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Pedro Henrique Doleski

SINALIZAÇÃO PURINÉRGICA EM TECIDO HEPÁTICO E ESPLÊNICO DE CAMUNDONGOS INFECTADOS COM *Toxoplasma gondii* E TRATADOS COM DISSELENETO DE DIFENILA

Santa Maria, RS
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Dissertação apresentada ao Curso de Pós-Graduação Em Ciências Biológicas (Bioquímica Toxicológica), da universidade Federal de Santa Maria (UFSM, RS), como requisito parcial para obtenção do título de **Mestre em Ciências Biológicas (Bioquímica Toxicológica)**

Orientador: Aleksandro Schafer Da Silva

Co-Orientador: Daniela Bitencourt Rosa Leal

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RESUMO

AVALIAÇÃO DA SINALIZAÇÃO PURINÉRGICA EM TECIDO HEPÁTICO E ESPLÊNICO DE CAMUNDONGOS INFECTADOS COM *Toxoplasma gondii* E TRATADOS COM DISSELENETO DE DIFENILA

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A toxoplasmose é uma zoonose de distribuição mundial causada pelo parasito *Toxoplasma gondii*, o qual em geral desenvolve infecção de característica crônica e assintomática em mamíferos e aves. Neste período, o parasito se encontra encistado no tecido cerebral, entretanto, é possível que o processo inflamatório desenvolvido para combater o parasito possa ocasionar dano extracerebral. Entre os possíveis órgãos atingidos pela infecção estão o fígado e o baço. Nucleotídeos e nucleosídeos purínicos e sua regulação por enzimas conhecidas como nucleotidases e nucleosidases estão envolvidas em inúmeros processos fisiológicos através da sinalização purinérgica. Portanto, o objetivo deste trabalho é avaliar o efeito do tratamento subcutâneo com disseleneto de difenila $(PhSe)_2$, um composto com efeitos antioxidantes e imunomodulatórios, sobre a concentração de purinas e atividade de enzimas purinérgicas no tecido hepático e esplênico de camundongos infectados por *T. gondii* (cepa ME-49). Para o experimento, 40 camundongos Swiss foram divididos em quatro grupos: Grupo A (não infectado), Grupo B (não infectado e tratado com $(PhSe)_2$), Grupo C (infectado) e Grupo D (infectado e tratado com $(PhSe)_2$). A infecção (Grupo C e D) foi realizada pela inoculação de 50 cistos de *T. gondii* de cepa ME-49. Os animais do Grupo B e Grupo D receberam 5 μmol kg^{-1} de $(PhSe)_2$ por via subcutânea no 1º e 20º dia após infecção. A eutanásia dos animais foi realizada 30 dias após a infecção e as amostras foram devidamente coletadas. Os resultados histopatológicos revelaram processo inflamatório no tecido hepático e esplênico em camundongos infectados por *T. gondii*, como também hepato-esplenomegalia e infiltrados inflamatórios. A infecção alterou a atividade das enzimas purinérgicas no fígado, as quais estão envolvidas no controle fisiológico de purinas. A elevada atividade da NTPDase pode estar relacionada com a redução dos altos níveis de ATP encontrados no fígado, enquanto que, a elevada atividade da adenosina desaminase (ADA), pode ser responsável pelos baixos níveis de adenosina encontrados no tecido. De mesma maneira, a elevada atividade da xantina oxidase (XO), pode estar relacionada com a degradação dos altos níveis de xantina, e assim elevando os níveis de ácido úrico observados pela análise por HPLC. Nos animais infectados e não tratados foram encontradas no baço as mesmas alterações enzimáticas que no fígado. Por outro lado, no soro de animais infectados, não foram encontradas alterações na enzima NTPDase, mas, aumento a atividade das enzimas 5´nucleotidase e ADA, as quais podem levar a uma redução dos níveis de adenosina na corrente sanguínea. No entanto, o $(PhSe)_2$ foi eficaz em reduzir os danos induzidos pela infecção, observados pela análise histológica como

infiltrados inflamatórios leves. O $(PhSe)_2$ também foi capaz de alterar as enzimas purinérgicas, elevando ainda mais a atividade da NTPDase nos animais infectados e tratados e diminuindo a atividade da ADA e XO teciduais. Possivelmente, estas regulações estão envolvidas na redução dos níveis de ATP e aumento de adenosina e xantina, fato o qual foi confirmado pela análise de purinas por HPLC no tecido hepático. Os efeitos antioxidantes do $(PhSe)_2$ foram observados no tecido esplênico, onde baixos níveis de espécies reativas de oxigênio (ROS) foram observados nos animais infectados e tratados quando comparado com os animais infectados não tratados. Interessantemente, o $(PhSe)_2$ não foi capaz de alterar o perfil de degradação encontrado em animais infectados e não tratados no soro, fato que demonstra sua ação ineficaz na corrente sanguínea. Devido à infecção crônica por *T. gondii* ser capaz de induzir processo inflamatório e alterações fisiológicas, e o tratamento com $(PhSe)_2$ ser capaz de reverter tais processos, sugere-se que o uso de agentes imunomodulatórios possam ser benéficos contra o dano esplênico e hepático observados em animais infectados cronicamente por *T. gondii*.

Palavras-chave: Toxoplasmose, sistema purinérgico, disseleneto de difenila

ABSTRACT

PURINERGIC SIGNALING IN HEPATHIC AND SPLENIC TISSUE OF MICE INFECTED WITH *Toxoplasma gondii* AND TREATED WITH DIPHENYL DISELENIDE

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Toxoplasmosis is a zoonosis of worldwide distribution caused by the parasite *Toxoplasma gondii*, which in general develops asymptomatic and chronic characteristic infection in mammals and birds. In this period, the parasite is encysted in the brain tissue, however, it is possible that the inflammatory process developed to combat the parasite may cause extracerebral damage. Among the possible organs affected by the infection are the liver and the spleen. Purine nucleotides and nucleosides and their regulation by enzymes known as nucleotidases and nucleosidases are involved in numerable physiological processes through purinergic signaling. Therefore, the objective of this work is to evaluate the effect of subcutaneous treatment with diphenyl diselenide $(PhSe)_2$, a compound with antioxidant and immunomodulatory effects, on the purine concentration and activity of purinergic enzymes in hepatic and splenic tissue of mice infected by *T. gondii* (strain ME-49). For the experiment, 40 Swiss mice were divided into four groups: Group A (uninfected), Group B (uninfected and treated with $(PhSe)_2$), Group C (infected) and Group D (infected and treated with $(PhSe)_2$). The infection (Group C and D) was performed by the inoculation of 50 cysts of *T. gondii* strain ME-49. The animals of Group B and Group D received 5 μmol kg^{-1} of $(PhSe)_2$ subcutaneously on the 1^{st} and 20^{th} day after infection. After 30 days of infection, the mice were euthanized and the samples were collected. Histopathological results revealed an inflammatory process in the hepatic and splenic tissues in mice infected with *T. gondii*, that is, splenomegaly and inflammatory infiltrates. The infection altered the activity of the purinergic enzymes in the liver, which are involved in the physiological control of purines. The high activity of NTPDase may be related to the reduction of high levels of ATP found in the liver, whereas the high adenosine deaminase (ADA) activity may be responsible for the low levels of adenosine found in the tissue. Likewise, the high activity of xanthine oxidase (XO) may be related to the degradation of high xanthine levels, thus raising uric acid levels observed by HPLC analysis. In the infected and untreated animals the same enzymatic changes were found in the spleen as in the liver. On the other hand, in the serum of infected animals, no alterations were found in the NTPDase enzyme, but, the activity of the 5'nucleotidase and ADA enzymes increased, which may lead to a reduction of the levels of adenosine in the bloodstream. However, $(PhSe)_2$ was effective in reducing the damage induced by the infection, observed by histological analysis as mild inflammatory infiltrates. It was also able to alter purinergic enzymes, further elevating NTPDase activity in infected and treated animals and decreasing ADA and XO tissue

activity. Possibly, these regulations are involved in the reduction of ATP levels and increase of adenosine and xanthine, a fact that was confirmed by HPLC purine analysis in the hepatic tissue. The antioxidant effects of $(PhSe)_2$ were observed in splenic tissue, where low levels of reactive oxygen species (ROS) were observed in infected and treated animals compared to untreated infected animals. Interestingly, $(PhSe)_2$ was not able to alter the degradation profile found in infected and untreated animals in the serum, which demonstrates its ineffective action on the bloodstream. *T. gondii* chronic infection is able to induce inflammatory process and physiological changes, and $(PhSe)_2$ treatment is able to reverse such processes, it is suggested that the use of immunomodulatory agents may be beneficial against splenic injury and In animals chronically infected by *T. gondii*.

Key-Words: Toxoplasmosis, Purinergic system, Diphenyl diselenide.

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LISTA DE ABREVIAÇÕES

5´n	**5´nucleotidase**
$(PhSe)_2$	**Disseleneto de difenila**
-SH	**Grupamento tiol**
ADA	**Adenosina desaminase**
ADO	**Adenosina**
ADP	**Adenosina-difosfato**
AMP	**Adenosina-monofosfato**
AMPc	**Adenosina-monofosfato cíclico**
ATP	**Adenosina-trifosfato**
AU	**Ácido Úrico**
DAMP	**Padrão molecular associado ao dano**
H_2O_2	**Peróxido de hidrogênio**
GSH	**Glutationa reduzida**
I-CAM	**Molécula de adesão intercelular**
IL-2	**Interleucina 1**
IL-12	**Interleucina 12**
INF-γ	**Interferon gama**
L. amazonensis	***Leishmania amazonensis***
ME-49	**Cepa avirulenta de *Toxoplasma gondii***
NTPDase	**Nucleosideo tri-difosfohidrolase**
$O_2^{\cdot -}$	**Aníon superóxido**
OH^-	**Hidroxila**

P1	**Purinoreceptor tipo 1**
P2	**Purinoreceptor tipo 2**
PNP	**Purino nucleosídeo fosforilase**
RH	**Cepa virulenta de *Toxoplasma gondii***
T. gondii	***Toxoplasma gondii***
TNF-α	**Fator de necrose tumoral alfa**
XO	**Xantina oxidase**

ANEXOS

ANEXO 1– Carta de aprovação pelo Comitê interno de Ética em Experimentação animal – UFSM

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1 INTRODUÇÃO

O *Toxoplasma gondii* é um protozoário pertencente ao filo Apicomplexa, classe Sporozoa e família Sarcocystidae, agente etiológico da doença toxoplasmose (DUBEY, 2007, p. 1). A toxoplasmose é uma zoonose, onde o parasito pode infectar inúmeros mamíferos e aves, além de humanos (REY, 2008, p. 192). Em felídeos, os hospedeiros definitivos do parasito, infectam a parede do intestino delgado e se reproduzem sexuadamente (DUBEY, 2009, p. 100). Já nos hospedeiros intermediários, outros mamíferos e aves, quando infectados por cepas de baixa virulência do parasito *T. gondii*, como a cepa ME49, apresentam inicialmente um quadro de infecção aguda onde o parasita se reproduz assexuadamente nos órgãos dos hospedeiros (DUBEY et al., 1998, p. 267). Entretanto, de 10 a 15 após a infecção pelo parasito, a resposta apresenta característica crônica, já que o parasita entra em sua fase de latência, onde se protege da resposta imunológica encistando-se no tecido cerebral (FERGUNSON; HUTCHINSON, 1987, p. 483). Uma vez neste tecido, os cistos são resistentes a resposta imune do hospedeiro como também a quimioterápicos (DUBEY, 2007, p. 1).

Esta infecção crônica apresenta alta frequência na população humana, onde aproximadamente 1/3 da população mundial, e 1/2 da população brasileira apresentam sorologia positiva para a infecção por *T. gondii* (DUBEY, 2007, p. 1; REY, 2008, p. 192). A toxoplasmose crônica é considerada assintomática, e pode durar toda a vida do hospedeiro (FERGUNSON; HUTCHINSON, 1987, p. 483). Entretanto, estudos revelam que a infecção crônica por *T. gondii* é capaz de induzir alterações comportamentais e doenças neuronais em roedores e humanos (FOND et al., 2013, p. 38; PRANDOTA, 2014, p. 205). Além disso, sendo também capaz de induzir alterações imunológicas sistêmicas em pacientes com toxoplasmose crônica. Entre as alterações sistêmicas estão o aumento da resposta inflamatória (ISKANDAR et al., 2016, p. 1), redução nos níveis de testosterona (ESLAMIRAD et al., 2013, p. 622) e predisposição a doenças hepáticas (EL-REHIM et al., 2013, p. 784) em pacientes soropositivos para *T. gondii*.

Entre os órgãos que apresentam alterações imunológicas ou fisiológicas estão o fígado e o baço (NAN et al., 2011, p. 109). O baço é responsável pela

remoção de eritrócitos da corrente sanguínea, como também é considerado um linfonodo periférico (KRAAL; DEN HAAN, 2016, p. 407). Já o fígado é um órgão vital, isto devido a sua função metabólica, o qual metaboliza os nutrientes provindos da absorção gastrointestinal como também inúmeros compostos endógenos como a bilirrubina e o colesterol (CAMPBELL, 2006, p. 102). A fim de desenvolver a resposta imunológica, o tecido esplênico e hepático apresentam células imunes como, por exemplo, linfócitos (RACANELLI; REHERMANN, 2006, p. 54). Mas, além de células imunológicas, o organismo apresenta inúmeros mediadores químicos para realizar a sinalização da resposta imune no organismo. Entre estes mediadores, estão os nucleotídeos e nucleosídeo de adenina, os quais são regulados por enzimas purinérgicas (ALAM; GORSKA, 2003, p. 476).

Sistema Purinérgico é caracterizado pela interação de nucleotídeos ou nucleosídeos purínicos com receptores celulares (BURNSTOCK, 2007, p. 1471). Este sistema está envolvido em inúmeras funções do organismo como a resposta neurológica, a vascular e imunológica (JUNGER, 2011, p. 201). A fim de controlar a concentração destes sinalizadores moleculares, o organismo expressa enzima purinérgicas, as quais metabolizam nucleotídeos e nucleosídeos purínicos (ZIMMERMANN, 2000, p. 299). A E-NTPDase é considerada um biomarcador da ativação de leucócitos, assim como, um regulador das concentrações extracelulares de ATP, uma vez que desempenha hidrólise deste nucleotídeo (DWYER et al., 2007, p. 171; YEGUTKIN, 2008, p. 1328). Já a enzima 5´-nucleotidase (CD73, E.C. 3.1.3.5) é capaz de hidrolisar monofosfonucleotídeos como o AMP. Enquanto que, a enzima ADA (CD26, E.C. 3.5.4.4) é responsável pela desaminação de adenosina formando o produto inosina (ZIMMERMANN, 2001, p. 44). A enzima XO é capaz de metabolizar os nucleosídeos hipoxantina e xantina produzindo ácido úrico e espécies reativas de oxigênio. Alterações na atividade destas enzimas são encontradas em inúmeras patologias (SCHETINGER et al., 2007, p. 77), entre elas infecções parasitárias(DOLESKI et al., 2016, p. 551; FAVERO et al., 2016, p. 48).

O disseleneto de difenila $(PhSe)_2$ é um organocalcogênio que contém selênio em sua composição, e apresenta inúmeras atividades biológicas (NOGUEIRA et al., 2004, p. 6255). Este composto orgânico de selênio apresenta atividade imunomodulatória, com predominância anti-inflamatória (LEITE et al., 2015, p. 119, NOGUEIRA et al., 2004, p. 6255). Além disso, apresenta ação hepato-protetora,

sendo capaz de proteger o tecido hepático de danos causados por agentes hepatotóxicos ou patologias (WILHELM et al., 2009b, p. 31).

Alterações sistêmicas, como também, no baço e fígado são relatadas em hospedeiros infectados cronicamente pelo parasito *T. gondii* (EL-REHIM et al., 2013, p. 784; NAN et al., 2011, p. 109). Considerando que a toxoplasmose crônica possa induzir uma resposta pró-inflamatória nestes tecidos, propomos o uso do agente imunomodulador e antioxidante $(PhSe)_2$. Desta maneira, o presente estudo teve como objetivo avaliar possíveis alterações no sistema purinérgico em tecido esplênico ou hepático de camundongos infectados com *T. gondii* cepa ME49 e tratados com $(PhSe)_2$.

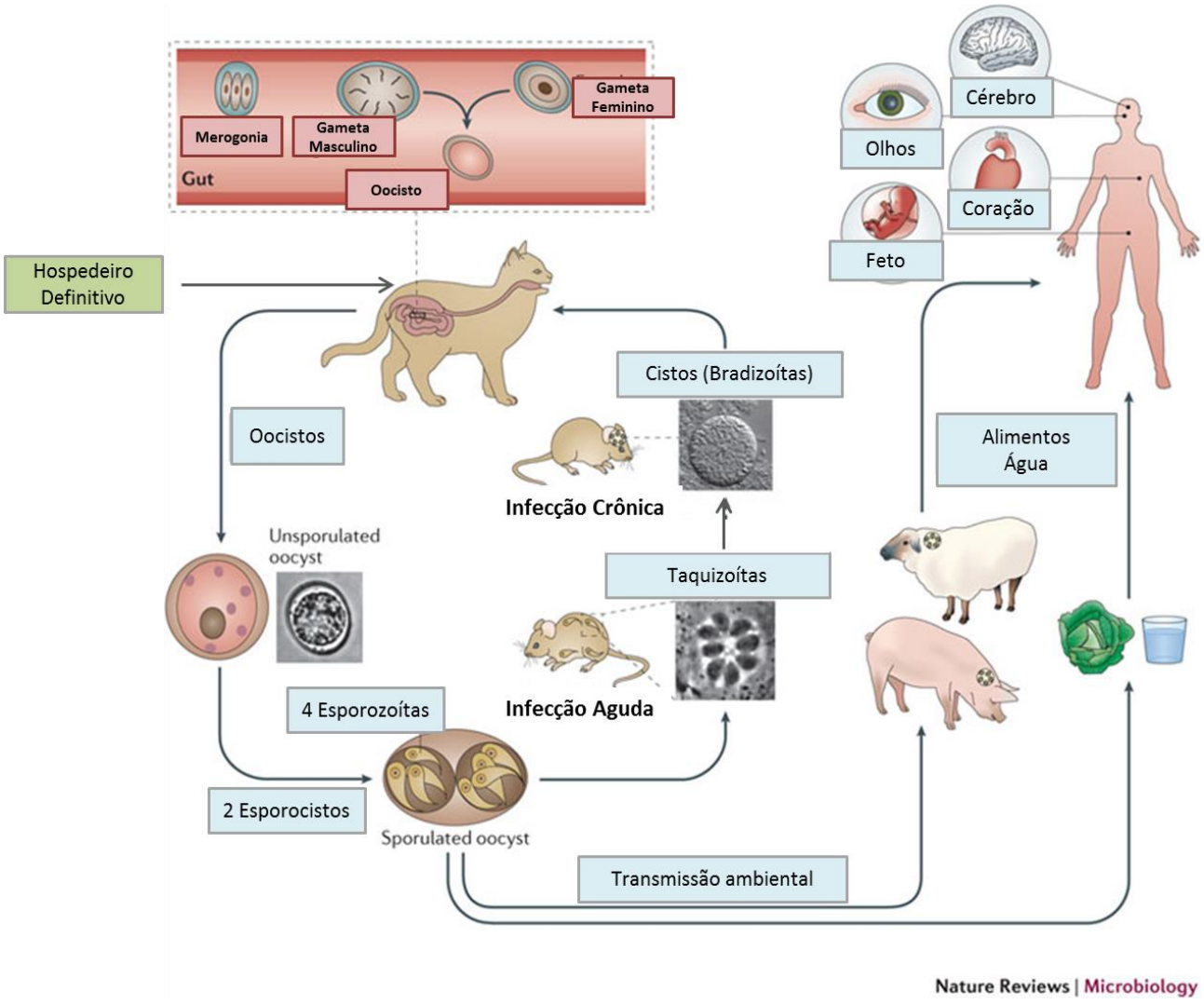
2 REVISÃO BIBLIOGRÁFICA

Toxoplasma gondii é um protozoário pertencente ao filo Apicomplexa, classe Sporozoa e família Sarcocystidae. Portanto, é considerado um esporozoário, e apresenta morfologia semelhante a coccidios e plasmódios, já que ambos possuem complexo apical, fato que lhes permitem invadir as células de seus hospedeiros (DUBEY, 2007, p. 1). O parasito é unicelular e apresenta forma oval ou alongada, alongando-se em forma de arco, fato que dá origem a seu nome genérico, do grego *toxon*, arco e *plasma*, molde. É um parasito intracelular, já que para sua sobrevivência no organismo hospedeiro, necessita penetrar nas células do mesmo. Penetram nas células do hospedeiro por processo de endocitose, onde a membrana da célula infectada não se rompe, mas sim forma-se um vacúolo onde o parasito se mantém realizando suas funções metabólicas com a célula infectada (REY, 2008, p. 192). O parasito é capaz de infectar inúmeros mamíferos e aves, sendo o agente etiológico único da toxoplasmose. Apresentam três formas infectantes distintas, sendo elas, (1) Taquizoítos: forma encontrada na infecção de fase aguda, onde o parasito apresenta alta reprodução e metabolismo energético acelerado, (2) Bradizoíto: é considerada a forma de resistência do parasito, sendo encontrados na fase crônica da infecção, e (3) Oocisto: produto da reprodução sexuada do parasito em felídeos, o qual se encontra nas fezes dos gatos e apresentam alta resistência físico-química no ambiente (DUBEY et a., 1998, p. 267).

O ciclo biológico do *T. gondii* inicia com a infecção de felídeos, os hospedeiros definitivos do parasito (Figura 1). Felídeos adquirem a infecção através da ingestão de órgãos ou carne contaminada por taquizoítos ou bradizoítos, provinda geralmente de pequenos roedores infectados ou pássaros, os quais se apresentam no ciclo biológico do *T. gondii* como hospedeiros intermediários (REY, 2008, p. 192). No intestino delgado os parasitos infectam células epiteliais, e nestas se multiplicam por merogonia, resultando na formação dos microgametas (gameta masculino) e macrogametas (gameta feminino), e consecutiva reprodução sexuada no epitélio intestinal. De acordo com a literatura, ocorrendo o encontro dos gametas, haverá a formação de um zigoto o qual evoluirá até a forma de um oocisto. Uma vez formado, o oocisto é liberado para a luz intestinal, e em conjunto com as fezes do animal é excretado para o ambiente (DUBEY et al., 1998, p. 267). No ambiente, o

oocisto necessita oxigênio para o seu amadurecimento, o qual ocorre pela formação de dois esporocitos, os quais formarão quatro esporozoítos cada. Ao apresentar esporozoítos, um oocisto é considerado maduro, e assim é infectante. Uma vez contaminando o ambiente, oocistos apresentam alta resistência físico-química em condições de temperatura e umidade adequadas (DUBEY, 2009, p. 100; REY, 2008, p. 192).

Figura 1 – Ciclo biológico do parasita *Toxoplasma gondii*.



Fonte: Adaptação de Hunter e Sibley (2012).

Além dos pequenos roedores e aves, presas comuns de felídeos, numerosos são os hospedeiros intermediários do *T. gondii*. Isto se deve a baixa especificidade do parasito por infectar células nucleadas, sendo capaz de infectar grande parte das

células do organismo de mamíferos e aves (HILL et al., 2005, p. 41; REY, 2008, p. 192; VIDOTTO, 1992, p. 69). A infecção dos hospedeiros intermediários ocorre pela ingestão de oocistos provenientes do ambiente contaminado, ou pelas outras formas do parasito através da ingestão de carne contaminada. Nestes, os parasitos atravessam o epitélio intestinal e infectam células sanguíneas e teciduais, e posteriormente se reproduzem assexuadamente por endodiogenia. Este tipo de reprodução ocorre uma vez estabelecido o vacúolo parasitóforo na célula infectada, onde um taquizoíto divide seu núcleo em dois e produz dois novos taquizoítos. Estes se disseminam no organismo do hospedeiro através da corrente sanguínea e linfática, podendo atingir vários órgãos (DUBEY, 2009, p. 100). Este período onde o parasito se reproduz rapidamente pelo organismo, é denominado fase proliferativa. A partir deste período o desenvolvimento e continuidade da infecção dependerá de certos fatores, sendo eles, a espécie infectada, o estado imunológico do hospedeiro e a cepa infecciosa. Infecções por cepas virulentas de *T. gondii* apresentam alta patogenicidade, isto porque desenvolvem um grave quadro de toxoplasmose aguda, onde o parasito prolifera-se rapidamente e invadem órgãos vitais como o cérebro, fígado, pulmões e coração (REY, 2008, p. 192). Caso o tratamento não seja feito adequadamente, o dano tecidual e a rápida proliferação parasitária podem levar o hospedeiro à morte em poucos dias. Entretanto, é importante ressaltar que a maior incidência de infecção por *T. gondii* ocorre por cepas de baixa virulência, como a cepa ME-49 (VIDOTTO, 1992, p. 69).

Hospedeiros intermediários infectados por cepas de *T. gondii* de baixa virulência apresentam inicialmente uma fase proliferativa, a qual é inibida rapidamente através da resposta imunológica do hospedeiro (DUBEY, 2007, p. 1). Em resposta a infecção, o sistema imunológico induz uma forte resposta pró-inflamatória contra o parasito. Esta resposta é desenvolvida principalmente pela ação das citocinas INF-γ e IL-12, as quais se encontram elevadas em animais infectados e são capazes de ativar células imunes do organismo, induzindo-as a um perfil pró-inflamatório sistêmico (HILL et al., 2005, p. 41; INNES, 1997, p. 131). Com o aumento da resposta imunológica do hospedeiro, a infecção pode seguir dois caminhos distintos; (1) eliminação completa do parasito no organismo, ou (2) os taquizoítos diminuem suas funções metabólicas e reproduzem-se muito lentamente, formando aglomerados parasitários no tecido cerebral, conhecidos como cistos

parasitários. Este estágio é denominado de infecção latente, onde os parasitos aglomerados são denominados bradizoítos e produzem um envoltório cístico que os protegem da resposta imunológica e também contra a ação de medicamentos (DUBEY et al.,1998, p. 267; SUZUKI et al., 2013, p. 755). A infecção latente pode durar por toda a vida do hospedeiro, entretanto, em quadros de imunodepressão, a diminuição na resposta inflamatória pode levar a reativação dos parasitas no tecido cerebral.

Infecções por cepas de *T. gondii* de baixa virulência podem levar a pequenos quadros de adenopatia, febre ou aumento hepato-esplênico em sua fase inicial (DUBEY, 2009, p. 100). Já a infecção latente apresenta característica assintomática, e desta maneira, a comunidade médica considera este período de latência infecciosa de pouca importância clínica em humanos (SUZUKI et al., 2013, p. 755). Sendo o maior risco deste período, a instalação de um quadro de imunodepressão. Entretanto, fatores que levam a falhas na resposta imunológica como a gravidez, senescência e abuso de anti-inflamatórios, como também os altos índices de infecção pelo vírus HIV, tornam a infecção latente um problema de saúde pública preocupante (DUBEY; JONES, 2008, p. 1257; INNES, 1997, p. 131 ; VIDOTTO, 1992, p. 69).

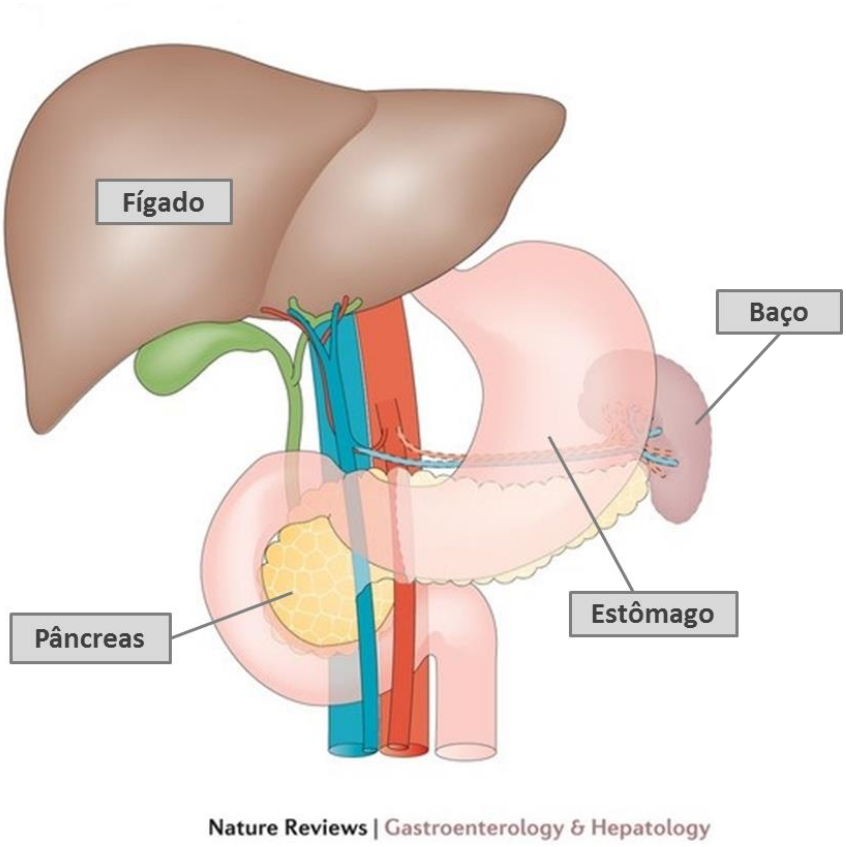
Quando comparado a humanos, camundongos apresentam maior fragilidade à infecção por cepas de *T. gondii* de baixa virulência, entretanto, é considerado um bom modelo experimental para o estudo da infecção latente (DUBEY; FRENKEL, 1998, p. 1). Aproximadamente 10 dias após a infecção experimental por cepa *T. gondii*, o organismo de um camundongo imunocompetente é capaz de induzir os parasitos a entrar em sua forma de latência no tecido cerebral. Estudos histológicos demonstram que 21 dias após a infecção, formam-se focos inflamatórios envolta dos cistos parasitários no cérebro, os quais se tornam maiores e induzem a produção de um envoltório fibroso sobre os cistos (FERGUNSON; HUTCHINSON, 1987, p. 483). Este processo inflamatório desenvolvido contra os cistos é considerado o fator desencadeante de alterações comportamentais em roedores (SUZUKI et al., 2013, p. 755). Além disso, pesquisas revelam que a infecção latente em camundongos induz a neuroinflamação, fato que reforça a teoria da alteração neuronal (FOND et al., 2013, p. 38; HENRIQUEZ et al., 2009, p. 122; TONIN et al., 2014, p. 526).

Estudos revelam que esta infecção latente por *T. gondii* pode também estar relacionada ao desenvolvimento de doenças neurodegenerativas em humanos (PRANDOTA, 2014, p. 205). Entre as doenças relacionadas com a infecção latente estão, desordens bipolares (DE BARROS et al., 2017, p. 59), esquizofrenia (ESSHILI et al., 2016, p. 327), perda de memória de trabalho (GAJEWSKI et al., 2016, p. 35), entre outras (PASSERI et al., 2016, p. 153). Além disso, estudos demonstram relação positiva entre distúrbios extracerebrais com a infecção latente por *T. gondii*. Sendo a infecção latente capaz de potencializar a resposta inflamatória crônica de pacientes obesos ou com artrite reumatoide, aumentando ainda mais a concentração dos mediadores inflamatórios e os marcadores clínicos encontrados nestas doenças (EL-SAYED et al., 2016, p. 767; ISKANDAR et al., 2016, p. 1). Mais ainda, é capaz de reduzir os níveis de testosterona em homens com infecção latente (ESLAMIRAD et al., 2013, p. 622). Interessantemente, El-Rehim et al. (2013, p. 784) demonstra em seu estudo uma possível relação entre infecção latente e o desenvolvimento de cirrose, o qual pode estar relacionado ao processo inflamatório desenvolvido contra a infecção latente. Entretanto, devido à baixa visibilidade dos possíveis danos causados a tecidos extracerebrais na infecção latente, pouco é conhecido sobre as possíveis alterações fisiológicas que poderiam levar ao desenvolvimento de patologias em órgãos como o baço e o fígado.

O baço é um órgão glandular localizado na região esquerda da cavidade abdominal, assim como, o maior órgão do sistema linfático, no qual atua como linfonodo periférico (Figura 2). Interessantemente, este órgão é capaz de produzir e armazenar células sanguíneas, entretanto, ao mesmo tempo é responsável por destruir e controlar estas células na corrente sanguínea (KRAAL; DEN HAAN, 2016, p. 407; LANZKOWSKY et al., 2016, p. 101). Isto ocorre devido a dois complexos tecidos presentes neste órgão, isto é, o tecido esplênico pode ser dividido em dois grandes grupos: (1) polpa vermelha: que consiste em células do sistema reticuloendotelial, o qual é responsável pela remoção e lise de eritrócitos danificados da corrente sanguínea, e (2) polpa branca: que realmente atua como linfonodo, armazenando linfócitos, além de produzir plaquetas (KRAAL; DEN HAAN, 2016, p. 407). Desta maneira, o baço apresenta importante função imunológica e hematológica. Em processos inflamatórios, o órgão é capaz de estimular a ativação e proliferação de leucócitos, como também a produção de citocinas e anticorpos

(LANZKOWSKY et al., 2016, p. 101). Este órgão está ligado vascularmente com o pâncreas e o fígado, e intrigantemente uma vez pró-inflamatório, é capaz de estimular o desenvolvimento pró-inflamatório em seus órgãos conectos.

Figura 2 – Anatomia e arranjo do baço e fígado, como também, suas conecções.



Fonte: Adaptação de De Rooij et al. (2016).

Já o fígado é responsável por funções vitais no organismo, como, o metabolismo de carboidratos, lipídios e proteínas (CAMPBELL, 2006, p. 102). Além disso, é responsável pela secreção de compostos como a bilirrubina, ácidos biliares e colesterol, e a excreção de compostos produzidos pelo metabolismo endógeno e exógeno. Mais ainda, o fígado atua na metabolização de fármacos e compostos tóxicos, conjugando-os a proteínas e excretando-os do organismo. Participa do controle de proteínas plasmáticas e produção de proteínas de fase aguda, as quais estão relacionadas ao desenvolvimento da febre no organismo (CAMPBELL, 2006, p. 102). Devido à alta complexidade das funções do tecido hepático, células de defesa se encontram estrategicamente no tecido para prevenir danos e proteger suas funções vitais. Entre as células de defesa estão macrófagos, células

dendríticas e uma considerável população de linfócitos (RACANELLI; REHERMANN, 2006, p. 54). Interessantemente, o fígado contém uma alta carga linfocitária, estimada em 10^{10} linfócitos, o que seria aproximadamente 25% do conteúdo celular hepático. Linfócitos hepáticos são capazes de modular a resposta imunológica no tecido através da liberação de citocinas e outros mediadores imunes, apresentando função fundamental no desenvolvimento inflamatório (SHETTY et al., 2008 p. 136). Desta maneira, através do isolamento e da avaliação celular destas, é possível obter informações valiosas da resposta imunológica do tecido hepático (GOOSSENS et al., 2000, p. 137).

Em muitos casos, a resposta imunológica desenvolvida nestes dois órgãos combate infecções e tumores, mas, em certos casos, uma resposta imunológica excessiva ocasiona as alterações patológicas. Por exemplo, o baço apresenta função crucial na remoção de eritrócitos infectados por parasitos como os do gênero *Plasmodium*, e a bactéria *Anaplasma marginale,* entre outros (HAA et al., 2015, p. 369; JASWAL et al., 2015, p. 495; KRAAL; DEN HAAN, 2016, p. 407). Isto ocorre devido ao sistema reticuloendotelial presente na polpa vermelha, o qual identifica as células infectadas e as remove da circulação inativando os parasitos (KRAAL; DEN HAAN, 2016, p. 407). Entretanto, por outro lado, certas alterações patológicas podem induzir uma excessiva resposta pró-inflamatória no órgão, a qual é capaz de aumentar o tamanho do tecido. Este evento é denominado esplenomegalia, e em geral ocorre pelo aumento do número celular influenciado pelo aumento da função do órgão, seja da polpa branca ou da polpa vermelha. Ao longo tempo, a esplenomegalia induz alterações como fibrose e dano celular, levando a disfunções teciduais sérias e a fragilidade mecânica do órgão (LANZKOWSKY et al., 2016, p. 101). O fígado não foge desta regra, onde a resposta inflamatória é importante para a proteção do tecido, como nos casos de hepatite viral (FÉNÉANT et al., 2014, p. 535) e infecções por parasitos do gênero *Schistosoma* (ELBAZ; ESMAT, 2013, p. 445). Entretanto, em certos casos a excessiva resposta imunológica é a alteração patológica, como nas doenças autoimunes hepáticas (MABEE; THIELE, 2000, p. 431) e nas rejeições a transplantes (CARBONE; NEUBERGER, 2014, p. 210). Como no baço, a resposta imune também é capaz de induzir um aumento no tecido hepático, denominado hepatomegalia (LINGURARU et al., 2012, p. 588). De acordo com a literatura, a excessiva resposta inflamatória por um longo período é capaz de

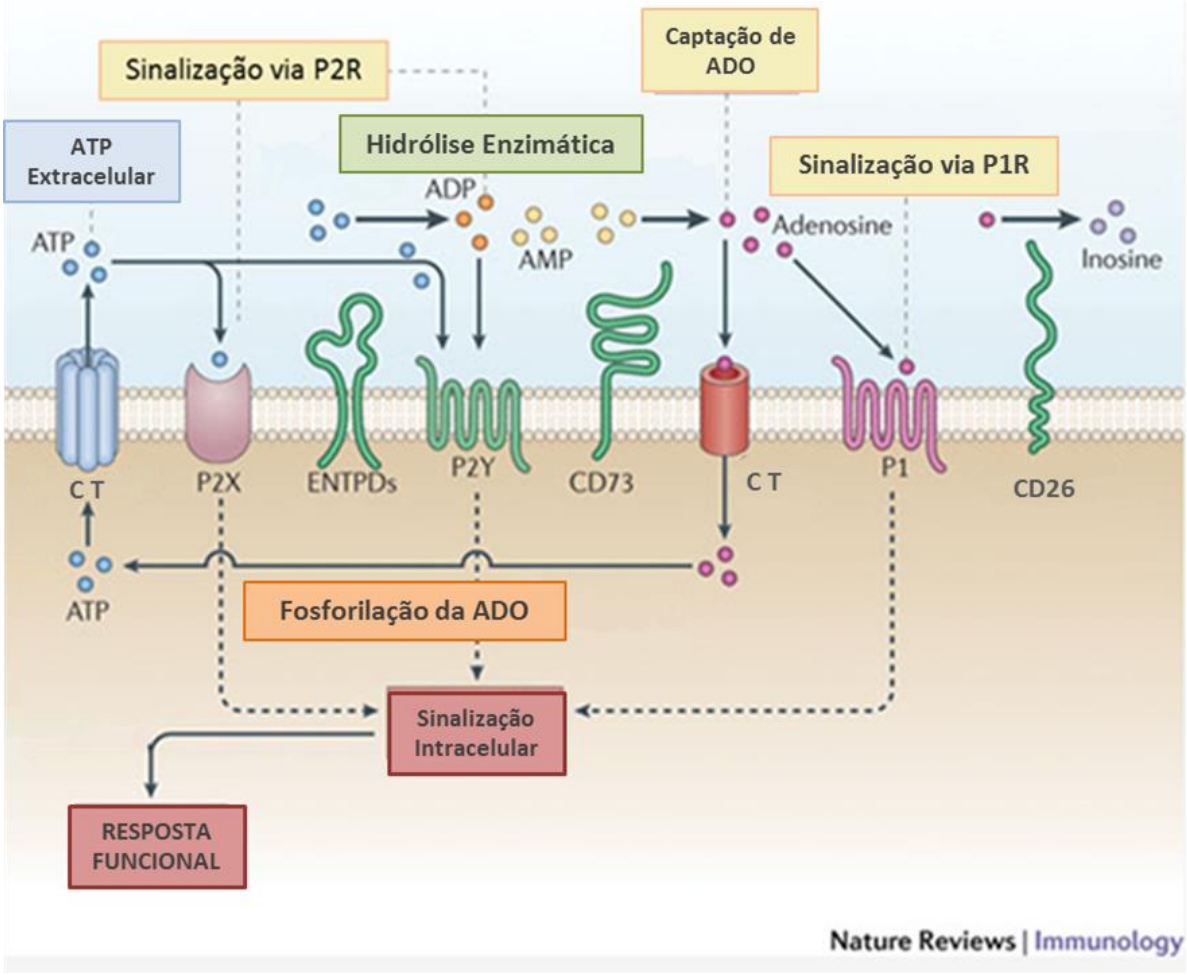
agravar alterações pré-existentes, ou até mesmo iniciar processos patológicos no tecido hepático, como a fibrose e a cirrose (RACANELLI; REHERMANN, 2006, p. 54; SHETTY et al., 2008, p. 136).

Para o desenvolvimento da resposta imunológica, além de células imunológicas, o organismo apresenta inúmeros mediadores químicos para realizar a sinalização da resposta imune no organismo. Entre estes mediadores, estão os nucleotídeos e nucleosídeo de adenina (ALAM; GORSKA, 2003, p. 476), regulados por enzimas purinérgicas.

Sistema Purinérgico (Figura 3), apresenta como sinalizador intercelular os nucleotídeos purínicos, bem como seus metabólitos, que interagem com receptores celulares específicos (BURNSTOCK, 2007, p. 1471). Estes sinalizadores moleculares e suas concentrações meio extracelular são controlados pela expressão e atividade de enzimas que metabolizam tais moléculas (ZIMMERMANN, 2000, p. 299). Este sistema está envolvido em inúmeras funções do organismo como a resposta neurológica, a agregação plaquetária e a resposta imunológica (JUNGER, 2011, p. 201). Os componentes do sistema purinérgico podem estar presentes na membrana extracelular de células endoteliais, plaquetas, macrófagos, eritrócitos, linfócitos, entre outras células, mas também no soro, com componentes solúveis, como as enzimas que degradam nucleotídeos extracelulares (ZIMMERMANN et al., 2012, p. 437).

Como representantes do sistema purinérgico estão às moléculas de sinalização ATP, ADP e adenosina. A adenosina é um nucleosídeo purínico composto por uma base nitrogenada bicíclica ligada a uma pentose, e o ATP e ADP são nucleotídeos, moléculas compostas por um nucleosídeo ligado a moléculas de fosfato (NELSON e COX, 2014, p. 103). Estes nucleotídeos e nucleosídeo de adenina estão em maior concentração no meio intracelular e apresentam inúmeras funções metabólicas nas células. Estas moléculas não atravessam livremente a membrana celular e estão em baixas concentrações no meio extracelular (NELSON e COX, 2014, p. 103). Em situações de dano celular, inflamação e estresse oxidativo ocorre um aumento da concentração plasmática de moléculas purínicas que acabam por alterar a sinalização purinérgica, a qual pode determinar as respostas fisiológicas dependentes deste sistema (DI VIRGILIO, 2001, p. 587).

Figura 3 – Representação dos componentes do sistema purinérgico: receptores (P1, P2X e P2Y), nucleotidases (NTPDase (ENTPDs), 5´nucleotidase (CD73) e ADA (CD26)), canais de transporte de nucleotídeos (CT) e sinalizadores (ATP, ADP e adenosina).



Fonte: Adaptação de Junger (2011).

Entre os receptores do sistema purinérgico, encontramos os receptores P1 e P2. Os receptores P1 são divididos em; A2 os quais estão ligados a proteína Gs, inibindo respostas celulares devido à ativação de AMPc, e A1 e A3; os quais estão acoplados à proteína Gq e Gi e promovem ativação celular. Já os receptores P2 se dividem em dois grandes grupos; o P2X que representa os receptores ligados a canais iônicos e o P2Y representando os receptores ligados à proteína G (RALEVIC e BURNSTOCK, 1998, p. 413). Estes receptores estão expressos na face

extracelular da membrana citoplasmática de inúmeras células, sendo assim possíveis regulações fisiológicas dependentes das concentrações extracelulares de sinalizadores. As moléculas de adenosina interagem com receptores P1 enquanto os nucleotídeos fosfatados interagem com os receptores P2 (JUNGER, 2011, p. 201).

O nucleotídeo ATP apresenta inúmeras funções biológicas no organismo, como, a contração muscular, neurotransmissão, inibição plaquetária e participação em processos inflamatórios (BOURS et al., 2006, p. 358). Células são capazes de secretar pequenas concentrações de ATP para o meio extracelular através de canais de transporte específicos. Entretanto, em casos de dano celular, estresse oxidativo e inflamação as células atingidas aumentam a expressão de canais de transporte e intensificam a secreção de ATP para o meio extracelular (JUNGER, 2011, p. 201). Ao interagir com receptores do tipo P2Y e P2X de linfócitos e outras células imunes, o nucleotídeo ATP estimula a ativação, proliferação e migração celular para focos inflamatórios (BARICORDI et al., 1996, p. 682; JAMIESON et al., 1996, p. 637). Mais ainda, induz a secreção de citocinas pró-inflamatórias, como a interleucina 2 (IL-2), interferon gama (INF-γ), fator de necrose tumoral alfa (TNF-α), entre outras (BOURS et al., 2006, p. 358; BUDAGIAN et al., 2003, p. 1549). A excessiva interação desencadeada pelo ATP extracelular com receptores P2X é capaz de induzir apoptose celular através da formação de poros de membrana. Estes eventos são dependentes da expressão de receptores P2 como também da expressão de ectoenzimas que degradam ATP em sua membrana plasmática (DI VIRGILIO, 1989, p. 587). Além disso, este nucleotídeo trifosfatado interage com células vasculares induzindo a vasodilatação e aumento na permeabilidade vascular em sítios inflamatórios (JUNGER, 2011, p. 201). Entretanto, é importante considerar que em um microambiente saudável, o efeito vascular do ATP dependerá de qual receptor for estimulado, apresentando ação vasodilatadora ao interagir com receptores do tipo P2Y, e vasoconstritora ao interagir com receptores P2X (RALEVIC, 2009, p. 413). Devido a seu potencial pró-inflamatório o nucleotídeo ATP é considerado uma molécula endógena chave para o desenvolvimento da resposta inflamatória (BOURS et al., 2006, p. 358). Desta maneira, imunologistas consideram altas concentrações de ATP um padrão molecular associado ao dano (DAMP),

podendo ser utilizado para fins diagnósticos em caso de dano celular ocasionado em patologias (JUNGER, 2011, p. 201).

O nucleotídeo ADP interage apenas com receptores P2Y, sendo um importante agregador plaquetário endógeno, apresentando também ação vasoconstritora (COLMAN, 1990, p. 1425; FURUKOJI et al., 2008, p. 583). Seu efeito na sinalização imune ainda é pouco conhecido, onde se acredita que participa sinergicamente com o nucleotídeo ATP nos receptores P2Y (BOURS et al., 2006, p. 358). O nucleotídeo AMP não apresenta função extracelular, pois não interage com nenhum receptor purinérgico. Mas, a degradação enzimática deste nucleotídeo é responsável pela formação de adenosina no meio extracelular, sendo assim importante para a regulação da sinalização dos receptores P1 (BOROWIEC et al., 2006, p. 269). A adenosina interage com receptores P1 desempenhando ação antagônica ao ATP (CROSTEIN, 1994, p. 1160). Em leucócitos, a sinalização via P1 mediada pela adenosina, induz a redução da adesão e transmigração destes para tecidos, devido à supressão da expressão de receptores I-CAM (MACKENZIE et al., 2002, p. 90). A estimulação dos receptores P1 com a adenosina também induz a supressão de linfócitos (MIRABET et al., 1999, p. 491) e reduz a liberação de citocinas pró-inflamatórias (DOS REIS et al., 1986, p. 213). Desta maneira esse nucleosídeo desempenha importante papel como agente imunossupressor e anti-inflamatório endógeno (CROSTEIN, 1994, p. 5). Já no tecido vascular apresenta importante função, já que é um inibidor da agregação plaquetária, e capaz de induzir vasodilatação, além de reduzir a frequência cardíaca (BIRK et al., 2002, p. 116; BOURS et al., 2006, p. 358; DE VENTE et al., 1984, p. 678). Portanto, a degradação extracelular de nucleotídeos produzindo adenosina, é considerada um importante evento fisiológico (BIRK et al., 2002, p. 116).

Uma complexa gama de ectoenzimas ancoradas a face extracelular da membrana celular, e enzimas solúveis presentes no soro e no liquido intersticial são, além dos canais de transporte, os responsáveis pela manutenção dos níveis de sinalizadores purínicos no meio extracelular (ZIMMERMANN, 2000, p. 299). O controle dos nucleotídeos e nucleosídeo de adenina no meio extracelular podem ser realizados pela degradação destas moléculas por enzimas chamadas nucleotidases. Entre estas enzimas estão, as nucleosídeo trifosfato difosfohidrolases (NTPDases), 5‘-nucleotidase (N´5) e a adenosina desaminase (ADA). A NTPDase inicia a

degradação do ATP a ADP e do ADP a AMP. Seguindo a cascata enzimática, o AMP é hidrolisado pela 5´nucleotidase à adenosina a qual é desaminada pela ADA, formando o nucleosídeo inosina (ZIMMERMANN et al., 2007, p. 537).

As NTPDases (CD39; E.C 3.6.1.5) são uma família de enzimas responsáveis pela hidrólise de nucleotídeos di- e tri-fosfatados, a seus monofosfonucleotídeos correspondentes. A família NTPDase é dividida em oito isoenzimas, sendo as NTPDases 1, 2, 3 e 8 ectoenzimas (CD39 ou E-NTPDase) as quais contêm seu sitio catalítico voltado ao meio extracelular. Enquanto que as NTPDase 4, 5, 6 e 7 são enzimas solúveis, presentes no meio intracelular como também no soro ou líquido intersticial (ZIMMERMANN, 2001, p. 44). A expressão e a atividade da NTPDase desempenham inúmeras funções biológicas por influenciar na sinalização dos nucleotídeos com receptores P2. Entre as funções biológicas influenciáveis está a proliferação celular, diferenciação, migração e morte celular (BURNSTOCK; VERKHRATSKY, 2010, p. 9).

A E-NTPDase é considerada um biomarcador da ativação de linfócitos, monócitos, macrófagos e células dendríticas, assim como, um regulador das concentrações extracelulares de ATP, uma vez que desempenha hidrólise deste nucleotídeo (DWYER et al., 2007, p. 171; YEGUTKIN, 2008, p. 1328). Através do controle do ATP extracelular, a E-NTPDase ancorada a membrana celular, desempenha importante papel no controle da resposta imune celular (DOMBROWSKI et al., 1997, p. 104; LEAL et al., 2005, p. 9). A liberação de altas concentrações de ATP para o meio extracelular é usualmente acompanhada pela secreção de certas enzimas do meio intracelular (WESTFALL et al., 2002, p. 439). Entre elas estão as NTPDase solúveis, as quais controlam os níveis de nucleotídeos extracelulares e consequentemente desempenham função imune e vascular na corrente sanguínea e em tecidos (YEGUTKIN et al., 2003, p. 1328). Considerando que altos níveis de ATP são liberados em células danificadas, a determinação da hidrólise enzimática pela NTPDase na corrente sanguínea e tecidos servem como ferramenta auxiliar no diagnóstico de dano celular em condições patológicas (DEAGLIO; ROBSON, 2011, p. 301; ENJYOJI et al., 1999, p. 1010; YEGUTKIN, 1997, p. 1328)

A enzima 5´-nucleotidase (CD73, E.C. 3.1.3.5) é capaz de desfosforilar monofosfonucleotídeos a seus nucleosídeos correspondentes, tendo maior afinidade pelo AMP. Devido a sua afinidade ao AMP, torna-se uma enzima importante, por ser responsável pela produção de adenosina extracelular (BOROWIEC et al., 2006, p. 269; ZIMMERMANN, 2001, p. 44). A 5´-nucleotidase apresenta uma importante função na sinalização mediada por receptores P1, mas apresenta outras funções biológicas não relacionadas com a ação catalítica, agindo também como molécula de adesão (SADEJ et al., 2008, p. 35).

A ADA (CD26, E.C. 3.5.4.4) é responsável pela desaminação de adenosina formando o produto inosina (ZIMMERMANN, 2001, p. 44), assim como é classificada em ADA-1 a isoenzima ancorada a membrana plasmática células (CD26 ou E-ADA) e ADA-2, isoforma solúvel da enzima presente no soro e no liquido intersticial (CRONSTEIN, 1994, p. 5). ADA-1 apresenta alta atividade em linfócitos e monócitos ativados, sendo assim a atividade e expressão desta enzima considerada um marcador da ativação destas células (FRANCO et al., 1997, p. 283; KALJAS et al., 2016, p. 105). Já a ADA-2 é essencial para o bom funcionamento do sistema imune e vascular na corrente sanguínea, sendo considerada um marcador de distúrbios imunológicos e cardiovasculares (GORRELL et al., 2001, p. 249; ABDI et al., 2016, p. 200; GIORGI et al., 2016, p. 400).

Alterações na atividade destas enzimas são encontradas em inúmeras patologias, isto devido à importância destas enzimas em relação ao controle da resposta fisiológica (SCHETINGER et al., 2007, p. 77). Inúmeras alterações em enzimas purinérgicas já foram encontradas em infecções parasitarias, estando estas alterações possivelmente relacionadas à patogênese da infecção. Acredita-se que a principal regulação desenvolvida por estas modulações enzimáticas seja sobre a resposta imunológica dos hospedeiros. Estas modulações encontradas apresentam padrões específicos, os quais dependem de fatores como, o parasito e sua patogenicidade, o órgão afetado e a fase da infecção.

Por exemplo, a infecção crônica do tecido hepático pelo helminto *Fasciola hepatica*, induz o aumento da atividade das enzimas purinérgicas hepáticas, resultando na redução de ATP e consecutivo controle negativo da resposta imunológica contra o parasita no tecido hepático. Por outro lado, nesta mesma

infecção, reduz a atividade enzimática no soro e eleva os níveis de ATP na corrente sanguínea, e desta forma sinaliza uma resposta pró-inflamatória sistêmica (BALDISSERA et al., 2016, p. 2363; DOLESKI et al., 2016, p. 551). Diferentemente, a infecção crônica pelo trematódeo *Eurytrema coeloticum*, o qual infecta o tecido pancreático, apresenta elevada atividade da enzima NTPDase no soro, e neste caso, está relacionada com a redução dos níveis de ATP na corrente sanguínea (FAVERO et al., 2016, p. 48). Mais ainda, neste mesmo estudo é possível observar correlação positiva entre a atividade da NTPDase sérica com o número de parasitas e o dano tecidual no pâncreas, resultados que reforçam a importância desta enzima na patogênese da infecção (FAVERO et al., 2016, p. 48).

Estudos com infecções por parasitos do gênero *Trypanosoma* e a sinalização purinérgica, demonstram a complexidade das respostas que este sistema pode apresentar. As infecções por *Trypanosoma cruzi* e *Trypanosoma evansi*, são capazes de reduzir a atividade das ectoenzimas E-NTPDase e E-ADA em linfócitos periféricos e plaquetas, como também, apresentam baixos níveis de ATP e elevados níveis de adenosina (DA SILVA et al., 2012, p. 80; OLIVEIRA et al., 2011, p. 9; SOUZA et al., 2012, p. 690). Esta complexa modulação é capaz de desenvolver um perfil anti-inflamatório, induzido principalmente pela ação da adenosina na corrente sanguínea. O nucleosídeo adenosina apresenta importante função no reparo tecidual, a qual é capaz de inibir a resposta pro-inflamatória, e reduzir citocinas e radicais livres (BOURS et al., 2006, p. 358; CRONSTEIN et al., 1983, p. 5). Entretanto, estudos revelam que a redução na resposta inflamatória induzida pela alta razão ATP/adenosina encontrada em infecções parasitárias, a qual protege o organismo de danos excessivos, também está relacionada com a continuidade do parasitismo no hospedeiro (MEYER, 2012, p. 299). A infecção por *Leishmania amazonensis* também é seguida de elevada produção enzimática de adenosina. Estudos com o uso de antagonistas adenosinérgicos para o tratamento da infecção por *L. amazonensis* foram capazes de reduzir o parasitismo no hospedeiro (DE ALMEIDA et al., 2008, p. 850). Existe receio ao uso de antagonistas adenosinérgicos, já que poderiam interferir na sinalização protetora desenvolvida pela adenosina. Mas, no caso da infecção por *L. amazonensis*, acredita-se que o estimulo dado a resposta pró-inflamatória seria capaz de eliminar os parasitos, e consequentemente reduzir o dano tecidual (DE ALMEIDA et al., 2008, p. 850).

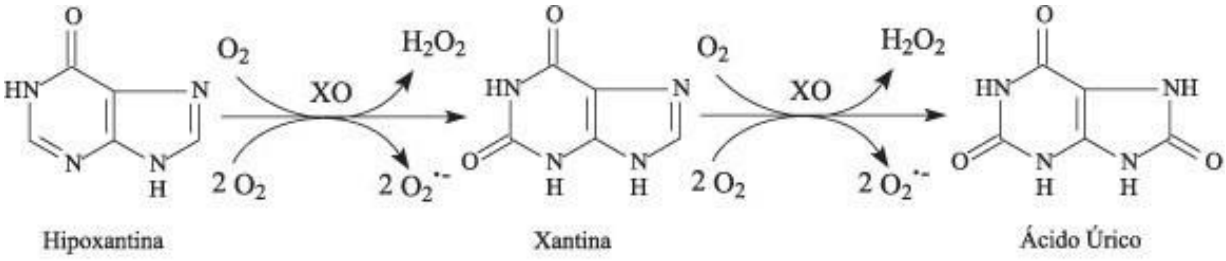
Interessantemente, a infecção por cepa virulenta (RH) de *T. gondii* também apresenta reduzida atividade das enzimas E-NTPDase e E-ADA em linfócitos periféricos, fato o qual sugere um possível envolvimento anti-inflamatório da adenosina, e consequentemente no parasitismo no hospedeiro (TONIN et al., 2013, p. 325; TONIN et al., 2014b, p. 51).

Como o sistema purinérgico confere desde a degradação do nucleotídeo ATP até o nucleosídeo inosina, a qual ocorre no meio extracelular, e apresenta importante sinalização intercelular (BURNSTOCK, 2007, p. 1471). Por outro lado, a degradação de inosina até a formação de ácido úrico ocorre no meio intracelular, não apresentando função sinalizadora conhecida no meio extracelular (NELSON; COX, 2014, p. 103). Exclusivamente no meio intracelular, o nucleosídeo inosina é degradado pela enzima purina nucleosídeo fosforilase (PNP), produzindo hipoxantina. A degradação enzimática continua com a degradação da hipoxantina pela enzima xantina oxidase (XO) formando xantina, a qual é novamente degradada pela XO formando ácido úrico (AU) (Figura 4). A atividade da enzima PNP, atua unicamente na metabolização de inosina, por outro lado, a atividade da enzima XO é capaz de produzir radicais livres e espécies reativas de oxigênio (NELSON; COX, 2014, p. 103). Ao degradar uma molécula de hipoxantina ou xantina, a enzima produz como subproduto da reação, peróxido de hidrogênio (H_2O_2) e anion superóxido ($O_2^{\cdot -}$). O H_2O_2 é uma espécie reativa não radicalares, isto porque, não apresentam desemparelhamento de orbital externo, como é o caso dos radicais livres como o anion superóxido (BIRBEN et al., 2012, p. 9). Entretanto, no organismo o H_2O_2 é capaz de reagir com biomoléculas e produzir radicais livres como o $O_2^{\cdot -}$ e hidroxilas (OH^-). O radical livre $O_2^{\cdot -}$ é considerado um dos radicais de oxigênio mais reativos presente no organismo, e como o H_2O_2 também é capaz de reagir com outras biomoléculas e produzir outros radicais livres agressivos (BIRBEN et al., 2012, p. 9). Radicais livres são necessários para a correta resposta imunológica no organismo, os quais são capazes de combater tumores e infecções microbianas. No entendo, quando em altas concentrações geram estresse oxidativo, onde biomoléculas são oxidadas pelos radicais livres, levando a perda de função da biomolécula oxidada (SOSA et al., 2013, p. 376).

A enzima XO é capaz de produzir radicais livres de alta instabilidade, e desta maneira esta relacionada ao desenvolvimento de estresse oxidativo em tecidos

(BIRBEN et al., 2012, p. 9). Entretanto, alterações na atividade da XO podem ser consideradas maléficas, e às vezes benéficas (KANG; HA, 2014, p. 1). Isto porque, o ácido úrico formado pela degradação de xantina pela XO, pode atuar como um sequestrador de radicais livres, como também é capaz de quelar íons metálicos, e inibir a produção de radicais livres (SOUZA-JUNIOR et al., 2014, p. 271). Logo, a atividade da enzima XO é capaz de melhorar a resposta imunológica em organismos saudáveis, já que produz ao mesmo tempo radical livre e agente antioxidante. Mas, em certas patologias a atividade da enzima e os níveis alterados de AU podem ser considerados um mal prognóstico da doença, fato que ainda gera conflitos na comunidade científica (BAGHERI et al., 2016, p. 27; MAHARANI et al., 2016, p. 395).

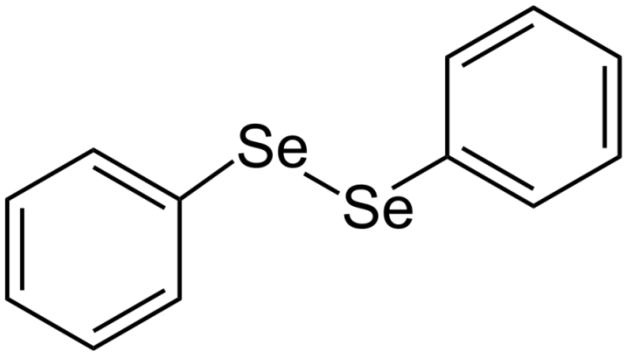
Figura 4 – Esquema da produção de Ácido Úrico através da degradação de Hipoxantina e Xantina pela enzima Xantina oxidase (XO). Como também a formação do peróxido de hidrogênio (H_2O_2) e ânion superóxido ($O_2^{\cdot-}$).



Fonte: (ALVES et al., 2010)

Uma atividade pró-inflamatória é capaz de gerar estresse oxidativo no organismo, apresentando importância no bom desenvolvimento da resposta imune (BIRBEN et al., 2012, p. 9; SOSA et al., 2013, p. 376). Mas, a longo prazo, o estresse oxidativo gerado pela resposta inflamatória é capaz de induzir ao dano e remodelação tecidual, e consequente perda de função (SOSA et al., 2013, p. 376). Logo, para prevenir danos oxidativos gerados por processos inflamatórios crônicos, tratamentos com agentes anti-oxidantes tem sido propostos para modelos *in vivo* (HUSSAIN et al., 2016, p. 1). Entre os antioxidantes com propriedades farmacológicas já bem definida pela comunidade cientifica está o disseleneto de difenila $(PhSe)_2$ (Figura 5).

Figura 5 – Estrutura molecular do disseleneto de difenila $(PhSe)_2$.



Fonte: (NOGUEIRA; ROCHA, 2004)

O $(PhSe)_2$ é um organocalcogênio que contém selênio em sua composição, e apresenta inúmeras atividades biológicas como também possíveis aplicações farmacológicas (NOGUEIRA et al., 2004, p. 6255). Estudos com animais de experimentação revelam que o $(PhSe)_2$ apresenta propriedade neuroprotetora e antidepressiva (BURGER et al., 2004, p. 339; NOGUEIRA et al., 2003b, p. 56), antihiperglicêmica (BARBOSA et al., 2006, p. 230), antiúlcera (SAVEGNAGO et a., 2007, p. 129), hepato-protetora (WILHELM et al., 2009a, p. 197), entre outros. Além disso, este composto orgânico de selênio apresenta atividade imunomodulatória, com predominância anti-inflamatória (LEITE et al., 2015, p. 119, NOGUEIRA et al., 2004, p. 6255). O seu efeito biológico mais estudado é sua atividade antioxidante, fato que pode explicar o ação benéfica do $(PhSe)_2$ sobre inúmeras patologias experimentais (NOGUEIRA; ROCHA, 2010, p. 2055). Entretanto, o verdadeiro mecanismo de ação do $(PhSe)_2$ ainda não está totalmente compreendido (NOGUEIRA et al., 2004, p. 6255).

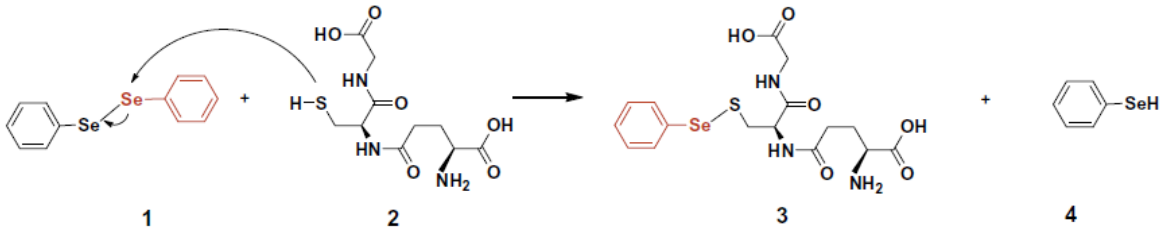
Um mecanismo de ação proposto para explicar os efeitos do $(PhSe)_2$ envolve a oxirredução de grupos tióis (-SH) de proteínas e biomoléculas ativas (NOGUEIRA; ROCHA, 2010, p. 2055; YOUNG et al., 1981, p. 1076). Desta maneira, $(PhSe)_2$ reage com grupos -SH da estrutura terciaria de proteínas, sendo esta interação capaz de modular a atividade e afinidade de enzimas ricas em grupos –SH (enzimas sulfidrílicas) em sua composição. Estudos toxicológicos demonstram que deste mecanismo o $(PhSe)_2$ é capaz de alterar importantes vias metabólicas, sendo este

não só seu provável mecanismo de ação, mas também seu mecanismo toxicológico (NOGUEIRA; ROCHA, 2010, p. 2055). Entretanto, efeitos tóxicos induzidos pelo $(PhSe)_2$ são observados apenas quando administrado em altas concentrações (NOGUEIRA et al., 2004, p. 6255). Entre as enzimas sulfidrílicas que o $(PhSe)_2$ é capaz de inibir estão a 5-lipoxigenase (BJORNSTEDT et al., 1996, p. 8511), alfa-aminolevulinato desidratase (NOGUEIRA et al., 2003a, p. 169) e a Na^+ K^+ ATPase (BORGES et al., 2005, p. 191).

A biomolécula glutationa reduzida (GSH) apresenta importante atividade antioxidante (NELSON; COX, 2014, p. 103), entretanto, por ser uma molécula sulfidrilica, ataca nucleofílicamente a molécula do $(PhSe)_2$ produzindo um aduto de GSH inativo e uma molécula de selenol (Figura 6) (PRINGOL et al., 2012, p. 65). Este efeito poderia ser capaz de reduzir o potencial antioxidante de um tecido, entretanto, com a queda dos níveis de GSH o organismo aumenta a produção de GSH, elevando seus níveis e consequentemente o potencial antioxidante (NOGUEIRA et al., 2004, p. 6255; PRINGOL et al., 2012, p. 65).

O $(PhSe)_2$ apresenta alta lipossolubilidade, apresentando assim afinidade por tecidos como o cérebro e fígado (NOGUEIRA; ROCHA, 2010, p. 2055). Inúmeros trabalhos demonstram o efeito benéfico do $(PhSe)_2$ sobre o tecido hepático; e neste tecido, o $(PhSe)_2$ é capaz de aumentar a atividade antioxidante (PRINGOL et al., 2012, p. 9), como também reduzir o estresse oxidativo induzido por metilmercúrio (DE FREITAS et al., 2009, p. 77). Apresenta importante ação hepato-protetora, sendo capaz de proteger o tecido hepático de danos causados pelo consumo excessivo de acetoaminofeno (WILHELM et al., 2009b, p. 31). Também protege o tecido hepático de roedores expostos experimentalmente aos agentes hepato-tóxicos 2-nitropropano (WILHELM et al., 2009a, p. 197) e paraquat (COSTA et al., 2013, p. 750). Mais ainda, apresenta efeito benéfico ao dano hepático causado pela indução experimental de diabetes em ratos (BARBOSA et al., 2006, p. 1761). Além do efeito antioxidante direto do $(PhSe)_2$ no tecido hepático, este é capaz de interagir com células imunes do tecido, como os macrófagos, induzindo-os a reduzir a produção de espécies reativas (HASSAN; ROCHA, 2012, p. 12287; STRALIOTTO et al., 2013, p. 106).

Figura 6 – Esquema de reação do $(PhSe)_2$ com moléculas sulfidrílicas. Na figura, a reação do $(PhSe)_2$ (1) com uma molécula de glutationa reduzida (2), com a produção de uma molécula de glutationa inativa (3) e uma molécula de selenol (4).



Fonte: Adaptação de Pringol et al. (2014).

Estudos demonstram que a administração de uma baixa dose de $(PhSe)_2$ em camundongos infectados por *T. gondii*, apresentam efeitos positivos. Em camundongos infectados por cepa avirulenta de *T. gondii* (ME-49), o tratamento com $(PhSe)_2$ em baixa dosagem é capaz de reduzir lesões no tecido cardíaco (MACHADO et al., 2016b, p. 25), como também, efeito antioxidante no tecido cerebral (MACHADO et al., 2016a, p. 51). Já em camundongos infectados por *T. gondii* de cepa virulenta (RH), o tratamento com $(PhSe)_2$ reduz o estresse oxidativo no soro. Entretanto, não foi evidenciado alterações nos níveis de citocinas relacionadas ao combate a infecção, como também, nenhum aumento ou reativação da infecção nos animais tratados (BARBOSA et al., 2014, p. 1761). O uso de agentes anti-inflamatórios na infecção por *T. gondii* são altamente controlados, a fim de evitar uma imunossupressão e ressurgimento da fase aguda. Desta maneira o tratamento subcutâneo com $(PhSe)_2$ a uma baixa dosagem é um possível candidato à terapia ao possível dano extracerebral gerado na infecção latente por *T. gondii*.

Receptores e enzimas purinérgicas apresentam em sua estrutura proteica inúmeros grupos –SH, sendo estes importantes para a estrutura e função destas proteínas (ENNION; EVANS, 2002, p. 303; IVANENKOV et al., 2003, p. 11726; IVANENKOV et al., 2005, p. 8998). Sabe-se que compostos orgânicos de selênio, com estrutura similar ao $(PhSe)_2$, são capazes de alterar proteínas relacionadas ao influxo de Ca^{2+} (MORETTO et al., 2003, p. 157). É importante relembrar, que a função dos purinoreceptores tipo P2X estão relacionados ao influxo de Ca^{2+} desenvolvido por este receptor, sendo possível que $(PhSe)_2$ altere estes receptores

e consequentemente suas funções. Modulações na sinalização realizada por purinoreceptores, em geral, induz a alterações na atividade enzimática de ectonucleotidases (BOURS et al., 2006, p. 358, JUNGER, 2011, p. 201). Desta maneira, é possível que o $(PhSe)_2$ interage com receptores e enzimas do sistema purinérgico, alterando a modulação fisiológica desenvolvida por estas proteínas.

Camundongos saudáveis infectados com *T. gondii* de cepa ME-49 apresentam a forma crônica da toxoplasmose, capaz de induzir dano ao tecido cerebral e possivelmente a órgãos como o fígado e o baço. Neste contexto, considerando que toxoplasmose latente está amplamente presente na sociedade, são necessárias pesquisas que demonstrem e confirmem possíveis alterações no tecido hepático e esplênico. Mais ainda, propor o agente protetor $(PhSe)_2$ como um tratamento para prevenir ou amenizar as possíveis alterações induzidas pela infecção latente por *T. gondii* de cepa avirulenta.

3 OBJETIVOS

3.1 Objetivo Geral

Avaliar o efeito do tratamento com disseleneto de difenila sobre componentes da sinalização purinérgica em tecido hepático e esplênico de camundongos infectados com a cepa ME-49 de *Toxoplasma gondii*.

3.2 Objetivos Específicos

Em camundongos saudáveis e cronicamente infectados por T. gondii, cepa ME49, e tratados com $(PhSe)_2$, se avaliou:

- A histopatologia do tecido hepático e esplênico.

- A atividade de enzimas séricas marcadoras de dano hepático alanina aminotransferase (ALT), aspartato aminotransferase (AST), fosfatase alcalina (FAL) e lactato desidrogenase (LDH).

- A atividade das enzimas NTPDase, 5´nucleotidase e ADA em homogeneizado de tecido hepático e esplênico.

- A atividade das enzimas E-NTPDase, e E-ADA em linfócitos hepáticos.

- A quantificação de nucleotídeos e nucleosídeos purínicos em homogeneizado de tecido hepático.

- A determinação dos níveis de espécies reativas de oxigênio em homogeneizado esplênico.

4 RESULTADOS

Os resultados que fazem parte desta dissertação estão apresentados sob a forma de três artigos. Os itens Materiais e Métodos, Resultados, Discussão e Referências Bibliográficas encontram-se compondo cada manuscrito e representam a íntegra deste estudo.

O artigo 1 foi submetido a revista Purinergic Signalling
O artigo 2 foi submetido a revista Experimental Parasitology.
O artigo 3 publicado na revista Microbial Pathogenesis.

4.1 ARTIGO 1

Diphenyl disselenide modulates nucleotidases, reducing inflammatory responses in the liver of *Toxoplasma gondii* infected mice

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Abstract

The aim of this study was to verify the effect of diphenyl disselenide $(PhSe)_2$ on hepatic nucleotidases and on the concentration of purines in mice infected by *Toxoplasma gondii*. The animals were divided into four groups: the Group A (uninfected), the Group B (uninfected and treated with $(PhSe)_2$), the Group C (infected), and the Group D (infected and treated with $(PhSe)_2$). The inoculation (groups C and D) was performed with 50 cysts of *T. gondii* (ME-49 strain). Mice from groups B and D were treated with 5 μmol kg^{-1} of $(PhSe)_2$. Liver tissue from infected mice showed less severe inflammation, elevated ATP\ADO ratio, elevated NTPDase, 5´nucleotidase and ADA activities compared to the uninfected group (the Group A; $P<0.05$). However, infected and treated mice showed decreased ATP levels and elevated ADO levels, as well as, higher NTPDase and 5´nucleotidase activities and decreased ADA activity in the hepatic tissue compared to the infected group ($P<0.05$). Moreover, the $(PhSe)_2$ treatment of infected mice reduced the hepatic inflammation and showed an immunomodulatory effect on ectonucleotidases of hepatic lymphocytes, which it returned to basal levels. Therefore, chronic infection by *T. gondii* induces hepatic inflammation in mice, and it is possible that purine levels and nucleotidase activities in hepatic tissue are related to the pathogenesis of the infection in this tissue. The treatment with $(PhSe)_2$ was able to reverse the hepatic inflammation in mice chronically infected, possibly due to the modulation of purinergic enzymes that produce an anti-inflammatory profile through the purinergic system in the liver tissue.

Keywords: Diphenyl disselenide, *Toxoplasma gondii*, hepatic lymphocytes, purines, nucleotidases.

Introduction

Toxoplasmosis is a zoonosis caused by *Toxoplasma gondii*, and felines are the definitive hosts. They may release oocysts of the parasite in the environment, contaminating accidental hosts, as humans and animals [1]. Usually healthy infected hosts do not show clinical signs, but this depends on the host immune response and also the strain of the parasite. The ME-49 strain is known for causing chronic toxoplasmosis, in which the parasite causes the latency form of the disease in the brain tissue [2]. However, studies demonstrated an association between infection and hepatic inflammation that leads to cirrhosis [3]. The liver plays an important role in controlling many metabolic routes of the body [4]. For protection, the organ has distinct immune cells such as Kupffer and stellate cells, and lymphocytes responsible for the maintenance of the immune response in the tissue. A health liver has approximately 10^{10} lymphocytes to protect the organ by responding to innumerable inflammatory mediators that induce cytokine release, and activation and recruitment of new immune cells [5]. Hepatic pathologies increase the number of lymphocytes that are activated through inflammatory signaling, which induce these cells to release pro-inflammatory cytokines, changing the immune response of this organ [6]. Besides cellular effectors, the immune system has numerous chemical mediators to perform the signaling of the immune response in the body [7].

The purinergic signaling is characterized by extracellular purine nucleotide interactions with specific cell receptors capable of modulating hepatic functions, leading to cell proliferation and vascularization, acting as an immune mediator [8].

When ATP interacts with receptor type P2, it triggers an increase in hepatocyte proliferation, causing vasodilation in sinusoidal vessels and enabling the immune response [9]. Whereas, when the ATP degradation product adenosine (ADO) interacts with receptor type P1, its induces the release of anti-inflammatory cytokines, enhancing the collagenolytic activity in the liver. In this way, ADO exerts anti-fibrotic effect and develop a protective cellular environment [9, 10]. Adenosine has a fundamental function in homeostasis of healthy tissue. However, low concentration of this molecule is related to the development of chronic liver diseases [11].

Healthy hepatic cells release low ATP concentrations into the extracellular medium through membrane channels, however this release is increased after stimuli, such as the interaction with inflammatory mediators, stress and cell damage. While low ATP levels have important physiological functions, high extracellular concentration of it can act as a damage-associated molecular patterns (DAMP), inducing pro-inflammatory events through lymphocyte activation [12]. Due to these events, the nucleotide concentration in the extracellular space is regulated by nucleotidases, enzymes expressed on a soluble form in the interstitial tissue or bound in the plasma membrane of the cells, so called ecto-enzymes [13]. This regulation starts with NTPDase that hydrolyses ATP and ADP into AMP. The second phase occurs by 5´nucleotidase, that hydrolyses AMP into Ado. Adenosine deaminase (ADA) is the third regulatory enzyme that deaminates Ado into inosine. Nucleotidase enzymes regulate nucleotide interactions through the increase or decrease of its concentration in the medium, and countless studies have shown the importance of this enzymatic regulation in physiological and pathological processes [14, 15].

Diphenyl diselenide $(PhSe)_2$ is an organoselenium compound with antioxidant, antiviral and immunomodulatory activities [16]. Several *in vivo* experiments show that $(PhSe)_2$ reduces the levels of pro-inflammatory cytokines and reactive species in various pathologies, however, the true mechanism for its protective and anti-inflammatory action still not fully understood [17, 18]. Studies indicated that $(PhSe)_2$ reacts with thiol groups in the tertiary protein structure and this interaction modulates the enzymatic activity and affinity to substrates, causing alterations on important metabolic pathways, and this is possibly the molecular mechanism of action [19].

In the chronic phase of toxoplasmosis of immunocompetent mice, the parasite is latent in the brain, but the infection is still able to induce damage to other tissues such as the liver. It is possible that components of the purinergic system are involved in the pathology of *T. gondii* infection, whereas the damage caused to the body is due to a pro-inflammatory signaling in non-infected tissues. Therefore, the aim of this study was to investigate the concentration of purines and the activity of nucleotidases in hepatic tissue of mice infected by *T. gondii* treated with diphenyl disselenide $(PhSe)_2$, as well as the effect of this treatment on ectonucleotidase activity in hepatic lymphocytes, which act as a biomarker of the immune response.

Materials and Methods

Chemicals

The substrates ATP, ADP, AMP, Ado, Coomassie Blue, $(PhSe)_2$ and Ficoll-Hypaque (1.077 g/mL) were obtained from Sigma Chemical Co. (St. Luis, MO, USA). All other reagents used in the experiments were of analytical grade with high purity.

Study design

Forty 60-day-old Swiss albino mice, weighing approximately 25-30g were used in the experiments. The *T. gondii* cystogenic strain (ME-49) used was maintained by consecutive passages in the brain of mice every 30 days. The animals were divided into four groups with 10 mice each: the Group A (uninfected), the Group B (uninfected treated with $(PhSe)_2$), the Group C (infected), and the Group D (infected treated with $(PhSe)_2$). Twenty mice (Groups C and D) were infected orally with 250 µL of brain homogenate containing 50 parasitic cysts of *T. gondii*. Mice from groups B and D were treated on days 1 and 20 post-infection (PI) with 5 µmol kg^{-1} of $(PhSe)_2$ by subcutaneous injection [19].

Sampling

Thirty days PI all animals were anesthetized in an isoflurane chamber. For seric markers, blood samples were collected by cardiac puncture without anticoagulant, and sera samples were obtained after centrifugation at 3000 rpm for 15 min. Livers were removed and divided into four parts with approximately 3g each. Two parts were used to histopathology and lymphocyte isolation. Another part was used for the determination of purine levels, and for that, the sample was gently homogenized in sterile flasks, and the resultant supernatant was mixed with 0.6M perchloric acid and 1M potassium hydroxide, as described previously [20]. And the last fragment was manually homogenized in 10mL of physiological solution (PS) with a syringe plunger, and centrifuged at 1500 rpm for 15 minutes. The supernatant was removed and used for hepatic lymphocyte isolation. Protein was measured by the Comassie Blue method according to Bradford [21] using serum albumin as the standard.

Histopathology

At the necropsy, tissue samples were collected from the right hepatic lobes, fixed in 10% buffered formalin in 0.1M phosphate buffer. The fixed tissue was dehydrated and embedded in paraffin. Tissue sections were stained with haematoxylin and eosin (H&E) for histopathological examination.

Seric markers of hepatic damage

Seric activities of alanine aminotransferase (ALT), aspartate aminotransferase (AST), alkaline phosphatase (ALP), and lactate dehydrogenase (LDH) were evaluated using commercial kits (Analisa®).

Hepatic purine levels

ATP and its breakdown products were analyzed by HPLC according to Voelter et al [21]. The proteins were denaturated using 0.6 mol/L of perchloric acid. All samples were centrifuged (16,000 x g for 10 min at 4ºC), supernatants were neutralized with 4.0N KOH, and clarified with a second centrifugation (16,000 x g for 30 min at 4ºC). After the second centrifugation, the supernatants were collected and centrifuged again (16,000 x g for 30 min at 4ºC). Aliquots of 20 µL were applied to a reversed-phase HPLC (LC-20AT model, Shimadzu, Kyoto, Japan) using a C18 column (Ultra C18, 25 cm x 4.6 mm x 5 µm, Restek – USA). The elution was carried out applying a linear gradient from 100% solvent A (60 mM KH2PO4 and 5 mM of tetrabutylammonium chloride, 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). Mobile phases were filtered through a 0.22 µm Millipore filter prior to analysis, and all the reagents were HPLC

grade. The amount of purines and metabolic residues were measured by absorption at 254 nm. The retention time of the standards was used as a parameter for identification and quantification by comparison of the peak area. Purine levels were expressed as nmol of different compounds per g of tissue.

Isolation of hepatic lymphocytes

Hepatic lymphocytes were isolated from approximately 3g of liver tissue carefully homogenized in PBS (phosphate buffer solution), and separated on Ficoll-Hypaque density gradients as previously described by Doleski et al [22].

E-NTPDase activity in hepatic lymphocytes

Activity of E-NTPDase in hepatic lymphocytes was previously described by Doleski et al [22]. Twenty µL of intact lymphocytes were added to the reaction medium containing 50 mM Tris-HCl buffer (pH 8.0), supplemented with 0.5 mM $CaCl_2$, 120 mM NaCl, 5 mM KCl and 60 mM Glucose in a final volume of 200 µL. The reaction was initiated by the addition of the substrate (ATP or ADP) at a final concentration of 2.0 mM in 37ºC, and stopped after 40 min of incubation with 200 µL of 10% trichloracetic acid (TCA). The samples were chilled on ice and the amount of Pi released was measured by the method of Chan et al [23]. Enzymatic activities were expressed as nmol of Pi/min/mg protein.

NTPDase and 5´nucleotidase activities in liver homogenates

NTPDase and 5´nucleotidase activities in liver homogenates were determined using the method described by Rosemberg et al [24], modified by Doleski et al [25]. First, liver homogenates were centrifuged at 2500 rpm for 10 min to remove possible

impurities. The reaction mixture for NTPDase activity contained 1mM of ATP or ADP as substrate, 5 mM $CaCl_2$, and 50 mM Tris-HCl (pH 8.0). For 5´nucleotidase, the reaction mixture contained 10 mM of AMP as substrate, 5 mM $MgCl_2$ and 50 mM Tris-HCl (pH 7.5). The reaction mixtures were incubated with approximately 1.0 mg of homogenized protein at 37 ºC for 30 min on a final volume of 0.2 mL. The reaction was stopped by the addition of 200 µL of 10% TCA. The samples were chilled on ice and the amount of Pi liberated was measured by the green malaquite method [23]. Enzymatic activities were expressed as nmol of Pi/min/mg protein.

E-ADA activity in hepatic lymphocytes and homogenates

E-ADA activities were measured spectrophotometrically, based on the direct formation of ammonia produced when the enzyme acts on adenosine [26]. The reaction initiated by the addition of the substrate (adenosine) to a final concentration of 21 mM/L and incubated for 60 minutes at 37 ºC with 25 µL of liver homogenates or intact hepatic lymphocytes. The reaction was stopped by the addition of 1.5 mL of 106/ 0.16 mM phenol–nitroprusside to the reaction mixture, which was immediately mixed with 1.5 mL of 125/11 mM alkaline-hypochloride (sodium hypochlorite). Released ammonia reacts with alkaline-hypochlorite and phenol in the presence of a catalyst-sodium nitroprusside to produce indophenol (a blue color), and the concentration of ammonia is directly proportional to the absorbance of indophenol read at 620 nm. Ammonium sulphate of 75 µM was used as ammonium standard. The value of E-ADA activity was expressed as U/mg of protein.

Statistical analysis

All experiments were performed in triplicate. Data represent mean +- standard deviation. Differences were assessed by two-way analysis of variance (ANOVA),

followed by Tukey post-hoc test. The values obtained in the assays were considered statically different for $P<0.05$.

Results

Histopathology

Animals infected by the strain ME-49 of *T. gondii* showed bristling hair and weight loss (groups C and D), and the infection was confirmed by histopathology, i.e., there were parasitic cysts in the brain of all infected animals in both groups. Mice of groups A and B did not show histological lesions in the liver. All mice of the Group C showed mild inflammatory foci in the liver (Figure 1); and alterations of these kind were observed in only four mice of the Group D.

Seric markers of hepatic damage

Seric AST, ALT, LDH and ALP activities did not show significant difference between groups by two-way ANOVA (Table 1).

Hepatic purine levels

The levels of purine in liver samples are shown in Table 2. The $(PhSe)_2$ treatment (the Group B) caused an increase of 60.8% on ATP concentrations, and an decrease of 56% in ADP concentrations in the liver. Moreover, AMP levels did not show difference compared to the control group (the Group A), but interestingly, there was an increase of 236% in Ado concentrations. Infected group (the Group C) showed a great increase (150%) in ATP concentration and a decrease of 84.6% for ADP, 21% for AMP, and 51.2% for Ado. $(PhSe)_2$ treatment used in infected mice (the Group D) decreased (24.3%) ATP concentration compared to infected mice (the

Group C), but when compared to the control group (the Group A) it showed an increase of 88.8%. The group D showed an increase of 35% for AMP, and 81.2% decrease of ADP levels, while Ado concentration did not show significant difference compared to the control or infected groups.

E-nucleotidases in hepatic lymphocytes

ATP and ADP hydrolysis by E-NTPDase in plasma membrane of hepatic lymphocytes samples are shown in Fig. 2. E-NTPDase activity for ATP substrate decreased 22%, and it did not show significant difference for ADP substrate in the Group B. Animals of the Group C showed an increase of 49.5%, and 56.6% in E-NTPDase activity for ATP and ADP compared to uninfected animals (the Group A), respectively. Mice of the Group D did not show significant differences on E-NTPDase activity for both substrates (ATP and ADP) compared to the control group (the Group A).

E-ADA activity in the plasma membrane of lymphocytes is shown in Fig. 3. Mice of the Group B did not show significant difference for E-ADA activity compared to the Group A, but the infected group (the Group C) showed a decrease of 70.6% in this enzyme. The Group D (infected and treated with $(PhSe)_2$) did not show a significant difference compared with the Group A.

Hepatic nucleotidases

Nucleotidase activities in liver homogenates are shown in Table 3. NTPDase activities in the Group B showed an increase of 46.52% and 46.27% to ATP and ADP hydrolysis, respectively. An increase of 26% to AMP hydrolysis by 5´nucleotidase activity, and a decrease of 44.4% in ADA activity compared to the control (the Group

A). Infected animals (the Group C) showed increased nucleotidase activities such as NTPDase (37.3% and 35.2% to ATP and ADP hydrolysis, respectively), and 5´nucleotidase (44.8% to AMP hydrolysis), and ADA activity (50.5%) compared to the Group A. However, $(PhSe)_2$ treatment of infected mice (the Group D) increased NTPDase activity (93.2% and 89.6% in ATP and ADP hydrolysis, respectively), as well as 5´nucleotidase activity (57.6%). ADA activity did not show significant differences compared to uninfected (the Group A) or infected animals (the Group C).

Discussion

T. gondii infection was confirmed by histophatological examinations of the brain that showed parasitic cysts with bradyzoites. These findings are common for *T. gondii* ME-49 strain, since it develops chronic toxoplasmosis during the parasitic latent phase [1,2]. Histological examinations of the liver of infected animals revealed a slight inflammation, and $(PhSe)_2$ treatment it in 60% of the animals. Serological analyses for markers of hepatic injury (ALT, AST, LDH, ALP) did not confirm tissue damage, possibly due to a slight inflammatory process histologically found, which did not cause, cell lysis, and consequently, the release of such markers in the bloodstream.

Firstly, it is worthy to note that $(PhSe)_2$ is a Janus-faced molecule, so its pharmacological effect or toxicity depends on the concentration used. In this study, low concentration of this compound was used (5 μmol kg-1) subcutaneously as previously reported [17]. Treatment with $(PhSe)_2$ in healthy mice caused ATP increase compared to healthy untreated animals. The mechanism to explain this result is unknown, however, it is possible that the $(PhSe)_2$ stimulates a constant release of ATP by hepatic cells, and by a compensatory effect, it raises hepatic

NTPDase activity in order to control the levels of this phosphate nucleotide in the tissue. The powerful anti-inflammatory action of $(PhSe)_2$ was confirmed on the purinergic system by a significant increase in adenosine concentration in liver tissue, an important endogenous anti-inflammatory molecule [14], which may be associated with the removal of inflammatory infiltrates in liver found in the majority of treated animals. The mechanism for this effect was associated with an increase in 5'nucleotidase activity and decrease in the ADA activity, stimulating the production and inhibiting adenosine degradation. It is known that the $(PhSe)_2$ is able to interact with thiol groups, altering many metabolic processes, including enzymatic activity and its affinity for substrates, which can act as a possible pharmacological or toxicological mechanism [19]. Our hypothesis is that the $(PhSe)_2$ acts by two ways: (1) increasing the concentration of nucleotides, such as ATP, and modulating by a compensatory manner the enzymatic activity, or (2) interacting covalently with the enzymes in question, producing an anti-inflammatory effect through the purinergic signaling pathway. To evaluate the effect of $(PhSe)_2$ on modulating the immune response, ecto-nucleotidases were evaluated in hepatic lymphocytes since they act as biomarkers of the immune response, i.e. enzymatic activity in these cells depends on the immune microenvironment. Liver lymphocytes showed a low decrease in the E-NTPDase activity after $(PhSe)_2$ treatment of healthy mice, possibly by stimulating the interaction of ATP with P2 receptors, enhancing the immune activity in a controlled manner, which demonstrates an immunomodulatory effect of $(PhSe)_2$ [12].

Infected mice showed the highest concentration of ATP, and it is know that high ATP levels is usually presented as a DAMP related to the inflammatory process in the tissue [9]. It is possible that mediators present after *T. gondii* infection stimulate a cellular release of ATP by transport channels, in order to perform a local pro-

inflammatory signaling. High concentrations of ATP can activate the immune system leading to an increase in leukocyte recruitment, production of ROS and pro-inflammatory cytokines which, in the long term, can damage and harm liver functions. In an attempt to reduce high ATP levels, the tissue shows a large increase in NTPDase activity, although it is still unable to reduce exposure to the nucleotide. It is also possible to assure that *T. gondii* infection may increase all nucleotidases analyzed in this study, such as ADA and 5'nucleotidases, that in turn, eventually reduces the concentration of AMP and Ado in the tissue. The nucleoside Ado acts to protect and reverse liver damage, but low interaction of this molecule with the P1 receptor is associated with the development of chronic liver diseases [10]. Thus, we propose that hosts infected by *T. gondii* with reduced interactions by Ado, might be predisposed to cirrhosis and other liver chronic diseases, as previously reported [3]. Ecto-nucleotidase activity in lymphocytes helps to understand this complex mechanism, since increased E-NTPDase activity controls high ATP interaction with P2 receptors in the cell membrane, and decreased E-ADA activity stimulates the interaction of low Ado concentration with its P1 receptors. In this way, lymphocytes try to avoid an exacerbated pro-inflammatory activation triggered by ATP.

Interestingly, $(PhSe)_2$ treatment of infected mice showed a reduction in ATP concentration in liver homogenates. We propose that this effect is related to the high NTPDase activity found in the hepatic tissue of this group, which is enhanced by $(PhSe)_2$ compared to infected untreated mice and, consequently, reduced the nucleotide levels in the tissue. Furthermore, the consecutive increase in NTPDase and 5'nucleotidase activities along with decreased ADA activity in the tissue, resulted in increased adenosine concentration, and possibly, increased interaction with P1 receptors, which may protect the hepatic tissue. This low ratio ATP/adenosine helps

to understand the anti-inflammatory mechanism produced by $(PhSe)_2$, which is known to produce many benefits *in vivo* [16]. $(PhSe)_2$ used to treat *T. gondii* infection was also able to modulate ecto-nucleotidase in lymphocytes to baseline levels, and thus, this fact can be interpreted as a reduction in markers of the immune response due to the re-establishment of the extracellular environment of nucleotides.

The increase in the activity of enzymes responsible for the regulation of extracellular nucleotides during *T. gondii* infection is in agreement with some studies that demonstrated similar results in several parasitic diseases. Experiments carried out by our research group with rats chronically infected by *Fasciola hepatica* demonstrated a considerable increase in the concentration of ATP in the liver and also, in the activity of NTPDase and 5'nucleotidase enzymes [25, 27]. However, unlike this present study, infection by *F. hepatica* decreased ADA activity, helping to maintain and control hepatic damage [28]. Certainly low concentrations of adenosine found in *T. gondii* infection is a bad prognosis of liver disease, and any treatment that may protect affected organs should be encouraged.

Our results demonstrated a change in some components of the purinergic signaling system in the liver of *T. gondii* infected mice, as well as signs of healing through subcutaneous treatment with $(PhSe)_2$. These results help to understand that some mechanisms involved in fighting the infection can be dangerous to the body itself, where the high ratio of ATP/adenosine found in infected mice elucidates one possible mechanism that leads to chronic liver disease in patients with chronic toxoplasmosis. We emphasize that $(PhSe)_2$ treatment has high capacity to elevate adenosine concentration in healthy liver tissue, as well as to re-induce a healthy extracellular nucleotide environment in an inflammatory process. For this, $(PhSe)_2$ is a potential drug that can be used to treat chronic liver diseases, as well as cirrhosis

and fibrosis, however, more studies should be conducted to evaluate its clinical efficacy for chronic diseases.

Compliance with ethical standards

Conflict of interest. Authors declares that she has no conflict of interest.

Ethical approval. This study was approved by the Ethic Committee on Animal Use of the Federal University of Santa Maria (UFSM) under protocol number 7787270815.

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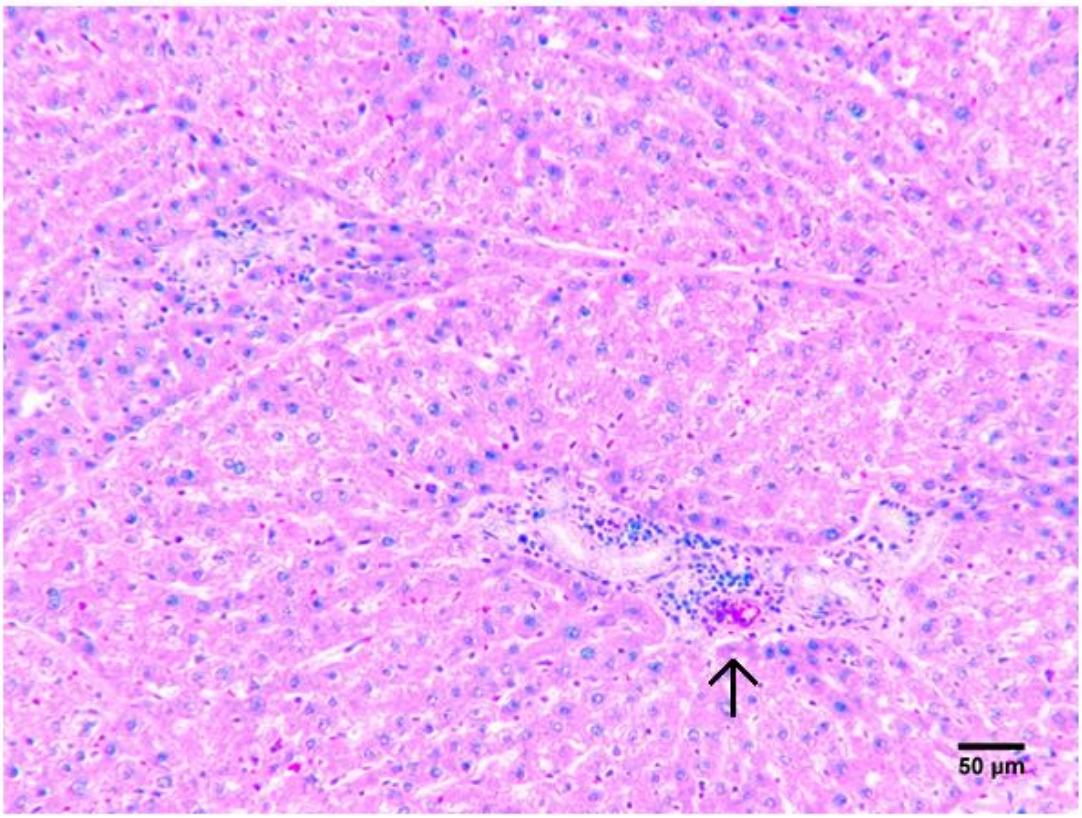

Figure 1: Mice experimentally infected by *Toxoplasma gondii*. Focal inflammatory infiltration in the liver of infected mice (the Group C). H&E staining.

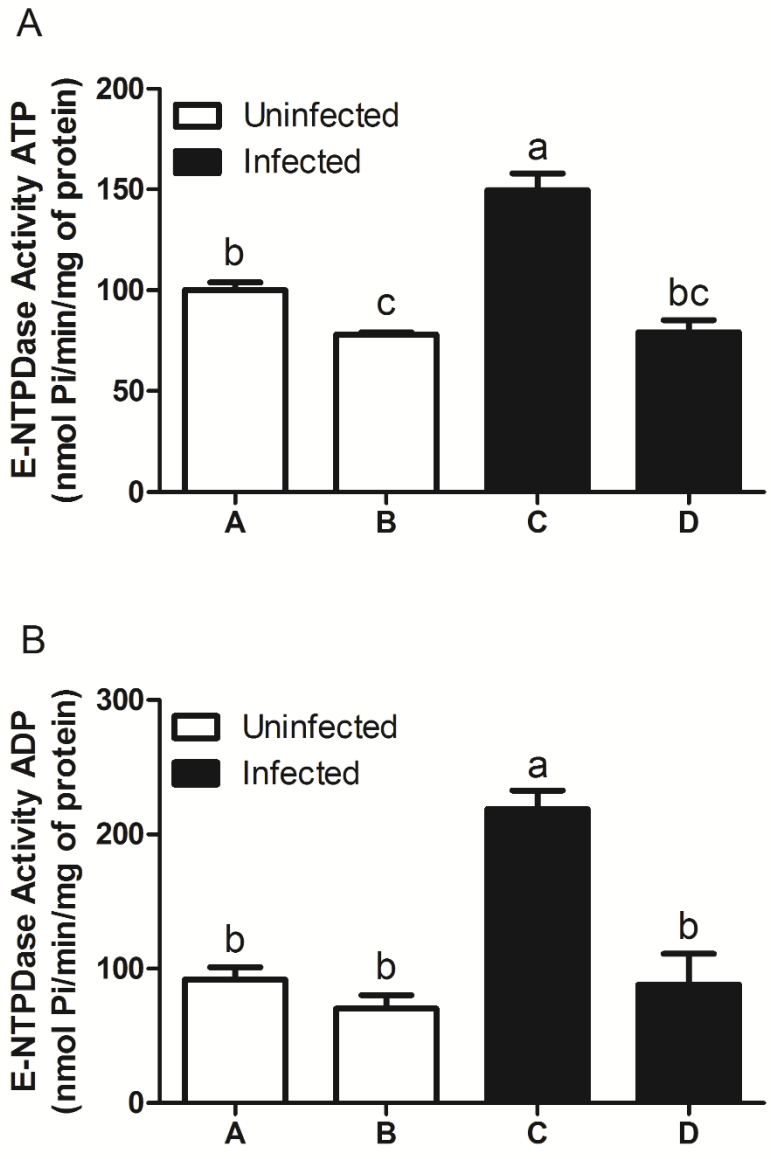

Figure 2: Mice infected by *Toxoplasma gondii* and treated with diphenyl disselenide $(PhSe)_2$. Hepatic lymphocytes E-NTPDase activity to ATP (A) and ADP (B) substrates. Columns represent mean ± SEM (n = 10). Different letters in the same graph denote significance for $P<0.05$ by two way ANOVA followed by Tukey post-hoc test. Note: the Group A (uninfected), the Group B (uninfected and treated with

$(PhSe)_2$), the Group C (infected), and the Group D (infected and treated with $(PhSe)_2$).

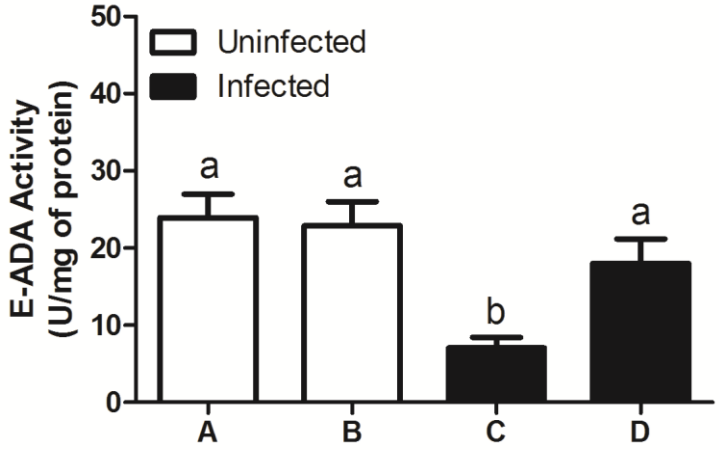

Figure 3: Mice infected by *Toxoplasma gondii* and treated with diphenyl disselenide $(PhSe)_2$. Hepatic lymphocytes E-ADA activity to adenosine (Ado) substrate. Columns represent mean ± SEM (n = 10). Different letters in the same graph denote significance for $P<0.05$ by two way ANOVA followed by Tukey post-hoc test. Note: the Group A (uninfected), the Group B (uninfected and treated with $(PhSe)_2$), the Group C (infected), and the Group D (infected and treated with $(PhSe)_2$).

Table 1: Seric enzymatic activity of hepatic damage markers (alanine aminotransferase (ALT), aspartate aminotransferase (AST), alkaline phosphatase (ALP), and lactate dehydrogenase (LDH)) in mice infected by *Toxoplasma gondii* and treated with diphenyl disselenide $(PhSe)_2$[a]

Markers	Group A	Group B	Group C	Group D	*P*
ALT (U/L)	49.20 ± 2.261	53.74 ± 3.652	70.20 ± 6.948	67.89 ± 8.237	>0.05
AST (U/L)	146.40 ± 16.78	178.70 ± 16.01	189.50 ± 25.44	177.80 ± 13.84	>0.05
LDH (mg/dL)	978.2 ± 117.5	1544 ± 188.7	1420 ± 206.4	1026 ± 95.59	>0.05
ALKP (U/L)	112.0 ± 10.41	150.3 ± 19.45	127.8 ± 26.06	107.7 ± 13.88	>0.05

[a] Results are expressed as mean ± SEM (n = 10). Data were analyzed statistically by two way analysis of variance (ANOVA). Post-hoc comparisons were made using Tukey test and differences were considered significant at $P<0.05$. Note: the Group A (uninfected), the Group B (uninfected and treated with (PhSe)2), the Group C (infected), and the Group D (infected and treated with (PhSe)2).

Table 2
Purine concentrations in the liver of mice infected by *Toxoplasma gondii* and treated with diphenyl disselenide $(PhSe)_2$[a]

Purines	Group A	Group B	Group C	Group D
ATP (nmoles/g)	1.43 ± 0.67[c]	2.30 ± 0.93[b]	3.57 ± 0.47[a]	2.70 ± 0.23[b]
ADP (nmoles/g)	10.2 ± 3.41[a]	4.49 ± 1.87[b]	1.57 ± 0.78[c]	1.92 ± 1.02[c]
AMP (nmoles/g)	136.3 ± 25.7[b]	132.1 ± 28.4[b]	107.5 ± 14.9[c]	184.1 ± 45.4[a]
Adenosine (nmoles/g)	19.8 ± 4.8[b]	66.5 ± 14.9[a]	9.66 ± 3.7[c]	13.4 ± 5.1[bc]

[a] Results are expressed as the mean ± SEM (n = 10). Data are analyzed statistically by two way analysis of variance (ANOVA). Post-hoc comparisons were made using Tukey test, and differences were considered significant at a $P<0.05$. Groups with equal letters in the same line, do not differ statistically. Note: the Group A (uninfected), the Group B (uninfected and treated with $(PhSe)_2$), the Group C (infected) and the Group D (infected and treated with (PhSe)2).

Table 3
Hepatic NTPDase (nmol of Pi/min/mg protein), 5´nucleotidase (nmol of Pi/min/mg protein), and ADA (U/mg of protein) activity in infected mice by *Toxoplasma gondii* and treated with diphenyl disselenide $(PhSe)_2$ [a]

Nucleotidases	Group A	Group B	Group C	Group D
NTPDase activity				
ATP substratum	5.341 ±0.735[c]	7.826 ±0.555[b]	7.334 ±0.178[b]	10.320 ±0.420[a]
ADP substratum	5.469 ±0.572[c]	8.003 ±0.559[b]	7.395 ±0.256[b]	10.370 ±0.354[a]
5´nucleotidase activity				
AMP substratum	5.362 ±0.203[c]	6.763 ±0.428[b]	7.766 ±0.127[ab]	8.449 ±0.143[a]
ADA activity				
Ade substratum	1.285 ±0.108[b]	0.714 ±0.093[c]	1.934 ±0.079[a]	1.641 ±0.190[ab]

[a] Results are expressed as mean ± SEM (n =10). Data were analyzed statistically by two way analysis of variance (ANOVA). Post-hoc comparisons were made using Tukey´s test and diference were considered significant at a $P<0.05$. Groups with letters equal the same line not statistically different. Note: Group A (uninfected), Group B (uninfected and treated with (PhSe)2), Group C (infected) and Group D (infected and treated with (PhSe)2).

4.2 ARTIGO 2

Toxoplasmosis treatment with diphenyl diselenide in infected mice modulates the activity of purinergic enzymes and reduces inflammation in spleen

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ABSTRACT

The protozoan *Toxoplasma gondii* may cause chronic infection in the brain tissue, which induces a systemic pro-inflammatory profile in the host. Chronic infections can induce numerous physiological changes in the body, such as alterations in the immune and oxidative profiles. Diphenyl diselenide $(PhSe)_2$, an organoselenium compound, show antioxidant and immunomodulatory activities. So, the aim of this study was to investigate the activity of purinergic enzymes and reactive oxygen species (ROS) in serum and spleen of mice chronically infected by *T. gondii,* untreated and treated with $(PhSe)_2$. For this experiment, forty mice were divided into four groups: Group A (healthy mice), Group B (healthy mice treated with $(PhSe)_2$), Group C (infected mice) and Group D (infected mice treated with $(PhSe)_2$). Group C and group D were infected through the inoculation of 50 cysts of *T. gondii*, strain ME-49. Groups B and D were treated subcutaneously with 5 μmol kg^{-1} of $(PhSe)_2$ at days 1 and 20 post-infection (PI). Chronic *T. gondii* infection induced splenomegaly and physiological changes in the spleen and raised inflammatory markers in histology, ROS levels and the activity of purinergic enzymes activity such as NTPDase, 5´nucleotidase and adenosine deaminase (ADA). In serum, the infection increased 5´nucleotidase and ADA activities. $(PhSe)_2$ *per se* has managed to decrease ROS levels and ADA activity and increase NTPDase and 5´nucleotidase in spleen. In infected mice, treatment with $(PhSe)_2$ was able to reverse the splenomegaly, reduce histological inflammatory markers, ROS levels and ADA activity in the spleen. Our results prove that chronic toxoplasmosis can induce splenomegaly, heightens ROS levels and purinergic enzyme activity in mice. These results suggest that $(PhSe)_2$ is an potential therapy for spleen changes found in chronic *T. gondii* infection.

Keywords: Diphenyl diselenide, oxidative stress, purinergic enzymes, spleen, *Toxoplasma gondii*.

1. INTRODUCTION

Toxoplasmosis is a disease caused by *Toxoplasma gondii*, protozoan that infect intestinal cells of felines in order to complete their biological cycle. Mammals and others warm-blooded animals can also be infected, however they display a different pathology which will depend on the parasite strain, and the immune status of the host (Dubey, 2007). Immunocompetent animals, when infected with avirulent strains of *T. gondii* experience a short acute phase where the parasite may be either eliminated from the host or quickly form small cysts in the brain tissue. This latent infection by *T. gondii* is denominated chronic toxoplasmosis, and is considered an asymptomatic disease (Fergunson and Hutchinson, 1987). Some studies reveal that the small brain inflammation connected to the cysts can induce neurological disorders in rodents (Machado et al., 2016) and humans (Ene et al., 2016). Moreover, the infection reduces serum levels of testosterone (Eslamirad et al., 2013) and increases the levels of pro-inflammatory cytokines, as INF-γ, to prevent a reactivation of the parasites (Barbosa et al., 2014). This systemic inflammatory profile may induce physiological changes in the body, as in the liver and spleen; organs that exhibit immune function and display pro-inflammatory cells in *T. gondii* infection (Nan et al., 2011). Spleen has two different tissues; (1) a red pulp that consists in the reticuloendothelial system responsible to remove damaged erythrocytes from the bloodstream, and (2) a white pulp, that act as a peripheral lymph node, and presents important immune functions such as storing lymphocytes and producing platelets. In inflammatory processes, the tissue can induce lymphocytes to produce and release

antibodies and cytokines (Kraal and den Haan, 2016). In some pathologies, the immune response can increase the size of the spleen,a condition called splenomegaly, which can result in tissue damage and dysfunction such as spleen enlargement and frailty. (Kraal and den Haan, 2016). Besides the systemic pro-inflammatory profile developed against the parasite, little is known about the status and functions of the spleen in the chronic infection by *T. gondii*.

The interaction of extracellular purines with specific cell receptors is denominated purinergic signaling, which is involved in numerous physiological functions (Ralevic and Burnstock, 1998). Stimulation of leukocytes through P2 purinergic receptors with ATP (adenosine triphosphate) may induce a pro-inflammatory profile in tissues. This interaction can lead to leukocyte activation and increased cell proliferation, reactive oxygen species (ROS) and cytokines production (Burnstock and Verkhratsky, 2010; Kuroki and Minakami, 1989). Therefore, the interaction of adenosine (ADO) with P1 receptors induces the antagonist effects of ATP. ADO is produced after tissue damage in order to reverse the pro inflammatory profile and recover damaged tissues (Bours et al., 2006; Ward et al., 1988). Purine compounds can be released by cells through membrane channels, which can be stimulated in inflammatory processes (Junger, 2011). In order to regulate the concentration of purines, cells express nucleotidases, which metabolize phosphate nucleotides such as ATP in their nucleosides derivatives (Zimmermman, 2010). NTPDase is responsible for hydrolyzing ATP and ADP to AMP, while 5'nucleotidase hydrolyzes AMP to the nucleoside ADO. The enzyme responsible for metabolizing ADO is a nucleosidase named ADA (adenosine deaminase), which deaminates ADO into inosine. These enzymes control the levels of purines, playing an important role in

physiological processes, and are altered in inflammatory diseases (Schetinger et al., 2007).

Chronic diseases may cause cell damage and oxidative stress, which over time can cause irreversible tissue damage, thus treatments with antioxidant compounds have been proposed to reverse the harm that may occur (Hussain et al., 2016). In this way, the organoselenium compound, Diphenyl diselenide $(PhSe)_2$, which possesses antioxidant and immunomodulatory activities, may be an effective therapy against chronic diseases (Nogueira et al., 2014). Studies reveal that $(PhSe)_2$ can induce an anti-inflammatory profile and protects tissues by reducing pro-inflammatory cytokines and ROS production (Leite et al., 2015; Nogueira and Rocha, 2010). Chronic toxoplasmosis is considered a silent infection in immunocompetent patients, which show no symptoms or signs of major damage to the body in the short term. In this study, we aimed to analyze the activities of purinergic enzymes and the levels of ROS in serum and spleen of immunocompetent mice infected by *T. gondii* strain ME49 in an attempt to evaluate possible physiological changes induced by the chronic infection with this parasite. Moreover, we present the antioxidant and immunomodulatory compound $(PhSe)_2$ as a potential treatment to the changes caused by this infection.

2. MATERIALS AND METHODS

2.1. Chemicals

Purinergic substrates ATP, ADP, AMP, ADO as well $(PhSe)_2$ were obtained from Sigma Chemical Co. (St. Luis, MO, USA). All other reagents used in the experiments were of analytical grade and of highest purity.

2.2. *Toxoplasma gondii* strain

Cystogenic ME-49 strains of *T. gondii* stored in liquid nitrogen were used to inoculate one single *Swiss* mouse. Thirty-two days after the infection, the mouse was euthanized the brain containing cysts of *T. gondii* were homogenized in saline solution and orally inoculated in another three mice; this procedure was done in order to reactivate the parasite´s virulence. The mice were then euthanized for brain collection 30 days post-infection (PI), and parasitic cysts counted and separated in order to infect the other animals of the experiment.

2.3. Experimental design

For this experiment, we used forty female mice weighing an average of 25±5g. The animals were divided into four groups (A, B, C and D) with ten animals each. Groups C and D were orally infected with 0.25 mL of brain homogenate containing 50 cysts of *T. gondii*. Animals from group B and C were subcutaneously treated with $(PhSe)_2$. For the subcutaneous treatment, $(PhSe)_2$ was dissolved in 0.1% dimetilsulfoxide and administered on the first and 20^{th} day post-infection (PI), using doses of 5 μmol kg^{-1} (Barbosa et al., 2014). After 30 days PI, all animals were euthanized in an isoflurane chamber, the spleens were dissected and blood samples were collected through cardiac puncture and allotted in tubes without anticoagulant to obtain serum samples.

2.4. Histopathology

Tissue samples were collected from the right hepatic lobes at necropsy and fixed in 10% buffered formalin in 0.1M phosphate buffer. The fixed tissue was then dehydrated and embedded in paraffin. Tissue sections were stained with

haematoxylin and eosin (H&E) for histopathological examination.

2.5. Spleen weight

After dissection, the spleen was cleaned with saline and weighed in an analytical balance. To prevent possible variations in spleen weight, the spleen weight (g) to body weight (g) ratio was employed.

2.6. NTPDase and 5´nucleotidase activity in serum

NTPDase and 5´nucleotidase activities in serum were determined as previously described by Oses et al. (2004). A reaction mixture containing 3mM of ATP or ADP as substrate and 112,5 mM Tris-HCl (pH 8.0) was used to measure the NTPDase activity, while for 5´Nucleotidase the reaction mixture consisted of 3 mM of AMP as substrate and 100 mM Tris-HCl (pH 7.5). The reaction mixtures were incubated with approximately 1.0 mg of homogenized protein at 37ºC for 40 minutes in a final volume of 200 µL. The reaction was stopped by the addition of 200 µL of 10% trichloroacetic acid (TCA). All samples were centrifuged at 5000x g for 5 min to eliminate precipitated protein and the supernatant was use for the colorimetric assay. The samples were chilled on ice and the amount of inorganic phosphate (Pi) liberated was measured by the method of Chan et al. (1986). In order to correct non-enzymatic hydrolysis, the homogenate was added to control samples after the reaction was stopped with TCA. Enzyme activities were expressed as ηmoles of Pi released per minute per milligram of protein (ηmol of Pi/min/mg protein).

2.7. NTPDase and 5´nucleotidase activity in spleen homogenized

NTPDase and 5´nucleotidase activities in homogenized spleen were determined using a methodology described by Rosemberg et al. (2010), modified by Doleski et al. (2016). First spleen homogenates were centrifuged at 2500 rpm for 10 minutes to remove impurities. The reaction mixture for measuring the NTPDase activity contains 1mM of ATP or ADP as substrate, 5 mM $CaCl_2$ and 50 mM Tris-HCl (pH 8.0), while for of 5´Nucleotidase activity a reaction mixture containing 10 mM of AMP as substrate, 5 mM $MgCl_2$ and 50 mM Tris-HCl (pH 7.5) was used. The reaction mixtures were incubated with approximately 1.0 mg of homogenized protein at 37ºC for 30 minutes in a final volume of 200 µL. The reaction was stopped by the addition of 200 µL of 10% trichloroacetic acid (TCA). The samples were chilled on ice and the amount of inorganic phosphate (Pi) liberated was measured by the method of Chan et al. (1986). Enzyme activities were expressed as ηmoles of Pi released per minute per milligram of protein (ηmol of Pi/min/mg protein).

2.8. ADA activity in serum and spleen homogenized

ADA activity was measured in serum and homogenized spleen samples according to Giusti and Gakis (1971). The methodology is based on the direct measurement of ammonia produced once the enzyme acts in presence of ADO, A volume of 25 µL of serum or spleen homogenate was used. The enzymatic reaction was started by addition of 500 µL of adenosine (21 mM) as substrate. The reaction was stopped by adding 1.5 mL of 106/0.16 mM phenol-sodium nitroprusside solution to the reaction mixture, which was immediately mixed with 1.5 mL of 125/11 mM alkaline-hypochlorite solution. Ammonium sulfate of 75 µM was used as ammonium standard. All experiments were performed in triplicate and the values of ADA activity serum and spleen homogenized were expressed in U/L/mg of protein.

2.9. ROS levels in serum and spleen homogenized

2´-7´-Dichlorofluorescein (DCFH) levels were determined as an index of the peroxide production by the cellular components (Halliwell and Gutteridge, 2007). Serum (10µL) and spleen homogenate (10µL) samples were added to a medium containing 10mM Tris-Hcl buffer (pH 7.4) and 1mM of DCFH-DA. The results were expressed by UDCF/mg protein.

2.10. Statistical analysis

All experiments were performed in triplicate. Data represent mean ± standard errors mean (SEM). Differences were assessed by two way analysis of variance (ANOVA), followed by Tukey´s post-hoc test. The values obtained in the assays were considered statistically different when $P<0.05$.

3. RESULTS

3.1. Infection course, spleen weight and histopathology

T. gondii chronic infection was confirmed by the presence of cysts in brain tissue (Figure 1a). The spleen weight to body weight ratios are presented in Figure 2. Group B did not show significant difference when compared with the control group (Group A). However, untreated infected mice (Group C) shown an increase of 92.20% when compared with the Group A. The treatment with $(PhSe)_2$ was able to decrease the ratio in 53.44% when compared with the untreated infected mice (Group C) and reverse the spleen weight to control values (Group A).

The histological analysis is shown in Figure 1b. Control mice (Group A) and treated $(PhSe)_2$ control group (Group B) did not show histological lesions in the

spleen. Presence of diffuse pulp hyperplasia was observed in untreated infected mice (Group C) and infected mice treated with $(PhSe)_2$ (Group D). Untreated infected mice (Group C) showed mild and diffuse spleen congestion. Presence of moderate to large numbers of megakaryocytes was observed in the spleen of untreated infected mice (Group C). The animals of group D, showed megakaryocytes countings ranging from mild to moderate.

3.2. ROS levels in serum and spleen homogenate

ROS levels are presented in Figure 3. Seric ROS levels (Figure 3a) did not differ significantly between the groups. Splenic ROS levels (Figure 3b) are decreased in 36.31% in Group B compared with the Group A (control group). However, an increase of 28.75% in spleen ROS levels in Group C when compared with the Group A. The treatment of infected mice (Group D) revealed a decrease of 69.27% of spleen ROS levels when compared with the control group (Group A).

3.3. NTPDase and 5´nucleotidase activity in serum

NTPDase and 5´nucleotidase activities in serum are displayed in Figure 4. NTPDase activity did not vary significantly among the groups. 5´nucleotidase in Group B did not show significant difference when compared to the Group A (control mice), However, an increase of 70.49% in 5´nucleotidase activity was observed in infected mice (Group C) when compared with Group A ($P<0.05$).

3.4. NTPDase and 5´nucleotidase activity in spleen homogenate

NTPDase and 5´nucleotidase activities in spleen homogenate are demonstrated in Figure 5. NTPDase activities in Group B show an increase of 41.75% and 31.92%

in ATP and ADP hydrolysis by NTPDase, respectively, compared group A. An increase of 17.16% to AMP hydrolysis by 5´nucleotidase activity in Group B, were observed when compared with control group (Group A). Infected group (Group C) shows an increase in the activity of all nucleotidases, being that an increase of 67.08% and 51.82% in ATP and ADP hydrolysis by NTPDase, respectively, and 38.91% to AMP hydrolysis by 5´nucleotidase compared to Group A. However, the treatment of $(PhSe)_2$ in infected animals (Group D) showed greater NTPDase, with an increase of 103.26% and 77.45% in ATP and ADP hydrolysis by NTPDase, respectively, when compared with uninfected animals (Group A).

3.5. ADA activity in serum and spleen homogenized

ADA activity in serum is shown in Figure 6.A. Serum ADA activity did not show significant difference in Group B when compared with the control group (Group A). An increase of 200.37% in ADA activity is found in untreated infected mice (Group C) when compared with control mice (Group A). Treated infected mice (Group D) showed an increase of 189.50% in ADA activity when compared with the control group (Group A), no a significant difference was found when compared with infected animals (Group C).

ADA activity in spleen homogenate is shown in Figure 6.B. Infected mice (Group C) showed an increase of 40.87% in ADA activity when compared with the control group (Group A). Treated control mice (Group B) and treated infected mice (Group D) showed a reduction of 28.96% and 38.09%, respectively, when compared with the untreated control group (Group A). Moreover, the Group B showed a reduction of 55.88% in ADA activity when compared with the Group C.

4. DISCUSSION

ME49 is an avirulent and cystogenic strain of *T. gondii*, thus is common to infect mice with this strain for experimental induction of chronic toxoplasmosis. 30 days PI, the chronic infection was confirmed through the histological analysis that revealed small cysts in the brain of infected mice. Considering that chronic toxoplasmosis induces a pro-inflammatory profile in the host, we evaluated potential systemic and splenic physiological changes that may occur during this condition. Our results revealed that serum and spleen show different physiologic responses to the chronic infection.

Elevated ROS levels in serum can be an indicative of oxidative stress throughout the body (Hussain et al., 2016). This parameter is increased in parasitic infection, as visceral leishmaniasis (Gupta et al., 2009) as well as acute infection by *Trypanosoma cruzi* (Melo et al., 2014) and *T. gondii* (Barbosa et al., 2014). However, serum ROS levels did not change in the chronic infection by *T. gondii*. NTPDase activity is considered an inflammatory marker in serum (Zimmermman, 2010), and is increased in some parasitic diseases, as *Fasciola hepatica* (Doleski et al., 2016) and *Anaplasma marginale* (Doyle et al., 2016) infection. In the same manner of ROS levels, serum NTPDase activity did not change in the *T. gondii* chronic infection. These results match the silent pattern of the chronic toxoplasmosis in serum, which shows high levels of pro-inflammatory cytokines but no increase in damage markers. *T. gondii* infected group showed elevated 5´nucleotidase activity, which can be related with the increase of ADO levels in serum, but the increased ADA activity found in this group, refutes this theory. ADA is the nucleotidase responsible for the reduction of ADO levels whose elevated ADA activity is considered a marker for leukocytes activation (Bours et al., 2006). High ADA activity in serum can improve the

pro-inflammatory profile by reducing ADO signaling in blood immune cells. Thus, it is possible that the high serum 5´nucleotidase activity found in this study is a physiologic response to the elevated ADO deamination by the high ADA activity in serum of infected mice.

Eventhough only slight changes were observed in serum of infected mice, the analysis of splenic tissue showed a pro-oxidative and pro-inflammatory profile, which were reversed by the treatment with $(PhSe)_2$. However, before discussing the changes induced by the chronic infection and it's the reversal by the treatment, we would like to point out important *per se* effects of subcutaneous $(PhSe)_2$ treatment in healthy mice. $(PhSe)_2$ is a lipophilic compound that shows high affinity for tissues (Nogueira et al., 2004), and its antioxidant action has already been described in literature (Nogueira and Rocha, 2010). Thus, it is possible to observe its effects on splenic tissue, and its antioxidant action was confirmed, in this study, through the reduction of splenic ROS levels in treated healthy mice. Moreover, the compound *per se* was able to modulate purine enzymes, perhaps to increase the extracellular ADO levels by increasing the NTPDase and 5´nucleotidase activities and decreasing ADA activity. This enzymatic profile induced by $(PhSe)_2$ may increase this anti-inflammatory nucleoside, and reveal a possible action mechanism for the $(PhSe)_2$ immunomodulatory effects.

Chronic toxoplasmosis prompts the immune system to establish a pro-inflammatory profile in order to maintain a continuous response against the parasite (Dubey, 2007). The spleen responds to *T. gondii* infection by increasing the release of Th1 cytokines by their immune cells and stimulating leukocyte activation and antibody production (Nam et al., 2014). Macrophages, the primary functional cells in the spleen, increase ROS production after activation by Th1 cytokines (Kraal and den

Haan, 2016). In this way, it is possible that the high ROS levels and inflammatory foci observed by the histological analysis of spleen are related to the immune response against chronic *T. gondii* infection. Splenomegaly is observed in some immune and hematological disorders, such as parasitic infection by *Plasmodium berguei* (Haa et al., 2015) and *Schistosoma manzoni* (Maia et al., 2007). Acute and chronic diseases can induce splenomegaly and, in this study, we show that chronic toxoplasmosis is not an exception. However, little is known about the factors that lead to the development of splenomegaly in this particular chronic infection, besides the increased production of pro-inflammatory cytokines in the tissue (Nan et al., 2011).

The activity of purinergic enzymes might help us to understand the factors that lead to the development of a pro-inflammatory profile in the tissue. In inflamed tissues, leukocytes can release immunomodulatory compounds, as well as grown factors, cytokines and damage-associated molecular patterns (DAMP´s) (Junger, 2011). Damaged cells or activated leukocytes release high levels of ATP in the extracellular space that act as a DAMP, stimulating local immune response through the increase of ROS levels and pro-inflammatory cytokines production. Thus, is possible that splenic NTPDase activity is increased in an attempt to control the high ATP levels stemmed by the pro-inflammatory immune signaling against the infection. Increased NTPDase activity has been described in other pathologies, revealing the NTPDase activity as a biological marker of endogenous levels of ATP (Doleski et al., 2016; Baldissera et al., 2016). Moreover, high 5´nucleotidase activity was observed in spleen, which could increase ADO levels, and consecutively reduce the inflammatory processes. However, the spleen has elevated ADA activity, which can remove possible elevated ADO levels. Therefore, the high NTPDase and ADA

activities found in infected mice disclosed the involvement of the purinergic enzymes in developing the pro-inflammatory and pro-oxidative profile in the spleen.

The subcutaneous $(PhSe)_2$ treatment performed in this study had been previously used by our research group to treat mice infected by a virulent strain of *T. gondii* (RH strain). In the previous study, treatment $(PhSe)_2$ reversed the serum pro-oxidant profile but no changes in the pro-inflammatory cytokines levels (Barbosa et al., 2014). In the current study, the benefit of (PhSe)2 treatment could be explained by its anti-inflammatory effects like the reversion of splenomegaly, histological congestion and splenic ROS levels in infected mice. Moreover, treatment with (PhSe)2 was able to cause several changes in the purinergic enzymes profile profile in splenic tissue. First, the NTPDase activity observed in treated infected nice was unexpectedly high, even higher than the one found in untreated infected mice. This finding could be accounted for the synergistic effect of the *per se* effects of (PhSe)2 and the elevated NTPDase activity induced by the infection. This high hydrolytic rate can reduce the concentration of ATP to a moderate pro-inflammatory profile, and assist with the reduction of the inflammation observed in this group. Another important change is the increase in ADO enzymatic production by the high 5´nucleotidase and decreased ADA activities. In contrast to high ATP levels, ADO can stimulate immune cells to release anti-inflammatory cytokines, which decreases ROS production and aids in the recovery of inflamed tissue (Bours et al., 2006). Nevertheless, the spleen histological analysis in treated infected animals showed a small white pulp hyperplasia, a fact which we consider positive, since the establishment of a controlled pro-inflammatory activity is able to prevent a reactivation of *T. gondii* in the body.

Chronic infection by *T. gondii* strain ME49 did not change the ROS levels or ATP hydrolysis in serum. Elevated 5´nucleotidase and ADA activities in serum can act in conjunction to reduce ADO levels, and consequently maintain the systemic immune response against the parasite. It is worthy to note that the $(PhSe)_2$ treatment was not able to reverse the changes induced by the infection in serum. *T. gondii* infection induces a pro-inflammatory profile in the spleen, with increased ROS levels, splenomegaly and inflammatory foci by the histological analysis. The splenic purinergic enzymes showed elevated activity, possibly to control the high ATP levels stemmed by activated cells in the inflamed tissue. However, elevated ADA activity reduces ADO levels, which can stimulate the inflammation in the spleen. Thus, is possible that the purinergic enzymes are involved in the pathogenesis of the chronic toxoplasmosis.

The $(PhSe)_2$ treatment brought remarkable results in *T. gondii* infected mice, as the reversion of splenomegaly, congestion and ROS levels. The anti-inflammatory effects of $(PhSe)_2$ can be observed in the changes of purinergic enzyme profile in the spleen. The unusual enzymatic profile, found in infection treated with this compound, stimulates the ATP hydrolysis and increases the ADO levels in the tissue. These findings reinforce our theory that the purinergic enzymes may be associated to the pathogenesis of *T. gondii* infection. Moreover, they reveal the effect of $(PhSe)_2$ in controlling the excessive immune response produced in the spleen of *T. gondii* ME49 infected mice.

Ethics Committee

This study was approved by the Ethic Committee on Animal Use of the Federal University of Santa Maria (UFSM) under protocol number 7787270815.

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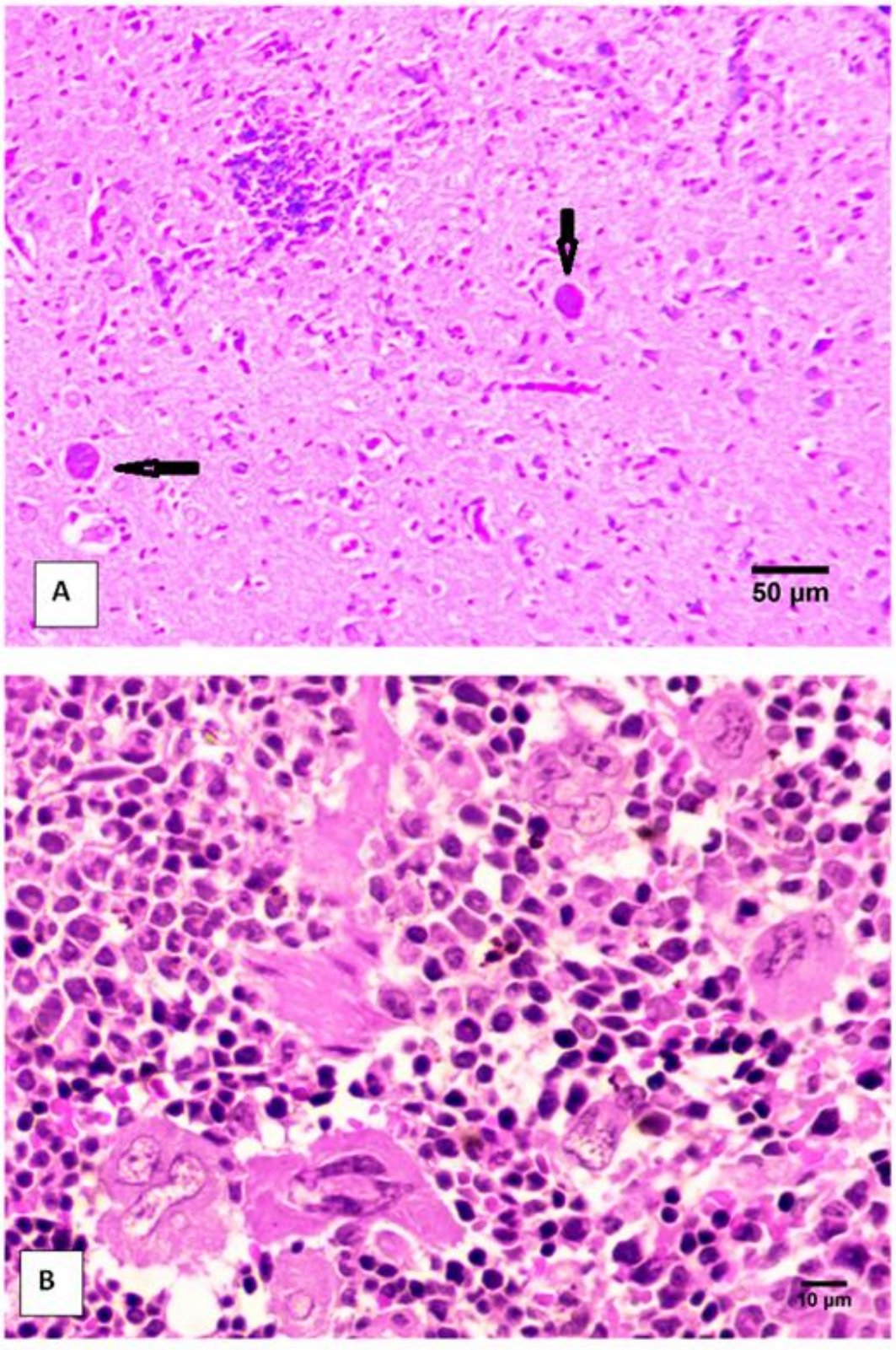

Figure 1. Cysts (arrow) in the brain of mice experimentally infected by Toxoplasma gondii (Figure 1.A). Presence of large numbers of megakaryocytes in the spleen of untreated T. gondii infected mice (Figure 1.B).

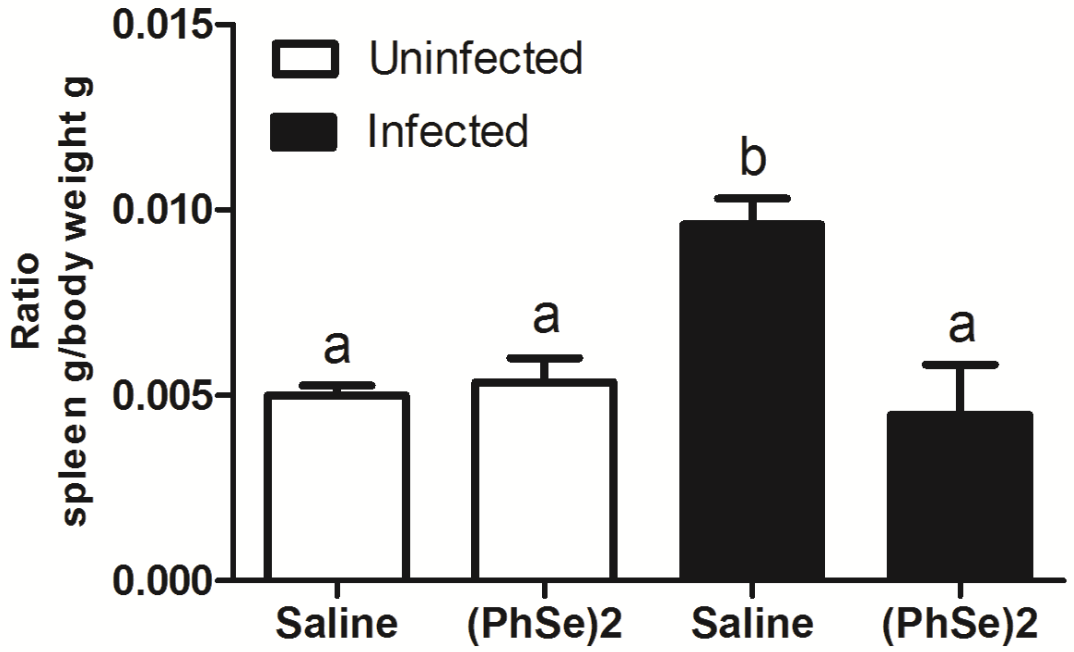

Figure 2. Spleen weight to body weight ratio of *Toxoplasma gondii* infected mice and $(PhSe)_2$-treated infected mice. Ratio is expressed as (Spleen g/body weight g). Columns represent mean ± SEM (n=10). Different letters in the same graph denote significance $P<0.05$ by two way ANOVA followed by Tukey´s post-hoc test

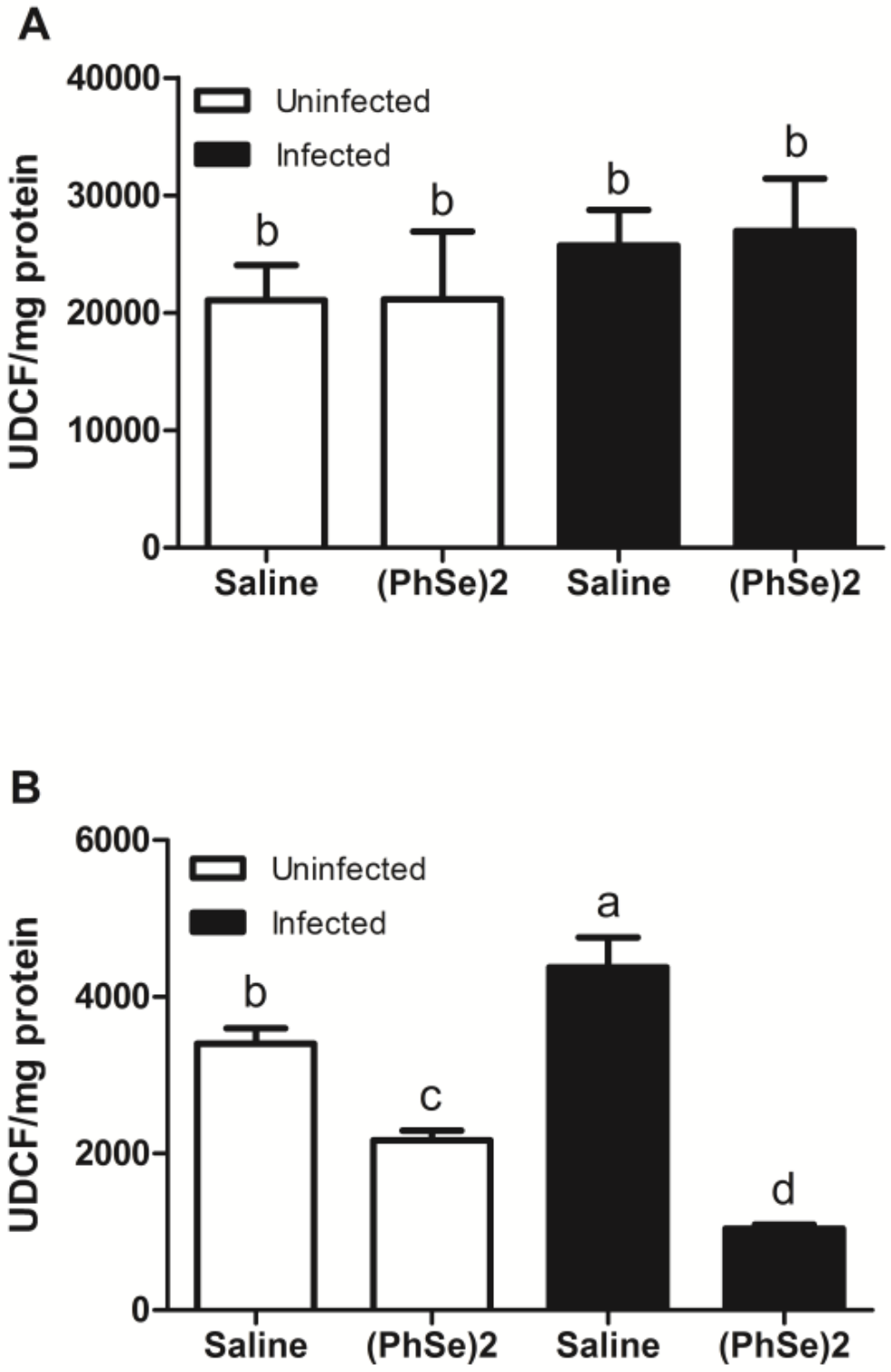

Figure 3. ROS levels in serum (Fig.3A) and spleen homogenate (Fig.3B) of *Toxoplasma gondii* infected mice and PhSe)$_2$-treated infected mice. ROS levels are expressed as UDCF/mg protein. Columns represent mean ± SEM (n=10). Different letters in the same graph denote significance $P<0.05$ by two way ANOVA followed by Tukey´s post-hoc test

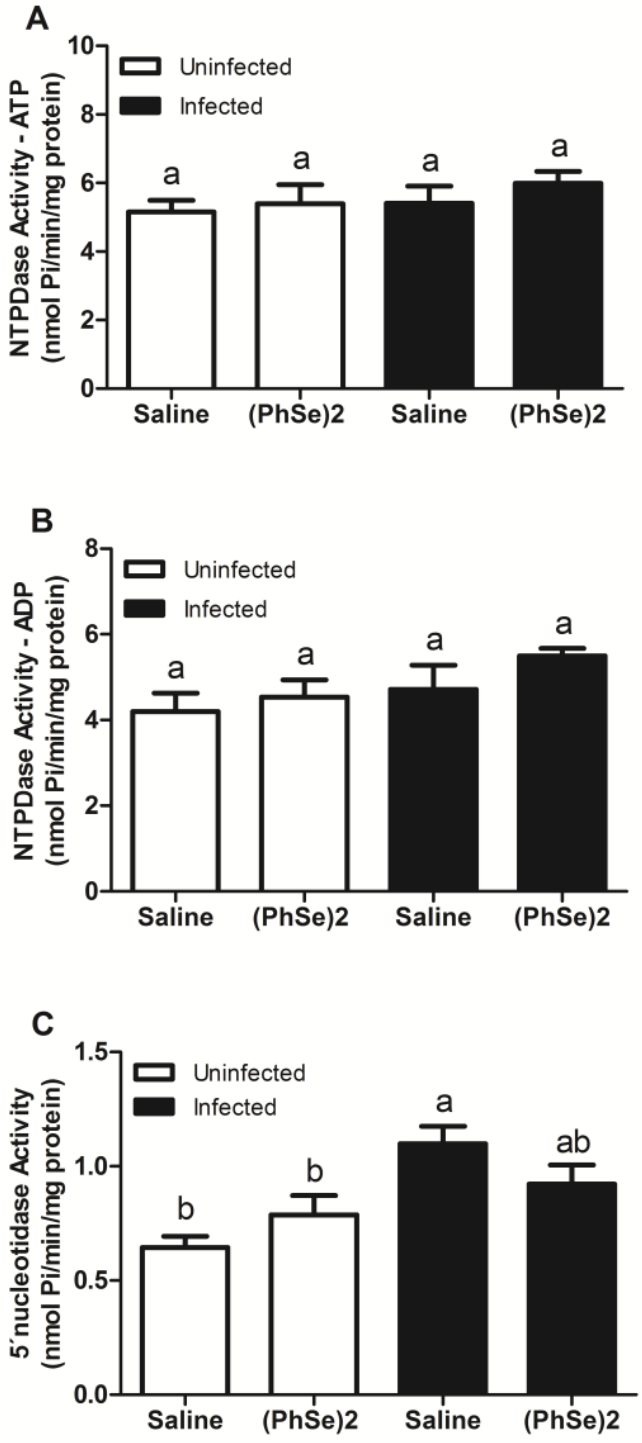

Figure 4. NTPDase activity to ATP (Fig.4A) and ADP (Fig.4B) substrate and 5´nucleotidase activity (Fig.4C) in serum of *Toxoplasma gondii* infected mice and $PhSe)_2$-treated infected mice. Enzymatic activities are expressed as ηmol Pi/min/mg protein. Columns represent mean ± SEM (n=10). Different letters in the same graph denote significance $P<0.05$ by two way ANOVA followed by Tukey´s post-hoc test.

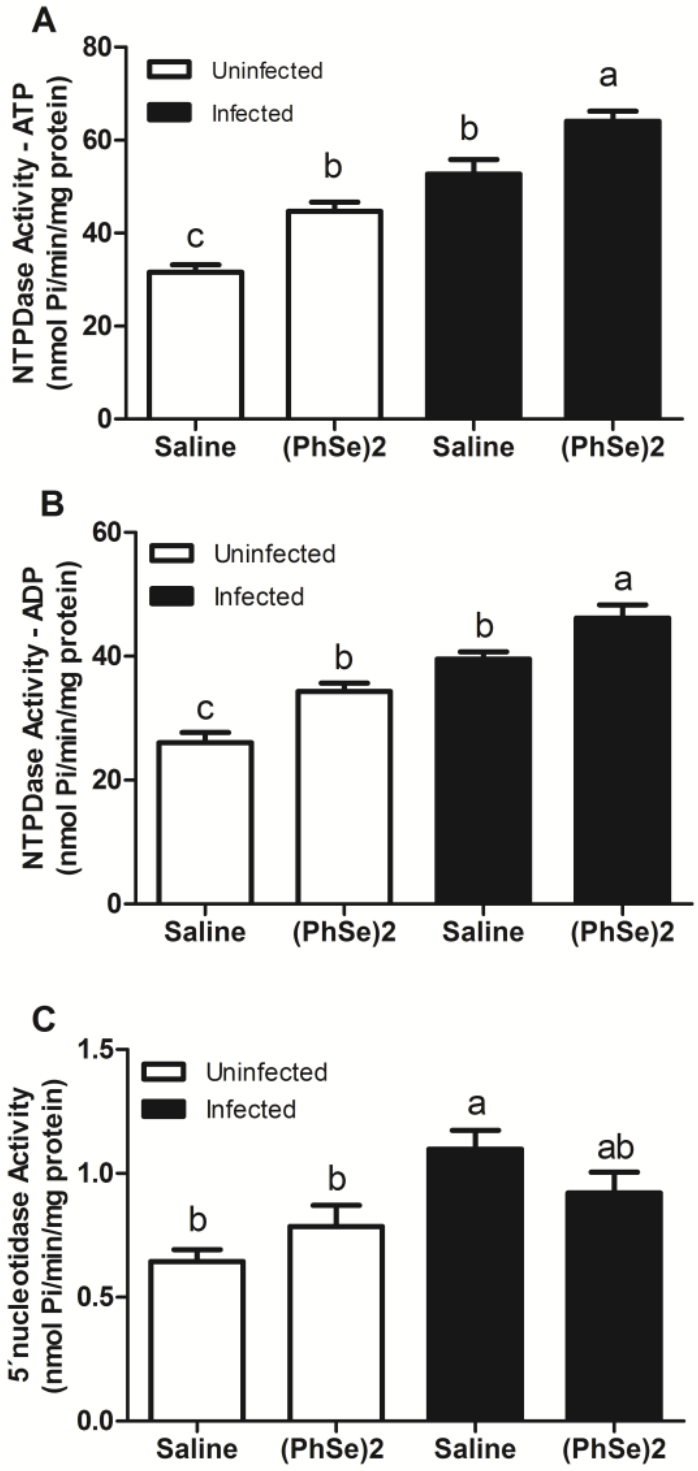

Figure 5. NTPDase activity to ATP (Fig.5A) and ADP (Fig.5B) substrate and 5´nucleotidase activity (Fig.5C) in spleen homogenate of *Toxoplasma gondii* infected mice and treated with $(PhSe)_2$. Enzymatic activities are expressed as ηmol Pi/min/mg protein. Columns represent mean ± SEM (n=10). Different letters in the same graph denote significance $P<0.05$ by two way ANOVA followed by Tukey´s post-hoc test

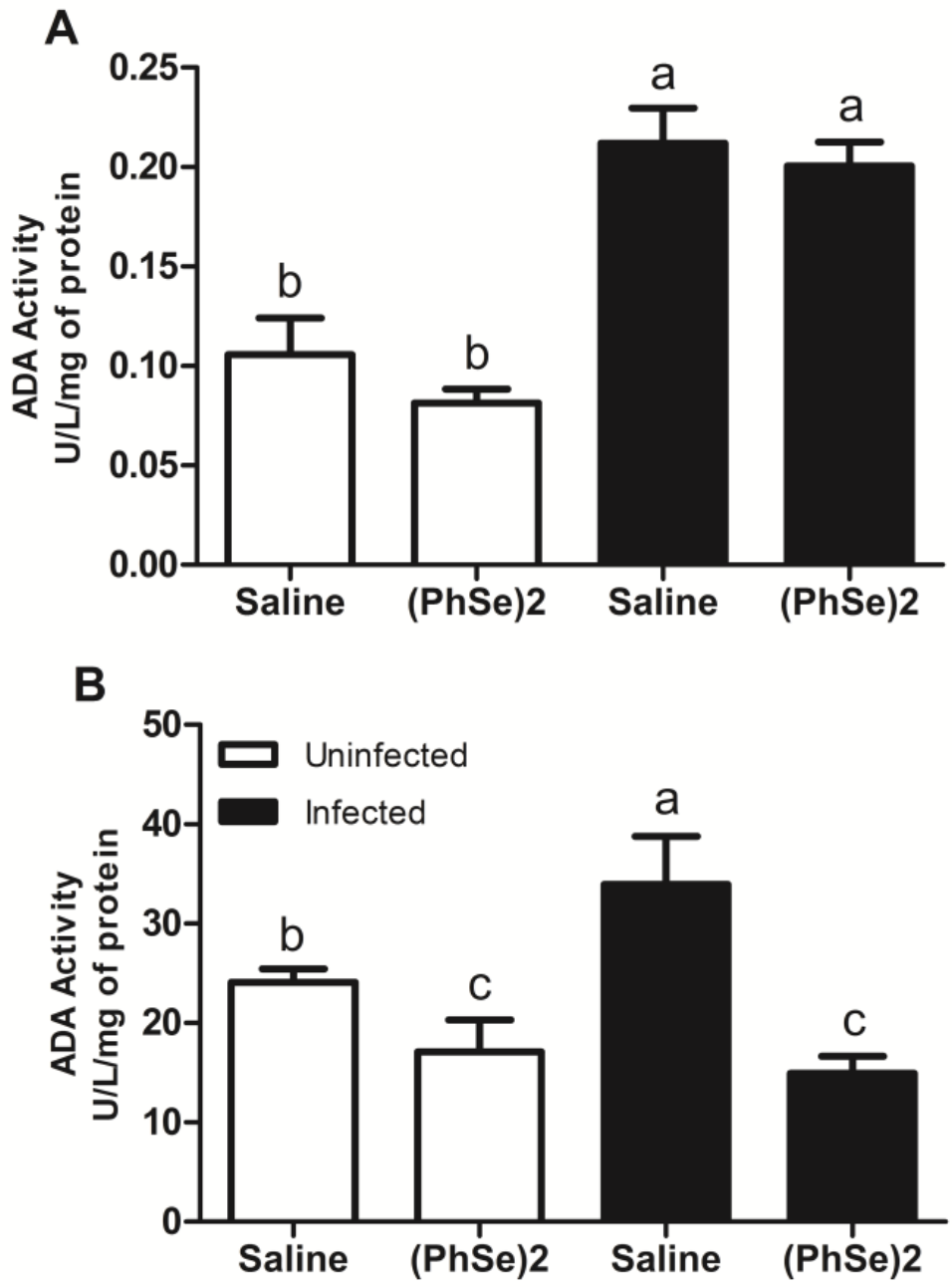

Figure 6. ADA activity in serum (Fig.6A) and spleen homogenate (Fig.6B) of *Toxoplasma gondii* in infected mice and PhSe)$_2$-treated infected mice. Enzymatic activities are expressed as U/L/mg protein. Columns represent mean ± SEM (n=10). Different letters in the same graph denote significance $P<0.05$ by two way ANOVA followed by Tukey´s post-hoc test.

4.3 ARTIGO 3

Hepatic xanthine oxidase activity and purine nucleosides levels as physiological mediators to analyze an subcutaneous treatment with $(PhSe)_2$ in mice infected by *Toxoplasma gondii*

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ABSTRACT

The aim of this study was to evaluate the levels of purine nucleosides and xanthine oxidase (XO) activity in the liver of mice chronically infected by *Toxoplasma gondii* and treated with diphenyl diselenide $(PhSe)_2$. For this experiment, forty Swiss mice were used. Twenty animals were orally infected by approximately 50 bradizoites of a cystogenic ME-49 strain of *T. gondii*, and the same number of uninfected mice was used as a control group. Ten infected and ten uninfected mice were subcutaneously treated twice (days 1 and 20 post-infection (PI)) with 5 μmol kg^{-1} of $(PhSe)_2$. On day 30 PI, liver samples were collected to measure the levels of hypoxanthine (HYPO), xanthine (XAN), uric acid (UA), and XO activity. Infected animals showed increased (P<0.05) levels of hepatic XAN and UA, as well as XO activity compared to uninfected animals. The use of $(PhSe)_2$ in healthy mice increased the levels of all nucleosides, but decreased XO activity compared to healthy untreated animals. The group of infected and treated animals showed increased XAN and UA levels, and XO activity compared to the healthy control group, however infected and treated mice showed a decrease in the XO activity compared to the infected untreated group. We conclude that chronic infection caused by *T. gondii* can induce hepatic changes, such as increased UA levels and XO activity, that can increase the pro-oxidative profile. The $(PhSe)_2$ treatment of healthy animals altered the levels of nucleosides, possibly due to low XO activity that decreased nucleoside degradation. Finally, $(PhSe)_2$ treatment decreased XO activity in the infected group and increased nucleoside levels; however it was unable to reduce the UA levels found during the infection.

Keywords: Diphenyl disselenite, hypoxanthine, xanthine, uric acid, toxoplasmosis.

INTRODUCTION

Toxoplasmosis is a disease caused by *Toxoplasma gondii*, a protozoon that infects animals and humans [1,2]. Chronic toxoplasmosis is characterized by the latent infection of the brain by the parasite, which induces neurological and behavioral disorders in mice and humans [3,4]. This chronic infection is considered asymptomatic, however the latency phase can improve the pro-inflammatory profile by increasing cytokines and others immune factors [5]. Some studies showed that the latent infection can cause some physiological changes in the host, leading to chronic liver disease [1,6]. The liver is considerable the most important metabolizing organ, and its proper functioning is important to maintain homeostasis through the body [7]. Tonin et al. [8] found purine nucleosides changes in the brain of mice chronically infected by *T. gondii*, and these changes might be related to the chronic pathology of the brain. However, the level of purine nucleosides in the liver of mice chronically infected by *T. gondii* remains unknown.

Purines are important molecules to the synthesis of nucleotides, and consequently DNA and RNA production in the cells. Some studies revealed that these compounds play an important role maintaining the physiological status, as well as in controlling the homeostasis during some diseases [9]. Xanthine oxidase (XO) is the enzyme responsible to oxidize nucleoside hypoxanthine (HYPO) and xanthine (XAN) in hydrogen peroxide (H_2O_2) and uric acid (UA), which is excreted into urine. The XO activity can produce oxidant and anti-oxidant products [10-12], and the XO activity may also improve the immune response, and increases the anti-oxidant defense, however in some cardiac and hepatic diseases higher XO activity and elevated levels of UA can be indicators of bad prognosis of the disease [11,13]. Therefore, it is possible that the real reason for the inflammatory action

showed by high XO activity might be the release of pro-inflammatory cytokines and DAMPs (damage associated molecular patterns) from leukocytes stimulated by the increase of this enzyme [14]. Anti-inflammatory compounds, and XO inhibitors are clinically administered in some diseases to avoid higher activity of this enzyme and, consequently, its pro-inflammatory action [10,11,14].

Diphenyl diselenide $(PhSe)_2$ is an organoselenium compound with antioxidant and immunomodulatory activities [15]. *In vivo* experiments have shown that $(PhSe)_2$ reduces the levels of pro-inflammatory cytokines, and reactive oxygen species in some pathologies [16, 17]. In this way, Barbosa et al. [18] demonstrated that a subcutaneous treatment with 5 μmol kg^{-1} of $(PhSe)_2$ can reduce the oxidative damage and the levels of pro-inflammatory cytokines in mice acutely infected by *T. gondii*. Moreover, this low dosage was unable to decrease the immune responses against the parasite. Considering that the chronic infection caused by *T. gondii* can induce some physiological changes in the host, the aim of this study was to evaluate whether changes in purine nucleosides levels and the XO activity in the liver tissue may have occurred in mice infected by a cystogenic strain of *T. gondii*.

MATERIALS AND METHODS

Toxoplasma gondii strain

This study used standard strain (ME-49; genotype II) of *T. gondii* [8] kept in liquid nitrogen in the laboratory to inoculate one mouse (Swiss). Thirty-two days later, brain homogenate (in saline solution) containing cysts with bradyzoites was collected and inoculated orally in other three mice; this procedure was done in order to reactivate the parasite´s virulence. The mice were euthanized for brain collection 30

days PI, and parasitic cysts were counted and separated in order to be used later in the experiment.

Experimental design

Forty 60-day-old female mice weighing an average of 25±5g were used for the experiment. This study was approved by the Ethic Committee on Animal Use of the Federal University of Santa Maria (UFSM) under protocol number 7787270815. The animals were divided into four groups (A, B, C and D) with ten animals each. The groups A and B were used as controls (uninfected). Animals in groups C and D were orally infected with 0.25 mL of brain homogenate containing 50 cysts of *T. gondii* bradyzoites. For the subcutaneous treatment, $(PhSe)_2$ (SIGMA; St. Louis, MO, USA) was dissolved in 0.1% dimetilsulfoxide and administered on day 1 and 20th post-infection (PI) using doses of 5 μmol kg^{-1} [18] in mice of groups B and D. On day 30th PI, the animals were euthanized with an overdose of isoflurane.

Tissue preparation

After the euthanasia, liver samples were collected and separated into two fragments (3g). One fragment was manually homogenized in 10mL of physiological solution (PS) with a syringe plunger and centrifuged at 1500 rpm for 15 min. The supernatant was collected, and used to measure XO activity. The other liver fragment was used to measure the levels of purine by gently homogenization in a sterile flask, and the resultant supernatant was mixed with 0.6M perchloric acid and 1M potassium hydroxide, as described previously [19].

Analysis of purine levels in the liver

Purine compounds and metabolic residues were analyzed by HPLC according to Voelter et al. [19]. The proteins were denaturized using 0.6 mol/L perchloric acid. All samples were centrifuged (16000 x g for 10 min at 4ºC), supernatants were neutralized with 4.0N KOH, and clarified with two more centrifugations (16000 x g for 30 min at 4ºC). Aliquots of 20 µL were applied to a reversed-phase HPLC (LC-20AT model, Shimadzu, Kyoto, Japan) using a C18 column (Ultra C18, 25 cm x 4.6 mm x 5 µm, Restek – USA). The elution was carried out applying a linear gradient from 100% solvent A (60 mM KH_2PO_4 and 5 mM of tetrabutylammonium chloride, 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 [19]. Mobile phases were filtered through a 0.22 µm Millipore filter prior to analysis, and all the reagents used were HPLC grade. The amount of purine and metabolic residues were measured by absorption at 254 nm. The retention time of the standards was used as a parameter for identification and quantification by comparison of the peak area. Purine levels were expressed as nmol of different compounds per g of tissue.

Xanthine oxidase activity

Hepatic XO activity was determined using the method previously described [20]. First, liver homogenates were centrifuged at 2500 rpm for 10 min to remove impurities. The reaction mixture contained 1 mM of xanthine as substrate, and 50 mM phosphate buffer (pH 7.4). The reaction mixture was incubated with approximately 0.5 mg of homogenized protein at 37 ºC for 60 min in a final volume of 0.5 mL. The rate of urate formation from xanthine degradation was determined by measuring the increased absorbance at 290 nm. The activity was expressed as UI/mg of protein.

Histopathology

At the necropsy, tissue samples were collected from the right hepatic lobe, fixed in 10% buffered formalin in 0.1M phosphate buffer. The fixed tissue was dehydrated and embedded in paraffin. Tissue sections were stained with haematoxylin and eosin (H&E) for histopathological examination.

Statistical analysis

Differences were assessed by two way analysis of variance (ANOVA) followed by Tukey´s post-hoc test. The values obtained in the assays were considered statically different when $P<0.05$. Data represent mean ± standard deviation.

RESULTS

Xanthine oxidase activity

The activities of XO in liver homogenates are shown in Fig 1. The treatment of healthy animals (the group B) showed 33% decrease in XO activity compared to the control group (the group A), while the infected group (the group C) showed 91% increase on the hepatic XO activity. The treatment of infected mice (the group D) caused an increase of 52% in XO activity compared to the control group (the group A), and a significant decrease of 43% compared to the group D.

Hepatic purine nucleosides levels

The levels of purine nucleosides in liver samples are shown in Table 1. The treatment of healthy mice (the group B) caused an increase of 76% of HYPO, 59% of XAN, and 33% of UA levels in the liver tissue compared to the control mice (the group A). The infected group (the group C) showed a significant increase of 17% in

XAN and 174% in UA concentrations. Infected and treated mice (the group D) showed an increase of 33% in HYPO, 45% in XAN, and 155% in UA levels.

Histopathology

The brain tissue of animals infected with the strain ME-49 of *Toxoplasma gondii* (groups C and D) had parasitic cysts. Mice of groups A and B did not show histological lesions in the liver and brain. The mice of the groups C and D showed inflammatory foci in the liver (Figure 2).

DISCUSSION

New scientific studies have shown that chronic toxoplasmosis can cause neuronal damage and behavioral changes in mice [3], and in this study, we have demonstrated that chronic infection can change the metabolization of nucleosides in the hepatic tissue, and may cause significant changes in liver. Firstly, the infection increased XO activity in the tissue, which probably led to high levels of XAN and UA found in the liver. This profile is observed in some chronic pathologies, and are considered a bad prognosis of the disease [10, 21, 22]. High XO activity can increase the inflammatory response and the levels of H_2O_2, which are capable of generating cellular damage and, in the long-term, it may increase the risk of developing chronic diseases [11]. On the other hand, the high XO activity observed in *T. gondii* infected mice induced the production of UA, that can act like a free radical scavenger, reducing the oxidative damage [12]. However, some studies revealed that high levels of UA caused losses of the anti-oxidant effects, inducing oxidative damage, cellular apoptosis, and senescence [13,14]. The high XO activity and UA levels possibly have a pro-inflammatory profile in the tissue of infected mice, which in a long term, may

increase the risk of chronic disease in the liver tissue, as observed in correlated studies [7].

Many clinical studies were carried out with antioxidants molecules such as $(PhSe)_2$ in order to increase the antioxidant capacity of the body or to reduce the oxidative stress in some diseases [15,17]. In this study, the treatment with $(PhSe)_2$ was able to alter XO activity and, consequently, the nucleoside metabolism. In uninfected treated and infected treated mice, we observed that a reduction in the activity of XO may lead to increased anti-inflammatory and anti-oxidant profile in liver tissue. Low XO activity reduces the production of H_2O_2, which acts as pro-oxidant, and reduces the degradation of HYPO and XAN, resulting in high levels of these nucleosides in the hepatic tissue. Interestingly, the treated control group showed a small increase in the concentration of UA compared to the control group, which we considered an antioxidant effect of $(PhSe)_2$. To reinforce this theory, some studies in the literature have demonstrated the anti-oxidant effects of $(PhSe)_2$ in the liver tissue, but did not consider the small increase of UA as a pro-inflammatory signal [14,15]. Unfortunately, when we analyzed UA levels between the infected treated and infected untreated group, no statistical differences were observed. It is possible that higher doses of $(PhSe)_2$ may result in a significant reduction in the levels of UA, but nevertheless we believe that the treatment with $(PhSe)_2$ may exhibit the antioxidant effect by inhibiting XO activity in infected mice. Furthermore, it is important to consider that the higher levels of UA found in both infected groups should not only be due to purine metabolism, but also a disorder in the excretion of UA in the liver tissue.

Thus, we can conclude that mice infected by a cystogenic strain ME-49 of *T. gondii* have increased XO activity and, consequently, increased nucleoside levels, as

well as xanthine and uric acid. These changes can induce a pro-inflammatory and pro-oxidative profile in the liver of the infected host. Considering that, the pro-oxidative profile can increase the risk of chronic hepatic disease, when some anti-oxidants treatment can be proposed. The treatment with low concentrations of $(PhSe)_2$ showed interesting effects, decreasing XO activity and the oxidative profile. However, this treatment did not change the high levels of UA found in the liver tissue. Therefore, more studies should be conducted to prove the pro-inflammatory profile in the liver tissue of toxoplasmosis, and the anti-oxidant effects of $(PhSe)_2$ in this disease.

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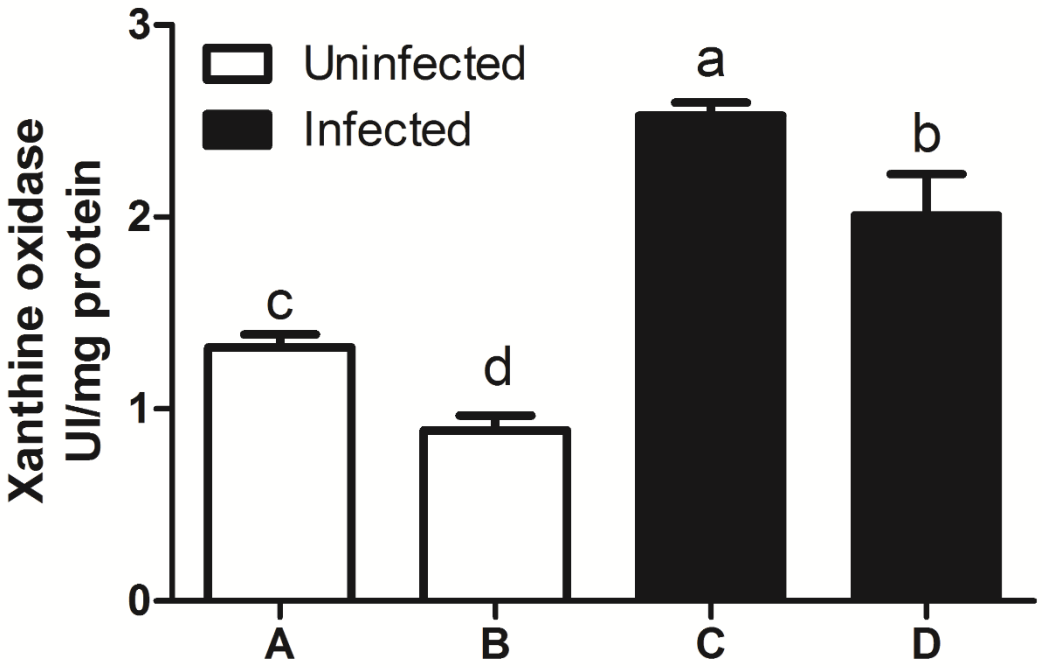

Fig. 1: Hepatic xanthine oxidase (XO) activity. Columns represent mean ± standard deviation (n=10). Different letters (lowercase) in the same graph denote significance ($P<0.05$) by two way ANOVA followed by Tukey´s posthoc test. Note: the group A (uninfected), the group B (uninfected and treated with $(PhSe)_2$), the group C (infected), and the group D (infected and treated with $(PhSe)_2$).

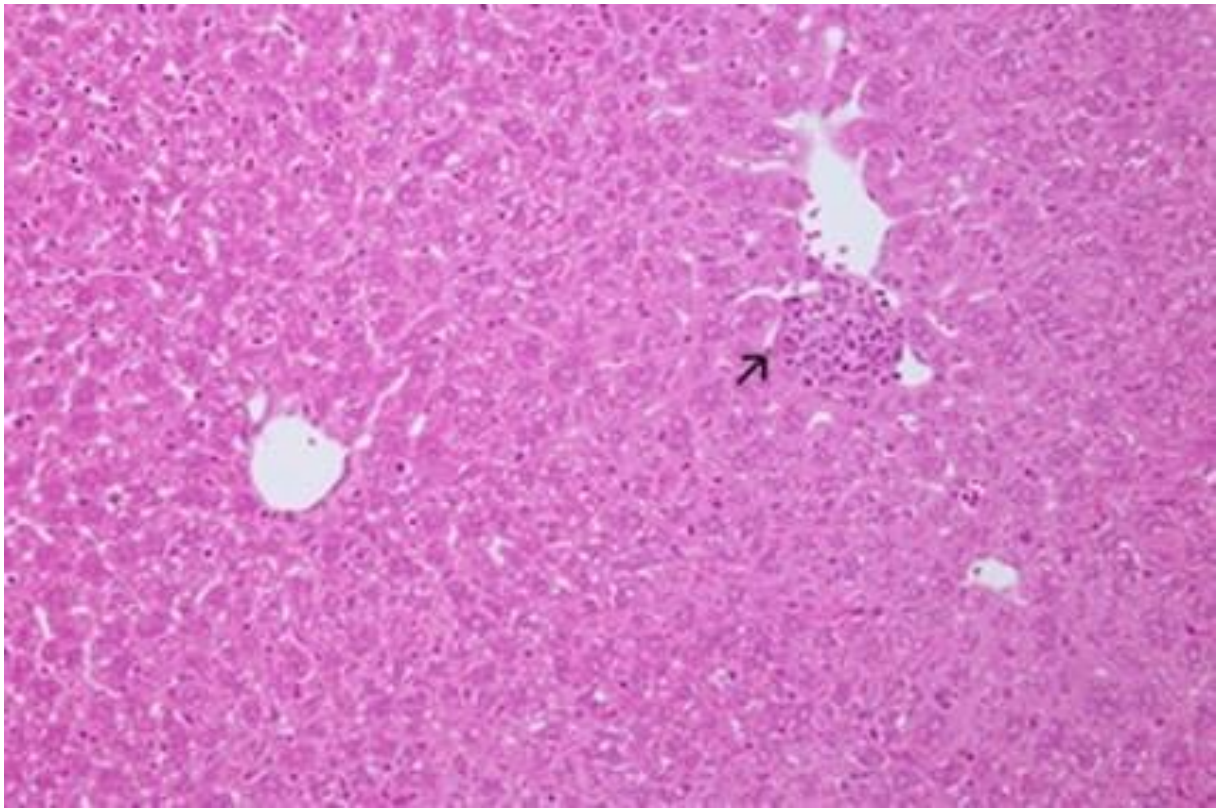

Fig. 2: Focal inflammatory infiltration in the liver of mice experimentally infected by *Toxoplasma gondii* (the group C). H&E staining.

Table 1. Mean and standard deviation of purine levels in liver homogenate: hypoxanthine, xanthine, and uric acid (n=10).

Purines	Group A	Group B	Group C	Group D
Hypoxanthine (nmol/g)	122.3 ± 37.9[b]	215.8 ± 81.3[a]	131.7 ± 24.8[b]	163.7 ± 56.4[ab]
Xanthine (nmol/g)	235.2 ± 12.7[c]	372.3 ± 40.4[a]	274.7 ± 17.6[b]	342.6 ± 21.7[a]
Uric acid (nmol/g)	105.4 ± 15.3[c]	141.8 ± 21.9[b]	288.7 ± 27.2[a]	268.0 ± 45.1[a]

[#]Different letters in the same line denote significance ($P<0.05$) by two way ANOVA followed by Tukey´s post-hoc test. Note: the group A (uninfected), the group B (uninfected and treated with (PhSe)2), the group C (infected), and the group D (infected and treated with (PhSe)2).

5 DISCUSSÃO

O tecido esplênico e hepático de animais infectados cronicamente pelo parasito *T. gondii* (cepa ME-49) apresentaram processo inflamatório leve. Sendo que, no tecido hepático o tratamento foi capaz de reverter o processo inflamatório em 60% dos animais analisados, e no tecido esplênico o tratamento reverteu parcialmente os achados inflamatórios em todos os animais analisados. Demonstrando neste trabalho o efeito hepatoprotetor do $(PhSe)_2$, efeito o qual já foi relatado em outros estudos (BARBOSA et al., 2006)

No tecido hepático dos animais infectados, a elevada atividade das enzimas purinérgicas desenvolveu uma alta razão ATP/adenosina no tecido, a qual estimula a sinalização dos receptores P2 e consequentemente processos inflamatórios (JUNGER, 2011). Desta maneira é possível relacionar a sinalização purinérgica com a inflamação observada neste tecido. Por outro lado, o tratamento da infecção crônica com $(PhSe)_2$ estimulou as enzimas purinérgicas a reverter a razão ATP/adenosina encontrada na infecção não tratada, estimulando uma sinalização via receptores P1, e os efeitos anti-inflamatórios desenvolvidos por esta sinalização (BOURS et al., 2006).

As ectoenzimas de linfócitos hepáticos, nos animais infectados, revelam uma tentativa destas células imunes em evitar uma excessiva sinalização via receptor P2 em suas membranas, a qual poderia estimular a resposta pró-inflamatória no tecido. Enquanto que, em animais infectados e tratados, as ectoenzimas voltaram a valores basais, possivelmente devido ao controle nos níveis de purinas exercido pelo tratamento (DOMBROWSKI et al, 1997). O tratamento não foi capaz de reduzir a concentração de ácido úrico no tecido hepático, mas reduziu a elevada atividade da XO, perfil qual é capaz de estimular uma resposta antioxidante nos animais tratados e infectados (KANG; HA, 2014, p. 1).

A sinalização purinérgica no tecido esplênico parece apresentar o mesmo padrão observado no tecido hepático, pois os resultados obtidos pela análise das enzimas purinérgicas foram semelhantes aos observados no tecido hepático. Onde a atividade das enzimas revelam uma sinalização pró-inflamatória via receptor P2 na infecção, e uma sinalização anti-inflamatória via sinalização P1 na infecção tratada com $(PhSe)_2$. Esta sinalização desenvolvida pelo tratamento apresenta ação

antioxidante, a qual foi observada pela redução dos níveis de ROS no tecido (BOURS et al., 2006, p. 358).

O soro de animais infectados não demonstrou alteração nos níveis de ROS ou na atividade da enzima NTPDase. Por outro lado, as enzimas 5´nucleotidase e ADA apresentaram elevada atividade sérica, as quais podem estar relacionadas com a redução dos níveis de adenosina e a regulação da sinalização do receptor P1 na corrente sanguínea, estimulando a resposta imunológica sistêmica contra o parasita (CRONSTEIN, 1994, p. 5). O tratamento com $(PhSe)_2$ dos animais tratados, não reverteu as alterações induzidas pela infecção no soro. Desta maneira, não alterando as possíveis regulações da sinalização do receptor P1 desenvolvidas pelo hospedeiro.

Possivelmente pela sua alta lipossolubilidade, o $(PhSe)_2$ tende a se concentrar nos tecidos, e desta maneira apresentou efeito no tecido hepático e esplênico neste estudo, enquanto que no soro não (NOGUEIRA et al., 2004, p. 6255). Uma vez nos tecidos, é possível que o $(PhSe)_2$ interaja com os grupos –SH presentes nos receptores e enzimas purinérgicas, estimulando as modulações observadas neste estudo (ENNION; EVANS, 2002, p. 303).

6 CONCLUSÃO

A partir dos resultados obtidos neste estudo pode se concluir que, em camundongos, a infecção crônica por *Toxoplasma gondii*, além de induzir alterações no tecido cerebral, é capaz de induzir inflamação, estresse oxidativo e alterações purinérgicas no baço e fígado de camundongos infectados. Uma vez que, a longo prazo, estas alterações são capazes de desenvolver dano e disfunções teciduais, o uso de agentes imunomodulatórios e antioxidantes, como o $(PhSe)_2$, podem ser sugeridos como tratamento de suporte ou preventivo para estes achados extracerebrais da infecção crônica por *T. gondii* em camundongos.

7 REFERÊNCIAS

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8 ANEXO

8.1 ANEXO 1

Comissão de Ética no Uso de Animais
da
Universidade Federal de Santa Maria

CERTIFICADO

Certificamos que o Projeto intitulado "Efeitos do disseleneto de difenila sobre o sistema purinérgico no fígado e enzimas do metabolismo energético no coração e cérebro de camundongos infectados com Toxoplasma gondii.", protocolado sob o CEUA nº 7787270815, sob a responsabilidade de **Aleksandro Schafer da Silva** - que envolve a produção, manutenção e/ou utilização de animais pertencentes ao filo Chordata, subfilo Vertebrata (exceto o homem), para fins de pesquisa científica ou ensino - está de acordo com os preceitos da Lei 11.794 de 8 de outubro de 2008, com o Decreto 6.899 de 15 de julho de 2009, bem como com as normas editadas pelo Conselho Nacional de Controle da Experimentação Animal (CONCEA), e foi **aprovado** pela Comissão de Ética no Uso de Animais da Universidade Federal de Santa Maria (CEUA/UFSM) na reunião de 31/03/2016.

We certify that the proposal "Eeffects of Diselenide diphenyl about purinergic system for Liver Enzymes and do energy metabolism in the heart and brain of mice infected with Toxoplasma gondii .", utilizing 40 Heterogenics mice (40 females), protocol number CEUA 7787270815, under the responsibility of **Aleksandro Schafer da Silva** - which involves the production, maintenance and/or use of animals belonging to the phylum Chordata, subphylum Vertebrata (except human beings), for scientific research purposes or teaching - is in accordance with Law 11.794 of October 8, 2008, Decree 6899 of July 15, 2009, as well as with the rules issued by the National Council for Control of Animal Experimentation (CONCEA), and was **approved** by the Ethic Committee on Animal Use of the Federal University of Santa Maria (CEUA/UFSM) in the meeting of 03/31/2016.

Finalidade da Proposta: Pesquisa

Vigência da Proposta: de 09/2015 a 07/2016 Área: Bioquimica Toxicológica

Procedência:	Biotério Central UFSM						
Espécie:	Camundongos heterogênicos	sexo:	Fêmeas	idade:	60 a 60 dias	N:	40
Linhagem:	Swiss			Peso:	25 a 30 g		

Resumo: A doença causada pela infecção por Toxoplasma gondii é mundialmente conhecida por toxoplasmose. Desde a primeira descrição, foram relatados casos de infecção em praticamente todos os continentes. Esta afeta um grande número de animais domésticos e selvagens, entre eles: bovinos, gatos, cães, e pequenos roedores. Humanos também são susceptíveis e desenvolver diferentes patologias em consequência da doença. A infecção pode causar um quadro agudo (raro) e crônico (frequente) da patogenia, onde a última leva a graves lesões e consequentemente sequelas em alguns casos. As enzimas creatina quinase, piruvato quinase e adenilato quinase são enzimas envolvidas na produção de ATP, necessárias para um ótimo funcionamento do sistema bioenergético celular, sendo suas atividades responsáveis pela produção e entrega adequada de ATP aos seus locais de consumo. Já as enzimas NTPDase, 5´nucleotidase e Adenosina desaminase, possuem a função de degradar ATP e outros nucleotídeos purínicos, os quais podem interagir com purinoreceptores desencadeando inúmeros processos fisiológicos. Neste estudo objetivamos avaliar o efeito do disseleneto de difenila sobre o sistema purinérgico no fígado e enzimas do metabolismo energético no coração e cérebro de camundongos

infectados com T. gondii, além de testes moleculares, objetivando verificar se o selênio

modulou as variáveis de forma favorável a minimizar os efeitos patológicos da doença.

Três dias antes das coletas, serão realizados os testes comportamentais.

Local do experimento: Laboratório de Biologia Molecular e Parasitologia Humana, Prédio 20, Sala 4227.

Santa Maria, 24 de junho de 2016

Profa. Dra. Daniela Bitencourt Rosa Leal
Coordenadora da Comissão de Ética no Uso de Animais
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8.2 ANEXO 2

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Hepatic xanthine oxidase activity and purine nucleosides levels as physiological mediators to analyze a subcutaneous treatment with $(PhSe)_2$ in mice infected by *Toxoplasma gondii*

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ABSTRACT

The aim of this study was to evaluate the levels of purine nucleosides and xanthine oxidase (XO) activity in the liver of mice chronically infected by *Toxoplasma gondii* and treated with diphenyl diselenide $(PhSe)_2$. For this experiment, forty Swiss mice were used. Twenty animals were orally infected by approximately 50 bradizoites of a cystogenic ME-49 strain of *T. gondii*, and the same number of uninfected mice was used as a control group. Ten infected and ten uninfected mice were subcutaneously treated twice (days 1 and 20 post-infection (PI)) with 5 μmol kg^{-1} of $(PhSe)_2$. On day 30 PI, liver samples were collected to measure the levels of hypoxanthine (HYPO), xanthine (XAN), uric acid (UA), and XO activity. Infected animals showed increased ($P < 0.05$) levels of hepatic XAN and UA, as well as XO activity compared to uninfected animals. The use of $(PhSe)_2$ in healthy mice increased the levels of all nucleosides, but decreased XO activity compared to healthy untreated animals. The group of infected and treated animals showed increased XAN and UA levels, and XO activity compared to the healthy control group, however infected and treated mice showed a decrease in the XO activity compared to the infected untreated group. We conclude that chronic infection caused by *T. gondii* can induce hepatic changes, such as increased UA levels and XO activity, that can increase the pro-oxidative profile. The $(PhSe)_2$ treatment of healthy animals altered the levels of nucleosides, possibly due to low XO activity that decreased nucleoside degradation. Finally, $(PhSe)_2$ treatment decreased XO activity in the infected group and increased nucleoside levels; however it was unable to reduce the UA levels found during the infection.

1. Introduction

Toxoplasmosis is a disease caused by *Toxoplasma gondii*, a protozoon that infects animals and humans [1,2]. Chronic toxoplasmosis is characterized by the latent infection of the brain by the parasite, which induces neurological and behavioral disorders in mice and humans [3,4]. This chronic infection is considered asymptomatic, however the latency phase can improve the pro-inflammatory profile by increasing cytokines and others immune factors [5]. Some studies showed that the latent infection can cause some physiological changes in the host, leading to chronic liver disease [1,6]. The liver is considerable the most important metabolizing organ, and its proper functioning is important to maintain homeostasis through the body [7]. Tonin et al. [8] found purine nucleosides changes in the brain of mice chronically infected by *T. gondii*, and these changes might be related to the chronic pathology of the brain. However, the level of purine nucleosides in the liver of mice chronically infected by *T. gondii* remains unknown.

Purines are important molecules to the synthesis of nucleotides, and consequently DNA and RNA production in the cells. Some studies revealed that these compounds play an important role maintaining the physiological status, as well as in controlling the homeostasis during some diseases [9]. Xanthine oxidase (XO) is the enzyme responsible to oxidize nucleoside hypoxanthine (HYPO) and xanthine (XAN) in hydrogen peroxide (H_2O_2) and uric acid (UA), which is excreted into urine. The XO activity can produce oxidant and anti-oxidant products [10–12], and the XO activity may also improve the immune response, and increases the anti-oxidant defense, however in some cardiac and hepatic diseases higher XO activity and elevated levels of UA can be indicators of bad prognosis of the disease [11,13]. Therefore, it is possible that the real reason for the inflammatory action showed by high XO activity might be the release of pro-inflammatory cytokines and DAMPs (damage associated molecular patterns) from leukocytes stimulated by the increase of this enzyme [14]. Anti-inflammatory compounds, and XO inhibitors are clinically

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