizade, pelo apoio e confiança;
- Às grandes "friends" e irmãs Danieli Urach, Carla Weiblen, Tatiana Correa Ribeiro, Vanessa Shopf Machado, pela amizade, paciência e apoio nos momentos difíceis;
- Aos amigos do Laboratório de Pesquisas Micológicas – LAPEMI, pela amizade, pelo apoio e toda ajuda de sempre;
- Ao amigo, Gustavo Wissmann, pela colaboação e incetivo ao meu perído sanduíche em Sevilla.
- As "hermanas" e companheiras María Torralbo Castiñeira, Patricia Luque Fernández e Maria Madalena Lozano. Sevilla foi muito melhor com a amizade de vocês.
- Ao grupo pesquisa do IBIS/Sevilla Enrique Calderón, Carmen de La Horra, Vicente Friaza, Rúben Morilla e Amaia Gonzáles, pela amizade e pelo acolhida.
- À Universidade Federal de Santa Maria e ao LAPEMI, por oportunizar a realização deste trabalho;
- À Universidade Federal do Rio Grande do Sul, por oportunizar a realização dos meus estudos de doutorado;
- Ao Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq) pelo apoio financeiro através de uma bolsa de doutorado;

- À CAPES pela bolsa PDSE que viabilizou a realização de um sonho. Um período de grande conhecimento técnico/profissional, mas também uma vivência cultural indescritível.

RESUMO

A criptococose é uma infecção fúngica sistêmica, predominantemente oportunista, causada por leveduras encapsuladas do gênero *Cryptococcus*. As infecções por *Cryptococcus neoformans* são comuns em nível mundial, e as formas graves são observadas nos pacientes imunocomprometidos. A principal fonte de infecção da criptococose são fezes de pássaros (principalmente pombos) contaminadas com o fungo, sendo a inalação de basidiósporos a principal via de infecção. O estabelecimento e a propagação da infecção são altamente dependentes da imunidade do hospedeiro, sendo o sistema imune celular o mecanismo primário de defesa do organismo contra *C. neoformans*. Nos últimos anos têm-se descrito outros elementos como ativadores e moduladores da resposta imune, destacando o sistema purinérgico e o sistema colinérgico. Desta maneira, este estudo buscou avaliar a influência da criptococose experimental sobre a atividade do sistema purinérgico e colinérgico, através de três objetivos: (1) avaliar a atividade da ecto-difosfoidrolases (E-NTPDase) e ecto-adenosina deaminase (E-ADA) em linfócitos e soro; (2) atividade da acetilcolinesterase (AChE) no cérebro e linfócitos, e butirilcolinesterase (BChE) no soro; e (3) avaliar os níveis de purinas no soro. Os resultados da avaliação do sistema purinérgico demonstraram que a hidrólise do trifosfato de adenosina (ATP) e difosfato de adenosina (ADP) foram diminuídas, bem como a atividade da E-ADA também esteva diminuída. Em relação a dosagem das colinesterases, observou-se um aumento na atividade da AChE nos linfócitos e no cérebro, e diminuição da BChE. Na dosagem do nível de purinas no soro, verificou-se um aumento nos níveis de ATP e adenosina (ADO) no dia 20 pós-infecção (PI), aumento de ATP e diminuição da ADO, inosina e ácido úrico no dia 50 PI. A avaliação da atividade da E-NTPDase e E-ADA levou a conclusão de que seus comportamentos hidrolíticos seriam compensatórios enquanto a E-NTPDase teria uma ação pró-inflamatória a E-ADA teria uma ação anti-inflamatória, gerando mecanismo de proteção contra danos teciduais secundários, possivelmente gerados respostas exacerbadas à infecção por *C. neoformans*. Adicionalmente, os dados da atividade da AChE, em amostras correspondentes, comprovaram o estabelecimento de uma resposta pró-inflamatória, corroborando com a hipótese da necessidade de um mecanismo de modulação. Por fim, observou-se um aumento nos níveis extracelulares de ATP caracterizando uma resposta pró-inflamatória. Desta forma, foi possível observar que existe uma participação direta dos sistemas purinérgico e colinérgico na imunomodulação da criptococose experimental, contribuindo para a instalação de uma resposta imune celular adequada para combater a proliferação da levedura, e um mecanismo de redução de danos teciduais associados à resposta imune exacerbada.

Palavras-chave: *Cryptococcus neoformans*, enzimas, sistema purinérgico, sistema colinérgico.

ABSTRACT

Cryptococcosis is a systemic fungal infection predominantly opportunistic, caused by encapsulated yeast from Cryptococcus genus. Cryptococcus neoformans infections are common worldwide, and the severe forms are observed in immunocompromised patients. The main source of cryptococcosis infection are bird droppings (especially pigeons) contaminated with the fungus, and the inhalation of basidiospore is the main route of infection. The establishment and spread of infection are highly dependent of the host immunity, and the cellular immune system is the primary mechanism for defense against C. neoformans. In recent years it has been described other elements as activators and modulators of the immune response, highlighting the purinergic and the cholinergic system. Thus, this study aimed to evaluate the influence of experimental cryptococcosis on the activity of purinergic and cholinergic systems through three objectives: (1) to evaluate the activity of the ecto-diphosphohydrolases (E-NTPDase) and ecto-adenosine deaminase (E-ADA) in lymphocytes and serum; (2) the activity of acetylcholinesterase (AchE) in the brain and lymphocytes, and butyrylcholinesterase (BChE) in serum; and (3) evaluate the serum levels of purines. The results of the evaluation in the purinergic system demonstrated that the hydrolysis of adenosine triphosphate (ATP) and adenosine diphosphate (ADP) was decreased as well as the E-ADA activity. For the dosage of the cholinesterase, there was an increase in AChE activity in lymphocytes and in the brain, and a decreased in BChE. The measurement of serum purine level demonstrate an increase in the levels of ATP and adenosine) on day 20 post-infection (PI), an increased in ATP and decreased in ADO, inosine and uric acid on day 50 PI. The assessment of the E-NTPDase and E-ADA activity led the conclusion that their hydrolytic behavior would be compensatory while the E-NTPDase would have a pro-inflammatory action, E-ADA would have an anti-inflammatory action, generating protective mechanism against secondary damage tissue, producing possibly exacerbated responses to C. neoformans infection. In addition, data of AChE activity in corresponding samples confirmed the establishment of a pro-inflammatory response, corroborating the hypothesis of the need for a modulation mechanism. Finally, there was an increase in extracellular levels of ATP featuring a pro-inflammatory response. In this way, it was observed a direct involvement of the purinergic and cholinergic systems in immunomodulation of experimental cryptococcosis, contributing to the installation of an immune cell response suitable to combat the proliferation of yeast, and a reduction mechanism of tissue damage associated with response immune exacerbated.

***Keywords**: Cryptococcus neoformans, enzymes, purinergic system, cholinergic system.*

LISTA DE ABREVIATURAS E SIGLAS

AIDS	Síndrome da Imunodeficiência Adquirida
Acetil-CoA	Acetil Coenzima-A
ACh	Acetilcolina
AChE	Acetilcolinesterase
ADO	Adenosina
ADP	Difosfato de adenosina
AMP	Monofosfato de adenosina
ATP	Trifosfato de adenosina
BCh	Butirilcolina
BChE	Butirilcolinesterase
E-ADA	Ecto-adenosina aeaminase
E-NTPDases	Ecto-difosfoidrolases, CD39 ou Ecto-Apirases
HYPO	Hipoxantina
IgG	Imunoglobulina da classe G
IgM	Imunoglobulina da classe M
IL	Interleucina
INF-ɣ	Interferon-gama
INO	Inosina
iNOS	Óxido Nítrico-Sintase induzida
LCR	Líquido Cefalorraquidiano
NK	*Natural killer*
NOS	Óxido nítrico-sintase
ON	Óxido nítrico
p.i./PI	Pós-infecção
SNA	Sistema nervoso autônomo
SNC	Sistema nervoso central
SNP	Sistema nervoso periférico
TNF-α	Fator de necrose tumoral-alfa
URIC	Ácido úrico
UTP	Uridina trifosfato
XAN	Xantina

LISTA DE FIGURAS

REVISÃO DE LITERATURA

ARTIGO 1

ARTIGO 2

ARTIGO 3

LISTA DE ANEXOS

LISTA DE TABELAS

ARTIGO 2

SUMÁRIO

APRESENTAÇÃO

Os resultados que fazem parte desta tese estão apresentados sob a forma de dois artigos científicos publicados em revistas científicas e um artigo submetido para publicação. A discussão traz um apanhado destes artigos, com suas interpretações discutidas sob um ponto de vista que buscou estabelecer uma conectividade entre os objetivos e resultados obtidos nos artigos que compuseram as bases científicas desta tese.

As **REFERÊNCIAS BIBLIOGRÁFICAS** se referem somente às citações que aparecem nos itens **INTRODUÇÃO**, **REVISÃO BIBLIOGRÁFICA** e **DISCUSSÃO** desta tese.

1 INTRODUÇÃO

As infecções fúngicas oportunística têm aumentado drasticamente nas últimas décadas, tendo se tornado uma importante causa de mortalidade entre os pacientes acometidos por estas patologias (VITALE et al., 2012). O problema geralmente se agrava ainda mais em pacientes imunossuprimidos, que são mais susceptíveis ao desenvolvimento infecções de fúngicas sistêmicas (SABLE et al., 2008; ESPINEL-INGROFF, 2009).

A criptococose é uma enfermidade micótica sistêmica, que acomete o sistema respiratório do homem, animais domésticos e silvestres, podendo disseminar-se para o sistema nervoso central (SNC), olhos, pele e outros órgãos. O estabelecimento e a disseminação desta infecção são altamente dependentes da imunidade do hospedeiro, os imunocomprometidos são mais afetados, embora a doença também ocorra em pacientes hígidos (NEGRONI, 2012).

A instalação de diversos patógenos em humanos e animais provoca o desenvolvimento de uma resposta imunológica na tentativa de controlar estes agentes infecciosos (ABBAS & LICHTMAN, 2005). O sistema de sinalização purinérgica desempenha um importante papel na modulação da resposta imunológica inflamatória através de biomoléculas extracelulares, como os nucleotídeos da adenina (adenosina trifosfato (ATP), adenosina difosfato (ADP) e adenosina monofosfato (AMP)) e seus derivados, os nucleosídeos da adenosina. Também outras purinas conhecidas como inosina, hipoxantina, xantina e acido úrico influenciam na resposta inflamatória (ZIMMERMANN, 2000; RALEVIC & BURNSTOCK, 2003).

Essas biomoléculas, em condições fisiológicas, são metabolizadas a fim de manter seus níveis extracelulares em concentrações normais. Para isto, existe um sistema constituído por enzimas ancoradas à membrana plasmática das células, como plaquetas, linfócitos e células endoteliais, denominadas ectoenzimas. Fazem parte desse sistema as enzimas ecto-nucleosídeo trifosfato difosfohidrolase (E-NTPDase) e ecto-adenosina deaminase (E-ADA), responsáveis pela regulação dos níveis de ATP e ADP (E-NTPDase) e de adenosina (E-ADA) (YEGUTKIN, 2008). A alteração na atividade destas enzimas tem sido descrita em várias condições patológicas, assim como em modelos experimentais, sugerindo que elas podem ser um importante parâmetro de diagnóstico patológico (BURNSTOCK, 2007).

Elevadas concentrações de nucleotídeos da adenina podem ser encontradas no meio extracelular, esta informação pode ser interpretada como um dano ou estímulo da proliferação célular por ação de patógenos (BURNSTOCK, 2006; DESROSIERS et al., 2007). Diversos estudos mostram que os nucleotídeos, ATP e ADP, secretados por linfócitos, leucócitos, plaquetas e células endoteliais danificadas, servem como mediadores capazes de modular o processo de inflamação e trombose vascular (RALEVIC & BURNSTOCK, 1998). A adenosina, que é um metabólito da degradação de nucleotídeos da adenina, desempenha funções importantes como efeitos neuromodulatórios, regulação de processos inflamatórios, inibição da agregação plaquetária e vasodilatação, sendo considerado também um agente cardioprotetor (ELY & BERNE, 1992; SOSLAU & YOUNGPRAPAKORN, 1997; BOROWIEC et al., 2006).

O sistema colinérgico é uma das mais importantes vias modulatórias do SNC (DESCARRIES et al., 1997; PERRY et al., 1999). A sinalização colinérgica também está notavelmente envolvida em ações anti-inflamatórias (BOROVIKOVA et al., 2000, DAS, 2007). A acetilcolina (ACh) é o neurotransmissor das sinapses e junções neuroefetoras colinérgicas dos SNC e sistema nervoso periférico (SNP), possuindo um papel crucial no SNC, associado com funções cognitivas, processamento de informações sensoriais, organização cortical do movimento e controle do fluxo sanguíneo cerebral (SCREMIN et al., 1997). Os níveis de ACh são controlados pela ação das enzimas acetilcolinesterase (AChE) e butirilcolinesterase (BChE) (DAJAS-BAILADOR & WONNACOTT, 2004), presentes em tecidos colinérgicos e não colinérgicos, assim como no sangue e outros fluídos corporais.

A criptococose possui grande importância na saúde humana e animal, é conhecida a sua habilidade de estimular as respostas imunoinflamatórias e a capacidade de colonizar órgãos e tecidos, estudos que buscam aprofundar os conhecimentos correlacionando esses aspectos são de extrema valia. Neste sentido, buscou-se estabelecer a participação dos sistemas purinérgico e colinérgico na criptococose experimental, em relação à modulação dos processos imunoinflamatórios. Os resultados obtidos em todos os estudos serão expostos e discutidos no decorrer desta tese.

2 REVISÃO DE LITERATURA

2.1 Agente Etiológico

2.1.1 Histórico e classificação

Cryptococcus neoformans é uma levedura encapsulada que causa criptococose, uma infecção fúngica de distribuição mundial, que acomete animais e humanos. Este fungo foi isolado inicialmente no final do século XIX por Busse e Buschke em 1894. O relato descreve o caso de uma mulher de 31 anos de idade com uma lesão da tíbia. Nesta ocasião o fungo isolado foi chamado de *Saccharomyces hominis*. No mesmo ano Sanfelice, a partir de suco de pêssego, também conseguiu isolar esta mesma levedura encapsulada denominando-a de *Saccharomyces neoformans* (BARNETT, 2010). Em 1895, Curtis relatou na França o segundo caso de criptococose, isolando esta levedura de uma lesão de quadril e denominando-a de *Saccharomyces subcutaneos tumefaciens* (HEITMAN et al., 2011).

Vuillemin, em 1901, reclassificou os isolados de Busse, Bushcke e Sanfelice colocando-os no gênero *Cryptococcus* baseado na inabilidade do mesmo em fermentar fontes de carbono e na incapacidade de formar ascósporos, que caracteriza as espécies do gênero *Saccharomyces*. Frothinham em 1902 reconheceu a patogenicidade da levedura através da lesão pulmonar em um cavalo nos Estados Unidos, similar ao fungo isolado por Busse e Buschke; com esses achados, foi comprovada a patogenicidade desta levedura, tanto para humanos quanto para animais (BARNETT, 2010).

A primeira metade do século XX marcou a consolidação de *C. neoformans* como uma levedura patogênica. Em 1935, Benham estudando vários isolados recuperados de humanos, incluindo aqueles obtidos por Busse, Curtis e Sanfelice, agrupou-os dentro do gênero *Cryptococcus* spp. Continuando seus estudos, Benham, em 1950, propôs a denominação *C. neoformans* para referir à forma imperfeita da levedura permanecendo após estudos realizados por KURTZMAN & FELL (1998).

Até a década de 1980, a criptococose era rara, assim como os estudos sobre este fungo. A partir deste momento, com o surgimento da síndrome da imunodeficiência adquirida (AIDS), bem como maior disponibilidade e utilização de fármacos e tratamentos imunossupressores, houve um aumento na incidência da criptococose (NEGRONI, 2012). Neste período os isolados de *C. neoformans* eram classificados em

três variedades, *C. neoformans* variedade *neoformans*, *C. neoformans* variedade *grubii* e *C. neoformans* variedade *gattii*.

Atualmente, o gênero *Cryptococcus* apresenta duas espécies patogênicas, *C. neoformans* e *C. gattii* que são classificadas em cinco sorotipos capsulares: A, D, AD, B e C, baseados nas reações de aglutinação dos antígenos dos polissacarídeos presentes na cápsula (HEITMAN et al., 2011). *C. neoformans* possui duas variedades, var. *grubii* e var. *neoformans*, com sorotipos A, D e hibrido AD. A espécie *C. gattii* corresponde aos sorotipos B e C (NEGRONI, 2012). *C. neoformans* é amplamente distribuído na natureza, especialmente em excrementos e ninhos de aves e casca de árvore; enquanto que a principal fonte de infecção de *C. gattii* são as folhas, cascas e frutos de árvores. Além dos cinco sorotipos capsulares, o complexo de espécies também foi dividido em nove distintos tipos moleculares com base em estudos das sequências de DNA, analisadas por RAPD (random amplified polymorphic DNA), AFLP (Amplified Fragment Length Polymorphism), RFLP (Restriction Fragment Length Polymorphism) e MLST (Multilocus Sequence Typing). O complexo *C. neoformans* e *C. gattii* está em constante discussão, a divisão atualmente aceita pode ser observada na Figura 1 (LIN & HEITMAN, 2006).

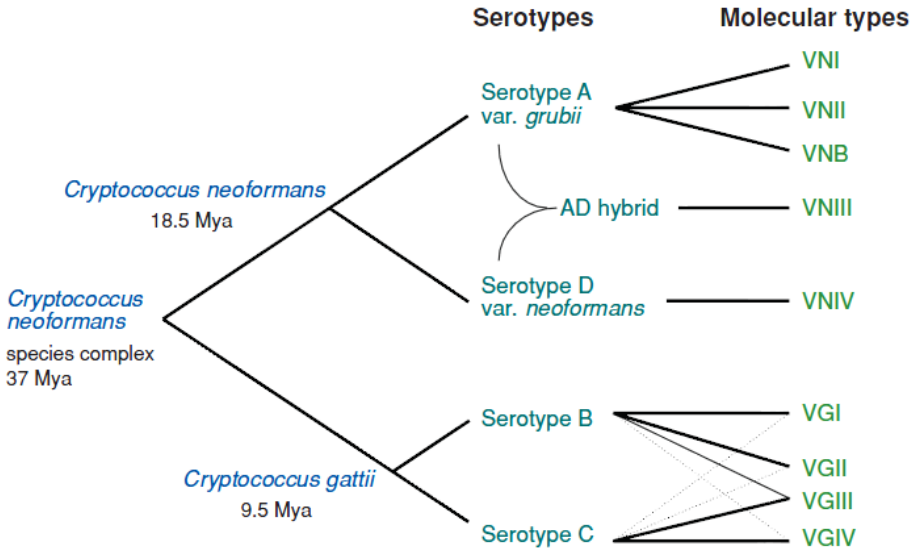

FIGURA 1- Evolução do complexo de espécies *C. neoformans*. As linhas sólidas e tracejadas indicam a prevalência do respectivo sorotipo em cada tipo molecular (LIN & HEITMAN, 2006).

Mundialmente os tipos moleculares VNI e VGI predominam como agentes da criptococose (MEYER et al., 2003; BOEKHOUT et al., 2001). Isolados clínicos e ambientais no Brasil mostram considerável diversidade genética de ambas as espécies, sendo o tipo molecular mais comum o VNI (64%), seguido por VGII (21%), VNII (5%), VGIII (4%), VGI e VNIV (3% cada), e VNIII (<1%) (TRILLES et al., 2008). A região sul do Brasil apresenta como tipo molecular predominante VNI, atingindo, sobretudo pacientes imunocomprometidos, principalmente com AIDS (IGREJA et al., 2004, MATSUMOTO et al., 2007). Diferentemente, no norte o agente mais comum é o tipo VGII sorotipo B, causando doença principalmente em pacientes imunocompetentes. O mesmo tipo molecular VGII foi o responsável pela epidemia no Canadá, identificado na grande maioria dos isolados clínicos humanos, veterinários e ambientais (STEPHEN et al., 2002).

2.1.2 Características da levedura

Cryptococcus spp. é uma levedura que microscopicamente se apresenta na forma arredondada e ovalada, com diâmetro variando de 3 a 7 μm de diâmetro. Possui uma cápsula de polissacarídeos complexos que varia de 1-30 μm de espessura, altamente antigênica, sendo um importante fator de virulência, responsável por diversos efeitos inibitórios sobre a resposta imune (GAZZONI et al., 2008; MCFADDEN et al., 2006). As colônias de *Cryptococcus* spp. são cremosas ou gomosas de consistência mole, lisa de cor branca, creme ou marrom, geralmente apresentando crescimento rápido (HEITMAN et al., 2011).

2.1.3 Fatores de virulência

A patogenia da criptococose é determinada pela expressão de fatores de virulência, dentre estes, podemos destacar: a formação de pigmentos melanínicos, presença de uma cápsula polissacarídica e termotolerância a 37˚C (HEITMAM et al., 2011). Estes fatores são regulados através de um controle genético, ou seja, vários genes estão envolvidos na capacidade do fungo em causar doença e garantir sua sobrevivência na natureza (ROSA & SILVA et al., 2008).

O gênero *Cryptococcus* segue a regra geral do Reino Fungi de que a maioria destes micro-organismos é incapaz de crescer à temperatura encontrada nos hospedeiros

mamíferos. A tolerância de *C. neoformans* e C. *gattii* à temperatura corpórea (37˚C) é uma pré-condição para o estabelecimento da doença em humanos e animais (LIN, 2009). No entanto, outras espécies como *C. laurentii*, *C. albidus*, *C. adeliensis* e *C. curvatus* tem sido ocasionalmente relatada como causa de infecção para humanos (CHAYAKULKEEREE & PERFECT, 2006; VIVIANI & TORTORANO, 2009). O desenvolvimento desta levedura em condições de temperatura elevada tem sido associado a vários genes, que estão envolvidos na manutenção da parede celular, na resposta ao estresse e no metabolismo oxidativo (NICHOLS et al., 2007). Estudos têm demonstrado que a manipulação destes genes pode resultar em perda completa ou atenuação da virulência destas cepas em modelos experimentais (PERFECT, 2006).

C. neoformans e *C. gattii* produzem uma enzima chamada fenoloxidase, a partir da qual é possível sintetizar um pigmento melanínico que tem função antioxidante e antifagocítica (HEITMAN et al., 2011). Quando a levedura é circundada pela melanina esta é capaz de neutralizar grandes porções de oxidantes produzidos pelo estímulo de macrófagos. A melanina é sintetizada a partir de precursores como dopamina, norepinefrina e epinefrina (EISENMAN et al., 2007). O cérebro é rico em substratos para fenoloxidase, tais como a dopamina, e esta poderia ser a responsável pelo tropismo deste micro-organismo pelo sistema nervoso. Além disso, há evidências de que no cérebro ocorra limitada ação de linfócitos T, o que ocasionaria um atraso no desenvolvimento da resposta inflamatória no tecido cerebral em relação a outros órgãos (KON et al., 2008). A melanina também interfere na ação e eficácia dos agentes antimicrobianos e antifúngicos utilizados no tratamento da criptococose. Este pigmento neutraliza a atividade de neutrófilos defensivos e limita avidamente drogas antifúngicas amplamente usadas no tratamento de infecções fúngicas graves (SABIITI & MAY, 2012).

A cápsula de *Cryptococcus* spp. é uma estrutura presente na superfície da levedura, composta majoritariamente por dois polissacarídeos, glucuronoxylomanana (GXM) e galactoxylomanana (Ga1XM), assim como por manoproteínas. Essa estrutura que é essencial para a virulência também auxilia no processo de identificação rápida e diagnóstico da meningite criptocócica, sendo a base do método clássico com tinta da China (ZARAGOZA et al., 2009). O tamanho da cápsula é variável, ou seja, essa estrutura possui a habilidade de alterar a sua dimensão, isso pode ocorrer entre diferentes cepas, mas também em uma mesma cepa, dependendo de condições ambientais como a presença de fosfolipídios, CO_2 e disponibilidade limitada de ferro

(O'MEARA & ALSPAUGH, 2012). Dentro de algumas horas após a infecção o aumento da cápsula é visível e a virulência é ressaltada por meio das propriedades capsulares. Já no seu isolamento direto do ambiente a cápsula apresenta tamanho reduzido (DAMBRÓS, 2005).

Os polissacarídeos capsulares inibem a fagocitose, consomem o complemento, absorvem e neutralizam as opsoninas. A cápsula torna a ingestão da levedura pelos fagócitos do hospedeiro extremamente ineficaz na ausência de opsoninas (HETIMAN et al., 2011). Estirpes com maior produção de cápsula são menos fagocitadas por macrófagos, além de proteger o fungo do estresse oxidativo pelo sistema imune e também contra a ação de medicamentos (ZARAGOZA et al., 2009; CORDOBA et al., 2011). Além dessa função protetora, a cápsula tem funções ofensivas. Os carboidratos capsulares também secretados pelo fungo para o meio alteram a atividade de linfócitos T e macrófagos e diminuindo a efetividade da resposta imune do hospedeiro (VECCHIARELLI, 2007).

Além dos três fatores clássicos abordados, outros potenciais determinantes da virulência do gênero *Cryptococcus* são investigados. Estudos realizados em modelos experimentais *in vivo* com cepas de *Cryptococcus* spp. que apresentam o gene da urease nulo mostram reduzida virulência (COX et al., 2000). Também as fosfolipases demonstram ser importante fator de virulência de *Cryptococcus* spp., uma vez que, são capazes de hidrolisar cadeia de ésteres degradando a membrana celular e levando à lise da célula (MA & MAY, 2009).

2.2 Criptococose

Criptococose é uma infecção fúngica com distribuição geográfica universal, que acomete principalmente o sistema respiratório e o SNC de humanos e animais (NEGRONI, 2012). As principais espécies do gênero *Cryptococccus* causadoras da doença são *C. gattii* e *C. neoformans*, sendo esta última espécie a mais frequentemente isolada independente da região geográfica e do sítio de infecção (FAVALESSA et al., 2009). *C. neoformans* var. *grubii* é a espécie mais comum e responsável por mais de 80% de todas as infecções criptocócicas, além disso, é o agente etiológico em 99% das infecções em pacientes com AIDS (CASADEVALL & PERFECT, 1998). No Brasil, *C. neoformans* foi responsável por 50% dos casos de morte devido a micoses oportunísticas em pacientes com AIDS entre 1996 a 2006 (PRADO et al., 2009).

C. neoformans ocorre em diversos substratos orgânicos, sendo frequentemente isolado da excreta dessecadas de aves, especialmente dos pombos urbanos (*Columba livia domestica*) (KON et al., 2008). A porta de entrada para *Cryptococcus* spp. é primariamente a inalação de basidiósporos do ambiente acometendo a via respiratória (Figura 2). A criptococose pulmonar pode variar desde formas assintomáticas a quadros sintomáticos de pneumonia com sinais como dor no peito, tosse, febre e até mesmo perda de peso (CHAYAKULKEEREE & PERFECT, 2006). A infecção também pode ocorrer por inoculação direta nos tecidos ou por meio de transplantes ou trauma na pele (PERFECT & CASADEVALL, 2002; IDNURM, 2010). Após a penetração no organismo do hospedeiro suscetível, os fungos podem produzir doença aguda ou latente. As manifestações iniciais da doença são provavelmente controladas pela quantidade do inóculo, o estado imune do hospedeiro e a virulência da cepa (MORETTI et al., 2008).

Esta levedura apresenta tropismo pelo SNC promovendo a colonização do mesmo, essa invasão ocorre por via hematógena (SILVA et al., 2006). Outros tecidos também podem ser afetados, especialmente em indivíduos acometidos pelo vírus da AIDS ou em terapia imunossupressora drástica (MITCHELL & PERFECT, 1995), tais como: pele, pulmões, próstata, trato urinário, olhos, miocárdio, ossos e articulações (CHAYAKULKEEREE & PERFECT, 2006).

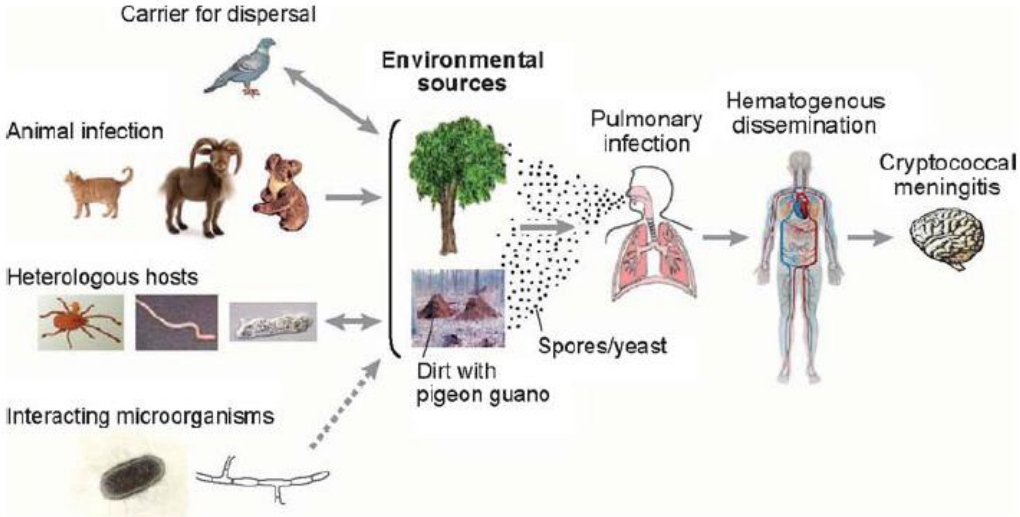

FIGURA 2- Ciclo de infecção de *Cryptococcus* (ambiente e hospedeiro). Adaptado de LIN & HEITMAN (2006).

Já em hospedeiros imunocompetentes, a imunidade humoral é ativada e a doença normalmente é contida na forma de um granuloma pulmonar, sendo que a infecção pode

tornar-se latente e ser reativada quando o hospedeiro torna-se imunossuprimido (STEENBERGEN & CASADEVALL, 2003). Investigações sorológicas mostram a presença de anticorpos contra *C. neoformans* em pessoas sem história clínica, demonstrando que o desenvolvimento da doença é menos frequente que a infecção (MORETTI et al., 2008).

2.2.1 Resposta imunológica do hospedeiro

Tanto o sistema imune inato quanto o sistema imune adaptativo protegem o hospedeiro de uma infecção por *Cryptococcus* spp. A imunidade celular é determinante na defesa dos pacientes contra a infecção pelo *C. neoformans.* O pulmão é a porta de entrada para este patógeno, sendo os macrófagos alveolares capazes de inibir e destruir o fungo. No recrutamento de macrófagos as células $CD4^+$ têm um importante papel, porém, esta é a principal população de células não funcionais em pacientes com AIDS, o que os torna mais suscetíveis à criptococose (ZHOU & MURPHY, 2006). A imunidade celular contra patógenos intracelulares requer a presença de interferon gama (IFNγ), fator de necrose tumoral alfa (TNFα), interleucina 2 (IL-2), interleucina 12 (IL-12) e ausência de interleucina 4 (IL-4), interleucina 10 (IL-10) e interleucina 13 (IL13) (OLSZEWSKI et al., 2010). Alterações nas células T e também nas células dendríticas têm sido identificadas como um fator predisponente para as formas mais graves da criptococose, com rápida progressão e disseminação (ZHOU & MURPHY, 2006).

Em paciente com AIDS há uma diminuição progressiva da imunidade celular em consequência da diminuição ou alteração do funcionamento de linfócitos T, o que leva à disfunção do sistema linfocítico-fagocitário, favorecendo o aparecimento de infecções fúngicas oportunistas. A incapacidade de monócitos de indivíduos com AIDS em internalizar *C. neoformans* é a provável causa da progressão da criptococose nestes indivíduos (MONARI et al., 1997). O vírus da AIDS determina múltiplos efeitos na resposta imune ao fungo como o distúrbio na produção de citocinas, queda na resposta Th1, queda da atividade anti-*Cryptococcus* spp. de monócitos e macrófagos alveolares com redução na produção de peróxido de hidrogênio (H_2O_2) (PIETRELLA et al., 2001; PIETRELLA et al., 2004).

O mecanismo de defesa do hospedeiro contra *C. neoformans* em infecção do SNC aparentemente é similar ao dos pulmões, via resposta Th1 mediada por células efetoras (HUFFNAGLE & McNEIL, 1999). O papel da resposta Th1 também é

reforçado em pacientes que sobreviveram à neurocriptococose. Estes indivíduos apresentam níveis mais elevados de IL-6, IFN-γ e IL-8 no líquido cefalorraquidiano (LCR) do que os pacientes que foram a óbito (SIDDIQUI et al., 2005). Além disto, a interação de *C. neoformans* com as células NK inibe a produção de citocinas como o fator de estimulação de colônias de macrófagos e granulócitos (GM-CSF) e TNF-α (MURPHY et al., 1997; BLACKSTOCK & MURPHY, 2004).

Em modelos experimentais existe uma baixa produção de TNF-α após a infecção por *C. neoformans*, o que poderia explicar o aumento observado na produção de IL-4, IL-5 e IL-10 (HERRING et al., 2005). Animais deficientes na produção de IL-12 e IL-6 apresentam maior suscetibilidade à infecção por *C. neoformans*, em contraste com animais deficientes em IL-10, que são mais resistentes. Estes dados reforçam a hipótese de que as citocinas Th1 contribuem para a resistência natural contra a infecção pela levedura (CHEN et al., 2005; LINDELL et al., 2005). Em contraste, a imunidade mediada por citocinas Th2 contribuem para o progresso da infecção, inibindo a propagação das células T (OLSZEWSKI et al., 2010). Todavia, a resposta mediada por anticorpos contra *C. neoformans* parece depender das associações entre citocinas Th1 e Th2 (BEENHOUWER et al., 2001). A resposta do tipo Th17 vem sendo estudada e caracterizada, porém, os primeiros estudos demonstram que ela colabora na prevenção da criptococose, visto que, ela aciona macrófagos e impede a proliferação intracelular da levedura (ANTACHOPOULOS & WALSH, 2012).

A habilidade de *C. neoformans* em causar doença depende do tipo de ativação de macrófagos. Durante o início do processo de infecção, células T produzem citocinas ativadoras de macrófagos com formação de granuloma e destruição do fungo intracelular ou contido no estado de dormência (FELDMESSER et al., 2001). Nos indivíduos com imunidade celular debilitada, *C. neoformans* cresce intracelularmente, podendo lisar o macrófago (reativação da disseminação) com a liberação do micro-organismo que pode então infectar outros fagócitos, promovendo o crescimento do fungo (proliferação) (MCQUISTON & WILLIAMSON, 2012).

C. neoformans também é capaz de crescer extracelularmente, evadindo da fagocitose através da produção de fatores específicos que bloqueiam a sua adesão e internalização pela célula fagocítica, incapacitando o reconhecimento e o desenvolvimento da resposta pelo sistema imune do hospedeiro (DEL POETA, 2004). Desta forma, *C. neoformans* é capaz de sobreviver e replicar-se intra e

extracelularmente, aumentando a dinâmica de contato com o sistema imune do hospedeiro (HEITMAN et al., 2011).

2.2.2 Criptococose em animais

A criptococose tem sido citada numa grande variedade de animais domésticos, porém dados desta infecção ainda são escassos quando comparados com humanos (HEITMAN et al., 2011). Os animais domésticos, assim como o homem, apresentam a criptococose associada a fatores predisponentes tais como diabetes, intervenções cirúrgicas, neoplasias e tratamentos com glicocorticoides. O vírus da imunodeficiência felina (FIV) da leucemia felina (FeLV) são fatores predisponentes da criptococose em gatos (TABOADA et al., 2005). A criptococose já foi descrita em cães, gatos (BOWLES & FRY, 2009; O'BRIEN et al., 2004, MARTINS et al., 2010) equinos (KOMMERS et al., 2005), bovinos (RIET-CORREA et al., 2011), caprino (CHAPMAN et al., 1990), ovino (SILVA et al., 2009), pássaros (RASO et al., 2004), macaco (HELKE et al., 2006), coala (KROCKENBERGER et al., 2003), boto (STEPHEN et al., 2002) e furão (WYRE et al., 2013).

Em felinos, os sinais clínicos são caracterizados pela formação de exsudato nasal e ocular, cegueira, incoordenação, febre, tosse, tumefação da cavidade nasal e faríngea por invasão de *C. neoformans* ou *C. gattii,* formando massas expansivas semelhantes às neoplasias, com posterior penetração na cavidade craniana e nervos óticos. Alguns gatos podem apresentar apenas infecção crônica de difícil tratamento (PEREIRA & COUTINHO, 2003). Não há confirmação de que haja predisposição quanto a idade, sexo ou raça, ainda que alguns estudos apontem uma maior ocorrência para gatos machos, na faixa de dois a três anos e da raça siamês (MALIK et al., 2006).

A criptococose canina geralmente afeta o cérebro, as meninges e os seios paranasais, ocasionando incoordenação, movimentos em círculos, rotação da cabeça, mudança de hábito, hiperestaxia e exsudação nasal (MARCASSO et al., 2005). Também pode ocorrer a formação de granulomas subcutâneos. Na necropsia, observa-se inflamação mucopurulenta dos seios paranasais, cavidade nasal, no cérebro e meninges (O'BRIEN et al., 2004).

Em equinos, a infecção por *C. neoformans* tem sido associada principalmente à rinite e meningite, mas também pode apresentar quadros de encefalite e pneumonia

(RILEY et al., 1992; KOMMERS et al., 2005; ZOPPA et al., 2008). Já em bovinos, a criptococose é rara e está associada à meningoencefalite (RIET-CORREA et al., 2011).

Assim como em humanos, as duas espécies mais comuns relatadas em furões são *C. neoformans* e *C. gattii*. Esta espécie animal vive perto do chão e passa a maior parte do seu tempo ativo, cheirando superfícies para investigar seu ambiente (BROWN, 2004). Isto os torna propensos a entrar em estreito contato com matéria orgânica em decomposição, principal tipo de substrato onde as células de *Cryptococcus* spp. podem ser encontradas (BOVERS et al., 2008). Os sinais clínicos apresentados por furões com criptococose podem variar de acordo com o sistema do órgão afetado. Geralmente são bastante similares aos relatados em cães e gatos, e incluem sistema respiratório, ocular, neurológico e sinais gastrointestinais, bem como a ocorrência de morte aguda (WYRE et al., 2013).

O coala (*Phascolarctos cinereus*), um marsupial arbóreo da Oceania é outro animal de extrema importância para o desenvolvimento da criptococose, esta espécie tem por característica obter seu alimento e abrigar-se em árvores de eucalipto, local de forte associação ao *C. gattii*. Estudos de patologia nesses animais revelam que a criptococose causa aproximadamente 3% da morte nesta espécie animal, tornando-se um modelo natural para estudos sobre a patogenia e epidemiologia desta doença (KROCKENBERGER et al., 2003).

2.2.3 Diagnóstico

O diagnóstico da criptococose, tradicionalmente, baseia-se nos aspectos clínicos da doença, na observação das leveduras encapsuladas pelo exame microscópico direto, no isolamento da levedura em cultivo, provas bioquímicas e na detecção do antígeno capsular no sobrenadante de diversos fluidos, incluindo soro e LCR através da aglutinação de partículas de látex (PERFECT & CASADEVALL, 2002; CHAYAKULKEEREE & PERFECT, 2006). Os tecidos e materiais utilizados para o diagnóstico são: soro, líquor, líquido pleural, linfa, amostras de nódulo linfático, pele e pulmão (BIANCHI et al., 2000).

O exame microscópico direto é realizado usando tinta de nanquim ou microscopia de contraste. É importante levar em consideração que no LCR as leveduras não viáveis mantêm a mesma morfologia das viáveis podendo ser visto durante vários

meses depois do sucesso do tratamento (CHAYAKULKEEREE & PERFECT, 2006; VIVIANI & TORTORANO, 2009). Já o cultivo pode ser efetuado a partir do material clínico semeado em meio *Sabouraud* acrescido de cloranfenicol, sem cicloheximida. As colônias irão se desenvolver em dois a três dias na temperatura entre 28˚C e 37˚C (PERFECT & CASADEVALL, 2002). Essa identificação pode ser complementada com algumas provas bioquímicas. O gênero *Cryptococcus* é capaz de hidrolisar ureia, sendo útil para diferenciar das espécies do gênero *Candida* que não possuem esta mesma propriedade. Além disso, a capacidade de assimilar inositol como única fonte de carbono é importante para diferenciar de espécies de *Rhodotorula* (HEITMAN et al 2011).

O teste sorológico do látex é altamente sensível e específico para a detecção dos antígenos de natureza polissacarídica presentes em casos de meningite criptocócica e nas formas disseminadas da doença, gerando melhores resultados do que a microscopia direta e o cultivo (HUSTON & MODY, 2009). A determinação do título de anticorpos pode ser utilizada para a avaliação da evolução da infecção, com o aumento do título significando a progressão da infecção e uma queda no título expressando uma regressão do processo infeccioso e, portanto, uma resposta positiva ao tratamento (MORETTI et al., 2008). Porém, podem ocorrer reações falso positivas devido à presença de células de *Trichosporon* spp. e bacilos Gram-negativos, devendo então estar sempre atento ao quadro clínico do paciente e outros testes relacionados. (HEITMAN et al., 2011).

O exame histopatológico também é utilizado no diagnóstico da criptococose. A coloração de hematoxilina-eosina é utilizada para visualização de *Cryptococcus* spp. que normalmente se apresenta com formatos ovais, esféricos ou elípticos, de coloração azul-pálido ou rosada. A cápsula não se cora devido a sua natureza mucopolissacarídica e é evidenciada como um halo claro ao redor da célula, podendo ser estreita ou espessa em relação ao seu diâmetro (PEDROSO & CANDIDO, 2006).

Atualmente as técnicas moleculares constituem uma ferramenta de grande utilidade em estudos investigativos e de diagnósticos da criptococose. Apesar da sua não utilização na rotina, estas técnicas apresentam elevada sensibilidade e especificidade para solucionar os obstáculos encontrados no diagnóstico convencional, podendo auxiliar tanto na identificação da espécie, como no sorotipo e no tipo molecular de cada cepa (MEYER, et al., 2003; BOVER et al., 2007). Também é importante ressaltar que estas técnicas podem ser empregadas diretamente na pesquisa

do material clínico sem a necessidade de isolamento, o que torna o diagnóstico final mais rápido e preciso.

2.2.4. Tratamento

O aumento no número de estudos que buscam identificar novos fármacos com ação antifúngica é decorrente da crescente ocorrência de doenças fúngicas como consequência das enfermidades e tratamentos imunossupressores (VITALE et al., 2012). Na clínica veterinária, o tratamento das enfermidades fúngicas tem se baseado principalmente no uso de agentes azólicos, utilizados de forma isolada ou em associação. Na terapia de doenças como a criptococose há necessidade de empregar antifúngicos sistêmicos eficientes e mesmo assim pode ocorrer refratariedade à terapêutica indicada (PFALLER et al., 2011).

O tratamento clássico da criptococose é baseado fundamentalmente na utilização de fármacos, necessitando ser iniciado com a maior brevidade possível e mantido por longo período. A terapêutica selecionada depende da imunidade do paciente, da doença primária apresentada por este e do local de infecção (PERFECT et al., 2010). Os fármacos mais utilizados em veterinária incluem: fluconazol, cetoconazol, itraconazol e anfotericina B. Na clínica humana os antifúngicos mais utilizados são: anfotericina B e suas formulações lipídicas, fluconazol, itraconazol, voriconazol e flucitosina. A ação do antifúngico pode ser fungistática ou fungicida, depende da concentração. Geralmente o paciente deve permanecer em acompanhamento clínico por um extenso período (PERFECT, 2012).

2.3 Sistema purinérgico

O sistema purinérgico é uma via de sinalização importante em diversos processos fisiológicos, envolvendo a resposta imune e inflamatória, dor, agregação plaquetária, a vasodilatação mediada pelo endotélio, proliferação e morte celular (BURNSTOCK, 2006). Três componentes principais fazem parte da sinalização do sistema purinérgico (YEGUTKIN, 2008):

- Nucleotídeos e nucleosídeos extracelulares, que são moléculas mediadoras da sinalização;

- Receptores purinérgicos específicos (P2X, P2Y e P1), através dos quais os nucleotídeos e nucleosídeos exercem seus efeitos;

- As ectoenzimas, responsáveis pela regulação dos níveis dos mediadores no meio extracelular.

Esse conjunto de componentes da sinalização purinérgica descritos acima (figura 3), são expressos nos diferentes tipos celulares, como linfócitos, plaquetas, células endoteliais entre outros, permitindo a formação de complexos distintos da sinalização purinérgica (JUNGER, 2011).

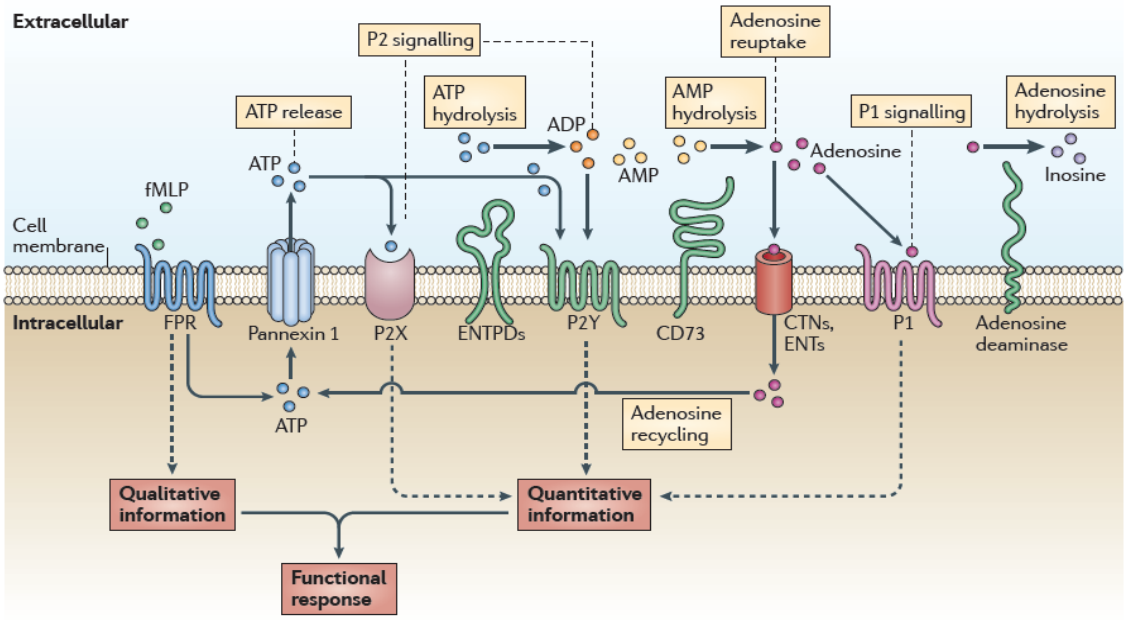

FIGURA 3- Representação esquemática dos componentes da sinalização purinérgica. Adaptado de JUNGER) (2011).

Os nucleosídeos são moléculas resultantes da união de uma base púrica ou pirimídica com uma pentose (ATKINSON et al., 2006). A formação do nucleotídeo ocorre quando os nucleosídeos são fosforilados por quinases específicas. São consideradas importantes moléculas sinalizadoras em tecidos os nucleotídeos: ATP, ADP e AMP (YEGUTKIN, 2008). Em processos inflamatórios e de estresse vascular mecânico ou oxidativo, ocorre um significativo aumento na concentração de nucleotídeos e nucleosídeos extracelulares a partir das células envolvidas nestes processos.

O ATP presente nos compartimentos extracelulares é responsável pela regulação de uma multiplicidade de processos biológicos, como a inflamação, a neurotransmissão, a função cardíaca, o metabolismo ósseo, entre outros. Os nucleosídeos difosfatados e trifosfatados ADP e ATP, juntamente com a adenosina, são conhecidos por controlar a resposta vascular, a injúria endotelial e os processos inflamatórios. No sistema vascular estas moléculas participam da atividade plaquetária, sendo que o ADP está envolvido no recrutamento e na agregação das plaquetas (ATKINSON et al., 2006). Além disso, estas moléculas também participam na ativação ou na inibição do sistema imunológico. Dependendo da concentração, o ATP tem funções pró-inflamatórias, pois é responsável pela estimulação e a proliferação de linfócitos, sendo estas células envolvidas na liberação de citocinas (BOURS et al., 2006). Enquanto isso, a adenosina apresenta-se como uma molécula anti-inflamatória (GESSI et al., 2007). Os nucleotídeos da adenina e a adenosina liberadas no espaço extracelular, desempenham suas funções biológicas através da sua ligação aos receptores purinérgicos presentes na membrana celular (JUNGER, 2011).

O controle dos níveis extracelulares de nucleotídeos e nucleosídeo de adenina são realizados por uma cascata de enzimas ancoradas na membrana celular ou meio intersticial. Dentre estas enzimas destacando-se: ecto-nucleosídeo trifosfato difosfohidrolase (E-NTPDase), ecto-nucleotídeo pirofosfatase (E-NPP), ecto-5'-nucleotidase e ecto-adenosina deaminase (E-ADA) (YEGUTKIN, 2008). Estas enzimas atuam em conjunto, formando uma cadeia enzimática (Figura 4) 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 enzima ecto-5'-nucleotidase formando adenosina. Finalmente, a adenosina é desaminada pela E-ADA em inosina (YEGUTKIN, 2008). Além disso, a cascata purinérgica continua controlada por enzimas que regulam os níveis de outras purinas conhecidas como hipoxantina, xantina e acido úrico, sendo o último uma potente molécula antioxidante (RALEVIC & BURNSTOCK, 2003).

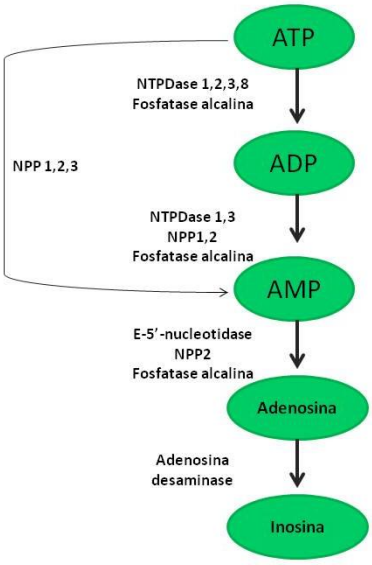

FIGURA 4 – Representação da ação das ectoenzimas no metabolismo dos nucleotídeos da adenina (ATP, ADP e AMP) e nucleosídeo adenosina (Adaptado de BOURS et al., 2006).

2.3.1 E-NTPDase

A classe de enzimas E-NTPDase; EC 3.6.1.5 representa uma conjunto de enzimas que se caracteriza pela capacidade de hidrolisar uma variedade de nucleosídeos 5'-trifosfato e nucleosídeos 5'-difosfato (como ATP e ADP, respectivamente) produzindo monofosfonucleotídeos correspondentes (como AMP) e fosfato inorgânico (Pi) (ZIMMERMAN et al., 2001).

Os membros desta família de enzimas são nomeados de E-NTPDase 1-8 que diferem quanto a localização na célula, distribuição tecidual e especificidade por substratos. Quatro enzimas estão localizadas na membrana celular com seu sítio catalítico voltado para o meio intracelular (E-NTPDase 4, 5, 6 e 7) e quatro para o meio extracelular (E-NTPDase 1, 2, 3 e 8) (ROBSON et al., 2006).

As ectoenzimas estão presentes em diversos tecidos, estando envolvidas em uma diversidade de processos fisiológicos e patológicos (BRUNS, 1990). A principal função da E-NTPDase tem sido principalmente atribuída a hidrólise extracelular dos nucleotídeos ATP e ADP em AMP e na geração de adenosina. Portanto, dependendo da localização tecidual a atividade enzimática possui diferentes papéis fisiológicos. Sendo assim, nos últimos anos, o papel destas enzimas tem sido avaliado em várias condições

patológicas como câncer (ARAÚJO et al., 2005), diabetes (LUNKES et al., 2003) e AIDS (LEAL et al., 2005).

2.3.2 E-ADA

A enzima E-ADA, EC 3.5.4.4 tem a função de regular a concentração de adenosina extracelular, incluindo a conversão da adenosina em inosina, seu metabólito inativo. A E-ADA é amplamente distribuída nos tecidos dos animais vertebrados e se divide em duas isoformas, ADA1 e ADA2. Tecidos contém predominantemente ADA1, no entanto a ADA2 é o principal componente do soro e é um suposto estimulador de células-T. A expressão de atividade desta enzima é heterogênea em tecidos periféricos e no SNC (FRANCO et al., 1997).

A E-ADA está presente em todos os tipos de células, mas a atividade é mais intensificada no timo e em linfócitos periféricos. Esta enzima desempenha um papel importante na função dos linfócitos, sendo também essencial para o crescimento normal, diferenciação e proliferação de linfócitos T (FRANCO et al., 1997; CODERO et al., 2001). Na superfície das células hematopoiéticas esta enzima geralmente pode atuar no processo de maturação eritrocítica (ARAN et al., 1991). A deficiência de E-ADA acarreta em um aumento nos níveis de adenosina extracelular, o que pode contribuir para condições patológicas (ALDRICH et al., 2000).

A atividade da E-ADA pode ser um marcador sensível na infecção e ser utilizada para o acompanhamento do curso na mesma. A atividade desta enzima se mostra elevada no soro de pacientes com tuberculose, malária e leishmaniose visceral (MELO et al., 2000; KHAMBU et al., 2007; ALTUG et al., 2008). A atividade da E-ADA foi dosada em infecção experimental por *Sporothriix schenckii* demonstrando que este fungo filamentoso altera a atividade da E-ADA no soro e linfócitos, o que demonstra o envolvimento desta enzima na patogênese da esporotricose (CASTRO et al., 2012). Uma elevada atividade da E-ADA também já foi relatada em pacientes com pleurite criptocócica (YOSHINO et al., 2010), justificando a necessidade de uma pesquisa mais aprofundada na tentativa de demonstrar seu efeito como um possível marcador na criptococose, auxiliando no diagnóstico desta enfermidade.

2.4 Sistema colinérgico

O sistema colinérgico está envolvido em várias funções vitais, no SNA controla a frequência cardíaca (MENDELOWITZ, 1999) e a contração da musculatura lisa gástrica (ROGERS et al., 1999); já no SNC apresenta envolvimento em funções cognitivas como atenção e memória (GOLD, 2003). A sinalização colinérgica ocorre através da regulação da concentração de acetilcolina (ACh), pelas enzimas acetilcolinesterase (AChE) e butirilcolinesterase BChE; este sistema pode levar a modulação fisiológica dos processos inflamatórios (DAS, 2007).

A síntese da ACh é realizada no citosol do neurônio a partir da acetil coenzima-A e da colina. Depois de sintetizada, a ACh é armazenada nas vesículas sinápticas, onde permanece até que ocorra um estímulo que a libere (ROSAS-BALLINA & TRACEY, 2009). A ACh é amplamente distribuída nos tecidos do SNC, onde seus principais efeitos são excitatórios, efetivados pela ativação de receptores específicos nicotínicos e muscarínicos.

As colinesterases desempenham papéis importantes na neurotransmissão colinérgica central e periférica. Estão presentes em tecidos colinérgicos e não colinérgicos assim como no sangue e outros fluídos corporais. Existem duas classes de colinesterases de acordo com suas propriedades catalíticas, especificidade aos substratos e distribuição tecidual (CHATONNET & LOCKRIDGE, 1989). A acetilcolinesterase (AChE; E.C 3.1.1.7) ou também chamada de colinesterase verdadeira, hidrolisa preferencialmente ésteres com grupamento acetil, e a butirilcolinesterase (BChE; E.C. 3.1.1.8) ou pseudocolinesterase, hidrolisa outros tipos de ésteres como a butirilcolina (TAYLOR & BROWN, 1999).

A AChE possui um papel regulatório na neurotransmissão colinérgica. Ela é responsável pela hidrólise rápida do neurotransmissor ACh (MASSOULIÉ et al., 1993). A AChE é amplamente distribuída no SNC, além disso, foi localizada e identificada em linfócitos, eritrócitos e plaquetas, onde provavelmente apresenta um importante papel na regulação das funções imunes (KAWASHIMA & FUJII, 2000). Esta enzima também tem potentes efeitos sobre a adesão celular, neurogênese, sinaptogênese e hematopoese, desta maneira um aumento ou inibição desta enzima pode resultar em consequências importantes no cérebro e outros órgãos (SOREQ & SEIDMAN, 2001).

A BChE é uma enzima sérica produzida no fígado (TAYLOR & BROWN, 1999), que possui capacidade de hidrolisar diversos ésteres de colina. A estrutura da

enzima BChE é codificada pelo gene BCHE, sendo encontrada nos principais sistemas corpóreos dos mamíferos, como massa branca do cérebro, sistema vascular, respiratório, digestório, urogenital e também em certas glândulas endócrinas e exócrinas. Entretanto sua função biológica ainda não foi claramente estabelecida, havendo sugestões de que esteja relacionada com o metabolismo de lipídeos, condução nervosa lenta e regulação dos níveis de colina no plasma (KUTTY, 1980).

Outra importante propriedade foi atribuída à AChE e BChE, demonstrando que estas enzimas podem atuar como marcadores inflamatórios (DAS, 2007). A atividade dessas enzimas no plasma e nos tecidos é elevada em pacientes com doença de Alzheimer (GIACOBINI, 2003), diabetes mellitus (SRIDHAR et al., 2005) e como resultado deste aumento, o nível de ACh no plasma e tecidos é reduzido. A via colinérgica anti-inflamatória é mediada pela ACh, que é o principal neurotransmissor envolvido e atua através da inibição da produção TNF-α,, IL-1 e uma série de outros mediadores inflamatórios e portanto, modula tanto a resposta imune como a neurotransmissão (DAS, 2007).

3 OBJETIVOS

3.1 Geral

Avaliar a influência da infecção experimental por *C. neoformans* sobre os sistemas purinérgico e colinérgico.

3.2 Específicos

- Avaliar a atividade das ectoenzimas E-NTPDase e E-ADA no soro de ratos infectados experimentalmente com *C. neoformans* e correlacionar com a resposta inflamatória;

- Avaliar a atividade das colinesterases AChE no sangue, cérebro e linfócitos e da BChE no soro de ratos infectados experimentalmente com *C. neoformans* e correlacionar com a resposta inflamatória;

- Determinar os níveis de purinas da cascata purinérgica em amostras de soro de ratos infectados experimentalmente por *Cryptococcus neoformans* e a sua participação na modulação da inflamação.

4 ARTIGOS

Os resultados deste trabalho apresentam-se na forma de artigos. Os artigos estão formatados nas normas do periódico, no qual foi publicado ou está submetido.

4.1 Artigo 1

E-NTPDase and E-ADA activities in rats experimental infected by *Cryptococcus neoformans*

Artigo publicado no periódico **Veterinary Microbiology**[1]

[1]A licença para a inclusão do artigo nesta tese encontrase- no anexo A.

Veterinary Microbiology 174 (2014) 206–213

Contents lists available at ScienceDirect

Veterinary Microbiology

journal homepage: www.elsevier.com/locate/vetmic

E-NTPDase and E-ADA activities in rats experimental infected by *Cryptococcus neoformans*

Maria Isabel de Azevedo [a,b], Laerte Ferreiro [a], Aleksandro S. Da Silva [c], Alexandre A. Tonin [d], Jader B. Ruchel [b], João F.P. Rezer [b], Raqueli T. França [b], Carine E.P. Zimmermann [b], Daniela B.R. Leal [b], Marta M.M.F. Duarte [e], Sonia T.A. Lopes [d], Mariana M. Flores [f], Rafael Fighera [f], Janio M. Santurio [b,*]

[a] *Laboratório de Micologia, Departamento de Patologia e Clinica Animal, Faculdade de Medicina Veterinária (UFRGS), Porto Alegre, RS, Brazil*
[b] *Departamento de Microbiologia e Parasitologia, Universidade Federal de Santa Maria, Santa Maria, RS, Brazil*
[c] *Departamento de Ciência Animal, Universidade do Estado de Santa Catarina, Florianópolis, SC, Brazil*
[d] *Departamento de Pequenos Animais, Universidade Federal de Santa Maria, Santa Maria, RS, Brazil*
[e] *Universidade Luterana do Brasil, Santa Maria, RS, Brazil*
[f] *Departamento de Patologia Veterinária, Universidade Federal de Santa Maria, Santa Maria, RS, Brazil*

ARTICLE INFO

Article history:
Received 6 January 2014
Received in revised form 15 July 2014
Accepted 9 September 2014

Keywords:
Cryptococcosis
Purinergic signaling
Enzyme activity
Lymphocytes

ABSTRACT

Cryptococcus neoformans, the etiological agent of cryptococcosis, is an opportunistic fungal pathogen of immunocompromised individuals. The aim of this study was to evaluate the activities of E-NTPDase and E-ADA in rats experimentally infected by *C. neoformans* var. *grubii*. Adult rats (35) were divided in two groups: 18 for the control group (uninfected) (A), and 17 for the infected group (B). Each group was separated into three sub-groups (A1, A2, A3—B1, B2, B3), and samples were collected on 10, 20, and 30 days post-infection (PI). Leukocyte counts, IFN-γ, TNF-α, IgM, IgG levels, and E-NTPDase and E-ADA activities were analyzed. It was possible to observe that IgG and IgM seric levels of infected rats were significantly elevated ($P < 0.01$) on days 10, 20 and 30 PI, as well as the levels of TNF-α and INF-γ when compared to uninfected rodents. Regarding E-NTPDase activity in lymphocytes, it was possible to observe that the ATP hydrolysis was significantly decreased on days 20 ($P < 0.01$) and 30 PI ($P < 0.05$), while ADP hydrolysis was significantly reduced only on day 20 PI ($P < 0.01$) when compared with uninfected group. Seric E-ADA activity had a significant reduction ($P < 0.01$) during all three evaluated periods when compared to the control group, while E-ADA activity in lymphocytes increased significantly ($P < 0.01$) when compared to the group A on day 10 PI; however on days 20 and 30 PI, its activity was considerable reduced in lymphocytes of infected animals ($P < 0.01$). Therefore, it is possible to conclude that the infection caused by *C. neoformans* in immunocompetent rats leads to changes in the purinergic signaling (NTPDase and E-ADA), concomitantly with an inflammatory response (increased levels of cytokines and immunoglobulins) associated with inflammatory infiltrates and histological lesions in the lung.

* Corresponding author. Tel.: +55 55 32208906; fax: +55 55 3220 8906.
E-mail addresses: janio.santurio@gmail.com, santurio@smail.ufsm.br (J.M. Santurio).

http://dx.doi.org/10.1016/j.vetmic.2014.09.007
0378-1135/

1. Introduction

Cryptococcosis is an invasive fungal infection caused inhalation of *Cryptococcus neoformans* or *Cryptococcus gattii* (Kidd et al., 2004). *C. neoformans* is an encapsulated yeast that causes life-threatening illness predominantly in immunocompromised hosts. *C. neoformans* var. *grubii* is the most common variety responsible for more than 80% of all cryptococcal infections and also for more than 99% of the infections in AIDS patients (Casadevall and Perfect, 1998). Previous studies show that intratracheal inoculation of rats with *C. neoformans* produces similarities to the course of pulmonary infection in immunocompetent humans. (Goldman et al., 1994a, 2000). The infection may be disseminated via blood or lymph from the lungs to other organs, especially to the central nervous system (CNS), if the primary infection is mild or asymptomatic (Mitchell and Perfect, 1995). The mechanism of host defense against *C. neoformans* infection in the CNS and lungs apparently occurs via Th1 cell-mediated effectors (Huffnagle and Mcneil, 1999). However, the response mediated by antibodies to *C. neoformans* seems to depend on the association between Th1 and Th2 cytokines (Beenhouwer et al., 2001).

The modulation of immune and inflammatory responses is signaled, among other systems, by the purinergic system through extracellular biomolecules such as ecto-enzymes, purinergic receivers, adenine nucleotides (ATP, ADP and AMP), and their derived nucleoside adenosine (Ralevic and Burnstock, 1998; Yegutkin, 2008). The ATP and ADP, which are released from many cells including leukocytes, platelets and damaged endothelial cells, are involved in the modulation of the inflammatory process. ATP has an essential role in lymphocyte function being necessary for the release of cytokines by T cells, such as interferon-γ (INF-γ) and interleukin-2 (IL-2), which are involved in triggering an immune response (Ralevic and Burnstock, 1998). However, a high ATP concentration may act as a cytotoxic signal capable of inducing cellular death through the opening of pores on the cellular membrane (Fillippini et al., 1990).

Extracellular levels of ATP and adenosine are controlled by ecto-enzymes such as ecto-nucleoside triphosphate diphosphohydrolase (E-NTPDase; CD39; EC 3.6.1.5) and ecto-adenosine deaminase (E-ADA; EC 3.5.4.4), which are anchored in the cellular surface with their active site facing the extracellular environment (Zimmermann, 2001). E-NTPDase is an enzyme that hydrolyzes extracellular nucleoside tri and/or diphosphates, both ATP and ADP into AMP. Ecto-5′-nucleotidase (CD73, EC 3.1.3.5) terminates the ectonucleotidasic cascade with the hydrolysis of monophosphated nucleotides (AMP) into adenosine, and adenosine deaminase converts adenosine into inosine (Franco et al., 1997; Zimmermann, 2001; Ralevic and Burnstock, 1998). The E-ADA is considered to be a key enzyme in purine metabolism, catalysing the irreversible deamination of adenosine (Franco et al., 1997). It has been demonstrated that this enzyme plays an important role in lymphocyte function and it is essential for the normal growth, differentiation, and proliferation of T lymphocytes (Franco et al., 1997; Codero et al., 2001).

The pathogenesis of cryptococcosis is not fully understood yet, especially regarding to situations involving various levels of immunocompetence. In this sense, considering that the enzymes of the purinergic system are closely involved in the modulation of the immune system and participate in the regulation of pro and anti-inflammatory events, this study aimed to investigate the E-NTPDase and E-ADA activities in rats experimentally infected by *C. neoformans*.

2. Material and methods

2.1. Animals

A total of 35 adult rats, males, with a mean age of 90 days, and weighing 298 (±29) grams were used in this study. They were kept in cages with 6 animals each in experimental facilities under controlled temperature and humidity (25 °C; 70%), fed with commercial feed, with water *ad libitum*, and submitted to a period of 7 days for adaptation. Wistar rats were chosen as the experimental model due to the large volume of samples that they may provide with the possibility of a large number of analyzes. The animals were obtained from the Animal Center of the Universidade Federal de Santa Maria.

This study was approved by the Ethics and Animal Welfare Committee of the Federal University of Santa Maria (UFSM) under protocol number 073/2012.

2.2. Cryptococcus neoformans strain and inoculum

2.2.1. Cryptococcus neoformans

The strain of *C. neoformans* used for animal inoculation was obtained from a clinical case of feline cryptococcosis (Martins et al., 2010). This strain was previously identified as *C. neoformans* var. *grubii* based on its micromorphology and molecular characteristics (GenBank accession number HQ148880).

2.2.2. Inoculum preparation

C. neoformans was isolated in Sabouraud Dextrose Agar (SDA) and incubated at 32 °C in order to obtain a viable and pure strain. Each animal was inoculated with 0.3 mL suspension of 1.7×10^7 cells of *C. neoformans*. The inoculum was prepared from young colonies diluted in phosphate-buffered saline (PBS) (Goldman et al., 1994b).

2.3. Experimental design

For the experimental design, 35 Wistar rats were divided in two groups: 18 composing the control group (the Group A: uninfected), and 17 animals were infected by *C. neoformans* (the Group B). After isoflurane anesthesia, the inoculation of 0.3 mL containing 1.7×10^7 cells of *C. neoformans* was performed intratrachealy as described previously (Goldman et al., 1994a). On a daily basis throughout the experiment all animals were clinically evaluated.

2.4. Sampling

Samples from the groups A and B were collected on days 10, 20, and 30 post-infection (PI) as follow: on day 10 (6 animals from subgroup A1; 6 animals from subgroup B1); on day 20 (6 animals from subgroup A2; 6 animals from subgroup B2) and on day 30 (6 animals from subgroup A3; 5 animals from subgroup B3). Blood samples were collected by cardiac puncture (8 mL) from anesthetized animals. Blood samples were allocated in tubes containing anticoagulant (ethylene-diaminetetraacetic acid—EDTA) for lymphocyte separation (4 mL) and hemogram (1 mL), and also stored (3 mL) in tubes without anticoagulant in order to obtain sera. All sera samples were stored at −20 °C.

2.5. Course of infection

Course of infection was assessed by the evaluation of colony-forming units (CFU) at days 10, 20, and 30 PI (sampling times). The brain and lungs of rats were collected and weighed after euthanasia. A fragment of each organ was mechanical grinded and homogenized for 5 to 10 s in PBS (1:10) containing 40 U of penicillin per mL. All samples were submitted to serial dilutions and 100 μL aliquots were added to plates containing SDA and incubated at 30 °C for 24–72 h. Data were reported as colony forming units (CFU)/g of organ analyzed. The results of CFU were presented in mean and standard deviation.

2.6. Histopathology

The animals were necropsied immediately after sampling (days 10, 20 and 30 PI). Right lung and brain of each rat were fixed in 10% buffered formalin. Sagittal sections at every 3 mm were obtained and stained with hematoxylin and eosin (HE).

2.7. Hematological parameters

Leukocyte counts were determined using an electronic counter (CC-550-Celm, São Paulo, Brazil). Blood smears were stained with *Romanowsky* (Panoptic method) to perform differential leukocyte counts.

2.8. Measuring cytokines and immunoglobulins

Cytokines (IFN-γ and TNF-α) quantification was assessed by ELISA using commercial kits (eBIOSCIENCE®, San Diego, USA) according to manufacturer's instructions. Briefly, 96-well microplates were sensitized with the primary antibody (dilution 1:10) at room temperature for 30 min; the serum sample was added and incubated (37 °C temperature for 30 min). After washing, the secondary antibody conjugated with peroxidase was added (dilution 1:5) and incubated. The presence and concentration of the cytokines were determined by the intensity of the color measured by spectrometry in a micro ELISA reader. Serum concentration of IgG and IgM were determined using an immunonephelometry on a Behring BN II Nephelometer (Dade Behring—USA), using Dade Behringe reagents. All tests were performed in duplicate.

2.9. Lymphocyte separation

Lymphocytes-rich mononuclear cells were isolated from peripheral blood collected with 7.2 mg dipotassium EDTA as anticoagulant and separated on FicoII–Histopaque density gradients as described by Böyum (1968). Lymphocyte viability was determined by measuring the activity of lactate dehydrogenase (LDH) using the kinetic method of the Labquest apparatus (Diagnostics Gold Analyzer).

2.10. Total protein levels

Protein level in serum and lymphocytes was measured by the Comassie Blue method according to Bradford (1976) using serum albumin as standard. These analyses were performed as described by Leal et al. (2005) in order to express the enzymatic activity.

2.11. Enzyme assays

2.11.1. E-NTPDase activity in lymphocytes

E-NTPDase activity was determined by a colorimetric assay in compliance with Leal et al. (2005). The reaction medium contained 0.5 mM $CaCl_2$, 120 mM NaCl, 5 mM KCl, 60 mM glucose, and 50 mM Tris–HCl buffer, pH 8.0 at a final volume of 200 μL. Twenty microliters of intact mononuclear cells suspended on saline solution were added to the reaction medium (2–4 μg/protein) and incubated for 10 min at 37 °C. The reaction was started by the addition of a substrate (ATP or ADP) at a final concentration of 2 mM and stopped with 200 μL 10% trichloroacetic acid (TCA) to provide a final concentration of 5%. The incubation proceeded for 70 min and the released inorganic phosphate was assayed by the method of Chan et al. (1986) using malachite green as a colorimetric reagent and KH_2PO_4 as standard. All samples were tested in triplicates on the same day of collection. E-NTPDase activity was expressed in μmol Pi released/min/mg protein.

2.11.2. E-ADA activity in serum and lymphocytes

E-ADA activity was measured spectrophotometrically in serum and lymphocytes 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 1.5 mL of 106/0.16 mM phenol–nitroprusside to the mixture, which was immediately mixed with 1.5 mL of 125/11 mM alkaline-hypochlorite (sodium hypochlorite). 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. All samples were tested in triplicates on the same day of collection. The specific activity is reported as units of E-ADA per mg of protein in serum and lymphocytes.

2.12. Statistical analysis

Firstly, data distribution was tested for normality of variance by Shapiro–Wilk test and the data showed a normal distribution. Then, the data was submitted to Student's t test ($P < 0.05$) by SAS/STAT program. We emphasize that the data was statistically analyzed on days 10, 20 and 30 of the experiment as independent variables, i.e., the three moments were not compared. The values were represented as mean ± standard deviation.

3. Results

3.1. Course of infection and clinical signs

Clinical respiratory distress was not observed throughout the experiment, but it was observed mild apathy and piloerection. *C. neoformans* was isolated from the lungs of infected animals after cultivation in SDA with chloramphenicol at 32 °C, resulting in the growth of white mucoid yeast colonies. Microscopical analysis of these colonies on India ink showed the presence of rounded cells of different sizes with and without budding surrounded by a clear halo.

Lung samples showed 4.2×10^4 ($\pm 1.5 \times 10^4$), 4.0×10^4 ($\pm 1.6 \times 10^4$) and $1.74 \pm x10^4$ CFU ($\pm 0.52 \times 10^4$) of *C. neoformans* per gram on days 10, 20 and 30 PI, respectively. Brain samples did not show positive results for CFU.

3.2. Histopathology

No detectable brain lesions at any timepoint in any replicates (i.e., no dissemination). Rats infected by *C. neoformans* developed histological lesions in the lung characterized by inflammatory response (Fig. 1). Lung lesions differed according to disease evolution and rats infected after 10 days PI showed a large number of foamy macrophages and neutrophil infiltrates in their lungs which was interpreted as neutrophilic and histiocytic pneumonia forming focally extensive areas. Occasionally, there was accumulation of mild lymphoplasmacytic infiltrate. There were small amounts of oval structures interpreted as yeasts. At day 20 PI, the lungs showed an elevated area, firm and whitish, with an irregular surface measuring approximately 0.8 cm × 0.3 cm. On cutting, the area was solid, firm, and homogeneously white. Histologically, the lungs showed a focal extensive area formed by multiple accumulations of amorphous material, characterized by a central area of caseous necrosis with multiple neutrophils. This area was surrounded by a great amount of epithelioid macrophages and giant cells. Externally, and in smaller number, there were lymphocytes and plasma cells. In the central area of the granuloma, there was a mixture of cellular debri and necrotic tissues with large amounts of round to oval structures classified as yeast cells. Lungs from infected rats after 30 days PI showed interstitial infiltration composed of macrophages (interstitial pneumonia) and rare yeasts. The bronchus-associated lymphoid tissue (BALT) was markedly hyperplastic showing two populations of lymphocytes: the majority composed of mature lymphocytes and lymphoblast as a minority.

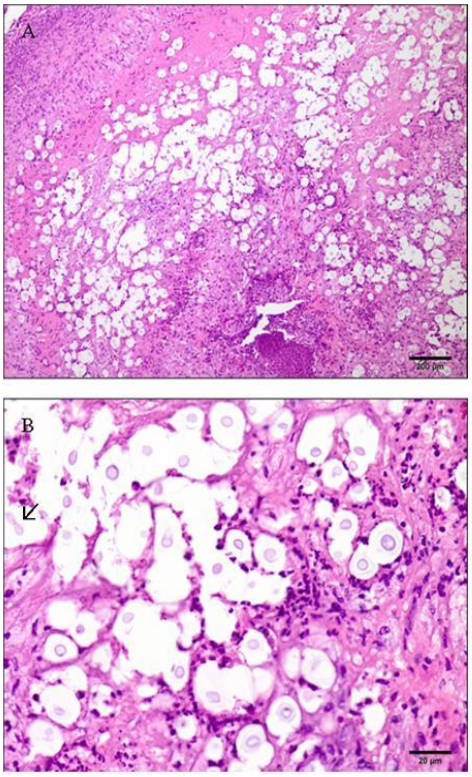

Fig. 1. Lung histology of rats experimentally infected by *Cryptococcus neoformans*. There is cellular infiltration in the alveolar space composed mainly by neutrophils. In the lesion it can be seen round to oval structures surrounded by an unstained capsule (A). The image below shows large amounts of round to oval structures of 20 to 60 µm of diameter with basophilic nuclei surrounded by an unstained coalescent capsule. Occasionally, it is possible to visualize budding (arrow). There are intact and degenerate neutrophils surrounding the yeast areas (B).

In lung was observed the presence of oval and round structures interpreted as yeast cells. These structures were basophilic with 20 to 60 µm of diameter and a core ranging from 10 to 20 µm, surrounded by an unstained capsule. There was small amount of yeasts when the inflammation was severe.

3.3. Leucogram

C. neoformans infected rats showed a significant ($P < 0.05$) increase in the total number of leukocytes on days 10 and 20 PI (Fig. 2A), in consequence of the increase in the number of lymphocytes (Fig. 2B), and neutrophils (Fig. 2C). On day 30 PI no significant ($P > 0.05$) differences were observed in the leucogram. The number of eosinophils and monocytes did not differ between groups during all three periods evaluated.

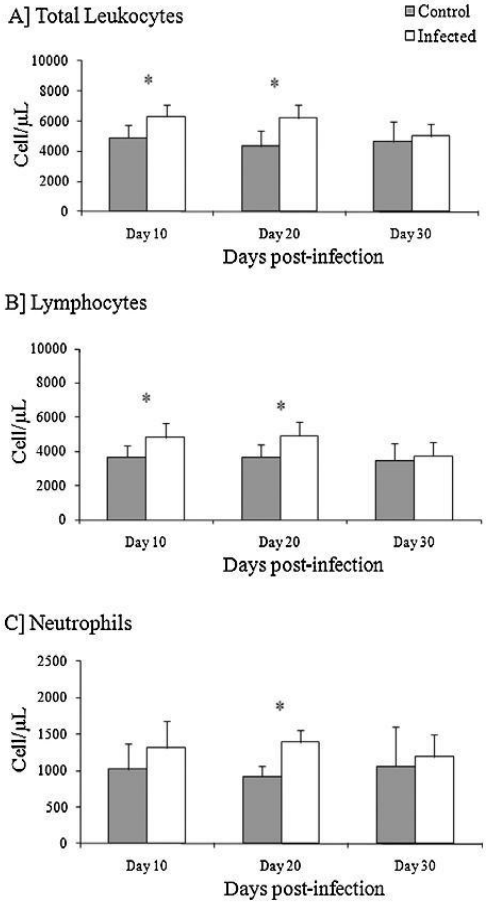

Fig. 2. Total leukocytes (A), lymphocytes (B), and neutrophils (C) of rats experimentally infected by *Cryptococcus neoformans* ($n = 6$) compared to the control group ($n = 6$) on days 10, 20, and 30 post-infection ($^{*}P < 0.05$).

3.4. Immunoglobulin and cytokine levels in the serum

Immunoglobulin and cytokine levels in the serum of rats infected by *C. neomorfans* are shown in Fig. 2. Infected animals showed levels of IgG (Fig. 3A), IgM (Fig. 3B), TNF (Fig. 3C) and INF-γ (Fig. 3D) significantly ($P < 0.01$) increased on days 10, 20 and 30 PI, when compared to healthy rodents. These variables were quantified just to see if infection by *C. neoformans* causes an immunological response, which was confirmed based on these results.

3.5. E-NTPDase activity in lymphocytes

The E-NTPDase activity in lymphocytes for ATP and ADP substrate is shown in Fig. 4. On day 10 PI no significant ($P > 0.05$) difference between groups for E-NTPDase activities was observed. ATP hydrolysis was significantly decreased on the day 20 ($P < 0.01$ – 37% – Fig. 4A) and 30 PI ($P < 0.05$ – 28% – Fig. 4A) in the infected groups, while ADP hydrolysis was significantly ($P < 0.01$) decreased (50%—Fig. 4B) only on day 20 PI.

3.6. E-ADA activity in serum and lymphocytes

E-ADA activity in serum and lymphocytes is shown in Fig. 5. Rats infected by *C. neoformans* presented a significant ($P < 0.01$) reduction in E-ADA activity in serum on days 10, 20 and 30 when compared to the control group (Fig. 5A). E-ADA activity in lymphocytes of infected rodents had a significant ($P < 0.01$) elevation only on day 10 PI, while on days 20 and 30 PI a considerable ($P < 0.01$) reduction was observed (Fig. 5B).

4. Discussion

We clearly observed that experimental infection by *C. neoformans* in rats was able to cause changes in the levels of leukocytes, cytokines, and immunoglobulins, as well as in E-NTPDase and E-ADA activities. *C. neoformans* is an environmental fungus with the ability to infect and cause disease in mammalian hosts, as it is capable of causing pulmonary cryptococcosis in rats (Casadevall and Perfect, 1998; Goldman et al., 1994b, 2000; Krockenberger et al., 2010), similar the present study. In addition, several organs and tissues have the potential to be affected by the disease, showing different degree of tissue damage. Our CFU results demonstrated the development of the disease in the lungs of infected animals on all three evaluated periods. The histopathology examination demonstrated multifocal to coalescing granulomas associated with intralesional yeasts in lungs of infected animals, a change typical of cryptococcosis and describes in rats (Goldman et al., 2000; Krockenberger et al., 2010).

Pro-inflammatory and anti-inflammatory activities are likely to be present at sites of cryptococcal infection (Wright et al., 2002). In our experimental infection, it was observed an increase of lymphocytes and neutrophils in rats infected, in response to the infection may in order to suppress the proliferation of *C. neoformans*. Lymphocytosis is the balance between the responses mediated by Th1 and Th2 cells and it is important for the evolution of the cryptococcosis, since when the cryptococcal infection is associated with a Th1 response (and consequent activation of phagocytes) usually there is resistance in the installation of infection (Huffnagle and Mcneil, 1999; Shoham and Levitz, 2005); whereas a Th2 response can lead to an inhibition of phagocytic activity, may increasing the susceptibility to *C. neoformans* (Koguchi and Kawakami, 2002). However, Jain et al. (2009) observed that that neither Th1 nor Th2 cytokines were correlated with changes in lung functions, probably explained by the relatively short life of cytokines and the fluctuation of their levels in infected animals, a finding that represents a different insight on Th2 role in *C. neoformans* infection. Neutrophilia may occasionally occur in animals infected by *C. neoformans* (Sherding, 2003), corroborating with our findings during this experiment. On day 30 PI, the number of colonies of fungus was lower to other days (10 and 20 PI), and the number of leukocytes in these animals did not differ between groups. However, the levels of

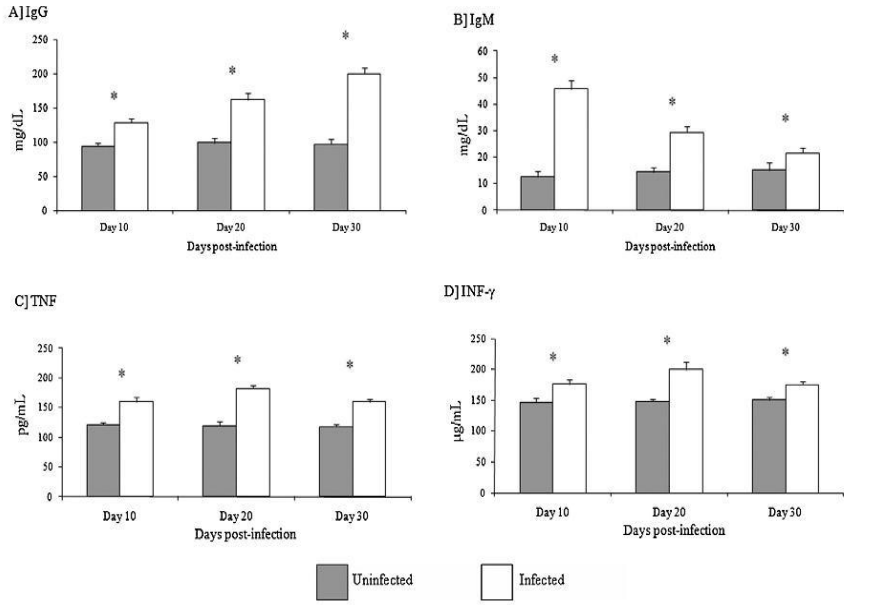

Fig. 3. Levels of immunoglobulins (IgG: (A) and IgM: (B)) and pro-inflammatory cytokines (TNF: (C) and INF-γ (D)) of rats experimentally infected by *Cryptococcus neoformans* ($n = 6$) compared to the control group ($n = 6$) on days 10, 20, and 30 post-infection ($^{*}P < 0.01$).

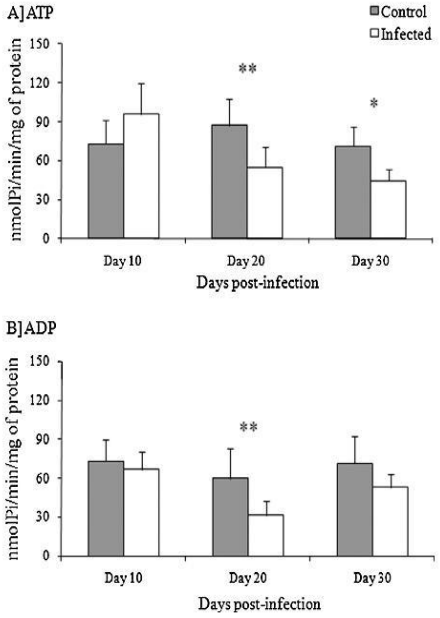

Fig. 4. E-NTPDase activity ((A) ATP hydrolysis; (B) ADP hydrolysis) in lymphocytes of rats experimentally infected by *Cryptococcus neoformans* ($n = 6$) compared to uninfected rats ($n = 6$) at days 10, 20, and 30 post-infection (Student's *t* test; $^{*}P < 0.05$; $^{**}P < 0.01$).

cytokines, immunoglobulins and activities of enzymes (E-NTPDase and E-ADA) changed in infected rats despite the reduction of fungi in the lung and histological lesions. The reduction in lung fungal burden is described, as it remained for several months at low levels (Goldman et al., 2000). Based on this information, future studies are being conducted to verify that the E-NTPDase and E-ADA enzymes may be new markers of pulmonary cryptococcosis asymptomatic.

In this study, it was observed an increase in the levels of IFN-γ and TNF-α along with evolution of the infection, cytokines also elevation in infected mice by *C. neoformans* (Jain et al., 2009). Chemokines and chemokine receptors are critical in T-cell polarization and microbial clearance in murine models of *C. neoformans* pulmonary infections (Olszewski et al., 2001; Traynor et al., 2002). IFN-γ and TNF-α stimulate the anti-*C. neoformans* activity of the macrophages; generally patients who survived to cryptococcosis have high levels of TNF-α when compared to patients who did not survived (Retini et al., 2001). Our results showed a characteristic immunological curve compatible with infectious process with an increase of IgM and IgG levels during the evaluated periods. In patients with cryptococcosis usually a response found in a serological evaluation is based predominantly in IgG, with IgM response less pronounced (Hamilton et al., 1997), similarly to what occurred in this study, demonstrating that infected animals exhibited an inflammatory response against the fungus.

The activity of the enzymes E-NTPDase and E-ADA has been related with the immune response in fungal diseases

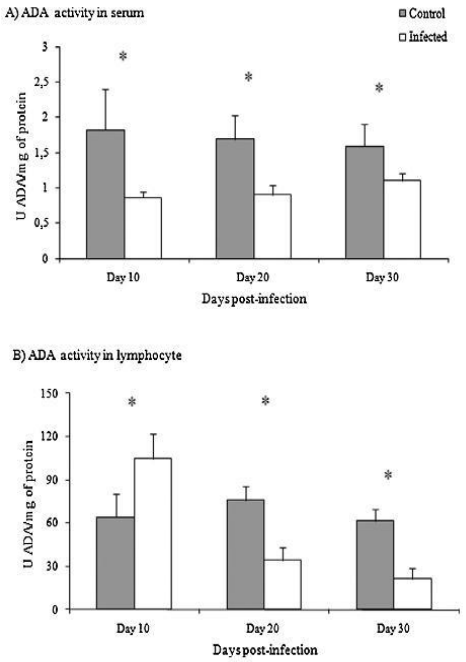

Fig. 5. Adenosine deaminase (E-ADA) activity in sera (A) and lymphocytes (B) of rats experimentally infected by *Cryptococcus neoformans* ($n = 6$) compared to uninfected ($n = 6$) animals at days 10, 20, and 30 post-infection (Student's t test; $^{*}P < 0.01$).

as pythiosis (Bach et al., 2010) and sporotrichosis (Castro et al., 2012). The results obtained in our experiment showed that during the experimental infection by *C. neoformans* occurred a decrease in the E-NTPDase activity when used ATP and ADP substrates during the infection. The roles of ADP in lymphocytes are still not well elucidated, since this nucleotide is mainly related to the coagulation processes (Zimmermann, 2001). However, it is well known that extracellular concentrations of ATP are dynamically controlled by ecto-enzyme E-NTPDase (Bours et al., 2006); therefore, it suggests that may have occured an increase in the concentration of extracellular ATP and ADP in the rats infected by *C. neoformans*. The increase of ATP in the extracellular medium may also lead to a pro-inflammatory reaction, mainly by the release of pro-inflammatory mediators (TNF-α, IL-12, IL1β and ROS), with differentiation of Th native cells into Th1 lymphocytes starting the inflammation process (Yegutkin, 2008). It is known that during the early infection by *C. neoformans* the T cells produce cytokines, which will activate the macrophages with formation of a granuloma leading to the destruction of intracellular fungus or contained it in a dormant condition (Feldmesser et al., 2001). For these reasons we believe that an increased level of extracellular ATP occurred in our experimental infection, mainly if considered the increase in the levels of TNF-α and INF-γ observed during the experiment.

Likewise, E-ADA plays an important role in lymphocyte function and is essential for its normal growth, differentiation, and proliferation (Franco et al., 1997; Codero et al., 2001). Clinically, high levels of E-ADA in the pleural fluid can be observed in patients with cryptococcal pleuritis (Yoshino et al., 2010), empyema, lymphoma, other malignancies, parapneumonic effusions and pleural effusions associated with collagen vascular diseases (Ocana et al., 1988; Burgess et al., 1996). In our results, it was possible to observe that the E-ADA activity in serum was inhibited on days 10, 20 and 30 PI compared to the control group. Decrease in serum E-ADA activity could be related to the need of a greater concentration of extracellular adenosine through interaction with purinergic receptors, playing a protective role in order to attenuate systemic changes may caused by infection (Franco et al., 1997). Regarding to E-ADA in lymphocytes, it was possible to observe an increase of its activity on day 10 PI, which can be related firstly to an inflammatory response against cryptococosis, being a similar response as observed in mice infected by *Sporothrix schenckii* (Castro et al., 2012). On the other hand, on the 20 and 30 PI its activity in lymphocytes was reduced compared to the control group. This reduction can lead to an interaction of adenosine with its receptors that exist in many cell types, with possible anti-inflammatory effects. Thus, during the last two moments of samples collection, E-ADA had its activity reduced, probably leading to a down regulation of inflammatory process, providing some protective effect against damages caused directly by the *C. neoformans*, as well as by the exacerbated inflammatory response.

Therefore, based on these results it is possible to conclude that experimental infection of immunocompetent rats by *Cryptococcus neoformans* leads to a regular immunologic response, mainly due to lymphocytes, neutrophils, cytokines, and immunoglobulin increases associated to the changes in the activity of E-NTPDase and E-ADA. Since the participation of these enzymes is well-known in other diseases, we believe that the alterations observed in activity of E-NTPDase (lymphocytes) and E-ADA (serum and lymphocytes) were correlated to the immune response against *C. neoformans*, and can serve in future as an important inflammatory marker for cryptococcosis.

Acknowledgements

The authors thank the National Council for Scientific and Technological Development of Brazil (CNPq) for financial support and Doctoral Scholarship.

References

Bach, B.C., Leal, D.B.R., Ruchel, J.B., Souza, V.C.G., Maboni, G., Dal Pozzo, M., Schlemmer, K.B., Alves, S.H., Santurio, J.M., 2010. Immunotherapy for pythiosis: effect on NTPDase activity in lymphocytes of an experimental model. Biomed. Pharmacothe. 64, 718–722.

Beenhouwer, D.O., Shapiro, S., Feldmesser, M., Casadevall, A., Scharff, M.D., 2001. Both Th1 and Th2 cytokines affect the ability of monoclonal antibodies to protect mice against *Cryptococcus neoformans*. Infect. Immun. 69, 6445–6455.

Böyum, A., 1968. Isolation of mononuclear cells and granulocytes from human blood. Isolation of monuclear cells by one centrifugation, and

of granulocytes by combining centrifugation and sedimentation at 1 g. Scand. J. Clin. Lab. Invest. Suppl. 97, 77–89.
Bours, M.J., Swennen, E.L.D.I., Virgilio, F., Cronstein, B.N., Dagnelie, P.C., 2006. Adenosine 5′-triphosphate and adenosine as endogenous signaling molecules in immunity and inflammation. Pharmacol. Ther. 112, 358–404.
Bradford, M.M., 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal. Biochem. 72, 248–254.
Burgess, L.J., Maritz, F.J., Le Roux, I., Taljaard, J.J., 1996. Combined use of pleural adenosine deaminase with lymphocyte/neutrophil ratio. Increased specificity for the diagnosis of tuberculous pleuritis. Chest 109, 414–419.
Casadevall, A., Perfect, J.R., 1998. Cryptococcus neoformans. ASM Press, Library of Congress, Washington, DC.
Castro, V.S., Pimentel, V.C., Da Silva, A.S., Thomé, G.R., Wolkmer, P., Castro, J.L., Costa, M.M., da Silva, C.B., Oliveira, D.C., Alves, S.H., Schetinger, M.R., Lopes, S.T., Mazzanti, C.M., 2012. Adenosine deaminase activity in serum and lymphocytes of rats infected with *Sporothrix schenckii*. Mycopathologia 174, 31–39.
Chan, K., Delfert, D., Junger, K.D., 1986. A direct colorimetric assay for the Ca^{2+}–ATPase activity. Anal. Biochem. 157, 375–380.
Codero, O., Salgado, F., Fernández-Alonso, C., Herrera, C., Lluis, C., Franco, R., Nogueira, M., 2001. Cytokines regulate membrane adenosine deaminase on human activated lymphocytes. J. Leukoc. Biol. 70, 920–930.
Feldmesser, M., Kress, Y., Casadevall, A., 2001. Dynamic changes in the morphology of *Cryptococcus neoformans* during murine pulmonary infection. Microbiology 147, 2355–2365.
Fillippini, A., Taffs, R.E., Agui, T., Sitkovsky, M.V., 1990. Ecto-ATPase activity in cytolytic t-lymphocytes: protection from the cytolytic effects of extracellular ATP. J. Biol. Chem. 265, 334–340.
Franco, R., Casado, V., Ciruela, F., Saura, C., Mallo, J., Canela, E.I., Llouis, C., 1997. Cell surface adenosine deaminase: much more than an ectoenzyme. Prog. Neurobiol. 52, 283–294.
Giusti, G., Gakis, C., 1971. Temperature conversion factors, activation energy, relative substrate specificity and optimum pH of adenosine deaminase from human serum and tissues. Enzyme 12, 417–425.
Goldman, D., Lee, S.C., Casadevall, A., 1994a. Pathogenesis of pulmonary *Cryptococcus neoformans* infection in the rat. Infect. Immun. 62, 4755–4761 (1994).
Goldman, D., Lee, S.C., Casadevall, A., 1994b. Pathogenesis of pulmonary *Cryptococcus neoformans* infection in the rat. Infect. Immun. 62, 4755–4761.
Goldman, D.L., Lee, S.C., Mednick, A.J., Montella, L., Casadevall, A., 2000. Persistent *Cryptococcus neoformans* pulmonary infection in the rat is associated with intracellular parasitism, decreased inducible nitric oxide synthase expression, and altered antibody responsiveness to cryptococcal polysaccharide. Infect. Immun. 68, 832–838.
Hamilton, A.J., Figueroa, J.I., Jeavons, L., Seaton, R.A., 1997. Recognition of cytoplasmic yeast antigens of *Cryptococcus neoformans* var. *neoformans* and *Cryptococcus neoformans* var. *gattii* by immune human sera. FEMS Immunol. Med. Microbiol. 17, 111–119.
Huffnagle, G.B., Mcneil, L.K., 1999. Dissemination of *C. neoformans* to the central nervous system: role of chemokines, Th1 immunity and leukocyte recruitment. J. Neurovirol. 5, 76–81.
Jain, A.V., Zhang, Y., Fields, W.B., McNamara, D.A., Choe, M.Y., Chen, G., Erb-Downward, J., Osterholzer, J.J., Toews, G.B., Huffnagle, G.B., Olszewski, M.A., 2009. Th2 but not Th1 immune bias results in altered lung functions in a murine model of pulmonary *Cryptococcus neoformans* infection. Infect. Immunol. 77, 5389–5399.
Kidd, S.E., Hagen, F., Tscharke, R.L., Huynh, M., Bartlett, K.H., Flye, M., Boekhout, T., Kwon-Chung, K.J., Meyer, W., 2004. A rare genotype of *Cryptococcus gattii* caused the cryptococcosis outbreak on Vancouver Island (British Columbia, Canada). Proc. Natl. Acad. Sci. U.S.A. 101, 17258–17263.
Krockenberger, M.B., Malik, R., Ngamskulringroj, P., Trilles, L., Ecandon, P., Dowd, S., Allen, C., Himmelreich, U., Canfield, P.J., Sorrell, T., Meyer, W., 2010. Pathogenesis of pulmonary *Cryptococcus gattii* infection: a rat model. Mycopathologia 170, 315–330.
Koguchi, Y., Kawakami, K., 2002. Cryptococcal infection and Th1–Th2 cytokine balance. Int. Rev. Immunol. 21, 423–438.
Leal, D.B.R., Streher, C.A., Neu, T.N., Bittencourt, F.P., Leal, C.A.M., Silva, J.E.P., Morsch, V.M., Schetinger, M.R.C., 2005. Characterization of NTPDase (NTPDase1; ecto-apyrase; ectodiphosphohydrolase; CD39; E.C. 3.6.1.5) activity in human lymphocytes. Biochim. Biophys. Acta 1721, 9–11.
Martins, D.B., Zanette, R.A., Françs, R.T., Howes, F., Azevedo, M.I., Botton, S.A., Mazzanti, C., Lopes, S.T.A., Santurio, J., 2010. Massive cryptococcal disseminated infection in an immunocompetent cat. Vet. Dermatol. 22, 232–234.
Mitchell, T.G., Perfect, J.R., 1995. Cryptococcosis in the era of AIDS—100 years after the discovery of *Cryptococcus neoformans*. Clin. Microbiol. Rev. 8, 515–548.
Ocana, I., Ribera, E., Martinez-Vazquez, J.M., Ruiz, I., Bejarano, E., Pigrau, C., Pahissa, A., 1988. Adenosine deaminase activity in rheumatoid pleural effusion. Ann. Rheum. Dis. 47, 394–397.
Olszewski, M.A., Huffnagle, G.B., Traynor, T.R., McDonald, R.A., Cook, D.N., Toews, G.B., 2001. Regulatory effects of macrophage inflammatory protein 1alpha/CCL3 on the development of immunity to *Cryptococcus neoformans* depend on expression of early inflammatory cytokines. Infect. Immun. 69, 6256–6263.
Ralevic, V., Burnstock, G., 1998. Receptores for purines and pyrimidines. Pharmacol. Rev. 50, 413–492.
Retini, C., Kozel, T.R., Pietrella, D., Monari, C., Bistoni, F., Vecchiarelli, A., 2001. Interdependency of interleukin-10 and interleukin-12 in regulation of T-cell differentiation and effector function of monocytes in response to stimulation with *Cryptococcus neoformans*. Infect. Immun. 69, 6064–6073.
Sherding, R.G., 2003. Micoses sistêmicas. In: Manual Saunders: clínica de pequenos animais. second ed. Roca, São Paulo, pp. 156–157.
Shoham, S., Levitz, S.M., 2005. The immune response to fungal infections. Br. J. Haematol. 129, 569–582.
Traynor, T.R., Herring, A.C., Dorf, M.E., Kuziel, W.A., Toews, G.B., Huffnagle, G.B., 2002. Differential roles of CC chemokine ligand 2/monocyte chemotactic protein-1 and CCR2 in the development of T1 immunity. J. Immunol. 168, 4659–4666.
Wright, L., Bubb, W., Davidson, J., Santangelo, R., Krockenberger, M.B., Himmelreich, U., Sorrell, T., 2002. Comparison of extracellular metabolite production by *Cryptococcus neoformans var. neoformans and var. gattii* and effects of metabolites on human neutrophils. Microb. Infect. 4, 1427–1438.
Yegutkin, G.G., 2008. Nucleotide and nucleoside-converting ectoenzymes: important modulators of purinergic signalling cascade. Biochim. Biophys. Acta 1783, 673–694.
Yoshino, Y., Kitazawa, T., Tatsuno, K., Ota, Y., Koike, K., 2010. Cryptococcal pleuritis containing a high level of adenosine deaminase in a patient with AIDS: a case report. Respiration 79, 153–156.
Zimmermann, H., 2001. Ectonucleotidases: some recent developments and note on nomenclature. Drug Dev. Res. 52, 44–56.

4.2 Artigo 2

Cholinesterase of rats experimentally infected by Cryptococcus neoformans: Relationship between inflammatory response and pathological findings

Artigo publicado no periódico **Pathology- Research and Pratice**[2]

[2]A licença para a inclusão do artigo nesta tese encontrase- no anexo B.

Pathology – Research and Practice 211 (2015) 851–857

Contents lists available at ScienceDirect

Pathology – Research and Practice

journal homepage: www.elsevier.com/locate/prp

Original Article

Cholinesterase of rats experimentally infected by *Cryptococcus neoformans*: Relationship between inflammatory response and pathological findings

Maria Isabel de Azevedo[a,d], Laerte Ferreiro[a], Aleksandro S. Da Silva[b], Alexandre A. Tonin[c], Maria Luiza Thorstenberg[d], Livia Gelain Catilhos[d], Raqueli T. França[e], Daniela B.R. Leal[d], Marta M.M.F. Duarte[f], Sonia T.A. Lopes[e], Manuela B. Sangoi[g], Rafael N. Moresco[g], Rafael Fighera[h], Janio M. Santurio[d,*]

[a] Laboratório de Micologia, Departamento de Patologia e Clínica Animal, Faculdade de Medicina Veterinária (UFRGS), Porto Alegre, RS, Brazil
[b] Departamento de Ciência Animal, Universidade do Estado de Santa Catarina, Brazil
[c] Universidade do Oeste de Santa Catarina (UNOESC), Xanxerê, SC, Brazil
[d] Departamento de Microbiologia e Parasitologia, Universidade Federal de Santa Maria, Santa Maria, RS, Brazil
[e] Departamento de Pequenos Animais, Universidade Federal de Santa Maria, Santa Maria, RS, Brazil
[f] Universidade Luterana do Brasil, Santa Maria, Brazil
[g] Departamento de Analises Clínicas Toxicológicas, Universidade Federal de Santa Maria, Santa Maria, RS, Brazil
[h] Departamento de Patologia Veterinária, Universidade Federal de Santa Maria, Brazil

ARTICLE INFO

Article history:
Received 7 October 2014
Received in revised form 18 May 2015
Accepted 3 August 2015

Keywords:
Cryptococcosis
Acetylcholine
Acetylcholinesterase
Butyrylcholinesterase
Inflammatory process
Oxidative stress

ABSTRACT

The aim of this study was to assess the role of the acetylcholinesterase (AChE) and butyrylcholinesterase (BChE) as biomarkers of inflammation and tissue injury on rats experimentally infected by *Cryptococcus neoformans*. For this purpose, 20 male rats were divided into two groups: 10 animals representing the uninfected control group (Group A) and 10 *C. neoformans* var. *grubii* infected animals (Group B). Blood and brain samples were collected on days 10 (A_{10} and B_{10}), and 30 (A_{30} and B_{30}) post-infection (PI) for hematological analyses; AChE (in lymphocytes and brain) and seric BChE activity; interleukins (IL-1, IL-6, and IL-10); nitrite/nitrate (NO_x) levels; and markers of protein oxidation (AOPP) and lipid peroxidation (TBARS). As a result, when animals of Group A were compared to animals of Group B, it was observed leukocytosis ($P<0.05$) on day 10 PI; AChE activity increase ($P<0.05$) in lymphocytes (day 30 PI) and in brain (days 10 and 30 PI); BChE activity decrease ($P<0.05$) on day 10 PI; IL-1 and IL-6 increase ($P<0.01$) in both periods, while IL-10 had reduced levels ($P<0.01$) in the same periods; NO_x levels increased ($P<0.05$) significantly on days 10 and 30 PI, while AOPP and TBARS levels increased significantly on day 30 PI; as well as pneumonia on infected rats. Therefore, based on the results obtained, it was possible to conclude that AChE and BChE behavior lead to a proinflammatory reaction evidenced by the enhancement of IL-1, IL-6, and NO_x throughout the experiment associated with reduction on IL-10 levels, and cellular damage.

1. Introduction

Cryptococcus neoformans is an encapsulated yeast and the causative agent of opportunistic mycoses called cryptococcosis, an infection predominantly occurring in immunocompromised hosts [1]. The infection occurs mainly in the respiratory tract, caused by inhalation of contaminated dust and debris, spreading from the lungs to other organs, especially the central nervous system (CNS) [2]. The immune defense against pathogens usually causes an inflammatory response associated with an anti-inflammatory response [3]. In this sense, studies indicate that passive antibody-mediated protection against *C. neoformans* requires both Th1 and Th2 associated cytokines, and reveal the complexity of the mechanisms through which antibodies modulate infection with this type of microorganism [4].

Regarding the mechanisms of immune response associated with the predilection of *C. neoformans* for the CNS, it is important to emphasize that the autonomic nervous system plays an

* Corresponding author at: Departamento de Microbiologia e Parasitologia, Universidade Federal de Santa Maria, Faixa de Camobi – Km 9, predio 20, Sala: 4139, CEP 97105900 Santa Maria, RS, Brazil.
E-mail address: janio.santurio@gmail.com (J.M. Santurio).

http://dx.doi.org/10.1016/j.prp.2015.08.005
0344-0338/

important role in controlling inflammation partially by the release of pro-inflammatory cytokines, through the secretion of adrenaline and noradrenaline, and by connecting these to α and β androgenic receptors on the immune cells [5]. In contrast, the vagal efferent pathway plays a very important role, modulating the inflammation and, for that, being called cholinergic anti-inflammatory pathway [3,5]. The main vagal neurotransmitter is acetylcholine (ACh), which is catalyzed by the cholinergic enzymatic system, composed of the cholinesterases. This group of enzymes is composed of acetylcholinesterase (AChE: E.C. 3.1.1.7), a membrane-bound enzyme mainly found in the brain, muscles, erythrocytes, lymphocytes and cholinergic neurons, that preferentially hydrolyzes esters with acetyl group [6,7] and butyrylcholinesterase (BChE: E.C. 3.1.1.8). BChE is a serine hydrolase that catalyzes the hydrolysis of esters of choline such as butyrylcholine and succinylcholine, found at different concentrations in the intestine, liver, kidney, heart, lung, brain, and serum [8,9]. ACh has an important role in attenuating the release of pro-inflammatory cytokines, such as tumor necrosis factor (TNF), interleukin-1 (IL-1), interleukin-6 (IL-6) and interleukin-18 (IL-18), and on endotoxin-activated macrophages without affecting the production of interleukin-10 (IL-10), an anti-inflammatory cytokine [5]. This inhibition is possible by ACh binding to α7 subunit-containing nicotinic acetylcholine receptor (α7nAChE) present on macrophages, being the dose-dependent inhibition [10].

In association with the evaluation of the participation of cholinesterases in the immune response against *C. neoformans*, some strategies to measure tissue and/or system damage, such as biomarkers of oxidative stress would be useful tools since they have the potential to help in the establishment of pathogenic stages, increasing the risk for disease [11–13]. Among these biomarkers, the assessment of thiobarbituric acid reactive substances (TBARS) is one of the most commonly applied methods for the measurement of lipid peroxidation [14], and the measurement of advanced oxidation protein products (AOPP) is another useful option. AOPP also helps to identify oxidative damage; however, specifically for proteins [15]. In this sense, another possibility is the measurement of nitric oxide (NO) levels, which allows the evaluation of both the immune responses [16] and oxidative stress status [17], acting as an extra tool for diagnosis and disease treatment [18,19].

Therefore, considering all the information mentioned above, the aim of this study is to evaluate the role of the cholinesterases as markers of inflammation and tissue injury in rats experimentally infected by *C. neoformans*.

2. Materials and methods

2.1. Animals

Twenty male Wistar rats (*Rattus norvegicus*) with 90 days of age and 298 (±29) g of average weight were kept in cages housed in a room with controlled temperature and humidity (25 °C; 70%). They were fed with commercial ration and received water *ad libitum*. All animals had a period of 7 days for adaptation and were clinically healthy in the beginning of the experiment (day 0). The procedure was approved by the Committee on Ethics in Animal Experimentation of Universidade Federal de Santa Maria (protocol number 073/2012).

2.2. Cryptococcus neoformans *var.* grubii *strain and inoculum*

2.2.1. Cryptococcus neoformans *var.* grubii

The strain of *C. neoformans* used for animal inoculation in this study was obtained from a clinical case of feline cryptococcosis [20]. This strain was previously identified as *C. neoformans* var. *grubii* based on its micromorphology and molecular characteristics, as well as by its genomic sequence data deposited in GenBank under the accession number HQ148880.

2.2.2. Inoculum preparation

C. neoformans was isolated on Sabouraud dextrose agar (SDA) and incubated at 32 °C in order to obtain a pure and viable strain. Each animal was inoculated with 0.3 ml of a suspension containing 1.7×10^7 cells of *C. neoformans* [21]. The inoculum was prepared from young fungal colonies diluted in phosphate-buffered saline (PBS).

2.3. Experimental design and infection

Rats were divided into two groups: 10 animals for the control group (Group A: uninfected) and 10 *C. neoformans* var. *grubii* infected animals (Group B). The inoculation was performed as previously described [21]. For inoculation, all animals were anesthetized with isoflurane. All animals underwent daily clinical evaluation throughout the experiment.

2.4. Progression of infection

Progression of infection was assessed by the evaluation of colony-forming units (CFU) at days 10 and 30 PI (sampling times). Briefly, brain and lungs of rats were collected and weighed after euthanasia. The organs were homogenized for 5–10 s in PBS containing 40 U of penicillin per ml. All samples were submitted to serial dilutions, and 100 μl aliquots were added to plates containing SDA and incubated at 30 °C for 24–72 h. Data were reported as CFU/g of organ.

2.5. Sampling

Blood samples were collected on days 10 and 30 PI from five anesthetized (isoflurane) animals of each group by cardiac puncture. Blood samples were stored in tubes containing anticoagulant (for separation of lymphocytes and hemogram) and in tubes without anticoagulant (to obtain sera to determine BChE activity and interleukins levels).

Brain samples from five rats of each group were also collected on days 10 and 30 PI, and they were divided into two hemispheres. The right hemisphere was weighed and homogenized in buffer Tris-HCl 10 mmol, pH 7.2 to verify the activity of AChE, along with NO_x, AOPP, and TBARS levels. All procedures described above were performed under refrigeration temperature (4 °C). Homogenates were stored and frozen at −20 °C until analyses. The left hemisphere was stored in 10% formalin for histological analyses.

2.6. Hematology

Hematocrit, total erythrocytes, hemoglobin concentration, and total leukocytes were evaluated using an automatic electronic counter. Smears were set and stained by the panoptic method to perform differential leukocyte counts. The hematocrit was obtained by centrifugation using a microcentrifuge (Sigma) at 18,600 × *g* for 5 min [22].

2.7. Cholinesterases activities

2.7.1. AChE in lymphocytes

Lymphocytes were obtained from whole blood with EDTA by gradient separation using Ficoll-Histopaque™ plus, according to the technique described by Böyum [23]. Lymphocyte viability and integrity were confirmed by determining the percentage of cells

excluding 0.1% trypan blue and measuring lactate dehydrogenase (LDH) activity [24].

In sequence, AChE activity was determined according to the method described by Fitzgerald and Costa [25]. Proteins of all samples were adjusted to 0.1–0.2 mg/ml. A volume of 0.2 ml of intact cells was added to a solution containing 1.0 mM acetylthiocholine (ATC), 0.1 mM 5,5′-dithiobis(2-nitrobenzoic acid) (DTNB), and 0.1 mM phosphate buffer (pH 8.0). Before and after incubation for 30 min at 27 °C, the absorbance was read on a spectrophotometer at 412 nm. AChE was calculated from the quotient between lymphocyte AChE activity and protein content. Results were expressed as μmol $AcSCh^{-1}$ mg^{-1} of protein.

2.7.2. Brain AChE activity

Brain homogenates were analyzed by the AChE enzymatic assay using a modified spectrophotometric method [26]. All tests were carried out in triplicate, and the enzyme activity was expressed in μmol $AcSCh\ h^{-1}$ mg^{-1} of protein.

2.7.3. Seric BChE activity

The method of Ellman et al. [27] was used for the determination of BChE activity in sera samples by using butyrylthiocholine as substrate. Results were expressed as μmol $BcSCh\ h^{-1}$ mg^{-1} of protein.

2.8. Interleukin levels

Interleukin quantification (IL-1, IL-6, and IL-10) was assessed by ELISA using commercial kits for rat (eBioscience®, San Diego, USA) according to the manufacturer's instructions. Briefly, microplates were sensitized with the primary antibody at room temperature (RT) for 30 min, then the sample was added and incubated (37 °C temperature, for 30 min). After washing, the secondary antibody conjugated with peroxidase was added and incubated. The presence and concentration of the interleukins were determined by the intensity of the color measured by spectrometry in a micro ELISA reader.

2.9. Measuring the levels of nitric oxide, protein oxidation, and lipid peroxidation in the brain

Nitric oxide levels were analyzed indirectly by quantifying seric NO_x (nitrite/nitrate) according to the technique described in detail by Tatsch et al. [28]. Thus, the NO_x was measured by the modified Griess method with the Cobas Mira automated analyzer using 50 μl of sample. NO_x results were expressed as μmol/l.

Protein oxidation concentration (AOPP) was determined using the semiautomated method [15]. The results were expressed as μmol/l. Lipid peroxidation in brain was determined by TBARS levels, measured by the absorbance of the red product at 532 nm [29] and expressed as μmol MDA/mg of protein.

2.10. Histopathology

The animals were necropsied immediately after sampling (days 10 and 30). Representative fragments of lung and brain were fixed in 10% buffered formalin. Sagittal sections of every 3 mm were obtained and stained with hematoxylin and eosin (HE).

2.11. Statistical analysis

First the data were tested for normality of variance by Shapiro–Wilk test, and the data with abnormal distribution were transformed to logarithms. The data were summarized as means and standard deviations, and were analyzed by the Student's *t*-test ($P < 0.05$). The relation between the number of lymphocytes and the AChE activity in lymphocytes was analyzed by linear correlation. The effect of AChE activity on IL-1 and IL-6 was analyzed by linear correlation. Analyses of correlation were also performed for BChE activity and seric IL-10. For all these analyses, the SAS software was used.

3. Results

3.1. Course of infection and clinical signs

During the experimental period, infected rats did not show clinical signs of respiratory distress, only mild apathy and piloerection. *C. neoformans* was isolated from lungs of infected animals using SDA at 30 °C, which resulted in the growth of white and mucoid yeast colonies. Microscopical analyses of these colonies with India ink showed the presence of rounded cells of different sizes with and without budding, surrounded by a clear halo.

Lung samples showed counts of 4.2×10^4 ($\pm 1.5 \times 10^4$) and 1.74×10^4 CFU ($\pm 0.52 \times 10^4$) of *C. neoformans* per gram of tissue on days 10 and 30 PI, respectively. There was no *C. neoformans* growth on brain samples.

3.2. Hematology

Hematology results are shown in Table 1. No significant ($P > 0.05$) difference was observed between groups for hematocrit, total erythrocytes, and hemoglobin results during the two analyzed periods. Infected rats showed a significant ($P < 0.05$) increase in total leukocyte counts, mainly due to an increase in the number of lymphocytes on day 10 PI when compared to the control group. On day 30 PI, there were no changes in hemogram among the studied groups ($P > 0.05$).

3.3. Cholinestarases

The results of AChE activity of lymphocyte and brain are shown in Fig. 1. Infected animals showed increased AChE activity in lymphocytes ($P < 0.05$) on day 30 PI when compared to uninfected animals (Fig. 1A). Moreover, AChE activity in the brain was also significantly increased ($P < 0.05$) in infected rats in both periods (Fig. 1B). On the other hand, seric BChE activity (Fig. 2) was reduced

Table 1
Means and standard deviation of the hematological parameters of rats experimentally infected with *Cryptococcus neoformans*.

Parameters	Days post-infection	Control group	Infected group
Hematocrit (%)	10	42.1[a] (±1.1)	40.1[a] (±2.3)
	30	41.4[a] (±0.8)	41.2[a] (±1.1)
Total erythrocytes ($\times 10^6/\mu l$)	10	6.43[a] (±0.30)	6.71[a] (±0.35)
	30	6.69[a] (±0.28)	6.81[a] (±0.32)
Hemoglobin (g/dl)	10	11.3[a] (±0.73)	11.6[a] (±0.61)
	30	11.9[a] (±0.32)	11.7[a] (±0.12)
Total leukocytes (/μl)	10*	4803[a] (±745)	6432[b] (±625)
	30	4602[a] (±1270)	5236[a] (±1003)
Neutrophils (/μl)	10	1001[a] (±340)	1330[a] (±325)
	30	1050[a] (±541)	1299[a] (±365)
Lymphocyte (/μl)	10*	3581[a] (±664)	4874[b] (±790)
	30	3470[a] (±980)	3766[a] (±821)
Eosinophils (/μl)	10	75.3[a] (±63)	101.2[a] (±51)
	30	53.1[a] (±45)	68.3[a] (±37)
Monocytes (/μl)	10	145.7[a] (±52)	126.8[a] (±54)
	30	28.9[a] (±23)	102.7[a] (±68)

[*] Means in the same line followed by different letters are statistically different among them by Tukey's test at 5% probability (*$P < 0.05$).

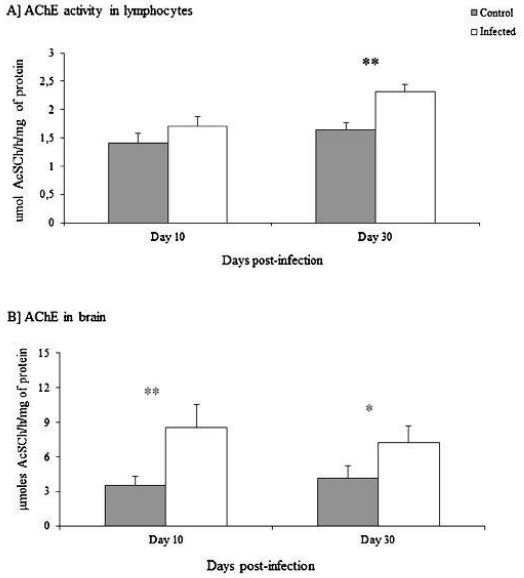

Fig. 1. AChE activity in lymphocytes (A) and brain (B) of rats infected by *Cryptococcus neoformans* (days 10 and 30 PI) when compared to the control group (*$P<0.05$, **$P<0.01$).

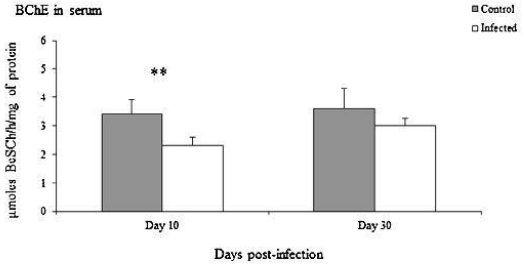

Fig. 2. BChE activity in serum of rats infected by *Cryptococcus neoformans* (days 10 and 30 PI) when compared to the control group (*$P<0.05$, **$P<0.01$).

in infected rats on day 10 PI when compared to the control group ($P<0.05$).

3.4. Interleukin levels

Seric pro-inflammatory cytokines levels increased ($P<0.01$) in infected rats compared to the control group (Fig. 3). On the contrary, anti-inflammatory cytokine (IL-10) was reduced in the same group when compared to uninfected animals during the two analyzed periods (Fig. 3C).

3.5. Levels of NO_x, AOPP and TBARS in brain

NO_x, AOPP, and TBARS results in brain samples are shown in Fig. 4. NO_x levels increased ($P<0.05$) significantly in infected animals when compared to uninfected ones on days 10 and 30 PI

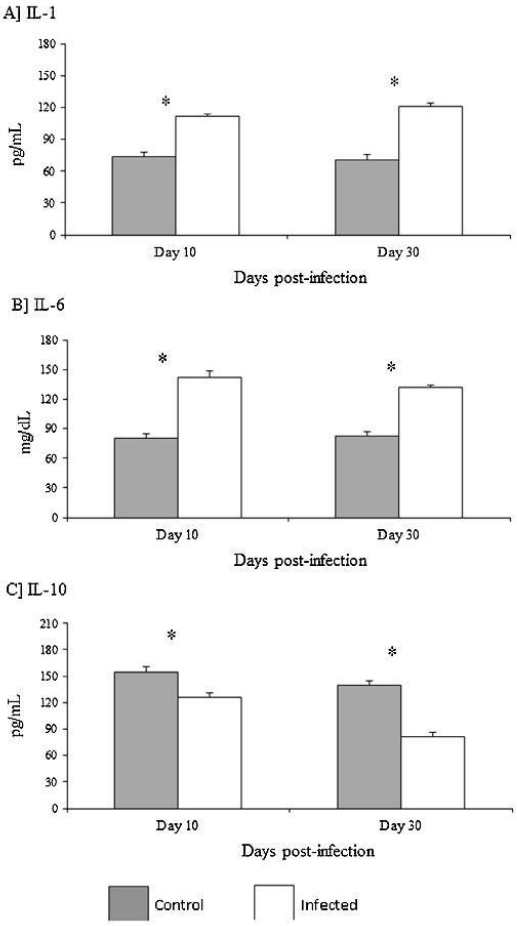

Fig. 3. Levels of interleukins in rats infected by *Cryptococcus neoformans* when compared to the control group at days 10 and 30 PI: IL-1 (A) IL-2 (B) and IL-10 (C). (*$P<0.01$; **$P<0.05$).

(Fig. 4A). AOPP (Fig. 4B), and TBARS (Fig. 4C) levels increased significantly on day 30 PI when these biomarkers were compared to healthy rats.

3.6. Correlation analysis

There was no significant correlation ($P>0.05$) between the number of lymphocytes and AChE activity in lymphocytes on day 10 ($r=0.38$) and 30 ($r=0.49$) PI. It was observed a strong positive correlation ($P<0.001$) between AChE activity in lymphocytes and the levels of IL-1 ($r=0.72$) and IL-6 ($r=0.80$) in the serum on day 30 PI, different from the results found on day 10 PI, where no correlation was observed. A strong positive correlation ($P<0.001$) was observed between BChE activity and levels of seric IL-10 ($r=0.79$) on day 10 PI, but not on day 30 PI.

3.7. Histology

Rats infected by *C. neoformans* developed histological lesions in the lung, characterized by inflammatory response (Fig. 5). Lung

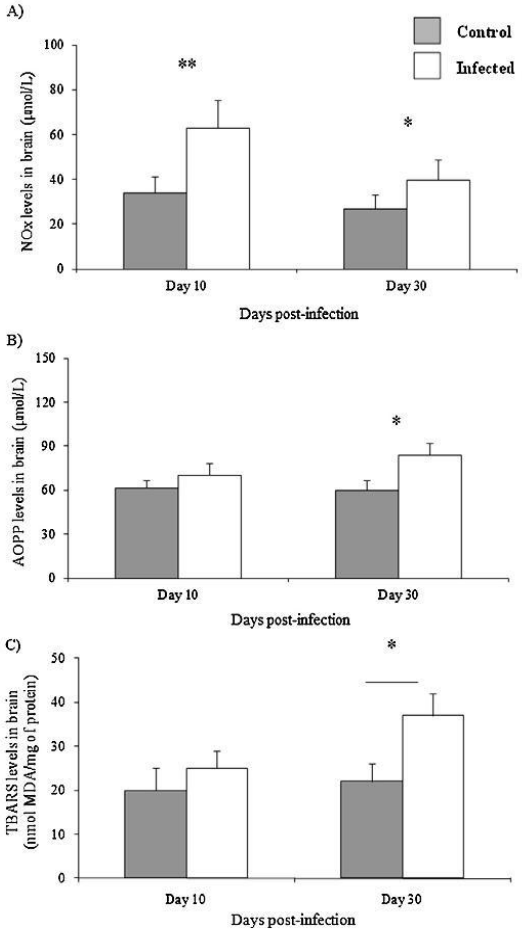

Fig. 4. Levels of NO_x (A), AOPP (B), and TBARS (C) in brain of rats infected by *Cryptococcus neoformans* (days 10 and 30 PI) when compared to the control group (*$P < 0.05$; **$P < 0.01$).

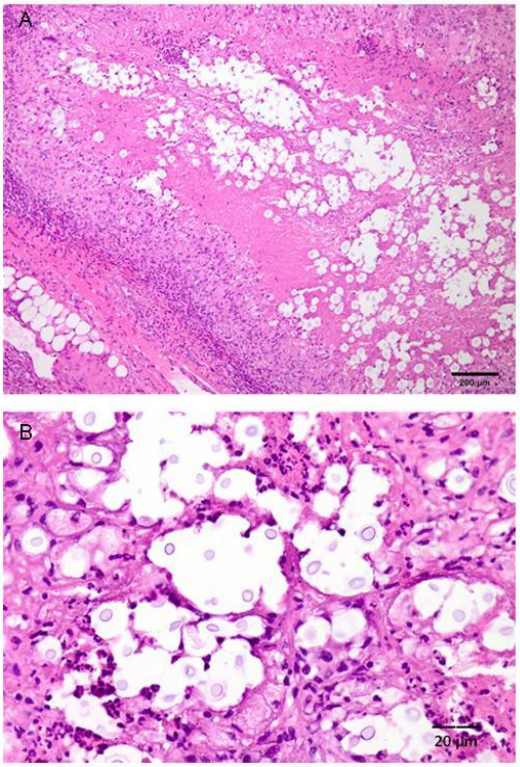

Fig. 5. Rats experimentally infected by *Cryptococcus neoformans* on day 10 PI. Alveolar spaces can be observed due to the edema and inflammatory infiltrate composed mainly by neutrophils and epithelioid macrophages (A). Under lower magnification, we observed large amounts of round to oval structures with 20–60 µm with basophilic nuclei surrounded by unstained capsule (B).

lesions differed according to disease progression, and rats infected after 10 days PI showed a large number of foamy macrophages and neutrophil infiltrates in their lungs, which was interpreted as neutrophilic and histiocytic pneumonia forming focally extensive areas (Fig. 5A). Occasionally, there was accumulation of mild lymphoplasmacytic infiltrate. There were small amounts of oval structures interpreted as yeasts (Fig. 5B). Lungs from infected rats after 30 days PI showed interstitial infiltration composed of macrophages (interstitial pneumonia) and rare yeasts (Fig. 6A). The bronchus-associated lymphoid tissue (BALT) was markedly hyperplastic, showing two populations of lymphocytes: the majority was composed of mature lymphocytes and lymphoblast as a minority. Lung samples showed oval and round structures interpreted as yeast cells. These structures were basophilic with 20–60 µm of diameter and a core ranging from 10 to 20 µm, surrounded by an unstained capsule. There was a small amount of yeasts associated with inflammation. There were no detectable brain lesions in all rats in this study (Fig. 6B).

4. Discussion

Intratracheal inoculation of *C. neoformans* in rats produces a similar pulmonary infection course of immunocompetent individuals [21]. In the present study, it was confirmed that animals with clinical signs compatible with lung disease developed lesions characteristic of pneumonia, as well as inflammatory infiltrates due to the presence of yeast, similarly to those reported in other studies [30]. The results obtained in our experimental infection by *C. neoformans* generated a pro-inflammatory reaction against this agent. There were no substantial changes in the behavior of the animals throughout the experiment; however, the hematological, biochemical and immunology analysis showed some important changes.

Despite many efforts, little is known about the origin or function of ACh in the blood [31]. However, the available data have demonstrated that ACh in the blood appears to be involved in the activation and clonal expansion of lymphocytes [32,33], seen as an anti-inflammatory effect by a decrease in TNF-α plasma levels [34] that down-regulates NO release by erythrocytes in the presence of fibrinogen [35]. Considering the data of hematological parameters, associated with the activity of AChE in lymphocytes, it is possible to assume that the lymphocytosis on day 10 PI was a consequence of fungal infection (as expected) associated with normal AChE activity, a situation that "preserves" ACh, allowing the lymphocyte

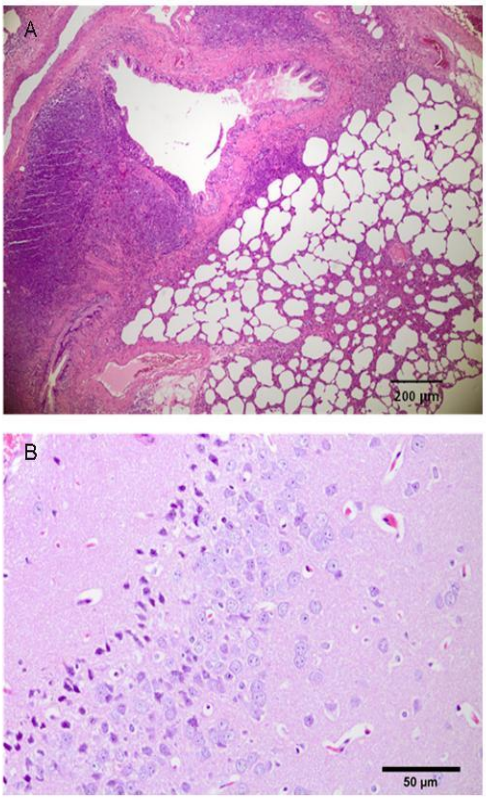

Fig. 6. Rats experimentally infected by *Cryptococcus neoformans* on day 30 PI. Lungs from infected rats after 30 days post-infection showed interstitial infiltration by macrophages and neutrophils (A). There were no detectable brain lesions on all rats in this study (B).

expansion, anti-inflammatory response and reduced NO delivery. On the other hand, on day 30 PI, the number of lymphocytes did not differ statistically from the negative control group; however, the AChE activity was increased. The increase in enzyme activity probably led to a reduction in the concentration of ACh, limiting the lymphocytes proliferation in this period, as well as to an increase in the levels of IL-1 and IL-6, since it is well known that ACh has an important role in attenuating the release of pro-inflammatory cytokines [5], characterizing, then, a pro-inflammatory pattern.

Brain activity of AChE was enhanced in both periods, on days 10 and 30 PI. [36] Researchers working with a murine model recently demonstrated that, unlike *C. gattii*, *C. neoformans* prefers the brain to the lung to establish infection [36]. Therefore, cryptococcal infection of the brain will depend on the successful maneuver by the fungus through the barriers involved in the early phase of infection. Increase in AChE activity in brain samples can be proportional to the reduction of ACh, a situation that favors pro-inflammatory events. Corroborating this hypothesis, our assessment of cytokines showed increased levels of IL-1 and IL-6 (pro-inflammatory cytokines) in both periods. According to the literature, ACh does not affect IL-10 production of IL-10 [5], an anti-inflammatory cytokine, but we observed (together with the increased activity of AChE) levels of IL-10 reduced on days 10 and 30 PI.

Additionally, if we analyze the AChE activity in brain along with the data from the biomarkers of oxidative stress (also assessed in brain tissue), it is possible to observe a correlation, especially regarding NO_x levels, since it increased in both periods (days 10 and 30 PI), while TBARS and AOPP levels increased on day 30 PI. When NO_x derives from inducible NOS (iNOS), it may work as a neurotoxin. iNOS is Ca^{2+}-independent and transcriptionally regulated. Once synthesized, iNOS is active for prolonged periods [36]. The increase in the pro-inflammatory cytokines observed throughout our experiment probably induced iNOS activity (generating NO_x), since *in vitro* studies show that NO or combined with inflammatory cytokines induced iNOS activity in glial cells [37–39]. Once TBARS and AOPP are reliable markers of cellular damage, it was expected that both of them showed increased levels on day 30 PI. Since in our experimental infection, the damage might be caused directly by the yeast or by the exacerbated inflammatory reaction (IL-1 and IL-6 with high levels in both analyzed periods), we considered both hypotheses as viable causes of increased levels of these two biomarkers at the end of the experiment.

Like AChE, BChE inactivates the ACh, but an important feature distinguishing them is its kinetic response to concentrations of Ach, reflected in their *Km* values [40]. In our results we observed BChE with reduced activity in serum on day 10 PI. As mentioned above, on day 10 PI, we found lymphocytosis associated with regular activity of AChE, which allowed us to believe that the concentrations of ACh were elevated at this point. BChE is less efficient in ACh hydrolysis at low concentrations but highly efficient at high quantities, at which AChE becomes substrate-inhibited [41]. Thus, the BChE activity was increased (due to the high concentration of ACh) to a certain point, where an enzymatic depletion occurred, causing the reduction of BChE observed on day 10 PI. As the infection progressed, the ACh showed reduced concentrations (promoting a pro-inflammatory reaction), favoring the activity of AChE, as observed on day 30 PI in lymphocytes.

In summary, based on the results obtained and discussed, it was possible to observe that AChE showed increased activity in brain tissue and lymphocytes, leading to a pro-inflammatory reaction evidenced by the enhancement of IL-1 and IL-6 and NO_x throughout the experiment (associated with IL-10 reduction), as well as by the increase of TBARS and AOPP at the end of the experiment, denoting cellular damage, even though biochemical abnormalities were not detected on histology of brain. BChE activity was reduced at the first sampling, probably due to its exhaustion by hydrolyzing the ACh, when it was in high concentrations. As a result, we conclude that the animals develop the disease, which leads to lipid peroxidation and protein oxidation associated with tissue lesions and inflammation, as well as increased inflammatory response in the blood. Moreover, we may also conclude that the AChE activity can be increased as an inflammatory marker of the disease, since this enzyme regulates the ACh levels (reduce), a powerful anti-inflammatory molecule. As a consequence of this, the immune response against the fungus is exacerbated. Therefore, the AChE is influenced by cryptococcosis, participating directly or indirectly in the events involved in the pathogenesis of the disease.

Acknowledgements

The authors thank the Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq) Brazil for their financial support for a Doctoral Scholarship.

References

[1] K. Neilsen, A.L. De Obaldia, J. Heitman, *Cryptococcus neoformans* mates on pigeon guano: implications for the realized ecological niche and globalization, Eukaryot Cell. 6 (2007) 949–959.
[2] M. Bovers, F. Hagen, T. Boekhout, Diversity of the *Cryptococcus neoformans-Cryptococcus gattii* species complex, Rev. Iberoam. Micol. 25 (2008) 4–12.

[3] L.V. Borovikova, S. Ivanova, M. Zhang, H. Yang, G.I. Botchkina, L.R. Watkins, H. Wang, N. Abumrad, J.W. Eaton, K.J. Tracey, Vagus nerve stimulation attenuates the systemic inflammatory response to endotoxin, Nature 405 (2000) 458–462.
[4] D.O. Beenhouwer, S. Shapiro, M. Feldmesser, A. Casadevall, M.D. Scharff, Both Th1 and Th2 cytokines affect the ability of monoclonal antibodies to protect mice against *Cryptococcus neoformans*, Infect. Immun. 69 (2001) 6445–6455.
[5] V.A. Pavlov, K.J. Tracey, The cholinergic anti-inflammtory pathway, Brain Behav. Immun. 19 (2005) 493–499.
[6] M.R.C. Schetinger, N.M. Porto, M.B. Moretto, V.M. Morsch, J.B.T. Rocha, V. Vieira, F. Moro, R.T. Neis, S. Bittencourt, H.G. Bonacorso, N. Zanatta, New benzodiazepines alter acetylcholinesterase and ATPase activities, Neurochem. Res. 25 (2000) 949–955.
[7] K. Kawashima, T. Fujii, The lymphocytic cholinergic system and its contributionto the regulation of immune activity, Life Sci. 74 (2003) 675–696.
[8] K.R. Dave, A.R. Syal, S.S. Katyare, Tissue cholinesterases. A comparative study of their kinetic properties, Z. Naturforsch. 55 (2000) 100–108.
[9] S. Darvesh, D.A. Hopkins, C. Ceula, Neurobiology of butyrylcholinesterase, Nat. Rev. Neurosci. 17 (2003) 131–138.
[10] L. Ulloa, The vagus nerve and the nicotinic anti-inflammatory pathway, Nat. Rev. Drug. Discov. 4 (2005) 673–684.
[11] M.S. Cooke, M.D. Evans, M. Dizdaroglu, J. Lunec, Oxidative DNA damage: mechanisms, mutation, and disease, FASEB J. 17 (2003) 1195–1214.
[12] D. Gackowski, E. Speina, M. Zielinska, J. Kowalewski, R. Rozalski, A. Siomek, T. Paciorek, B. Tudek, R. Olinski, Products of oxidative DNA damage and repair as possible biomarkers of susceptibility to lung cancer, Cancer Res. 63 (2003) 4899–4902.
[13] N. Ilhan, N. Ilhan, Vascular endothelial growth factor and oxidative damage in cancer, Clin. Biochem. 36 (2003) 225–228.
[14] H. Esterbauer, Cytotoxicity and genotoxicity of lipid-oxidation products, Am. J. Clin. Nutr. 57 (1993) 779–785.
[15] V. Witko-Sarsat, M. Friedlander, T.N. Khoa, C. Capeillère-Blandin, A.T. Nguyen, S. Canteloup, J.M. Dayer, P. Jungers, T. Drüeke, B. Descamps-Latscha, Advanced oxidation protein products as a novel mediators of inflammation and monocyte activation in a chronic renal failure, J. Immunol. 161 (1998) 2524–2532.
[16] C. Bogdan, Nitric oxide and the immune response, Nat. Immunol. 2 (2001) 907–916.
[17] J.S. Beckman, W.H. Koppenol, Nitric oxide, superoxide, and peroxynitrite: the good, the bad, and ugly, Am. J. Physiol. 5 (1996) 1424–1437.
[18] J.M. Chatkin, P. Djupesland, W. Qian, J. Haight, N. Zamel, Óxido nítrico exalado no diagnóstico e acompanhamento das doenças respiratórias, J. Bras. Pneumol. 26 (2000) 36–43.
[19] C.M. Santiago, C.H. Fernandes, M.S. Nogueira, E.V. Veiga, E.C. Cárnio, Utilização do óxido nítrico como terapêutica: implicações para a enfermagem, Rev. Latino-Am. Enfermagem. 8 (2000) 76–82.
[20] D.B. Martins, R.A. Zanette, R.T. França, F. Howes, M.I. Azevedo, S.A. Botton, C. Mazzanti, S.T.A. Lopes, J. Santurio, Massive cryptococcal disseminated infection in an immunocompetent cat, Vet. Dermatol. 22 (2010) 232–234.
[21] D. Goldman, S.C. Lee, A. Casadevall, Pathogenesis of pulmonary *Cryptococcus neoformans* infection in the rat, Infect. Immun. 62 (1994) 4755–4761.
[22] B.V. Feldman, J.G. Zinkl, N.C. Jain, Schalm's Veterinary Hematology, Lippincott Williams & Wilkins, Philadelphia, 2000.
[23] A. Böyum, Solation of mononuclear cells and granulocytes from human blood Isolation of mononuclear cells by one centrifugation, and of granulocytes by combining centrifugation and sedimentation at 1 g, Scand. J. Clin. Lab Invest. Suppl. 97 (1968) 77–89.
[24] W. Struber, Trypan blue exclusion test of cell viability, in: J. Coligan, A. Kruisbeek, D. Marguiles, E. Shevach, W. Strober (Eds.), Curr. Protoc. Immunol., 2001.
[25] B.B. Fitzgerald, L.G. Costa, Modulation of muscarinic receptors an acetylcholinesterase activity in lymphocytes and brain areas following repeated organophosphate exposure in rats, Fund. Appl. Toxicol. 20 (1993) 210–216.
[26] J.B.T. Rocha, T. Emanuelli, M.E. Pereira, Effects of early under nutrition on kinetic parameters of brain acetylcholinesterase from adult rats, Acta Neurobiol. Exp. 53 (1993) 431–437.
[27] G.L. Ellman, K.O. Coutney, V. Andres, R.M. Featherstone, A new and rapid colorimetric determination of acetylcholinesterase activity, Biochem. Pharmacol. 7 (1961) 88–95.
[28] E. Tatsch, G.V. Bochi, R.S. Pereira, H. Kober, J.R. Oliveira, R.N. Moresco, A simple and inexpensive automated technique for measurement of serum nitrite/nitrate, Clin. Biochem. 44 (2011) 348–350.
[29] H. Ohkawa, N. Ohishi, K. Yagi, Assay for lipid peroxides in animal tissues by thiobarbituric acid reaction, Anal. Biochem. 95 (1978) 351–358.
[30] K. Shibuya, A. Hirata, J. Omuta, M. Sugamata, S. Katori, N. Saito, N. Murata, A. Morita, K. Takahashi, C. Hasegawa, A. Mitsuda, T. Hatori, H. Nonaka, Granuloma and cryptococcosis, J. Infect. Chemother. 11 (2005) 115–122.
[31] V. Battisti, M.R.C. Schetinger, L.D.K. Maders, K.F. Santos, M.D. Bagatini, M.C. Correa, R.M. Spanevello, M.C. Araújo, V.M. Morsch, Changes in acetylcholinesterase (AChE) activity in lymphocytes and whole blood in acute lymphoblastic leukemia patients, Clin. Chim. Acta 402 (2009) 114–118.
[32] P. Masturzo, M. Salmona, O. Nordstrom, S. Consolo, H. Ladinski, Intact human lymphocyte membranes respond to muscarinic receptor stimulation by oxotremorine with marked changes in microviscosity and an increase in cyclic GMP, FEBS Lett. 192 (1985) 194–198.
[33] C. Ikeda, I. Morita, A. Mori, K. Fujimoto, T. Suzuki, K. Kawashima, S. Murota, Phorbol ester stimulates acetylcholine synthesis in cultures endothelial cells isolated from procine cerebral microvessels, Brain Res. 665 (1994) 147–152.
[34] A.S. Silva-Herdad, C. Saldanha, Effects of acetylcholine on an animal model of inflammation, Clin. Hemorheol. Microcirc. 53 (1–2) (2013).
[35] C. Saldanha, T. Freitas, J.P. Almeida, Fibrinogen effects on erythrocyte nitric oxide mobilization in presence of acetylcholine, Life Sci. 91 (21-22) (2012) 1017–1022.
[36] S.R. Jaffrey, S.H. Snyder, Nitric oxide: a neural messenger, Annu. Rev. Cell Dev. Biol. 11 (1995) 417–440.
[37] M.L. Simmons, S. Murphy, Cytokines regulate L-arginine-dependent cyclic GMP production in rat glial cells, Eur. J. Neurosci. 5 (1993) 825–831.
[38] L.I. Romero, J.B. Tatro, J.A. Field, S. Reichlin, Roles of IL-1 and TNF-α in endotoxin-induced activation of nitric oxide synthase in cultured rat brain cells, Am. J. Physiol. 270 (1996) 326–332.
[39] C.C. Chao, J.R. Lokensgard, W.S. Sheng, S. Hu, Peterson PK. IL-1-induced iNOS expression in human astrocytes via NF-kB, Neuroreport 8 (1997) 3163–3166.
[40] N.H. Greig, T. Utsuki, D.K. Ingram, Y. Wang, G. Pepeu, C. Scali, Q. Yu, J. Mamczarz, H.W. Holloway, T. Giordano, D. Chen, K. Furukawa, K. Sambamurti, A. Brossi, D.K. Lahiri, Selective butyrylcholinesterase inhibition elevates brain acetylcholine, augments learning and lowers Alzheimer-amyloid peptide in rodent, PNAS 102 (47) (2005) 17213–17218.
[41] A. Silver, The Biology of Cholinesterases, Elsevier Agricultural Research Council Institute, New York, 1974.

4.3 Artigo 3

Participation of purines in the modulation of inflammatory response in rats experimentally infected by *Cryptococcus neoformans*

Artigo submetido ao periódico **Microbial Pathogenesis**

Participation of purines in the modulation of inflammatory response in rats experimentally infected by *Cryptococcus neoformans*

Maria Isabel de Azevedo[a,c], Laerte Ferreiro[a], Aleksandro S. Da Silva[b], Alexandre A. Tonin[c], Danieli Urach Monteiro[c], Emerson A. Casali[d,e,f], Cesar E.J. Moritz[d,e], Gabriel H. Schirmbeck[d], Valesca V. Cardoso[d,f], Mariana M. Flores[g], Rafael Fighera[g], Lenita M. Stefani[b], Janio M. Santurio[c*]

[a] Laboratório de Micologia, Departamento de Patologia e Clinica Animal, Faculdade de Veterinária (UFRGS), Porto Alegre, RS, Brazil.

[b] Departamento de Zootecnia, Universidade do Estado de Santa Catarina, Chapecó, SC, Brasil

[c] Departamento de Microbiologia e Parasitologia, Universidade Federal de Santa Maria, Santa Maria, RS, Brazil.

[d] Departamento de Ciências Morfológicas, Instituto de Ciências Básicas da Saúde (ICBS), Universidade Federal do Rio Grande do Sul (UFRGS), Porto Alegre, RS, Brazil.

[e] Departamento de Bioquímica, Instituto de Ciências Básicas da Saúde (ICBS), Universidade Federal do Rio Grande do Sul (UFRGS), Porto Alegre, RS, Brazil.

[f] Laboratório de Mutagênese e Toxicologia, Centro Universitário Metodista- IPA, Porto Alegre, RS, Brazil.

[g] Departamento de Patologia, UFSM, Santa Maria, RS, Brazil.

*Corresponding author: janio.santurio@gmail.com

Address: Departamento de Microbiologia e Parasitologia da UFSM.

Faixa de Camobi - Km 9, Campus Universitário, Santa Maria - RS, Brazil.

97105-900, Prédio 20, Sala 4139 Fax: +55 55 3220-8906.

Abstract

The present study was carried out to assess the participation of purines in the activation and modulation of inflammatory response of rats experimentally infected by *Cryptococcus neoformans*. Twenty four Wistar rats were divided into two groups of 12 animals each: Group A - uninfected control group and Group B - infected by *C. neoformans*. Blood was collected 20 and 50 days post-infection (PI) from six animals of each group in order to verify purine levels (adenosine triphosphate (ATP), adenosine diphosphate (ADP), adenosine monophosphate (AMP), adenosine (ADO), inosine (INO), hypoxanthine (HYPO), xanthine (XAN) and uric acid (URIC)). ATP levels were significantly increased ($P<0.05$) in serum from infected animals on days 20 and 50 PI, as well as adenosine levels after 20 days PI on rats. However, on day 50 PI, levels of adenosine, inosine, hipoxantine and uric acid were reduced ($P<0.05$). Therefore, it was possible to conclude that the purine levels in serum were altered and that these changes may be able to influence the pathogenesis of the disease caused by *C. neoformans* due the participation of purines (ATP and adenosine main) in the activation and modulation of inflammatory response.

Keywords: Cryptococcosis; Purinergic signaling; pathology; immune response.

1. Introduction

Cryptococcus neoformans, the predominant etiological agent of cryptococcosis, is an encapsulated fungal pathogen that causes disease that ranges from an asymptomatic infection to a mild bronchopneumonia and even to a life-threatening infection of the central nervous system (CNS) [1]. The most common and serious type of cryptococcal disease is the pulmonary cryptococcosis that if uncontrolled may progress to cryptococcal meningitis (CM) or meningoencephalitis [2,3]. Pulmonary

cryptococcal infection will occur only when fungal cells are deposited deep into the lungs after inhalation. In the lungs alveolar macrophages (AMs) will be the first cells to encounter the fungus and will respond by internalizing them through phagocytosis [4]. When cryptococcal infection is not controlled at this stage, the microorganism may spread throughout the body, with particular preference for the CNS leading to meningitis and/or meningoencephalitis, a life-threatening disease with high mortality rates [1,5].

Resistance or susceptibility to *Cryptococcus* disease in humans and animals depend on the outcome of many host and pathogen derived factors. In this sense, purinergic mechanisms have been shown to be involved in various pathological conditions where nucleotides ATP, ADP, AMP and the nucleosides adenosine and inosine are secreted by hematological and endothelial cells and used as mediators able to modulate the inflammation process, vascular thrombosis, muscle contraction, neurotransmission and pain [6,7,8,9]. Additionally, purinergic mechanisms also play an important role in brain trauma and ischaemia, neurodegenerative diseases involving neuroimmune and neuroinflammatory reactions, as well as in neuropsychiatric diseases, including depression and schizophrenia [8]. The deamination of adenosine to inosine favors the maintenance and survival of invading microorganisms, since adenosine promotes chemotaxis, activation and degranulation of mast cells [10,11]. Hypoxanthine, xanthine, and uric acid are bioproducts of purine catabolism [12], being the uric acid a potent antioxidant. Therefore, the aim of this study was to access the purine levels of the purinergic cascade in serum samples from rats experimentally infected by *C. neoformans* and to evaluate the participation of purines in the modulation of inflammation.

2. Materials and methods

2.1. Animals

Twenty-four adult male rats with 90 days of age and 274 (±21) grams of weight were used. They were kept in cages with six animals each on an experimental facility under controlled temperature and humidity (25 °C; 70%), fed with commercial feed, with water *ad libitum*, and submitted to a period of 12 days for adaptation.

This study was approved by the Ethics and Animal Welfare Committee of the Federal University of Santa Maria (UFSM), under protocol number 6598280615.

2.2. Strain

2.2.1. Cryptococcus neoformans var. grubii

Cryptococcus neoformans used for inoculum preparation was obtained from a clinical case of a feline with cryptococcosis [13]. This strain had previously been identified as *Cryptococcus neoformans var. grubii* based on micromorphology and molecular characteristics.

2.2.2. Inoculum preparation

C. neoformans was grown in Sabouraud dextrose agar (SDA) medium for 24 h at 30°C to obtain a viable and pure strain. The fungal inoculum was prepared using young colonies diluted in phosphate-buffered saline (PBS).

2.3. Experimental design

In this study, Wistar rats were divided into two groups: 12 animals composed the control group A (group: uninfected), and 12 animals composed group B (group: infected) infected by *C. neoformans*. After isoflurane anesthesia, the inoculation of

0.3mL containing $1.7x10^7$ cells of *C. neoformans* was performed intratrachealy as described previously [14].

2.4. Course of infection

Course of infection was assessed by the evaluation of colony-forming units (CFU) on days 20 and 50 PI (sampling times). The lungs of rats were collected and weighed after euthanasia. A fragment of each organ was mechanical grinded and homogenized in PBS (1:10) containing 40 U of penicillin/mL. All samples were submitted to serial dilutions and 100µL aliquots were added to plates containing SDA and incubated at 30°C for 24-72 h. Data were reported as (CFU)/g of organ analyzed. The results of CFU were shown as mean and standard deviation.

2.5. Collecting samples

Samples from the groups A and B were collected on days 20 and 50 (PI) as follows: on day 20 (six animals of group A and six animals of group B); and on day 50 (six animals of group A and six animals of group B). Blood samples were collected by cardiac puncture (4mL) from anesthetized animals. Blood samples were allocated in tubes without anticoagulant in order to obtain serum to measure the levels of purines. To obtain the serum, all blood samples were centrifuged (5000 g for 5min at 37 °C). The serum was stored at −20 °C until analysis.

2.5. Serum preparation

The denaturation of serum sample proteins was performed using 0.6 mol L^{-1} perchloric acid. All samples were then centrifuged (14000 g for 10 min at 4 °C) and the supernatants were neutralized with 4.0 N KOH and clarified with a second

centrifugation (14000 g for 15 min at 4 °C). The serum of rat from each group (A and B) was used to measure the concentration of purines ATP, ADP, AMP, ADO, INO, HYPO, XAN and (URIC)) [15].

2.6. Analysis of purine levels in serum

Aliquots of 20 μL of serum samples were applied to a reversed-phase performance liquid chromatography (HPLC) system (Shimadzu, Japan) using a C_{18} column (Ultra C18, 25 cm × 4.6 mm x 5μm, Restek - USA). The elution was carried out applying a linear gradient from 100% of solvent A (60 mM KH_2PO_4 and 5 mM of tetrabutylammonium phosphate, pH 6.0) to 100% of solvent B (solvent A plus 30% methanol) over a 30 min period (flow rate at 1.4 mL/min) according to a method previously described [16]. The amounts of purines were measured by absorption measured at 260 nm. The retention time of standards was used as parameter for identification and quantification. Purines concentrations were expressed as nmol of different compounds per mL of serum.

2.7. Histopathology

Lungs tissues from three animals of each group (three of each period evaluated, days 20 and 50 PI) were collected, and then stored in 10% formalin buffered solution. Sagittal sections of every 3 mm were obtained and stained with hematoxylin and eosin.

2.8. Statistical analysis

The data were submitted to one-way analysis of variance followed by the Students t-test ($P<0.05$). The values were represented as mean ± standard error. All samples were processed in triplicate.

3. Results

3.1. Course of infection and clinical signs

Rats showed mild apathy and piloerection, and one rat died before the end of the experiment with macro-lesions in the lungs, and histological examinations revealed the presence of fungus associated with pneumonia. Similar findings were also found on other experimental animals described below.

C. neoformans was isolated from the lungs of infected animals on days 20 and 50 PI, resulting in the growth of white mucoid yeast colonies. Microscopical analysis of these colonies on India ink showed the presence of rounded cells of different sizes with and without budding surrounded by a clear halo. Lung samples showed 8.3 $x10^4$ (± $1.8x10^4$) and 2.8 ± $x10^4$ CFU (± $0.35x10^4$) of *C. neoformans* per gram on days 20 e 50 PI, respectively.

3.2. Purines levels in serum

The levels of purines in serum of rats were shown in Figure 1. Serum ATP levels increased significantly in infected animals on days 20 and 50 PI, as well as ADO on day 20 PI. Was observed the reduced levels of hypoxanthine in both periods in infected rats, and decreased levels of ADO, INO and URIC only in day 50 PI.

3.3. Histological evaluation

Rats infected by *C. neoformans* developed histological lesions in the lung characterized by inflammatory response (Figure 2). At day 20 and 50 PI, the lungs showed an elevated, firm and whitish area, with an irregular surface. On cutting, the area was solid, hard, and homogeneously white. Histologically, the lungs showed a

focal extensive area formed by multiple accumulations of amorphous material, characterized by a central area of caseous necrosis with multiple neutrophils. This area was surrounded by a great amount of epithelioid macrophages and giant cells. In the central area of the granuloma, there was a mixture of cellular debri and necrotic tissue with large amounts of round to oval structures classified as yeast cells. These structures were basophilic with 20 to 60μm of diameter and a core ranging from 10 to 20μm, surrounded by an unstained capsule.

4. Discussion

In cryptococcal infection, like in many others, the virulence of the microorganism relies on specific molecular factors that target host defenses, allowing or not, the establishment of the infection. In this context, rats were used as experimental model and intratracheal inoculation by *C. neoformans*, producing a local lung infection, similar to pulmonary infection in immunocompetent humans [14]. Importantly, the animals showed clinical signs of the disease, as well as death after complications caused by cryptococcosis.

Extracellular ATP levels were increased on serum sample of infected rats. According to the literature, the high levels of ATP in the extracellular milieu are directly linked to the inflammatory response against the infection, because ATP is a molecule pro-inflammatory, able to activate cell defense [17], regulating the activation, migration, phagocytosis and release of pro-inflammatory factors in immune and glial cells [18]. A tendency of pro-inflammatory response is supported by our histological results, since it was observed signs of inflammatory infiltrate in lungs of animals.

Considering the pro-inflammatory pattern mentioned earlier, it is imperative to highlight that in adaptive immune responses to pathogens, there is a critical balance

between pro and anti-inflammatory immune responses. Uncontrolled pro-inflammatory immune responses can result in damage to host tissues, whereas anti-inflammatory immune responses initiated prematurely can result in pathogen survival, which is deleterious for the host [19]. Among the molecules evaluated in our study, (ADO) is the main molecule being in charge of some anti-inflammatory responses. ADO receptor ligation on monocytes and macrophages strongly suppresses the production of IL-12 through TLR4 [20,23]. Since IL-12 is vital in directing a strong inflammatory response, the ADO suppression of IL-12 production is probably one of the central mechanisms whereby ADO receptor occupancy prevents inflammation-induced tissue injury. IL-12, a pro-inflammatory cytokine and a main inducer of Th1 responses and cell-mediated immunity, is suppressed by adenosine and its analogues, whereas secretion of IL-10, a protective cytokine that suppresses IL-12 and TNF-α release, is enhanced by ADO and $A2_A$ receptor agonists [21,22,24,25]. Thus, we observed increased levels of ADO in serum of rats on day 20 PI. Our research group had already observed an inhibition of E-ADA activity in serum of rats infected by *C. neoformans* early on the infection [26], corroborating the present results. As mentioned earlier, there was a greater concentration of extracellular ADO by interaction with purinergic receptors, playing a protective role in order to attenuate systemic changes caused by infection [27]. Therefore, we believe that enhanced levels of ADO on day 20 PI played a modulatory function on cryptococcal infection during the early infection, avoiding excessive tissue damage.

On the other hand, ADO levels on day 50 PI were statistically reduced compared to higher levels on day 20 PI for the same samples, showing an inverse pattern. Since histological examination on day 50 PI showed signs of the infection, we believe this ADO behavior was favoring a pro-inflammatory action, as an attempt to control the

infection during the late phase of experimental infection. This hypothesis is in accordance to the conclusions regarding to ATP levels, which still increased on day 50 PI signaling an inflammatory response. This counter-regulatory effect suggests that ADO enhances the inflammatory response at sites where ADO is present in low concentrations, or at sites where net ADO uptake and metabolism is greater than ADO production, as can occur at sites of microbial infection [28].

Evidences indicate that the adenosine breakdown products, INO and URIC, can influence many facets of the innate immune response [21,29]. Most of our results showed a consequence of the purinergic cascade, since the subsequent products depends on their precursors [30]. The oxidation of HYPO, XAN, catalyzed by xanthine oxidase, produces URIC, a potent antioxidant [31]. Our results showed that URIC was statistically reduced at day 50 PI (or with a tendency of reduction at day 20 PI) in samples of serum of rats, representing a potential reduction in free radical scavenging. Thus, because the relative levels of these various purinergic by-products, we can suggest a closely reflection on the metabolic status of the tissue, proposing that our results, on purine compounds assessment, could represent an ideal sensor system on providing the immune system with essential information about the ‘health’ of the tissue, in this case, regarding to a cryptococcal infection.

Based on these results, we can conclude that the experimental infection by *C. neoformans* in rats, led to a characteristic purinergic response to the infection, providing an increase on extracellular levels of ATP, which characterizes a pro-inflammatory response, followed by episodes of anti-inflammatory modulations when extracellular ADO levels were increased, or corroborating the pro-inflammatory events, when ADO was reduced. Also, products of ADO catabolism show different actions, serving as a “sensor of tissue health”. Therefore, the cryptococcosis changes purine levels, and

consequently it influences the pathogenesis of the disease, because the importance of the vital functions in which these molecules are involved.

Acknowledgements

The authors thank the National Council for Scientific and Technological Development of Brazil (CNPq) for their financial support for a Doctoral Scholarship.

References

[1] Negroni R. Cryptococosis. Clinics in Dematology 2012; 30:599-609.

[2] Christianson JC, Engber W, Andes D. Primary cutaneous cryptococcosis in immunocompetent and immunocompromised hosts. Medical Mycology 2003; 41:177-88.

[3] El-Kersh K, Rawasia WF, Chaddha U et al. Rarity revisited: cryptococcal peritonitis. BMJ Case Reports 10. 2013.

[4] Goldman DL, Lee SC, Mednick AJ et al. Persistent *Cryptococcus neoformans* pulmonary infection in the rat is associated with intracellular parasitism, decreased inducible nitric oxide synthase expression, and altered antibody responsiveness to cryptococcal polysaccharide. Infection and Immunity 2000; 68:832-838.

[5] Bovers M, Hagen F, Boekhout T. Diversity of the Cryptococcus neoformans-Cryptococcus gattii species complex. Revista Iberoamericana de Micologia 2008; 25:S4-12.

[6] Ralevic V, Burnstock G. Receptores for purines and pyrimidines. Pharmacological Reviews 1998; 50:413-92.

[7] Sitkovsky MV, Ohta A. The 'danger' sensors that STOP the immune response: the A2 adenosine receptors? Trends in Immunology 2005; 26:299-304.

[8] Burnstock G. Purinergic signaling – an overview. Novartis Foundation Symposia 2006; 276;26–48.

[9] Desrosiers MD, Cembrola KM, Fakir MJ et al. Adenosine deamination sustains dendritic cell activation in inflammation. The Journal of Immunology 2007; 179:1884-92.

[10] Jin X, Shepherd RK, Duling BR et al. Inosine binds to A3 adenosine receptors and stimulates mast cell degranulation. The American Society for Clinical Investigation 1997; 100:2849-57.

[11] Gounaris K, Selkirk ME. Parasite nucleotide-metabolizing enzymes and host purinergic signalling. Trends in Parasitology 2005; 21:17-21**.**

[12] Chen XB, Gomes MJ. Estimation of Microbial Protein Supply to Sheep and Cattle Based on Urinary Excretion of Purine Derivatives an Overview of Technical Details. Occasional publication, International Feed Research Unit. Rowett Research Institute, Aberdeen, UK. 1992.

[13] Martins DB, Zanette RA, França RT et al. Massive cryptococcal disseminated infection in an immunocompetent cat. Veterinary Dermatology 2010; 22:232–234.

[14]Goldman D, Lee SC, Casadevall A. Pathogenesis of pulmonary *Cryptococcus neoformans* infection in the rat. Infection and Immunity 1994; 62:4755–4761.

[15] Ryder JM, Determination of adenosine triphosphate and its breakdown products in fish products by high-performance liquid chromatography. Journal of Agricultural and Food Chemistry 1985; 33:678–680.

[16] Voelter W, Zech K, Arnold P et al. Determination of selected pyrimidines, purines and their metabolites in serum and urine by reversed-phase ionpair chromatography. Journal of Chromatography 1980; 199:345–354.

[17] Bours MJ, Swennen ELDI, Virgilio F et al. Adenosine 5'-triphosphate and adenosine as endogenous signaling molecules in immunity and inflammation. Pharmacology & Therapeutics 2006; 112:358-404

[18] Gandelman M, Peluffo H, Beckman JS et al. Research Extracellular ATP and the P2X7 receptor in astrocyte-mediated motor neuron death: implications for amyotrophic lateral sclerosis. Journal of Neuroinflammation 2010; 7:9-33.

[19] Bot A. Editorial –In this Issue: Innate Immunity in Normal and Pathologic Circumstances. International Reviews of Immunology 2014; 33:441-442.

[20] Haskó G, Németh ZH, Vizi ES et al. An agonist of adenosine A3 receptors decreases interleukin-12 and interferon-g production and prevents lethality in endotoxemic mice. European Journal of Pharmacology 1998; 358:261-268.

[21] Haskó G, Kuhel JF, Chen MA et al. Adenosine inhibits IL-12 and TNF- production via adenosine A2A receptor-dependent and independent mechanisms. The FASEB Journal 2000; 14: 2065-2074.

[22] Link AA, Kino T, Worth JA et al. Ligand-activation of the adenosine A2A receptors inhibits IL-12 production by human monocytes. The Journal of Immunology 2000; 164: 436-442.

[23] Khoa ND, Montesinos MC, Reiss AB et al. Inflammatory cytokines regulate function and expression of adenosine A2A receptors in human monocytic THP-1 cells. *J Immunol* 2001; **167**(7): 4026-4032.

[24] Le Moine O, Stordeur P, Schandene L et al. Adenosine enhances IL-10 secretion by human monocytesThe Journal of Immunology 1996; 156:4408-4414.

[25] Haskó G, Szabo C, Nemeth ZH et al. Adenosine receptor agonists differentially regulate IL-10, TNF-, and nitric oxide production in RAW 264.7 macrophages and in endotoxemic mice. The Journal of Immunology 1996; 157:4634-4640.

[26] Azevedo MI, Ferreiro L, Da Silva AS et al. E-NTPDase and E-ADA activities in rats experimental infected by *Cryptococcus neoformans*. Veterinary Microbiology 2014; 174: 206-213.

[27] Franco R, Casado V, Ciruela F et al. Cell surface adenosine deaminase: much more than an ectoenzyme. Progress in Neurobiology 1997; 52:283-294.

[28] Haskó G, Cronstein BN. Adenosine: an endogenous regulator of innate immunity. Trends in Immunology 2004; 25:33-39.

[29] Scott GS, Spitsin SV, Kean RB et al. Therapeutic intervention in experimental allergic encephalomyelitis by administration of uric acid precursors. PNAS, Proceedings of the National Academy of Sciences USA 2002; 99:16303-16308

[30] Ralevic V, Burnstock G. Involvement of purinergic signalling in cardiovascular diseases. Drug News & Perspectives 2003; 16:133-40.

[31] Santos CX, Anjos EI, Augusto O. Uric acid oxidation by peroxynitrite: multiple reactions, free radical formation, and amplification of lipid oxidation. Archives of Biochemistry and Biophysics 1999; 372:285-294.

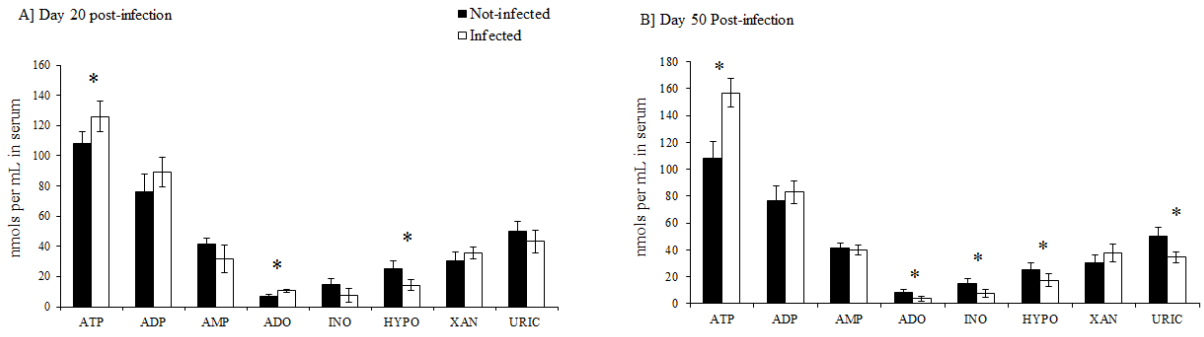

Fig. 1. Purine levels in serum of rats infected by *Cryptococcus neoformans* on days 20 (A) and 50 (B) post-infection. Serum levels of purines (right hemisphere): adenosine triphosphate (ATP), adenosine diphosphate (ADP), adenosine monophosphate (AMP), adenosine (ADO), inosine (INO), hypoxanthine (HYPO), xanthine (XAN) and uric acid (URIC), *P<0.05.

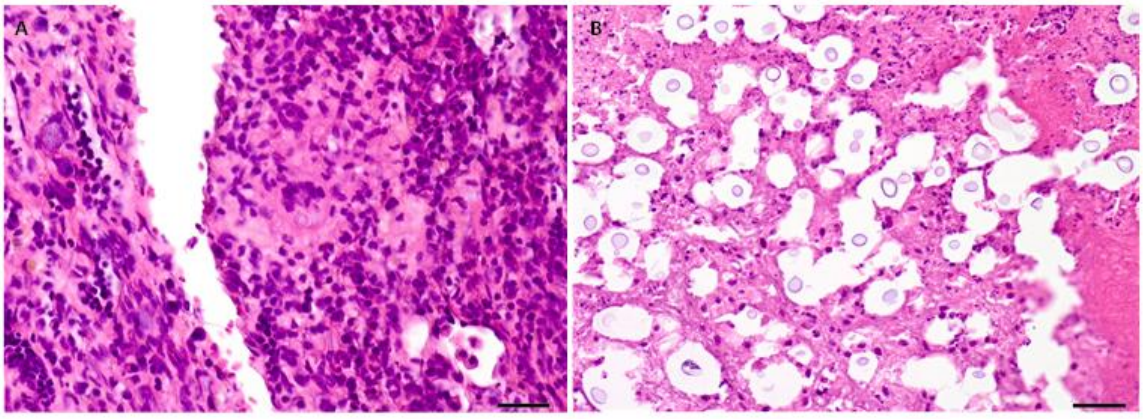

Fig. 2. Experimental infection by *Cryptococcus neoformans* in rats. Lung fragments of rats showed inflammatory infiltrate composed by neutrophils and epithelioid macrophages on day 20 post-infection (PI) (A: BAR 50 um); it is also possible to observe large amounts of round to oval structures with 20 to 60μm, with basophilic nuclei surrounded by unstained capsule on day 50 PI (B; BAR 20 um).

5 DISCUSSÃO

O mecanismo de defesa do organismo para combater *Cryptococcus* spp. conta com a participação do sistema imune inato e adaptativo, sendo a resposta imune mediada por células T o principal mecanismo de defesa imune (ZHOU & MURPHY, 2006). Em se tratando de resposta imune, tanto o sistema purinérgico (BURNSTOCK, 2006), quanto o colinérgico (DAS, 2007) possuem algumas propriedades imunomodulatórias bem definidas; porém, a relação destes sistemas com algumas importantes doenças ainda tende a ser muito explorada em pesquisas futuras. Desta maneira, sabendo-se que uma resposta imune eficiente é vital ao hospedeiro frente a uma infecção por *Cryptococcus* spp., buscou-se avaliar a participação dos sistemas purinérgico e colinérgico nesta enfermidade.

Foi avaliada inicialmente a influência da infecção por *C.* var. *grubii* em um panorama sistêmico, *in vivo*, uma vez que foram utilizadas avaliações de atividades enzimáticas em células sanguíneas, principalmente nos linfócitos. Observando as respostas à infecção, geradas pelo sistema purinérgico (sinalização purinérgica), foi possível observar que tanto a E-NTPDase quanto a E-ADA tiveram suas atividades afetadas pela criptococose. A E-NTPDase desempenha um importante papel no controle da função dos linfócitos, incluindo o reconhecimento do antígeno e ativação de funções efetoras das células T citotóxicas (FILIPPINI et al., 1990).

Quando a atividade desta enzima foi avaliada em linfócitos de ratos, experimentalmente infectados com *C.* var. *grubii*, observou-se uma diminuição da atividade da E-NTPDase quando utilizados substratos de ATP e ADP. É conhecido que as concentrações extracelulares de ATP são controladas dinamicamente pela ecto-enzima E-NTPDase (BOURS et al., 2006). Portanto, sugere-se que pode ter ocorrido um aumento na concentração de ATP e ADP extracelular nos ratos infectados experimentalmente. O aumento de ATP no meio extracelular pode também levar a um reação pró-inflamatória, principalmente pela liberação de mediadores pró-inflamatórios, com a diferenciação de células Th nativas em linfócitos Th1, iniciando o processo de inflamação (YEGUTKIN, 2008). Sabe-se que durante a fase inicial da infecção por *C. neoformans* as células T produzem citocinas, que ativam macrófagos levando à destruição intracelular de fungo ou mantendo este em uma condição dormente (FELDMESSER et al., 2001). Por estas razões acreditamos que um aumento do nível de

ATP extracelular ocorreu na infecção experimental, principalmente considerando o aumento nos níveis de TNF-α e INF-ɣ observado durante o experimento.

Para reforçar a hipótese da ocorrência de uma reação pró-inflamatória pelo ATP, foi avaliada a atividade da E-ADA nos linfócitos, visto que a E-ADA é responsável pela regulação da concentração de adenosina (ADO) no meio extracelular (RALEVIC & BURNSTOCK, 1998). A ADO pode atuar como um "sinalizador" fornecendo informações para o sistema imune sobre dano tecidual, principalmente em infecções agudas (KUMAR & SHARMA, 2009) dos neutrófilos e linfócitos (BOURS et al., 2006). Clinicamente, altos níveis de E-ADA no líquido pleural pode ser observado em pacientes com pleurite criptocócica (YOSHINO et al., 2010), empiema, linfoma, derrames parapneumônicos e derrames pleurais associado a doenças do colágeno (OCANA et al., 1988; BURGESS et al., 1996). Em nossos resultados, foi possível observar que a atividade de E-ADA no soro foi inibida nos dias 10, 20 e 30 PI em comparação com o controle grupo. Diminuição da atividade E-ADA sérica pode estar relacionada a necessidade de uma maior concentração de extracelular ADO através da interação com receptores purinérgicos, desempenhando um papel de proteção, a fim de atenuar alterações sistêmicas causadas pela infecção (FRANCO et al., 1997).

Em condições patológicas, a ADO desempenha um papel protetor, modulando a liberação de neurotransmissores e também atuando como um regulador endógeno da imunidade inata e na defesa do hospedeiro de lesão tecidual excessiva associada à inflamação (RATHBONE et al., 1999; BERAUDI et al., 2003; HASKO & CRONSTEIN, 2004; SITKOVSKY & OHTA, 2005; BURNSTOCK, 2006; DESROSIERS et al., 2007), sendo considerada uma molécula sinalizadora de dano celular (BOURS et al., 2006). Desta maneira, a ADO em altas concentrações pode atuar via receptores purinérgicos, atenuando a inflamação e os danos teciduais. Esses resultados corroboram a hipótese de que o aumento na atividade da E-NTPDase visou proporcionar um efeito estimulador da resposta imune, frente à infecção por *Cryptococcus neoformans*. Neste primeiro experimento, foram ainda avaliados alguns outros parâmetros visando a confirmação da infecção pelo agente, e assim, validando os resultados obtidos. Neste sentido é importante ressaltar que na avaliação sorológica foram observados níveis de IgM e IgG aumentados nos três períodos avaliados, caracterizando uma curva imunológica típica de um processo infeccioso.

No segundo estudo o objetivo foi estabelecer o papel das enzimas do sistema colinérgico na criptococose. Da mesma forma que na avaliação anterior, foram

estabelecidos parâmetros que validassem, ou confirmassem, nesse caso, a resposta inflamatória gerada pela resposta à infecção. Nesse sentido foi optado por se dosar as citocinas IL-1, IL-6 e IL10. As duas primeiras interleucinas foram detectadas em concentrações estatisticamente elevadas em relação ao controle negativo, em ambos os períodos, demonstrando o padrão pró-inflamatório próprio à infecção. Em relação à atividade enzimática colinérgica, foi observado um aumento da AChE nos linfócitos e no cérebro e diminuição da BChE no soro.

A AChE possui um papel regulatório na neurotransmissão colinérgica. Ela é responsável pela hidrólise rápida do neurotransmissor ACh, uma molécula mediadora da via colinérgica anti-inflamatória, cuja atuação se dá pela inibição da produção de TNF-α e IL-1, quando esta molécula se liga a receptores nicotínicos na superfície dos linfócitos (RAMIREZ et al., 1997). Considerando os dados de parâmetros hematológicos, associado com a atividade da AChE em linfócitos, é possível perceber que a linfocitose no dia 10 PI foi uma consequência da infecção fúngica (como esperado) associado com atividade AChE normal, uma situação que "preserva" ACh, permitindo a expansão do linfócito. No período que corresponde a 30 dias PI a atividade da AChE foi aumentada levando a uma redução na concentração de ACh, e limitando proliferação dos linfócitos neste período, uma vez que é bem conhecido que a ACh tem um papel importante na atenuação da liberação de citocinas pró-inflamatórias (PAVLOV & TRACEY, 2005). Atividade cerebral de AChE foi reforçada em ambos os períodos, nos dias 10 e 30 de PI. Aumento da atividade da AChE em amostras do cérebro pode ser proporcional à redução da ACh, uma situação que favorece eventos pró-inflamatórios.

A BChE foi dosada em amostras de soro desses animais, porém apresentando diminuição na sua atividade somente no dia 10 PI. BChE é menos eficiente na hidrólise de ACh em concentrações baixas, mas altamente eficiente em quantidades elevadas (DAJAS-BAILADOR & WONNACOTT, 2004). Assim, a atividade da BChE esteve diminuida (devido à baixa concentração de ACh), a atividade desta enzima é dependente do subtrato. Conforme foi possível observar que a AChE demonstrou aumento da atividade em tecido cerebral e linfócitos, levando a uma reativação pró-inflamatória, confirmada pelo aumento de IL-1 e IL-6. Além disso, também se pode concluir que a atividade de AChE pode ser aumentada como marcador inflamatório da doença, uma vez que esta enzima regula os níveis de ACh, uma potente molécula anti-inflamatória.

No estudo da avaliação dos níveis de purinas no soro foi possível observar que ocorreu um aumento nos níveis de ATP em ambos os períodos. Níveis elevados de ATP no meio extracelular são diretamente ligados a resposta inflamatória para combater a infecção, visto que, o ATP é capaz de ativar vários mediadores pro-inflamatórias (BOURS et al., 2006; GANDELMAN et al., 2010). Dentre as purinas analisadas a ADO é a principal molécula que desempenha atividade anti-inflamatória, seu pequeno aumento no dia 20 PI demostra um papel anti-inflamatório prematuro na tentativa de evitar dano excessivo aos tecidos. Quando avaliada a cascata purinérgica é importante lembrar que os produtos subsequentes dependem dos seus precussores (RALEVIC & BURNSTOCK, 2003). A oxidação de moléculas como a hipoxantina e xantina, quando catalisadas por ação da xantina oxidase, produzem o ácido úrico que é considerado como um potente antioxidante (SANTOS et al., 1999). Nosso estudo mostrou uma redução nos níveis deste antioxidante, representando redução no potencial de eliminação de radicais livres.

Portanto, na resposta imune frente a diferentes patógenos é crucial um equilíbrio entre respostas pró e anti-inflamatória. Resposta imune pró-inflamatórias não controladas podem resultar em danos ao tecido do hospedeiro, enquanto que a resposta imune anti-inflamatória precoce pode resultar em sobrevivência de agentes patogênicos prejudicando mais uma vez o hospedeiro. O sistema enzimático colinérgico e purinérgico parecem participar da modulação da resposta inflamatória à criptococose. Desta maneira o equilíbrio entre as respostas inflamatórias são determinantes para modular a resposta imune no hospedeiro frente à infecção por *C. neoformans*.

6 CONCLUSÕES

1. Os resultados apresentados nesta tese permitem concluir que a infecção experimental por *C. neoformans* foi capaz de alterar os sistemas purinérgico e colinérgico

2. Na avaliação da atividade das enzimas E-NTPDase e E-ADA, no soro e linfócitos, foi possível observar que tanto a E-NTPDase quanto a E-ADA tiveram suas atividades afetadas pela criptococose. A hidrólise do ATP e ADP foram diminuídas, bem como a atividade da E-ADA também esteve diminuída. Isso demonstra uma atividade pró-inflamatória da E-NTPDase e uma atividade anti-inflamatória da E-ADA, ou seja, fortalecendo a hipótese da existência de um mecanismo de modulação da resposta inflamatória;

3. Na avaliação da AChE nos linfócitos e no cérebro foi possível observar que a atividade da AChE esteve aumentada, diminuindo a ACh e estabelecendo uma resposta pró-inflamatória. Essa reação foi evidenciada pelo aumento IL-1 e IL-6. A atividade da BChE no soro esteve reduzida provavelmente devido a exaustão da hidrólise da ACh;

5. Na avaliação dos níveis de purinas no soro foi possível observar que ocorreu um aumento nos níveis de ATP em ambos os períodos, caracterizando uma resposta pró-inflamatória.

7 REFERÊNCIAS BIBLIOGRÁFICAS

ABBAS, A.K.; LICHTMAN, A.H. **Imunologia Celular e Molecular**, 5º ed. Rio de Janeiro: Elsevier, 2005.

ALDRICH, M.B.; BLACKBURN, M.R.; KELLEMS, R.E. The importance of adenosine deaminase for lymphocyte development and function. **Biochemical Biophysical Research Communications**, v. 272, p. 311-315, 2000.

ALTUG, N.; YÜKSEK, N.; AĞAOĞLU, Z.T.; KELEŞ, I. Determination of adenosine deaminase activity in cattle naturally infected with *Theileria annulata.* **Tropical Animal Health Production,** v.40, p.449-456, 2008.

ANTACHOPOULOS, C.; WALSH, T. J. Immunotherapy of Cryptococcus infections. **Clinical Microbiology and Infection**, v.18, p. 126-33, 2012.

ARAN, J.M.; COLOMER, D.; MATUTES, E.; VIVES-CORRONS, J.L.; FRANCO, R. Presence of adenosine deaminase on the surface of mononuclear blood cells: immunochemical localization using light and electron microscopy. **The Journal of Histochemistry and Cytochemistry**, v. 39, p. 1001-1008, 1991.

ARAÚJO, M.C.; MORSCH, A.; ZANIN, R.; BAUCHSPIESSR, R.J.B.; MORSCH, V.M.; SCHETINGER, M.R. Enzymes that hydrolyze adenine nucleotides in plateletes from breast cancer patients. **Biochimica et Biophysica Acta,** v. 1740, p.421-426, 2005.

ATKINSON, B.; DWYER, K.; ENJYOJI, K.; ROBSON, S.C. Ecto-nucleotidases of the CD39/NTPDase family modulate platelet activation and thrombus formation: Potential as therapeutic target. **Blood cells, Molecules and Diseases**, v.36, p.217-222, 2006.

BARNETT, J. A. A history of research on yeasts 14: medical yeasts part 2, *Cryptococcus neoformans*. **Yeast,** v. 27, p. 875-904, 2010.

BEENHOUWER, D.O.; SHAPIRO, S.; FELDMESSER, M.; CASADEVALL, A.; SCHARFF, M. D. Both Th1 and Th2 cytokines affect the ability of monoclonal antibodies to protect mice against *Cryptococcus neoformans*. **Infection and Immunity,** v. 69, p. 6445-6455, 2001.

BERAUDI, A.; TRAVERSA, U.; VILLANI, L.; SEKINO, Y.; NAGY, J.I.; POLI, A. Distribution and expression of A_1 adenosine receptors, adenosine deaminase and

adenosine deaminase-binding protein (CD26) in goldfish brain. **Neurochemistry International,** v.42, p.455–463, 2003.

BIANCHI, M.H.; ROBLES, A.M.; VITALE, R.; HELOU, S.; ARECHAVALA, A.; NEGRONI, R. The usefulness of blood cultures in diagnosing HIV-related systemic mycoses: evaluation of a manual lysis- centrifugation method. **Medical Mycology**, v. 38, p.77-80, 2000.

BLACKSTOCK, R.; MURPHY, J.W. Age-related resistance of C57BL/6 mice to *Cryptococcus neoformans* is dependent on maturation of NKT cells. **Infection and Immunity,** v. 72, n. 9, p. 5175-5180, 2004.

BOEKHOUT, T.; THEELEN, B.; DIAZ, M.; FELL, J.W.; HOP, W.C.; ABELN, E.C.; DROMER, F., MEYER, W. Hybrid genotypes in the pathogenic yeast *Cryptococcus neoformans.* **Microbiology,** v.147, p. 891-907, 2001.

BOROVIKOVA, L. V.; IVANOVA, S.; ZHANG, M.; YANG, H.; BOTCHKINA, G.I., WATKINS, L.R.; WANG, H.; ABUMRAD, N.; EATON, J.W.; TRACEY, K.J. Vagus nerve stimulation attenuates the systemic inflammatory response to endotoxin. **Nature**, v. 405, n. 6785, p. 458-462, 2000.

BOROWIEC, A.; LECHWARD, K.; TKACZ-STACHOWSKA, K.; SKLADANOWSKI, A.C. Adenosine as a metabolic regulator of tissue function: production of adenosine by cytoplasmic 5'-nucleotidases. **Acta Biochimica Polonica**, v. 53, p. 269-278, 2006.

BOURS, M.J.; SWENNEN, E.L.; DI VIRGILIO, F.; CRONSTEIN, B.N.; DAGNELIE, P.C. Adenosine 5'-triphosphate and adenosine as endogenous signaling molecules in immunity and inflammation. **Pharmacology & Therapeutics**, v.112, p.358-404, 2006.

BOVERS, M.; HAGEN, F.; KURAMAE, E.E.; BOEKHOUT, T. Six monophyletic lineages identified within Cryptococcus neoformans and Cryptococcus gattii by multi-locus sequence typing. **Fungal Genetics Biology,** 2008 v.45 p. 400-21, 2008.

BOVERS, M. R.; DIAZ, F.; HAGEN, L.; SPANJAARD, B.; DUIM, C. E.; VISSER,4 H. L.; HOOGVELD, J.; SCHARRINGA, I. M.; HOEPELMAN, J. W.; FELL, T.; BOEKHOUT T. Identification of Genotypically Diverse *Cryptococcus neoformans* and*Cryptococcus gattii* Isolates by Luminex xMAP Technology. **Journal of Clinical Microbiology**, v. 45 p. 1874–1883, 2007.

BOWLES, D. B.; FRY, D. R. Nasal cryptococcosis in two dogs in New Zealand. **New Zealand Veterinary Journal**, v. 57, p. 53-57, 2009.

BROWN, S.A. Basic anatomy, physiology and husbandry. In: Quesenberry KE, Carpenter JW, editors. Ferrets, rabbits, and rodents. **Clinical medicine and surgery**. 2nd ed. St Louis: Saunders; p. 2–12., 2004.

BURGESS, L.J.; MARITZ, F.J.; LE ROUX, I.; TALJAARD, J.J. Combined use of pleural adenosine deaminase with lymphocyte/neutrophil ratio. Increased specificity for the diagnosis of tuberculous pleuritis. **Chest**, v. 109, p. 414–419, 1996.

BRUNS, R.F. Adenosine receptors. Roles and pharmacology. **Annals of the New York Academy of Sciences**, v. 603, p. 211-225, 1990.

BURNSTOCK, G. Purine and pyrimidine receptors. **Cellular and Molecular Life Science,** v. 64, p. 1471-83, 2007.

BURNSTOCK, G. Purinergic signaling – an overview. **Novartis Found Symposium,** v.276, p.26–48, 2006

CASTRO, V.S.; PIMENTEL, V.C.; DA SILVA, A.S.; THOME , G.R.; WOLKMER, P.; CASTRO, J.L.; COSTA, M.M.; DA SILVA, C.B.; OLIVEIRA, D.C.; ALVES, S.H.; SCHETINGER, M.R.; LOPES, S.T.; MAZZANTI, C.M. Adenosine deaminase activity in serum and lymphocytes of rats infected with *Sporothrix schenckii*. **Mycopathologia** v.174, p. 31–39, 2012.

CASADEVALL, A.; PERFECT, J.R. ***Cryptococcus neoformans*.** ASM Press, Library of Congress, Washington, DC, 1998.

CHAYAKULKEEREE, M.; PERFECT, J.R. Cryptococcosis. **Infectious Disease Clinics of North America**, v. 20, p. 507-44, 2006.

CHAPMAN, H.M.; ROBINSON, W. F.; BOLTON, J.R.; ROBERTSON, J.P. *Cryptococcus neoformans* in goats. **Australian Veterinary Journal,** v. 67, p. 263-265, 1990.

CHATONNET, A.; LOCKRIDGE, O. Comparison of butyrylcholinesterase and acetylcholinesterase. **Biochemical Journal**, v. 260, n. 3, p. 625-634, 1989.

CHEN, G.H.; MCDONALD, R.A.; WELLS , J.C.; HUFFNAGLE, G.B.; LUKACS, N.W.; TOEWS, G.B. The gamma interferon receptor is required for the protective pulmonary inflammatory response to *Cryptococcus neoformans***. Infection Immunity**, v. 73, p. 1788-1796, 2005.

CODERO, O.; SALGADO, F.J.; FERNÁNDEZ-ALONSO, C.M.; HERRERA, C.; LLUIS, C.;FRANCO, R.; NOGUEIRA, M. Cytokines regulate membrane adenosine deaminase on human activated lymphocytes. **Journal of Leukocyte Biology,** v.70, p.920–930, 2001.

CORDOBA, S.; AFELTRA, J.; VITALE, R.G. Evaluation of the *in vitro* activity of amphotericin B by time-kill curve methodology against large and small capsulate *C. neoformans* isolates. **Diagnostic Microbiology and Infectious Disease**. v. 71, p. 260–262, 2011.

COX, G.M.; MUKHERJEE, J.; COLE, G.T.; CASADEVALL, A.; PERFECT, J.R. Urease as a virulence factor in experimental cryptococcosis, **Infection and Immunity**, v.68, p.443-448, 2000.

DAJAS-BAILADOR, F.; WONNACOTT, S. Nicotinic acetylcholine receptors and the regulation of neuronal signaling. **Trends in Pharmacological Sciences**, v. 25, n. 6, p. 317-324, 2004.

DAMBRÓS, B.P. Variabilidade genética de *Cryptococcus neoformans* isolado de pacientes HIV positivos atendidos no Hospital Nereu Ramos de Florianópolis, Santa Catarina. Dissertação (Mestrado em Biotecnologia) Universidade Federal de Santa Catarina, 2005.

DAS, U.N. Acetylcholinesterase and butyrylcholinesterase as possible markers of low-grade systemic inflammation. **Medical Science Monitor**, v. 13, n. 12, p. 214-221, 2007.

DEL POETA, M. Role of phagocytosis in the virulence of *Cryptococcus neoformans*. **Eukaryotic Cell,** v. 3, p. 1067-75, 2004.

DESCARRIES, L.; GISIGER, V.; STERIADE, M. Diffuse transmission by acetylcholine in the CNS. **Progress in Neurobiology**, v. 53, n. 5, p. 603-625, 1997.

DESROSIERS**,** M.D. ; CEMBROLA, K.M.; FAKIR, M.J.; STEPHENS, L.A.; JAMA, F.M.; SHAMELI, A.; MEHAL, W.Z.; SANTAMARIA, P.; SHI, Y. Adenosine deamination sustains dendritic cell activation in inflammation. **Journal of Immunology,** v.179, p.1884-1892, 2007.

EISENMAN, H. C.; MUES, M.; WEBER, S. E.; FRASES, S.; CHASKES, S.; GERFEN, G. & CASADEVALL, A. Cryptococcus neoformans laccase catalyses melain synthesis from both D- and L- Dopa. **Microbiology**, v. 53, p.3954 – 3962, 2007.

ELY, S.W.; BERNE, R.M. Protective effects of adenosine in myocardial ischemia. **Circulation**, v. 85, p. 893-904, 1992.

ESPINEL-INGROFF, A. Novel antifungal agents, targets or therapeutic strategies for the treatment of invasive fingal disesases: a review of the literature (2005-2009). **Revista Iberoamericana de Micologia**, v.26, p.15-22, 2009.

FAVALESSA, O.C.; RIBEIRO, L.C.; TADANO,T.; FONTES, C.J.F.; DIAS,F.B.; COELHO, B.P.A.; HAHN, R.C. First description of phenotypic profile and in vitro drug susceptibility of Cryptococcus spp yeast isolated from HIV-positive and HIV-negative patients in State of Mato Grosso. **Revista Sociedade Brasileira de Medicina Tropical**, v. 42(6), p.661-665, 2009.

FELDMESSER, M.; KRESS, Y.; CASADEVALL, A. Dynamic changes in the morphology of *Cryptococcus neoformans* during murine pulmonary infection. **Microbiology**,v. 147, p. 2355–2365, 2001.

FILIPPINI, A.; TAFFS, R. E.; SITKOVSKY, M. V. Extracellular ATP in T lymphocyte activation: possible role in effector functions. **Proceedings of the National Academy of Sciences,** v. 87, n. 21, p. 8267-8271, 1990.

FRANCO, R.; CASADÓ, V.; CIRUELA, F.; SAURA, C.; MALLOL, J.; CANELA, EI.; LLUIS, C. Cell surface adenosine deaminase: much more than na ectoenzyme. **Progress in Neurobiology**, v. 52, p. 283-294, 1997.

GANDELMAN, M.; PELUFFO, H.; BECKMAN, J.S.; CASSINA, P.; BARBEITO, L. Research Extracellular ATP and the P2X7 receptor in astrocyte-mediated motor neuron death: implications for amyotrophic lateral sclerosis. **Journal of Neuroinflammation**, v. 7, p. 9-33, 2010.

GAZZONI, A.F.; PEGAS, K.L.; SEVERO, L.C. Histopathological techniques for diagnosis cryptococosis deu to capsulate-deficient Cryptococcus: case report, **Revista Sociedade Brasileira de Medicina Tropical**, v 41, p. 76-78, 2008.

GESSI, S.; VARANI, K.; MERIGHI, S.; FOGLI, E.; SACCHETTO, V.; BENINI, A.; LEUNG, E.; MAC-LENNAN, S.; BOREA, P.A. Adenosine and lymphocyte regulation. **Purinergic Signalling**, v. 3, p. 109-16, 2007.

GIACOBINI, E. Cholinesterases: new roles in brain function and in Alzheimer's disease. **International Journal of Geriatric Psychiatry**, v. 18, (Suppl.1), p. S1–5, 2003.

GOLD, P. E. Acetylcholine modulation of neural systems involved in learning and memory. **Neurobiology of Learning and Memory**, v. 80, n. 3, p. 194 -210, 2003.

HASKO, G., CRONSTEIN, B.N. Adenosine: an endogenous regulator of innate immunity. **Trends Immunology,** v.25, p.33-39, 2004.

HEITMAN, J.; KOZEL, T.R.; KWON-CHUNG, K.J.; PERFECT, J.T.; CASADEVALL, A. ***Cryptococcus*** **from human pathogen to model yest** 2 nd. Washington: ASM Press, 2011, 620p.

HELKE, K.L.; DENVER, M.C.; BRONSON, E.; MANKOWSKI, J.L. Disseminate Cryptococcosis in a Guenon (*Cercopithecus ascanius*). **Veterinary Pathology,** v.43, p.75–78, 2006.

HERRING, A.C.; FALKOWSKI, N.R.; CHEN, G.H.; MCDONALD, R.A.; TOEWS, HUFFNAGLE, G.B. Transient neutralization of tumor necrosis factor alpha can produce a chronic fungal infection in an immunocompetent host: potential role of immature dendritic cells. **Infection and Immunity**, v. 73, p. 39–49, 2005.

HUFFNAGLE, G.B.; McNEIL, L.K. Dissemination of *C. neoformans* to the central nervous system: role of chemokines, Th1 immunity and leukocyte recruitment. **Journal For Neurovirology** . v. 5, p. 76–81, 1999.

HUSTON, S.M.; MODY, C.H. Cryptococcosis: an emerging respiratory mycosis. **Clinics in Chest Medicine**, v. 30, p. 253-264, 2009.

IDNURM, A. A. tetrad analysis of the basidiomycete fungus *Cryptococcus neoformans.* **Genetics**, v. 185, p.153–163, 2010.

IGREJA, R.P.; LAZÉRA MS, WANKE B, GALHARDO MC, KIDD SE, MEYER W 2004. Molecular epidemiology of *Cryptococcus neoformans* isolates from AIDS patients of the Brazilian city, Rio de Janeiro. **Medical Mycology** 42: 229-238.

JUNGER, W.G. Immune cell regulation by autocrine purinergic signalling. **Nature Reviews Immunology**, v. 11, p. 201-212, 2011.

KAWASHIMA, K.; FUJII, T. Extraneuronal cholinergic system in lymphocytes. **Pharmacology & Therapeutics**, v. 86, n. 1, p. 29-48, 2000.

KHAMBU, B.; MEHTA, K.D.; RIJAL, S.; LAMSAL, M.; MAJHI, S.; BARAL, N. Serum nitrite level and adenosine deaminase activity is altered in visceral Leishmaniasis. **Nepal Medical College journal,** v.9, p.40–43, 2007.

KOMMERS, G.D.; SOUZA, T.M.; SOUTO, M.A.M.; DE LA CORTE, F.D.; BARROS, C.S.L. Criptococose pulmonar granulomatosa em um eqüino. **Ciência Rural**, Santa Maria, v. 35, p. 938-940, 2005.

KON, A.S.; GRUMACH, A.S.; COLOMBO, A.L.; PENALVA, A.C.O.; WANKE, B.; TELLES, F.Q.; SEVERO, L.C. Consenso em criptocococse. **Revista da Sociedade Barsileira de Medicina Tropical**. V41, 524-544, 2008.

KROCKENBERGER, M.B.; CANDIELD, P.J.; MALIK, R. *Cryptococcus neoformans var. gattii* in the Koala (*Phascolaretos cinereus*): a review of 43 cases od cryptococcosis. **Medical Mycology,** v 41, p 225-234, 2003.

KUMAR, V.; SHARMA, A. Adenosine: an endogenous modulator of innate immune system with therapeutic potential. **The European Journal of Pharmacology**, v. 616, n. 1-3, p. 7-15, 2009.

KURTZMAN, C.P.; FELL, J.W. **The yeast: a taxonomic study**, 4 th ed. New York: ACM Press, 1998, 1055 p.

KUTTY, K. M. Review: Biological function of cholinesterase. **Annals of Clinical Biochemistry**, v. 13, n. 3, p. 239-243, 1980.

LEAL, D.B.R.; STREHER C.A.; NEU T.N.; BITENCOURT F.P.; LEAL C.A.M.; SILVA, J.E.P.; MORSCH V.M.; SCHETINGER M.R.C. Characterization of NTPDase (NTPDase1; ecto-apyrase; ecto-diphosphohydrolase; CD39; E.C. 3.6.1.5) activity in humans lymphocytes. **Biochimica et Biophysica Acta,** v. 1721, p. 9-11, 2005.

LIN, X & HEITMAN, J. The biology of the *Cryptococccus neoformans* species complex. **Annual Review of Microbiology**, v, 105, p. 60: 69, 2006.

LIN, X. *Cryptococcus neoformans*: Morphogenesis, infection, and evolution. Infection, **Genetics and Evolution**, v. 9, p. 401-416, 2009.

LINDELL, D.M.; MOORE, T.A.; MCDONALD, R.A.; TOEWS, G.B.; HUFFNAGLE, G.B. Generation of antifungal effector CD8+ T cells in the absence of CD4+ T cells during *Cryptococcus neoformans* infection. **The Journal of Immunology** v. 174, p. 7920–7928, 2005.

LUNKES, I.G.; LUNKES, D.; STEFANELLO, F.; MORCH, A.; MORCH, M.V.; MAZZANTTI, M.C.; SCHETINGER, M.C.R. Enzymes that hydrolyze adenine nucleotides in diabetes and associated pathologies. **Thrombosis Research,** v. 109, p. 189-194, 2003.

MA, H.; MAY, R.C. Virulence in Cryptococcus species. **Advances in Applied Microbiology**, v. 67, p. 131-90, 2009.

MALIK, R.; KROCKENBERG, M.; O'BRIEN, C.R.; MARTIN, P.; WIGNEY, D.; MEDLEAU, L. Cryptococosis. In: Greence, C. E. (Ed) **Infections diseases of the dog and cat**. 3rd ed. St. Louis: Saunders Elsevier, 2006, p. 584-598.

MARCASSO, R.A.; SIERRA, S.; ARIAS, M.V.B.; BRACARENSE, A.P.F.R.L.; YAMAMURA, A.A.M.; BIASI, F., LOPES, B.A.; AMUDE, A.M.; CORTÊZ D.E.A. Criptococose no sistema nervoso de cães - relato de três casos. **Ciências Agrárias**, v. 26, p. 229-238, 2005.

MASSOULIÉ, J.; PEZZEMENTI, L.; BON, S.; KREJCI, E.; VALLETTE, F.M. Molecular and cellular biology of cholinesterase. **Progress in Neurobiology**, v. 41, n. 1, p. 31-91, 1993.

MARTINS, D.B., ZANETTE, R.A.; FRANC¸S, R.T.; HOWES, F.; AZEVEDO, M.I.; BOTTON, S.A.; MAZZANTI, C.; LOPES, S.T.A.; SANTURIO, J.M. Massive cryptococcal disseminated infection in an immunocompetent cat. **Veterinary Dermatology.** V. 22, p. 232–234, 2010.

MATSUMOTO M.T.; FUSCO-ALMEIDA, A.M.; BAEZA, L.C.; MELHEM, M.S.; MEDES GIANNINI, M.J. Genotyping, serotyping and determination of mating-type of *Cryptococcus neoformans* clinical isolates from São Paulo State, Brazil. **Revista do Instituto de Medicina Tropical de São Paulo** v. 49: 41-47, 2007.

MCFADDEN, D.; ZARAGOZA, O.; CASADEVALL, A. The capsular dynamics of *Cryptococcus neoformans*. **Trends in Microbiology**, v. 14, p. 497-505, 2006.

MCQUISTON, T.J.; WILLIAMSON, P.R. Paradoxical roles of alveolar macrophages in the host response to Cryptococcus neoformans. **Journal of Infection and Chemotherapy,** v.18, p. 1-9, 2012.

MEYER, W.; CASTAÑEDA, A.; JACKSON, S.; HUYNH, M.; CASTAÑEDA, E. AND THE IBEROAMERICAN CRYPTOCOCCAL STUDY GROUP. Molecular typing of IberoAmerican *Cryptococcus neoformans* isolates. **Emerging Infectious Diseases**, v. 9, p. 189-195, 2003.

MELO, F.A.F.; AFIUNE, J.B.; SANTOS, M.L.; CASTRO FILHO, A. Diagnóstico da tuberculose pleural pela ADA, isolada ou combinada a outras variáveis, inclusive em HIV-positivos. **Folha Médica,** v.119, p.9-21, 2000.

MENDELOWITZ, D. Advances in parasympathetic control of heart rate and cardiac function. **News in Physiological Science**, v. 14, p. 155-161, 1999.

MITCHELL, T. G.; PERFECT, J.R. Cryptococcosis in the era of AIDS--100 years after the discovery of Cryptococcus neoformans. **Clinical Microbiology Review,** v. 8, p. 515-48, 1995.

MONARI, C.; BALDELLI, F.; PIETRELLA, D.; RETINI, C.; TASCINI, C.; FRANCISCI, D.; BISTONI, F.; VECCHIARELLI, A. Monocyte dysfunction in patients with acquired immunodefi ciency syndrome (AIDS) versus *Cryptococcus neoformans*. **Journal of Infection**, v. 35, p. 257–263, 1997.

MORETTI, M.L.; RESENDE, M.R.; LAZERA, M.S.; COLOMBO, A.L,; SHIKANAI-YASUDA, M.A. Guidlines in cryptococosis – 2008. **Revista da Sociedade Brasileira de Medicina tropical**, v. 41, p. 524-544, 2008.

MURPHY, J. W.; ZHOU, A.; WONG, S.C. Direct interactions of human natural killer cells with *Cryptococcus neoformans* inhibit granulocyte-macrophage colony-stimulating factor and tumor necrosis factor alpha production. **Infection and Immunity,** v. 65, p. 4564-4571, 1997.

NEGRONI, R. Cryptococosis. **Clinics in dermatology**, v. 30, p. 599–609, 2012.

NICHOLS, C.B.; PERFEC, Z.H.; ALSPAUGH, J.A. A *Ras1*-Cdc24 signal transduction pathway mediates thermotolerance in the fungal pathogen *Cryptococcus neoformans*. **Molecular Microbiology**, v. 63, p. 1118-1130, 2007.

O'BRIEN, C.R.; KROCKENBERGER, M.B.; WIGNEY, D.I.; MARTIN, P.; MALIK, R. Retrospective study of feline and canine cryptococcosis in Australia from 1981 to 2001: 195 cases. **Medical Mycology**, v. 42, p.449–460, 2004.

OCANA, I., RIBERA, E., MARTINEZ-VAZQUEZ, J.M., RUIZ, I., BEJARANO, E., PIGRAU, C., PAHISSA, A. Adenosine deaminase activity in rheumatoid pleural effusion. **Annals of the Rheumatic Diseases**, v. 47, p. 394–397, 1988.

OLSZEWSKI, M. A.; ZHANG, Y.; HUFFNAGLE, G.B. Mechanisms of cryptococcal virulence and persistence. **Future Microbiology**, v. 5, p. 1269-88, 2010.

O'MEARA, T.R.; ALSPAUGH, J.A. The Cryptococcus neoformans capsule; a sword and a shield. **Clinical Microbiology Reviews**, v. 25, p. 387-408, 2012.

PEDROSO, R.S.; CANDIDO, R.C. Diagnóstico laboratorial da criptococose. **NewsLab**, v. 77, p. 94-102, 2006.

PAVLOV, V.A.; TRACEY, K. J. The cholinergic anti-inflammatory pathway. **Brain, Behaviour, and Immunity**, v.19, n.6, p.493-499, 2005.

PEREIRA, A.P.C.; COUTINHO, S.D.A. Criptococose em cães e gatos – revisão. **Revista Clínica Veterinária**, v. 8, p. 24-32, 2003.

PERRY, E.; WALKER, M.; GRACE, J.; PERRY, R. Acetylcholine in mind: a neurotransmitter correlate of consciousness? **Trends in Neuroscience**, v. 22, n. 6, p. 273-280, 1999.

PERFECT, J. R. *Cryptococcus neoformans*: the yeast that likes it hot. **FEMS Yeast Research**, v. 6, p. 463-468, 2006.

PEFECT, J R. The impact of the host on fungal infections. **American Journal of Medicine, v.** 125, p. 39-51, 2009.

PERFECT, J.R.; CASADEVALL, A. Cryptococcosis. **Infectious Disease Clinics of North America,** v. 16, p. 837-874, 2002.

PERFECT, J.R.; DISMUKES, W.E.; DROMER, F.; GOLDMAN, D.L.; GRAYBILL, J.R; HAMILL, R.J, Clinical pratice guidelines for the management of cryptococcal disease: 2010 update by the Infectious Diseases Society of America. **Clinical Infectious Disease**, v. 50, p. 291-322, 2010.

PFALLER, M.A.; LODGE, J.K.; GHANNOUM, M.A. **Drug resistance in *Cryptococcus*: Epidemiology and molecularmechanisms.** In: Heitman J, Kozel TR, KWON-CHUNG, K.J.; PERFECT, J.R.; CASADEVALL A. *Cryptococcus : From Human Pathogen to Model Yeast.* 1st ed. Washington, DC: ASM Press, 2011:203–216.

PIETRELLA, D.; PERITO, S.; BISTONI, F.; VECCHIARELLI, A. Cytotoxic T lymphocyte antigen costimulation influences T-cell activation in response to *Cryptococcus neoformans*. **Infection and Immunity,** v. 69, p. 1508-1514, 2001.

PIETRELLA, D.; LUPO, P.; BISTONI, F.; VECCHIARELLI, A. An early imbalance of interleukin 12 influences the adjuvant effect of mannoproteins of *Cryptococcus neoformans*. **Cellular Microbiology ,** v. 6, p. 883-891, 2004.

PRADO, M.; DA SILVA, M.B.; LAURENTI, R.; TRAVESSOS, L.R.; TABOADA, C.P. Mortality due to systemic mycoses as a primary cause of death or in association with AIDS in Brazil: a review from 1996 to 2006. **Mémórias do Instituto Oswaldo Cruz**, 104:513-21, 2009.

RALEVIC, V.; BURNSTOCK, G. Receptores for purines and pyrimidines. **Pharmacological Reviews**, v. 50, p. 413-492, 1998.

RALEVIC, V.; BURNSTOCK, G. Involvement of purinergic signaling in cardiovascular diseases. **Drug News & Perspectives**, v. 16, p. 133-140, 2003.

RAMIREZ, M. J.; CENARRUZABEITIA, E.; LASHERAS, B.; DEL RIO, J. 5-HT2 receptor regulation of acetylcholine release induced by dopaminergic stimulation in rat striatal slices. **Brain Research**, v. 757, n. 1, p. 17-23, 1997.

RASO, T.F.'; WERTHER, K.; MIRANDA, E.T.; MENDES-GIANNINI, M.J.S. Cryptococcosis outbreak in psittacine birds in Brazil. **Medical Mycology,** v.42, p.355–362, 2004.

RATHBONE, M..P.; MIDDLEMISS, P.J.; GYSBERS, J.W.; ANDREW, C.; HERMAN, M.A.; REED, J.K.; CICCARELLI, R.; DI IORIO, P.; CACIAGLI, F.

Trophic effects of purines in neurons and glial cells. **Progress Neurobiology,** v.59, p.663–690, 1999.

RIET-CORREA, F.; KROCKENBERGER, M.; DANTAS, A.F.; OLIVEIRA, D.M. Bovine cryptococcal meningoencephalitis. **Journal of Veterinary Diagnostic Investigation**, v. 23, p.1056-1060, 2011.

RILEY, C. B.; BOLTON, J.R.; MILLS, J.N.; THOMAS, J.B. Cryptococcosis in seven horses. **Australian Vetetrinary Journal**, v.69, p.135-139, 1992.

ROBSON, S.C.; SÉVIGNY, J.; ZIMMERMANN, H. The NTPDase family of ectonucleotidases: structure function relationship and pathophysiological significance. **Purinergic Signalling**, v.2, p.409-430, 2006.

ROGERS, R. C.; HERMANN, G. E.; TRAVAGLI, R. A. Brainstem pathways responsible for oesophageal control of gastric motility and tone in the rat. **Journal of Physiology**, v. 514, n. 2, p. 369-383, 1999.

ROSA E SILVA, L. K.; STAATS, C. C.; GOULART, L. S.; MORELLO, L. G.; FUNGARO, M. H. P.; SCHRANK, A.; VAINSTEIN, M. H. Identification of novel temperature-regulated genes in the human pathogen *Cryptococcus neoformans* using representational difference analysis. **Reserch Microbiology**, v. 159, p. 221-229, 2008.

ROSAS-BALLINA, M.; TRACEY, K. J. Cholinergic control of inflammation. **Journal of Internal Medicine**, v. 265, p. 663–679, 2009.

SABLE, C.A.; STROHMAIER, K.M.; CHODAKEWITZ, J.A.; Advance in antifungal therapy. **Annual Review of Medicine,** v. 59, p.361-79, 2008.

SABIITI, W.; MAY, R.C. Mechanisms of infection by the human fungal pathogen Cryptococcus neoformans. **Future Microbiology**, p. 1297-1313, 2012.

SANTOS, C.X.; ANJOS, E.I.; AUGUSTO, O. Uric acid oxidation by peroxynitrite: multiple reactions, free radical formation, and amplification of lipid oxidation. **Archives of Biochemistry and Biophysics**, v. 372, p. 285-294, 1999.

SCREMIN, O.U.; LI, M.G.; SCREMIN, A.M.; JENDEN, D.J. Cholinesterase inhibition improves blood flow in the ischemic cerebral cortex. **Brain Research Bulletin**, v. 42, n. 1, p. 59-70, 1997.

SIDDIQUI, T.J.; ZAMANI, T.; PARADA, J.P. Primary cryptococcal prostatitis and correlation with serum prostate specific antigen in a renal transplant recipient. **Journal of Infection,** v. 51, p. e153-7, 2005.

SILVA, E.G.; BARONI FDE, A.; VIANI, F.C.; RUIZ LDA, S.; GANDRA, R.F.; AULER, M. E.; DIAS, A.L.; GAMBALE, W.; PAULA, C.R. Virulence profile of strains of *Cryptococcus neoformans* var. *grubii* evaluated by experimental infection in BALB/c mice and correlation with exoenzyme activity. **Journal of Medical Microbiology,** v. 55, p. 139-42, 2006.

SILVA, S.T.G.; SOUZA, J.C.A.; IZAEL, M.A.; RIET-CORREA, F.; PORTELA, R.; DANTAS, A.F., MENDONÇA, C.L.; AFONSO, J.A.B. Criptococose em ovino – relato de caso. **Ciência Animal Brasileira**, v.1, p. 707-713, 2009.

SILVER, A. **The Biology of Cholinesterases,** Elsevier Agricultural ResearchCouncil Institute, New York, 1974.

SITKOVSKY, M.V.; OHTA, A. The 'danger' sensors that STOP the immune response: the A2 adenosine receptors? **Trends Immunology,** v.26, p.299-304, 2005.

SRIDHAR, G.R.; NIRMALA, G.; APPARAO, A.; MADHAVI, A.S.; SREELATHA, S.; RANI, J.S.; VIJAYALAKSHMI, P. Serum butyrylcholinesterase in type 2 diabetes mellitus: a biochemical and bioinformatics approach **Lipids in Health and Disease**, v. 4, p. 18, 2005.

STEENBERGEN, J.N.; CASADEVALL, A. The origin and maintenance of virulence for the human pathogenic fungus Cryptococcus neoformans. **Microbes and Infection** v. 7, p. 667-75, 2003.

STEPHEN, C.S.; LESTER, W.; BLACK, M.; FYFE, A.; RAVERTY, S. Multispecies outbreak cryptococcosis on Southern Vancouver Island, British Columbia. **Canadian Journal of Veterinary Research**, v.43, p.792–794, 2002.

SOREQ, H.; SEIDMAN, S. Acetylcholinesterase – new roles for an old actor. **Nature Reviews in Neuroscience**, v. 2, p. 29302, 2001.

SOSLAU, G.; YOUNGPRAPAKORN, D. A possible dual physiological role of extracellular ATP in the modulation of platelet aggregation. **Biochimica et Biophysica Acta**, v. 1355, p. 131-140, 1997.

TABOADA, J.; GROOTERS, A.M. Systemic mycoses, In: ELTINGERS. S.J.: FELDMAN. E.C. (Ed). **Text book of veterinary internal medicine:** disease of the dog and cat, 6th ed. Philadelphia:WB Sauders, 2005.

TAYLOR, P.; BROWN, J. H. **Acetylcholine**. In: SIEGEL, G. J. et al (eds) **Basic neurochemistry: molecular, cellular, and medical aspects**. Lippincott-Raven Publishers, Philadelphia, p. 213-242, 1999.

TRILLES, L.; SANTOS, M.; WANKE, L. B.; OLIVEIRA, V. R.; BARBOSA, G. G.; NISHIKAWA, M. M. ; MORALES, B. P.; MEYER, W. Regional pattern of the molecular types of *Cryptococcus neoformans* and *Cryptococcus gattii* in Brazil. **Memórias Instituto Oswaldo Cruz**, v. 103, p. 455 - 462, 2008.

VECCHIARELLI, A. Fungal capsular polysaccharide and T-cell suppression: the hidden nature of poor immunogenicity. **Critical Reviews in Immunology**, v.27, p 547-557, 2007.

VITALE, R.G., PASCUCCELLI, V., AFELTRA J. Influence of capsule size on the in vitro activity of antifungal agents against clinical *Cryptococcus neoformans* var. *grubii* strains. **Journal of Medical Microbiology**, v. 61, p.384–388, 2012.

VIVIANI, M.A.; TORTORANO, A.M.. **Cryptococcosis**. In: Anaisse EJ, Mc Ginnis MR, Pfaller MA, editors. **Clinical Mycology**. 2nd ed. New York: Churchill Livingstone Elsevier; p. 231-49, 2009.

VOELTER, W.; ZECH, K.; ARNOLD, P.; LUDWIG, G. Determination of selected pyrimidines, purines and their metabolites in serum and urine by reversed-phase ionpair chromatography. **Journal of Chromatography ,** v. 199, p. 345-354, 1980.

WYRE, N. R., MICHELS, S.C., CHEN, S. Select Emerging Diseases in Ferrtes. **Veterinary Clinics of North America: Exotic Animal Practice,** v. 16, v. 469-493, 2013.

YEGUTKIN, G.G. Nucleotide and nucleoside converting ectoenzymes: important modulators of purinergic signalling cascade. **Biochimica et Biophysica Acta**, v.1783, p.673-694, 2008.

YOSHINO, Y.; KITAZAWA, T.; TATSUNO, K.; OTA, Y.; KOIKE, K. Cryptococcal Pleuritis Containing a High Level of Adenosine Deaminase in a Patient with AIDS: A Case Report. **Respiration**, v.79**,** 153–156, 2010.

ZARAGOZA, O.; RODRIGUES, M.L.; DE JESUS, M.; FRASES, S.; DADACHOVA, E.; CASADEVALL, A. The capsule of the fungal pathogen Cryptococcus neoformans, **Advances in Applied Microbiology**, v 68, p. 133-216, 2009.

ZHOU, Q., MURPHY, J. Immune response and immunotherapy to Cryptococcus infections. **Immunology Research**, v. 35, p. 191-208, 2006.

ZIMMERMANN, H. Extracellular metabolism of ATP and other nucleotides. **Naunyn-Schmiedeberg's Archives of Pharmacology**, v. 362, p. 299-309, 2000.

ZIMMERMANN, H. Ectonucleotidases: some recent developments and note on nomenclature. **Drug Developmental Research**, v. 52, p. 44-56, 2001.

ZOPPA, A.L.V.; CRISPIM, R.; SINHORINI, I.L.; BENITES, N.R.; SILVA, L.C.L.C.; ACCARIN, R.Y.A. Obstrução nasal por granuloma fúngico em eqüino: relato de caso. **Arquivo Brasileiro Medicina Veteterinária e Zootecnia**, v. 60, p. 315-321, 2008.

8. ANEXOS

Anexo A – Licença Elsevier para reprodução do Artigo 1

ELSEVIER LICENSE
TERMS AND CONDITIONS

Feb 22, 2016

This is a License Agreement between Maria Isabel Azevedo ("You") and Elsevier ("Elsevier") provided by Copyright Clearance Center ("CCC"). The license consists of your order details, the terms and conditions provided by Elsevier, and the payment terms and conditions.

All payments must be made in full to CCC. For payment instructions, please see information listed at the bottom of this form.

Supplier	Elsevier Limited The Boulevard,Langford Lane Kidlington,Oxford,OX5 1GB,UK
Registered Company Number	1982084
Customer name	Maria Isabel Azevedo
Customer address	Avenida Roraima, 1000, prédio 20 Santa Maria, 97105140
License number	3814230258859
License date	Feb 22, 2016
Licensed content publisher	Elsevier
Licensed content publication	Veterinary Microbiology
Licensed content title	E-NTPDase and E-ADA activities in rats experimental infected by Cryptococcus neoformans
Licensed content author	Maria Isabel de Azevedo,Laerte Ferreiro,Aleksandro S. Da Silva,Alexandre A. Tonin,Jader B. Ruchel,João F.P. Rezer,Raqueli T. França,Carine E.P. Zimmermann,Daniela B.R. Leal,Marta M.M.F. Duarte,Sonia T.A. Lopes,Mariana M. Flores,Rafael Fighera et al.
Licensed content date	7 November 2014
Licensed content volume number	174
Licensed content issue number	1-2
Number of pages	8
Start Page	206
End Page	213
Type of Use	reuse in a thesis/dissertation
Portion	full article
Format	both print and electronic
Are you the author of this Elsevier article?	Yes
Will you be translating?	No
Title of your thesis/dissertation	INFECÇÃO EXPERIMENTAL POR Cryptoccocus neoformans: INFLUÊNCIA DA DOENÇA SOBRE OS SISTEMAS PURINÉRGICO E COLINÉRGICO
Expected completion date	Feb 2016

Anexo B – Licença Elsevier para reprodução do Artigo 2

ELSEVIER LICENSE
TERMS AND CONDITIONS

Feb 22, 2016

This is a License Agreement between Maria Isabel Azevedo ("You") and Elsevier ("Elsevier") provided by Copyright Clearance Center ("CCC"). The license consists of your order details, the terms and conditions provided by Elsevier, and the payment terms and conditions.

All payments must be made in full to CCC. For payment instructions, please see information listed at the bottom of this form.

Supplier	Elsevier Limited The Boulevard,Langford Lane Kidlington,Oxford,OX5 1GB,UK
Registered Company Number	1982084
Customer name	Maria Isabel Azevedo
Customer address	Avenida Roraima, 1000, prédio 20 Santa Maria, 97105140
License number	3814241057920
License date	Feb 22, 2016
Licensed content publisher	Elsevier
Licensed content publication	Pathology - Research and Practice
Licensed content title	Cholinesterase of rats experimentally infected by Cryptococcus neoformans: Relationship between inflammatory response and pathological findings
Licensed content author	Maria Isabel de Azevedo,Laerte Ferreiro,Aleksandro S. Da Silva,Alexandre A. Tonin,Maria Luiza Thorstenberg,Livia Gelain Catilhos,Raqueli T. França,Daniela B.R. Leal,Marta M.M.F. Duarte,Sonia T.A. Lopes,Manuela B. Sangoi,Rafael N. Moresco et al.
Licensed content date	November 2015
Licensed content volume number	211
Licensed content issue number	11
Number of pages	7
Start Page	851
End Page	857
Type of Use	reuse in a thesis/dissertation
Portion	full article
Format	both print and electronic
Are you the author of this Elsevier article?	Yes
Will you be translating?	No
Title of your thesis/dissertation	INFECÇÃO EXPERIMENTAL POR Cryptoccocus neoformans: INFLUÊNCIA DA DOENÇA SOBRE OS SISTEMAS PURINÉRGICO E COLINÉRGICO

Anexo C – Carta de aprovação da Comissão de ética no uso de animais

UNIVERSIDADE FEDERAL DE SANTA MARIA
PRÓ-REITORIA DE PÓS-GRADUAÇÃO E PESQUISA
COMISSÃO DE ÉTICA NO USO DE ANIMAIS-UFSM

CARTA DE APROVAÇÃO

A Comissão de Ética no Uso de Animais-UFSM, analisou o protocolo de pesquisa:

Título do Projeto: "Avaliação dos colinesterases NTPDase e adenosina desaminase como marcadores inflamatório em ratos experimentalmente infectados por Cryptococcus neofarmans"

Numero do Parecer: 073/2012 (2)

Pesquisador Responsável: Jânio Moraes Santurio

Este projeto foi **APROVADO** em seus aspectos éticos e metodológicos. Toda e qualquer alteração do Projeto, assim como os eventos adversos graves, deverão ser comunicados imediatamente a este Comitê.

Os membros da CEUA-UFSM não participaram do processo de avaliação dos projetos onde constam como pesquisadores.

DATA DA REUNIÃO DE APROVAÇÃO:

Santa Maria, 08 de outubro de 2012

André Vasconcelos Soares
Vice- Coordenador da Comissão de Ética no Uso de Animais-UFSM