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**Vanessa Schopf Machado**

# APLICAÇÃO DE DISSELENETO DE DIFENILA SUBCUTÂNEO EM CAMUNDONGOS INFECTADOS COM *Toxoplasma gondii*: EFEITOS IMUNOMODULATÓRIOS E ANTIOXIDANTES

Santa Maria, RS
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Dissertação apresentada ao Curso de Pós-Graduação em Ciências Biológicas: Bioquímica Toxicológica do Centro de Ciências Naturais e Exatas, da Universidade Federal de Santa Maria (UFSM,RS), como requisito parcial para obtenção do título de **Mestre em Ciências Biológicas: Bioquímica Toxicológica.**

Orientador: Profº Drº. Aleksandro Schafer da Silva

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*“Tudo que é seu encontrará uma maneira de chegar até você”*

**(Chico Xavier)**

**RESUMO**

# APLICAÇÃO DE DISSELENETO DE DIFENILA SUBCUTÂNEO EM CAMUNDONGOS INFECTADOS COM *Toxoplasma gondii*: EFEITOS IMUNOMODULATÓRIOS E ANTIOXIDANTES

AUTOR: Vanessa Schopf Machado
ORIENTADOR: Aleksandro Schafer Da Silva

*Toxoplasma gondii* é capaz de parasitar qualquer tipo de célula nucleada, podendo causar alterações bioquímicas, funcionais e estruturais nas células dos hospedeiros. Os quimioterápicos para o tratamento não são totalmente eficazes e apresentam efeitos colaterais, sendo necessário a busca por formas alternativas. Com isso, o objetivo deste estudo foi avaliar o efeito da administração subcutânea do disseleneto de difenila $(PhSe)_2$ sobre enzimas do metabolismo energético no coração e encéfalo de camundongos experimentalmente infectados com *T. gondii,* assim como verificar o seu efeito modulatório sobre variáveis bioquímicas e comportamentais minimizando os efeitos patológicos da doença. Os animais infectados com *T. gondii* apresentaram comportamento ansiolítico e perda de memória, de forma similar ao observado nos animais infectados e tratados com $(PhSe)_2$. Nas análises histopatológicas de encéfalo foi possível observar a presença de cistos de *T. gondii* e infiltrado inflamatório moderado; já no coração foi observada severa necrose e hemorragia em ambos grupos infectados, porém de menor intensidade nos tratados. Uma redução significante na atividade das enzimas creatina quinase (CK) e adenilato quinase (AK) foi observada no encéfalo dos animais infectados (controle positivo) quando comparado ao grupo não infectado (controle negativo), porém o tratamento com $(PhSe)_2$ não foi capaz de reverter essa diminuição quando comparado com o controle positivo. Foi observada uma redução significativa na atividade da AK no coração, ao passo que a atividade da CK cardíaca aumentou nos animais infectados quando comparado aos animais não infectados. O tratamento com $(PhSe)_2$ não foi capaz de prevenir estas alterações enzimáticas de CK e AK quando comparado ao controle positivo. Foi observado um aumento significante nos marcadores séricos da função cardíaca, como a CK, mioglobina e lactato desidrogenase (LDH) nos animais infectados quando comparados aos animais não infectados. O tratamento com $(PhSe)_2$ foi capaz de prevenir o aumento dos níveis séricos da CK e LDH comparado com o grupo controle positivo. A atividade da acetilcolinestarase aumentou significativamente nos animais infectados comparado ao grupo controle negativo, mas o tratamento com $(PhSe)_2$ não foi capaz de prevenir este aumento. Um aumento significante nos níveis das espécies reativas ao ácido tiobarbitúrico (TBARS), bem com um aumento na atividade da glutationa peroxidase (GPx) e glutationa reductase (GR) foi observado nos animais infectados comparado ao controle negativo, ao passo que a atividade da glutationa S-transferase (GST) diminui nesses animais. O tratamento com $(PhSe)_2$ foi capaz de prevenir o aumento dos níveis de TBARS e GR, bem como prevenir a depleção na atividade da GST no encéfalo de animais infectados comparado ao controle positivo. Pode-se concluir que a infecção crônica por *T. gondii*, além de induzir alterações no tecido encefálico, é capaz de induzir inflamação, estresse oxidativo e alterações no coração de camundongos infectados, e o uso de $(PhSe)_2$ apresentou efeito protetor no coração, além de efeito antioxidante, porém não foi capaz de reverter os achados comportamentais.

**Palavras-chave:** Toxoplasmose. Antioxidante. Disseleneto de Difenila. AChE.

**ABSTRACT**

# APPLICATION OF SUBCUTANEOUS DIPHENYL DISSELENIDE IN MICE INFECTED WITH *Toxoplasma gondii*: IMMUNOMODULATORY AND ANTIOXIDANT EFFECTS

AUTOR: Vanessa Schopf Machado
ORIENTADOR: Aleksandro Schafer Da Silva

*Toxoplasma gondii* is able to parasitize any type of nucleated cell, which can cause biochemical, functional and structural changes in the host cells. The chemotherapy for the treatment is not totally effective and has side effects, being necessary the search for alternative forms. The objective of this study was to evaluate the effect of subcutaneous administration of diphenyl diselenide $(PhSe)_2$ on energetic metabolism enzymes in the heart and brain of mice experimentally infected with *T. gondii*, as well as to verify its modulatory effect on biochemical variables and behavioral, minimizing the pathological effects of the disease. The animals infected with *T. gondii* showed anxiolytic behavior and memory loss, similar to that observed in animals infected and treated with $(PhSe)_2$. In the histopathological analyzes of encephalon it was possible to observe the presence of *T. gondii* cysts and moderate inflammatory infiltrate; severe necrosis and hemorrhage were observed in both infected and non-treated groups. A significant reduction in the activity of the enzymes creatine kinase (CK) and adenylate kinase (AK) were observed in the brain of infected animals (positive control) as compared to the uninfected group (negative control), but the treatment with $(PhSe)_2$ was not able to reverse this decrease when compared to the positive control. A significant reduction in AK activity was observed in the heart, while cardiac CK activity increased in infected animals compared to uninfected animals. Treatment with $(PhSe)_2$ was not able to prevent these enzymatic alterations of CK and AK when compared to the positive control. A significant increase was observed in serum markers of cardiac function, such as CK, myoglobin and lactate dehydrogenase (LDH) in infected animals when compared to uninfected animals. Treatment with $(PhSe)_2$ was able to prevent the increase of serum levels of CK and LDH compared to the positive control group. Acetylcholinesterase (AChE) activity increased significantly in infected animals compared to the negative control group, but treatment with $(PhSe)_2$ was not able to prevent this increase. A significant increase in the levels of thiobarbituric acid reactive species (TBARS), as well as an increase in the activity of glutathione peroxidase (GPx) and glutathione reductase (GR) was observed in infected animals compared to the negative control, whereas glutathione S-transferase (GST) decreases in these animals. Treatment with $(PhSe)_2$ was able to prevent the increase of TBARS and GR levels, as well as to prevent the depletion of GST activity in the brain of infected animals compared to the positive control. We can conclude that chronic *T. gondii* infection, besides inducing changes in brain tissue, is capable of inducing inflammation, oxidative stress and changes in the heart of infected mice, and the use of $(PhSe)_2$ had a protective effect on the heart, besides antioxidant effect, but it was not able to reverse the behavioral findings.

**Key-words:** Toxoplasmosis. Antioxidant. Diphenyl diselenide. AChE.

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| | |
|---|---|
| **ACh** | **Acetilcolina** |
| **AChE** | **Acetilcolinesterase** |
| **AK** | **Adenosina Quinase** |
| **CK** | **Creatina Quinase** |
| **PK** | **Piruvato Quinase** |
| **AMP** | **Monofosfato de Adenosina** |
| **ADP** | **Difosfato de Adenosina** |
| **ATP** | **Trifosfato de Adenosina** |
| **SOD** | **Superóxido Dismutase** |
| **CAT** | **Catalase** |
| **GR** | **Glutationa Reductase** |
| **GPx** | **Glutationa Peroxidase** |
| **GST** | **Glutationa S-Transferase** |
| **GSH** | **Glutationa Reduzida** |
| **EROs** | **Espécies Reativas de Oxigênio** |
| **LDH** | **Lactato Desidrogenase** |
| **$(PhSe)_2$** | **Disseleneto de Difenila** |
| **TBARS** | **Espécies Reativas ao Ácido Tiobarbitúrico** |
| **SNC** | **Sistema Nervoso Central** |
| **SNP** | **Sistema Nervoso Periférico** |
| **DNA** | **Ácido Desoxirribonucléico** |
| **ME-49** | **Cepa avirulenta de *T. gondii*** |
| ***T. gondii*** | ***Toxoplasma gondii*** |

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# 1 INTRODUÇÃO

*Toxoplasma gondii* é um protozoário parasito intracelular, pertencente ao filo Apicomplexa, com grande capacidade de parasitar diversos tipos de células e infectar praticamente qualquer animal de sangue quente (DUBEY, 2012), podendo causar doença severa em humanos e animais (HUNTER e SIBLEY, 2012). Os felídeos, são os hospedeiros definitivos do parasito, infectam a parede do intestino delgado e se reproduzem sexuadamente (DUBEY, 2009), já no hospedeiro intermediário, mamíferos e aves, quando infectados por cepas avirulentas do parasito *T. gondii*, como exemplo a cepa ME-49, podem desenvolver quadros de infecção aguda onde o parasito se desenvolve nos órgãos dos hospedeiros (DUBEY et al., 1998). No entanto, no período de 10 a 15 dias após a infecção, a doença apresenta característica crônica, onde o parasito entra em sua fase de latência, protegendo-se da resposta imunológica, podendo encistar em diversos órgãos, principalmente no SNC (FERGUNSON; HUTCHINSON, 1987).

A doença causada pela infecção por *T. gondii* é mundialmente conhecida por toxoplasmose, a qual é de grande relevância no âmbito da saúde pública, quando não tratada ou em pacientes imunodeprimidos, a toxoplasmose pode evoluir para uma doença sistêmica grave, ocasionando sérios danos principalmente no sistema nervoso central e olho (BARRAGAN e SIBLEY, 2003). Já a toxoplasmose crônica é considerada assintomática e pode durar toda vida do hospedeiro (FERGUNSON; HUTCHINSON, 1987). Porém, estudos relatam que a infecção crônica é capaz de induzir alterações comportamentais e doenças neuronais em roedores e humanos (FOND et al., 2013; PRANDOTA, 2014), além de provocar alterações sistêmicas aumentando a resposta inflamatória (ISKANDAR et al., 2016).

O sistema colinérgico é visto como uma das vias mais importantes do sistema nervoso central (SNC) (PERRY et al., 1999), estando envolvida em ações anti-inflamatórias (DAS, 2007). A acetilcolina (ACh) é um importante neurotransmissor, possui papel fundamental no sistema nervoso central (SNC), associado com funções cognitivas, processamento de informações sensoriais entre outras (SCREMIN et al., 1997). Os níveis de ACh são regulados pela ação da enzima acetilcolinesterase (AChE), presente tanto nos tecidos colinérgicos e não colinérgicos, assim como no sangue e fluidos corporais (DAJAS-BAILADOR e WONNACOTT, 2004). Em um estudo realizado por Tonin et al. (2014a), a atividade da AChE em cérebro aumentou em animais infectados com *T. gondii*, essa alteração pode estar diretamente relacionada com a resposta inflamatória contra a infecção pelo parasito, uma vez

que o aumento da atividade da AChE provoca uma maior hidrólise da Ach (DAJAS-BAILADOR e WONNACOTT, 2004).

Outra importante molécula sinalizadora para um bom funcionamento do sistema bioenergético celular é o trifosfato de adenosina (ATP) produzido e entregue aos locais de consumo em uma velocidade correspondente à sua demanda (SEGAL et al., 2007; GLORIA-BOTTINI, et al., 2011). Com isso, a rede de transferência de grupos fosforil, catalisada pelas enzimas creatina quinase (CK), adenilato quinase (AK) e piruvato quinase (PK) que atuam entre os locais de produção e consumo de ATP, desenvolvem um papel crucial na homeostase energéticas das células (VALENTIN et al., 2000). Estudo com camundongos infectados com *T. gondii*, demostraram que os níveis de ATP no cérebro têm picos de acordo com o estágio da doença, isto é, aumento de ATP na fase aguda e diminuidos durante a fase crônica da doença (TONIN et al., 2014a). A deficiência na AK e CK, pode acarretar distúrbios nas funções celulares e comprometimento das regiões que necessitam de maior demanda de ATP (RECH et al., 2008), também atuam na regulação da resposta imune do hospedeiro e nos processos de replicação, transcrição e síntese proteica no ciclo biológico do *T. gondii* (MILLER et al., 2011; SAUVAGE et al., 2009; TONIN et al., 2014).

Devido à importância da toxoplasmose para a saúde pública, e sua capacidade de estimular a resposta imunoinflamatória e colonização de órgãos e tecidos, em especial o SNC, estudos que busquem aprofundar os conhecimentos correlacionando esses aspectos, além de novas alternativas de tratamento mais eficazes e sem efeitos colaterais são extremamente importantes. Apesar de existir uma lista considerável de medicamentos com ação reconhecida contra o *T. gondii*, como por exemplo, a sulfadiazina em uso combinado com a pirimetamina, além dos diaminopiridinas, lincosaminas e antibióticos macrolídeos (FRENKEL e BERMUDEZ, 2010; LEVI e MENDONÇA, 2003), nenhum desses é totalmente eficaz contra o parasito e apresentam diversos efeitos colaterais. Hussain et al.(2016), sugeriram para prevenção de danos oxidativos gerados por processos inflamatórios crônicos, tratamentos com agentes antioxidantes em modelos *in vivo*. A associação de quimioterápicos com antioxidantes gera um aumento na eficácia terapêutica e redução nos marcadores de lesão celular quando comparado aos animais somente tratados com quimioterápicos (NOBREGA, 1991). Baseado nisso, surge nossa hipótese que o selênio poderia ser um imunomodulador e antioxidante na toxoplasmose.

O disseleneto de difenila $(PhSe)_2$, é um composto orgânico derivado do selênio com propriedades antioxidantes e farmacológicas já bem elucidadas (NOGUEIRA et al., 2004). Possui inúmeras propriedades farmacológicas, dentre as quais destacam-se a sua ação antioxidante (GHISLENI et al., 2008), além de suas propriedades anti-inflamatórias e antinociceptivas (NOGUEIRA et al., 2003), sendo capaz de proteger o SNC, musculatura esquelética e cardíaca de danos causados por agentes tóxicos ou patologias (WILHELM et al., 2009). Alterações sistêmicas são relatadas em hospedeiros infectados cronicamente com *T. gondii* (EL-REHIM et al., 2013; NAM et al., 2011). Considerando todas as complicações causadas pelo parasito e a falta de opções medicamentosas mais seguras e com menos efeitos colaterais, propomos para este estudo o uso do composto $(PhSe)_2$ com o objetivo de avaliar seus benefícios ao metabolismo energético, sistema antioxidante e colinérgico em camundongos infectados com *T. gondii*.

# 2 REVISÃO BIBLIOGRÁFICA

## 2.1 *Toxoplasma gondii*

*Toxoplasma gondii* é um protozoário, parasito intracelular, pertencente ao reino *Protista*, filo *Apicomplexa*, classe *Conoidasida*, ordem *Eucoccidiida*, família *Sarcocystidae* e gênero *Toxoplasma* (NICOLLE e MANCEAUX, 1909). O gênero *Toxoplasma* do grego *taxon* = arco e *plasma* = corpo foi usado pelos seus descobridores Nicolle e Manceaux em 1909 pelo fato deste parasito apresentar morfologia crescente. O ciclo biológico heteroxênico do *T. gondii* compreende o ciclo obrigatório entre o hospedeiro definitivo (felídeos) que desenvolvem em seu organismo a fase sexuada e os intermediários (mamíferos e aves) que desenvolvem em seu organismo a fase assexuada (FERREIRA e VITOR, 2014).

Em mamíferos e aves este parasito é capaz de reconhecer e invadir células nucleadas e se reproduzir no interior destas (DUBEY, 2012). As formas evolutivas do *T. gondii* responsáveis pela infecção dos hospedeiros intermediários e definitivos são: cistos teciduais (contendo bradizoítos) presentes em carnes e vísceras de organismos infectados; taquizoítos encontrado em diversos tecidos e fluídos corporais e os esporozóitas presentes no interior dos oocistos eliminados juntamente com as fezes do hospedeiro definitivo (DUBEY, 2002). Os taquizoítos possuem formato elíptico com aproximadamente 6 μm de comprimento e 2 μm de largura, e são obrigatoriamente intracelulares de todas as células nucleadas (Figura 1 A). São capazes de invadir as células preferencialmente por penetração ativa, encontrados no interior de vacúolos parasitóforos e, eventualmente, são intracelulares (JONES e DUBEY, 2010).

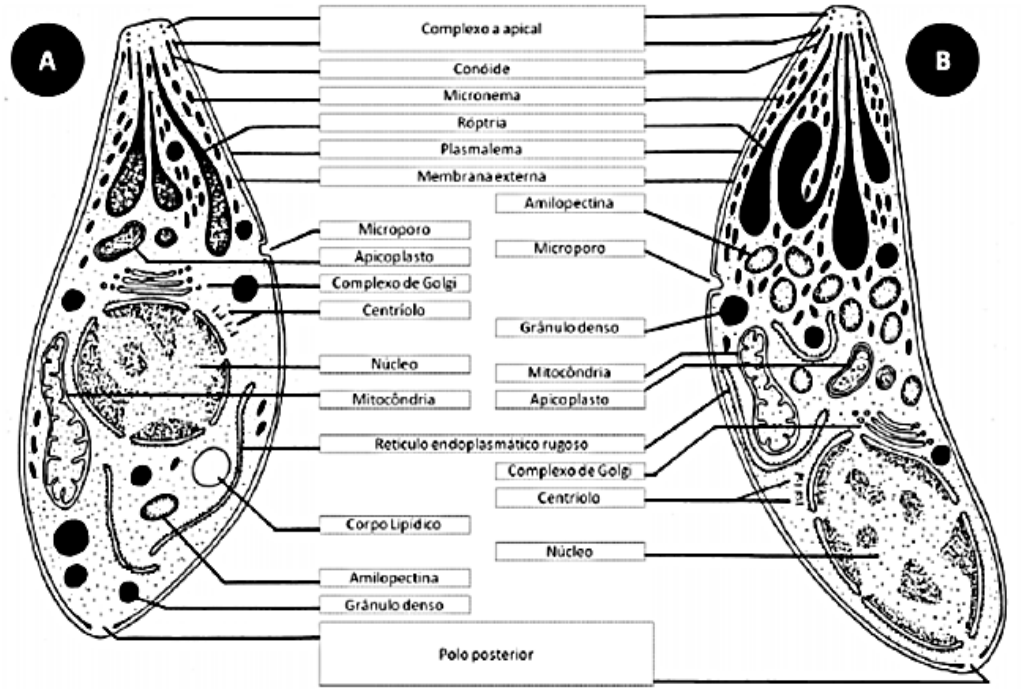
Os taquizoítos são considerados a forma de multiplicação e de disseminação rápida encontrada na fase aguda da infecção, capazes de se multiplicar rapidamente dentro de muitos tipos celulares do hospedeiro intermediário e nas células epiteliais não intestinais do hospedeiro definitivo (BARBOSA et al., 2005; JONES e DUBEY, 2010; ROBERT-GANGNEUX e DARDÉ, 2012). A fase aguda ocorre geralmente entre 8 a 12 dias pós-infecção, onde os taquizoítos se multiplicam de forma assexuada dentro da célula hospedeira por endodiogenia, uma forma especial de reprodução, onde duas progênies são formadas dentro de uma célula mãe (DE SOUZA, 1974; DUBEY, LINDSAY e SPEER, 1998). Terminada a replicação, os taquizoítos completam seu ciclo deixando o vacúolo e alcançando o meio extracelular devido ao rompimento da membrana plasmática da célula hospedeira, disseminando-se pela via hematogênica ou linfática para vários tecidos (DUBEY, 1998).

Os taquizoítos são capazes de permear as barreiras teciduais como hematoencefálica atingindo o SNC, além de causar danos nos olhos, musculatura esquelética, cardíaca (HOWE e SIBLEY, 1995) e placenta, podendo causar complicações severas ao feto (WEISS e KIM, 2007). Os taquizoítos são a forma infectante menos resistente às condições ambientais adversas, ao suco gástrico, desidratação e variações osmóticas (NEVES, 1995). Os taquizoítos de *T. gondii* do interior das células dão origem a um novo estágio evolutivo denominado bradizoíto (Figura 1 B).

A variação no ritmo de multiplicação do parasito depende da resposta imunológica do hospedeiro relacionada à regulação na produção principalmente das citocinas (IL-10, TGF-β, IL-27 e IL-12) e linfócitos TCD4+, responsáveis por modular a patogênese da toxoplasmose sistêmica e local (GADDI e YAP, 2007). Estudos sugerem que, embora os fatores imunológicos do hospedeiro participem decisivamente na modulação da multiplicação e interconversão de *T. gondii*, existem fatores genéticos relacionados com a virulência do parasito, ou seja, enquanto cepas dos tipos II e III tem baixa virulência e favorecem o enquistamento e cronificação da infecção (APPLEFORD e SMITH, 2000), a forma tipo I causa casos de doença agudas.

As cepas de *T. gondii* são classificadas com base na taxa de mortalidade e o tempo de sobrevida dos camundongos infectados com inóculos graduais de taquizoítos, com base nesses critérios as cepas podem ser definidas como virulentas, não virulentas ou de virulência intermediária. A cepa tipo I, incluem as RH, CAST e VEL são altamente virulentas exibindo uma dose letal de 100% ($DL_{100\%}$) equivalente a um único taquizoito viável, enquanto que cepas não virulentas, como a ME-49, exibem uma $DL_{100} > 10^3$ taquizoítos, sendo as infecções crônicas facilmente estabelecidas em camundongos, já as cepas intermediárias apresentam um fenótipo variável de $DL_{100} > 10$ taquizoítos (FERREIRA e VITOR, 2014).

Figura 1 - (A) Taquizoíto e (B) Bradizoíto de *Toxoplasma gondii*.



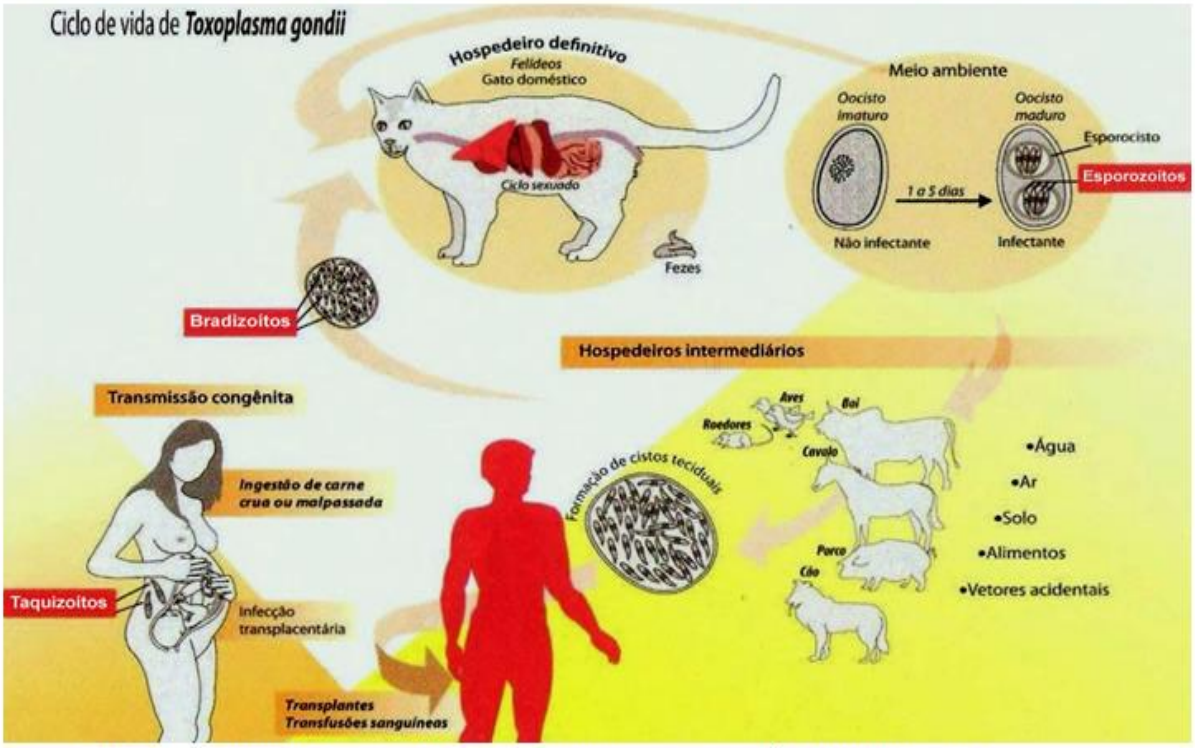

Fonte: Adaptada de DUBEY et al. 1998.

Os bradizoítos (do Grego *brady* = lento), também conhecido como cistozoítos, são a forma de resistência do *T. gondii* nos tecidos, assim como são a fase de multiplicação lenta representam a fase crônica da infecção e diferem morfologicamente pouco dos taquizoítos. Os eventos que se seguem após a diferenciação dos bradizoítos são as alterações morfológicas da membrana e da matriz do vacúolo parasitóforo, constituindo a parede cística dando origem ao cisto tecidual, estrutura característica da fase crônica da infecção (GUIMARÃES et al., 2007), podendo se desenvolver em diversos órgãos (DUBEY, 2010). Estudos relataram um maior tropismo para a formação de cistos cerebrais em pequenos roedores, independente da cepa de *T. gondii*, enquanto que em grandes mamíferos como bovinos, ovinos e caprinos os cistos são predominantes no tecido muscular (DUBEY et al., 2008).

Os cistos teciduais podem variar de tamanho dependendo do tempo, sendo que os cistos jovens podem medir cerca de 5 μm de diâmetro, enquanto que os mais velhos podem medir em média 60 μm. Hill e Dubey (2005) relataram que os cistos teciduais conseguem permanecer latentes por toda a vida do hospedeiro sem apresentar uma resposta inflamatória ou imunológica, evitando, assim, sua destruição. Porém, se os cistos teciduais se romperem durante o curso da infecção, os bradizoitos podem se converterem em taquizoitos (conversão),

estes podem invadir células hospedeiras e se rediferenciar em bradizoítos (interconversão), formando um novo cisto tecidual (TENTER, 2000) (Figura 2).

Figura 2- Ciclo biológico do *T. gondii* e vias de transmissão entre os hospedeiros intermediários e definitivos.



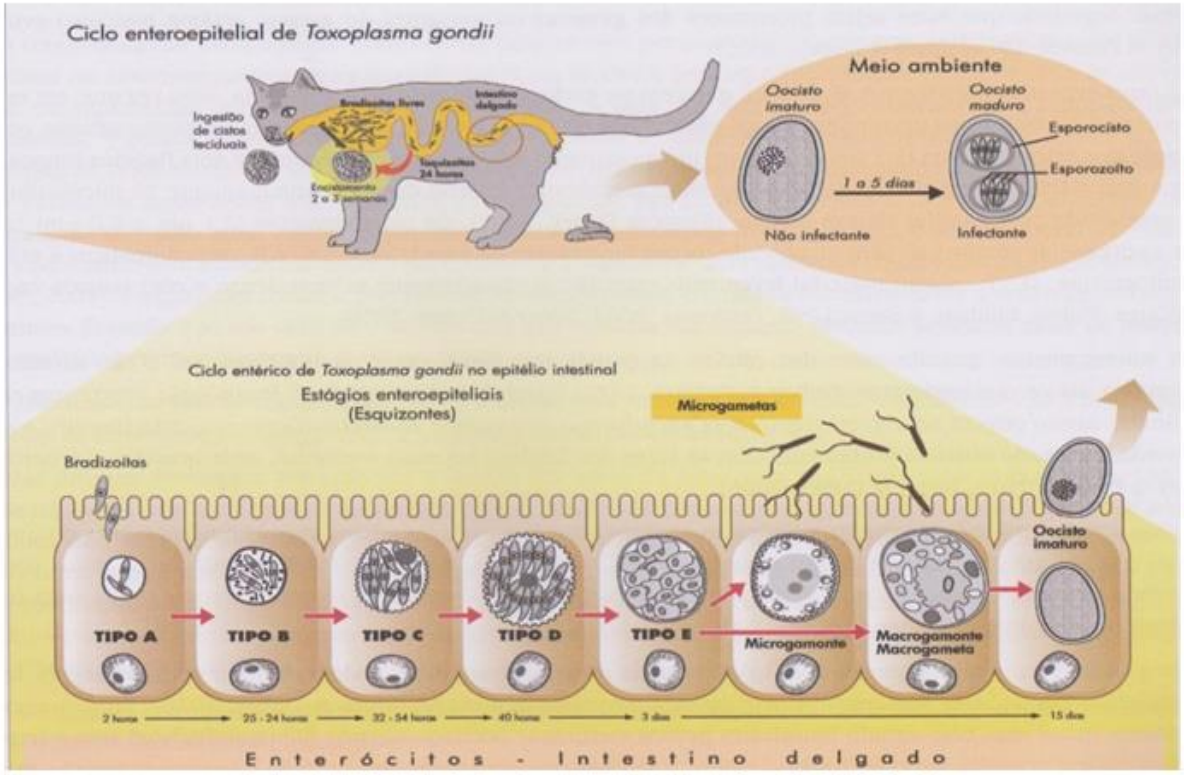

Fonte: MOURA, AMENDOEIRA E BARBOSA, 2009. Ilustração: GETÚLIO VILANOVA.

O *T. gondii* tem capacidade de afetar o tecido neural de camundongos infectados fazendo com que estes se tornem menos neofóbicos, levando a uma menor aversão ao odor de gatos. Esse comportamento faz com que esses roedores infectados sejam facilmente capturados e ingeridos pelos gatos e o ciclo de vida do parasito seja preservado (COX e HOLLAND, 2001). No epitélio intestinal dos felídeos os cistos teciduais perdem sua parede cística devido à ação de enzimas proteolíticas, liberando os bradizoitos que penetram no epitélio intestinal, iniciando o desenvolvimento de várias gerações de *T. gondii*. Em seguida ocorre cinco estágios enteroepitelial também chamados de esquizontes distintos, tipos A, B, C, D e E (DUBEY, 2009). Segundo Dubey (2005), os esquizontes estão localizados entre a superfície interna e o núcleo dos enterócitos.

Os estágios enteroepiteliais se iniciam pelo esquizonte do tipo A e ocorrem de forma sequencial, ou seja, o tipo A conduz à formação dos tipos B e C, e estes amadurecem

rapidamente e desaparecem conforme vão surgindo às formas do tipo D. Os gametócitos só poderão ser observados na ausência das fases evolutivas do tipo A, B e C, já as fases evolutivas do tipo D e E ocorrem de forma simultânea com os gametas, sugerindo que estes sejam precursores dos gametas ou variantes do mesmo estágio biológico evolutivo (Figura 3) (SPEER e DUBEY, 2005).

Figura 3- Estágios enteroepiteliais do Ciclo de *T. gondii* nas células epiteliais superficiais do intestino delgado de gatos domésticos.




Fonte: MOURA, AMENDOEIRA E BARBOSA, 2009. Ilustração: GETÚLIO VILANOVA

Os oocistos produzidos no epitélio intestinal dos felídeos (HD) são eliminados ainda imaturos, junto com as fezes dos felídeos no meio ambiente, onde sofrem maturação (TENTER, HECKEROTH e WEISS, 2000). A maturação ou esporulação dos oocistos ocorre no período de um a cinco dias, sob a influência da oxigenação, umidade e temperatura adequadas (DUBEY, 2004). Os oocistos esporulados têm aspectos subesféricos e elípticos e medem 11 a 13 µm de diâmetro, cada oocisto esporulado apresentam em seu interior dois esporocistos e cada esporocisto apresenta quatro esporozoítas (SPEER e DUBEY, 2005). Os

oocistos são resistentes a vários processos de inativação, são altamente resistentes ao meio ambiente e suas influências, podendo permanecer viáveis em água fria por até 54 meses (DUBEY, 1998). Além disso, não são inativados quando submetidos a tratamentos físicos e químicos tradicionais, como por exemplo, em estações de tratamento de água, incluindo a cloração, o tratamento de ozônio e raios ultravioleta (DUBEY et al., 2010).

Pesquisadores acreditam que um terço, ou mais, da população mundial esteja cronicamente infectado e apresentam anticorpos para o parasito (HUNTER e SIBLEY, 2012; MONTOYA e LIESENFELD, 2004; TENDER et al., 2000), visto que os índices de soropositividade no Brasil variam de 23 a 83%, dependendo de alguns fatores climáticos, socioeconômicos e culturais (FIALHO et al., 2009). A toxoplasmose é geralmente assintomática em indivíduos saudáveis (80%-90%), porém, linfadenopatia, meningoencefalite, miocardite, miosite ou distúrbios oculares podem ocorrer em alguns pacientes.

A neurotoxoplasmose, também chamada de toxoplasmose cerebral é uma infecção no cérebro que ocorre em pessoas com a imunidade baixa, apresentando múltiplos sinais e sintomas e com composições diferentes para cada caso. Como por exemplo, uma das formas de apresentação é a meningoencefalite; por conta disso é possível observar os sinais clínicos como: rigidez de nuca, sinais de Kernig e Brudzinski, além de confusão mental, convulsões focais ou generalizadas e até coma (SU et al., 2010).

A toxoplasmose ocular é reconhecida como a principal complicação associada à infecção por *T. gondii* congênita ou adquirida (HADI e RAMEH, 2007). A toxoplasmose adquirida é responsável pela maioria dos casos oftalmológicos, os quais também são provenientes da reativação do *T. gondii* em pacientes imunocomprometidos (DUPOUY-CAMET et al., 2012). Por outro lado, a toxoplasmose congênita ocorre quando a mulher adquire a infecção durante o período de gravidez, a parasitemia provoca placentite seguida por propagação de taquizoítos para o feto caracterizando a transmissão vertical podendo causar complicações graves ao feto como, por exemplo, retardo mental e perda visual (DUBEY et al., 2009, RESENDE et al, 2010).

## 2.2 TRATAMENTO TOXOPLASMOSE

Os medicamentos existentes com ação reconhecida contra o *T. gondii*, são a sulfadiazina em uso combinado com a pirimetamina representam o padrão-ouro no tratamento da toxoplasmose, porém existem outros medicamentos com ação sobre o parasito que são as

diaminopiridinas, lincosaminas e antibióticos macrolídeos (FRENKEL e BERMUDEZ, 2010). No entanto, a pirimetamina e a sulfadiazina, são antagonistas do folato, e atuam inibindo a duplicação do DNA (ácido desoxirribonucleico) e a enzima dihidrofosfato redutase. Já a clindamicina é uma droga que apresenta efeitos tóxicos ao parasito através da ligação a açúcares presentes na membrana celular, além de inibir a síntese proteica e apresenta relativa boa experiência clínica na toxoplasmose humana (MENDONÇA, 2014).

Muitos estudos continuam em busca de novas alternativas para combater a toxoplasmose e diminuir os efeitos colaterais dos medicamentos quimioterápicos utilizados, no entanto faz-se necessário à busca por novas drogas capazes de minimizar os efeitos colaterais e de combater o parasito. Em um estudo realizado por Bottari et al. (2015) demostrou-se que o resveratrol um potente antioxidante presente no vinho tinto e na casca de uva, quando utilizado na sua forma livre e nanoencapsulado em associação com sulfametoxazol e trimetropim foram capazes de modular a atividade da (AChE) em animais infectados com *T. gondii*. Portanto, como a associação de quimioterápicos com antioxidantes gera um aumento na eficácia terapêutica e redução nos marcadores de lesão celular (NOBREGA, 1991; BOTTARI et al., 2015) nossa hipótese é que o selênio poderia ser um imunomodulador e antioxidante na toxoplasmose, similar ao resveratrol.

## 2.3 DISSELENETO DE DIFENILA $(PhSe)_2$

O $(PhSe)_2$ é um organocalcogênio derivado do selênio (Figura 4) que tem sido alvo de interesse devido suas inúmeras propriedades farmacológicas (NOGUEIRA et al., 2004) e potencial atividade antioxidante (NOGUEIRA e ROCHA, 2010). O selênio (Se) é um calcogênio do grupo 16 da tabela periódica e foi descoberto pelo químico sueco Jons Jacob Berzelius no ano de 1917 (STADTMAN, 1980; LU e HOLMGREN, 2009). O selênio apresenta capacidade de compartilhar propriedades químicas e físicas com o enxofre, permitindo que o selênio substitua o enxofre, promovendo interações selênio-enxofre nos sistemas biológicos (YOUNG et al., 1981). Entretanto, as diferenças nas propriedades físico-químicas entre selênio e enxofre constituem a base de seus papéis biológicos específicos (STADTMAN, 1980). O selênio apresenta grande importância nutricional. De acordo com literatura, baixos níveis de selênio podem levar à predisposição para o desenvolvimento de algumas doenças, como exemplo, câncer, esclerose, doença cardiovascular, cirrose e diabetes (NAVARRO-ALARCÓN e LÓPEZ-MARTINEZ, 2000). A ingesta diária de selênio para

uma pessoa adulta deve ser entre 50-200µg, porém, pesquisas científicas revelam que a concentração alimentar requerida de selênio é muito próxima da dose que pode ser tóxica (OLDFIELD, 1987), devido sua habilidade de oxirredução de grupos tióis (–SH) de proteínas e biomoléculas ativas e capacidade de gerar radicais livres (BARBOSA et al., 1998; NOGUEIRA et al., 2004).

Sabe-se que as moléculas contendo selênio, como por exemplo, o $(PhSe)_2$ são considerados nucleófilos, portanto apresentam maior potencial antioxidante do que os antioxidantes clássicos (ARTEEL e SIES, 2001), além de serem bastante lipofílicos (NOGUEIRA et al., 2004). O $(PhSe)_2$ é capaz de reagir com grupos –SH da estrutura terciária de proteínas, sendo esta interação capaz de modular a atividade e afinidade de enzimas ricas em grupos –SH em sua composição. Estudos toxicológicos relatam 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 provável mecanismo toxicológico (NOGUEIRA; ROCHA, 2010). No entanto, o aparecimento de tais efeitos neurotóxicos estão relacionados à dose, ao veículo, a rota de administração, bem como a idade e a espécie dos animais (BRITO et al., 2006; NOGUEIRA et al., 2004; PRIGOL et al., 2009; SAVEGNAGO et al., 2007).

Outras pesquisas buscam entender a função e a biologia molecular de selenoproteínas. O selênio está presente como resíduo de selenocistéina no sitio ativo de várias enzimas como podemos destacar: glutationa peroxidase (WINGLER e BRIGELIUS-FLOHÉ, 1999), tioredoxina reductase (HOLMGREN, 1985) e selenoproteína P (URSINI et al., 1990). De fato, estudos realizados em laboratório têm demostrado que o $(PhSe)_2$ apresenta propriedades antiúlcera (SAVEGNAGO et al., 2006), anti-inflamatório e antinociceptiva (SAVEGNAGO et al., 2007), antidepressiva e ansiolítica (SAVEGNAGO et al., 2008), neuroprotetor (GHISLENI et al., 2003), hepato-protetora (BORGES et al., 2006), anti-hiperglicêmica (BARBOSA et al., 2006) e pode retardar a progressão do câncer (BARBOSA et al., 2008).

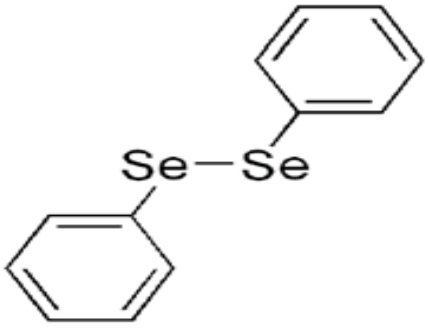
Em um estudo realizado por Nogueira et al. (2004) sugerem que, pelo fato do $(PhSe)_2$ possuir atividade semelhante a glutationa peroxidase, este composto é um bom candidato a ser um agente antioxidante. Porém, alguns estudos relatam que o composto apresenta efeitos tóxicos causando inibições na atividade das enzimas delta-aminolevulínico desidratase (δ-ALA-D) (NOGUEIRA et al., 2003b) no sangue humano e inibe a atividade da $Na^+$, $K^+$ ATPase cerebral (BORGES et al., 2005). Além disso, Nogueira et al. (2003c) relataram que o composto $(PhSe)_2$ apresentou efeito neurotóxico, induzindo convulsões (PRIGOL at al., 2008), e também é capaz de afetar o sistema glutamatérgico em plaquetas humanas (BORGES et al., 2004) e de ratos (NOGUEIRA et al., 2001). Pouco se sabe sobre o verdadeiro

mecanismo de ação do $(PhSe)_2$ relacionados com seus efeitos antioxidantes e anti-inflamatórios (NOGUEIRA; ROCHA, 2010), porém uma hipótese para explicar seus efeitos sobre as enzimas envolvidas no metabolismo energético e antioxidante em encéfalo e coração seria pelo fato deste composto ser bastante lipofílico e ultrapassar com facilidade a barreira hematoencefálica e membranas biológicas (MACIEL et al., 2003), além de compartilhar propriedades químicas e físicas com o enxofre (S) (STADTMAN, 1980).

Sendo assim, o selênio (Se) substituindo o S promove uma interação Se-S nos sistemas biológicos podendo atuar diretamente ou indiretamente sobre enzimas do metabolismo energético, pois o selênio desempenha papel fisiológico na estrutura de muitas enzimas envolvidas na decomposição de peróxidos, incluindo glutationa peroxidase e fosfolípideos os quais contêm selênio na forma se selenocisteína (URSINI et al., 1987; NEVE et al., 1989).

Neste contexto, a suplementação de dietas com selênio tanto para animais quanto para humanos, tem sido aceita na comunidade científica. A partir da descoberta do papel eficaz do selênio nos sítios ativos de enzimas e do conceito de que moléculas contendo selênio podem ser melhores nucleófilos (e, portanto antioxidantes) que os clássicos antioxidantes (ARTEEL e SIES, 2001). Portanto, apesar das ótimas propriedades já descritas para essa forma de selênio, cuidados são necessários, assim como pesquisas para definir a dose efetiva e não tóxica para humanos e animais.

Figura 4- Estrutura do Disseleneto de Difenila $(PhSe)_2$.



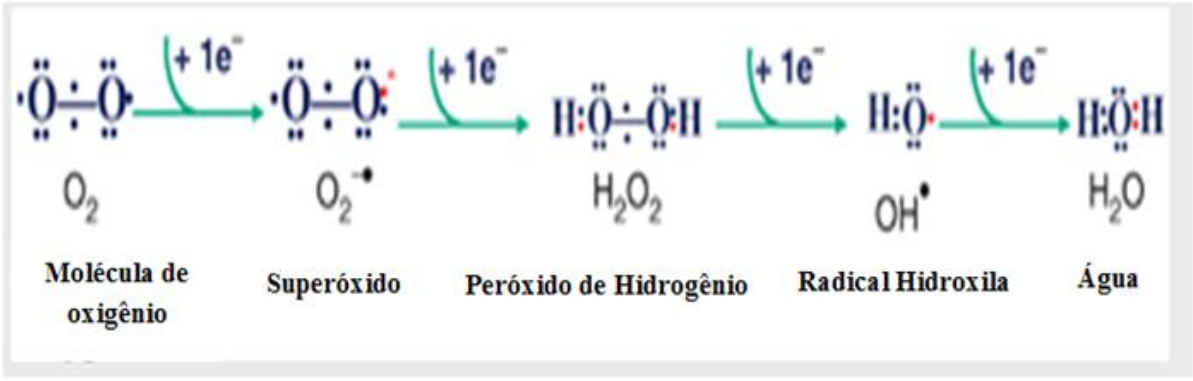

Fonte: NOGUEIRA; ROCHA, 2004.

## 2.3 SISTEMA ANTIOXIDANTE

O oxigênio molecular é um elemento essencial para a vida, porém a redução incompleta ou a excitação deste elemento durante o metabolismo aeróbico pode levar a produção de espécies reativas de oxigênio (EROs). EROs incluem o anion superóxido, radical hidroxila, oxigênio singlete e peróxido de hidrogênio. A produção de ERO em condições fisiológicas normais é importante para as reações de oxigenação celular (TAOKA et al., 1997; LIU et al., 2004) e necessária para uma correta resposta imunológica no organismo, os quais são capazes de combater tumores e infecções microbianas. Porém, uma produção excessiva de ERO, como em condições fisiopatológicas, como por exemplo, lesões no SNC, podem levar ao estresse oxidativo (LIU et al., 2001; SOSA et al., 2013).

Em longo prazo, este estresse oxidativo gerado pela resposta inflamatória pode induzir ao dano e remodelação tecidual, e consequente perda de função (SOSA et al., 2013). Assim, o estado oxidativo das células representa um equilíbrio complexo entre moléculas pró-oxidantes e antioxidantes (LI et al., 2005), demostrando que quando o organismo é exposto a uma fonte de estresse, o aumento da geração de ERO pode levar a um estado oxidativo desequilibrado em favor das moléculas oxidantes, resultando em aumento de reações oxidativas e danos moleculares (KRAFT et al., 2007). O peróxido de hidrogênio não é um radical livre, mas é uma molécula prejudicial devido à sua capacidade de penetrar nas membranas biológicas e causar toxicidade as células devido a maior geração de radicais livres. O superóxido é o produto da redução de um elétron do oxigênio molecular, já o peróxido de hidrogênio é o produto da transferência de dois elétrons (TAOKA et al., 1997) (Figura 5) .

Figura 5- Ilustração esquemática da formação de espécies reativas de oxigênio (EROs).




Fonte: adaptado de JIA et al. (2012).

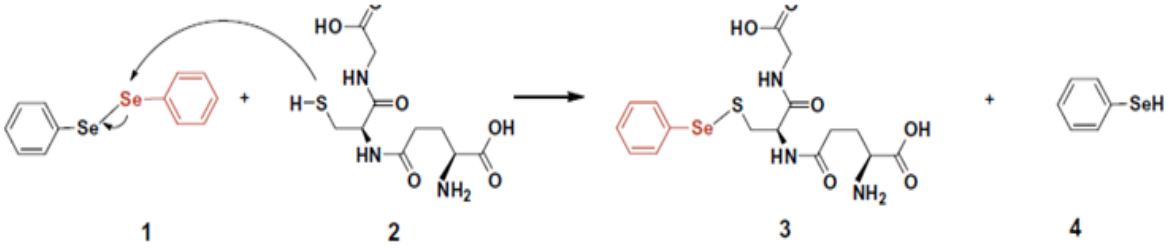
Para controlar a produção de ERO, o organismo aeróbico utiliza de vários mecanismos antioxidantes, incluindo antioxidantes enzimáticos e não-enzimáticos (STOREY, 1996). Os antioxidantes não-enzimáticos incluem as glutationas, vitamina C e E, β-caroteno e ácido úrico, diferente dos antioxidantes enzimáticos que incluem a superóxido dismutase (SOD), catalase (CAT), glutationa reductase (GR), glutationa peroxidase (GPx), glutationa transferase (GST) entre outras (FRIDOVICH, 1995). Níveis elevados de ERO podem sobrecarregar as defesas antioxidantes celulares podendo provocar danos diretos ou indiretos ao organismo (HALL, 1992). Os efeitos diretos estão relacionados a cadeia de reações de peroxidação envolvendo lipídios e outras macromoléculas, e os efeitos indiretos incluem caminhos metabólicos modificados e fisiopatologia alterada no sistema de órgãos devido ao dano oxidativo. O estresse oxidativo também ocorre quando as ERO são produzidas mais rapidamente do que podem ser removidos por mecanismos de defesa celular (HALL, 1992).

A glutationa (GST) possui papel crucial na biotransformação e eliminação de xenobióticos e na defesa das células contra o estresse oxidativo. A glutationa é um antioxidante hidrossolúvel reconhecido como o tiol não proteíco mais importante nos sistemas vivos. Pode ser encontrada na forma reduzida (GSH) ou oxidada (GSSG) e a razão GST/GSSG é utilizada para estimar o estado redox dos sistemas biológicos. Para que a atividade protetora da glutationa expressa pela redução de espécies oxidantes, e consequente oxidação da GSH à glutationa dissulfeto (GSSG) seja mantida, a GSH precisa ser regenerada através de um ciclo catalítico, onde é possível identificar a atividade de três grupos de enzimas: a glutationa oxidase (GO), a glutationa peroxidase (GPx) e a glutationa reductase (GR).

A GPx está envolvida em um importante sistema enzimático de defesa contra radicais livres encontradas em muitos tecidos de origem animal. A enzima glutationa S-transferase (GST) catalisa o ataque nucleofílico da forma reduzida da glutationa (GSH) a compostos que apresentam um carbono, um nitrogênio ou um átomo de enxofre eletrofílico (HAYES et al., 2005). Esta molécula apresenta um papel crítico na manutenção e no equilíbrio de oxidação-redução e na regulação das vias de sinalização induzidas por estresse oxidativo, bem como de EROs de desintoxicação e outros aldeídos reativos (DICKINSON e FORMAN, 2002). O SNC apresenta níveis reduzidos de glutationa em comparação com outros órgãos como o fígado, o que deixa o SNC mais vulnerável ao estresse oxidativo (LOGAN et al., 2005; SEKHON e FEHLINGS, 2001; VAZIRI et al., 2004). A biomolécula glutationa reduzida (GSH) devido suas características sulfidrílicas

ataca nucleofilicamente a molécula de $(PhSe)_2$ produzindo um aduto de GSH inativo e uma molécula de selenol (NELSON; COX, 2014; PRIGOL et al., 2012) (Figura 6).

Figura 6 – Esquema de reação do $(PhSe)_2$ com moléculas sulfidrilicas. 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. (2012).

## 2.5 SISTEMA COLINÉRGICO

O estresse oxidativo também afeta outros sistemas, como o colinérgico composto por enzimas, receptores e moléculas como ACh. As colinesterases estão presentes em diferentes tecidos, como encéfalo e em alguns fluidos corporais, e estão divididas de acordo com seus substratos específicos, comportamento na presença de excesso de substrato e susceptibilidade a inibidores (ÇOKUGRAS, 2003).

A AChE é uma colinesterase específica, presente em altas concentrações no SNC, sistema nervoso periférico (SNP), músculo estriado e eritrócitos (DAS, 2007; SOREQ e SEIDMAN, 2001), responsável pela regulação de ACh extracelular. ACh é um neurotransmissor das sinapses e junções neuroefetoras colinérgicas do SNC e SNP, possuindo papel crucial no SNC, relacionada com funções cognitivas, processamento de informações sensoriais, organização cortical do movimento e controle do fluxo cerebral (SCREMIN et al., 1997), além de estar envolvida em ações anti-inflamatórias (DAS, 2007). Assim, investigando a supressão da ACh no processo inflamatório e as colinesterases que as regulam, como por exemplo a AChE, mesmo que de forma indireta, os níveis de ACh e colinesterases podem servir como marcadores de processos inflamatórios sistêmicos (DAS, 2007).

A AChE é essencial para o funcionamento normal do sistema nervoso, sendo capaz de eliminar rapidamente a ação da ACh lançada na fenda sináptica (DOREQ e SEIDMAN, 2001), além de estar associada ao desenvolvimento cerebral, aprendizagem, memória, dano cerebrais (BALLARD et al., 2005; METZ e TRACEY, 2005; ZIMMERMAN e SOREQ,

2006), na modulação da atividade glial, fluxo sanguíneo cerebral, atuando também como uma proteína de adesão no desenvolvimento e manutenção sináptica (BALLART et al., 2005).

## 2.6 ENZIMAS DO METABOLISMO ENERGÉTICO

As enzimas creatina quinase (CK), piruvato quinase (PK) e adenilato quinase (AK) estão envolvidas na manutenção e homeostase dos tecidos (DE FRANCESCHI et al., 2013; JANSEN et al., 2003), regulando os niveis de ATP. A CK possui papel importante na homeostase energética de células com necessidades energéticas intermitentes, como o músculo cardíaco, sendo sua redução ou aumento relacionado a um vasto número de patologias (TOREN et al., 1994). A AK catalisa a reação de transferência de alta energia entre os nucleotídeos ATP, adenosina difosfato (ADP) e adenosina monofosfato (AMP), sendo responsável pela interconversão de ATP, ADP e AMP (DZEJA e TERZIC, 1998). A PK é uma das enzimas regulatórias da rota glicolítica, e catalisa a transferência de um grupo fosforil do fosfoenolpiruvato para o ADP formar piruvato e ATP, podendo ser considerada uma enzima chave para todo o metabolismo celular (VALENTIN et al., 2000). A deficiência na AK e CK, pode acarretar distúrbios nas funções celulares e comprometimento das regiões que necessitam de maior demanda de ATP (RECH et al., 2008), também atuam na regulação da resposta imune do hospedeiro e nos processos de replicação, transcrição e síntese proteica no ciclo biológico do *T. gondii* (SAUVAGE et al., 2009).

No meio extracelular, o ATP participa de uma série de processos biológicos como: neurotransmissão, contração muscular, função cardíaca, agregação plaquetária, metabolismo do glicogênio hepático e processos inflamatórios (TORRES et al., 2002). O ATP liberado no meio extracelular por dano ou estímulo celular por ação de patógenos, faz com que o sistema imunológico entenda essa liberação como um “sinal de perigo” participando em vários aspectos do estabelecimento de uma resposta inflamatória (VIRGILIO, 1998; LANGSTON et al., 2003).

Os sistemas biológicos usam energia química para manter os organismos vivos, e a oxidação dos combustíveis energéticos é regulada para manter a homeostase do ATP celular. Problema na manutenção destes nucleotídeos nas células pode causar hipotireoidismo, obesidade e infarto do miocardio (MARKS, SMITH e LIEBERMAN, 2007), no entanto, manter a homeostasia energética é fundamental para a sobrevivência de uma célula normal (FOO et al., 2012). Porém, para que ocorra a produção e consumo do ATP nas células é

necessário que ocorra a integração de reações enzimáticas de trocas de grupos fosforil de alta energia com metabolismo energético mitocondrial.

Para um bom funcionamento do sistema bioenergético celular, é necessário que o ATP seja produzido e entregue aos locais de consumo (SEGAL et al., 2007; GLORIA-BOTTINI, et al., 2011). Um estudo com camundongos infectados com *T. gondii*, demostrou que os níveis de ATP no encéfalo têm picos de acordo com o estágio da doença, isto é, aumentos de ATP na fase aguda e diminuidos durante a fase crônica da doença (TONIN et al., 2014a).

# 3 OBJETIVOS

## 3.1 OBJETIVO GERAL

Avaliar o efeito da aplicação subcutânea com disseleneto de difenila em camundongos experimentalmente infectados com *T. gondii* e seus benéficos à saúde dos animais evitando ou minimizando os efeitos colaterais da doença.

## 3.2 OBJETIVOS ESPECÍFICOS

Em camundongos saudáveis e cronicamente infectados com *T. gondii*, cepa ME-49, e tratados com $(PhSe)_2$, se objetivou:

- Investigar alterações comportamentais, referentes à memória, ansiedade e locomoção;

- Análisar a presença do parasito e lesões no coração e encéfalo;

- Determinar da atividade das enzimas AK e CK em encéfalo e coração;

- Mensurar a enzimas creatina quinase e creatina quinase-MB, troponina, mioglobina e lactato desidrogenase no soro;

- Avaliar a atividade da AChE em encéfalo de camundongos;

- Avaliar o efeito antioxidante (GR, GPx e GST) e os níveis de espécies reativas ao ácido tiobarbitúrico (TBARS) no encéfalo;

# 4 RESULTADOS

Os resultados que fazem parte desta dissertação estão apresentados sob a forma de dois Artigos, os quais se encontram no item Artigo Científico. As seções Materiais e Métodos, Resultados, Discussão e Referências encontram-se no próprio artigo e representam a íntegra deste estudo.

Os itens Discussão e Conclusão geral desse estudo encontra-se no final desta dissertação e apresenta interpretações e comentários gerais sobre os artigos contidos neste trabalho.

As Referências referem-se somente às citações que aparecem nos itens introdução, revisão bibliográfica e discussão desta dissertação.

Os artigos estão estruturados de acordo com as normas da revista científica para o qual foram submetidos e publicados: Experimental Parasitology

# 5 PUBLICAÇÕES CIENTÍFICAS

## 5.1 ARTIGO I:

*Toxoplasma gondii*: Effects of diphenyl diselenide in experimental toxoplasmosis on biomarkers of cardiac function

**Artigo Publicado na Revista Experimental Parasitology**

# *Toxoplasma gondii*: Effects of diphenyl diselenide in experimental toxoplasmosis on biomarkers of cardiac function

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**ABSTRACT**

This study aimed to investigate the effects of diphenyl diselenide $(PhSe)_2$ to treat mice experimentally infected by *Toxoplasma gondii* on seric biomarkers of cardiac function (creatine kinase, creatine kinase MB, troponin, and myoglobin), and lactate dehydrogenase, as well as to evaluate the enzymatic activity of creatine kinase (CK) and adenylate kinase (AK) in heart tissue. For the study, 40 female mice were divided into four groups of 10 animals each: the group A (uninfected and untreated), the group B (uninfected and treated), the group C (infected and untreated) and the group D (infected and treated). The inoculation was performed with 50 cysts of *T. gondii* (ME-49 strain). Mice from groups B and D were treated at days 1 and 20 post-infection (PI) with 5 μmol $kg^{-1}$ of $(PhSe)_2$ subcutaneously. On day 30 PI, the mice were anesthetized and euthanized for blood and heart collection. As a result, it was observed a decrease in AK activity ($P<0.01$) in the heart samples of groups C and D compared to the group A. Cardiac CK increased in the group C compared to the group A ($P<0.01$). CK levels increased in infected mice (the group C) compared to other groups (A and D). Regarding CK-MB level, there was a decrease in the group D compared to the group B, without statistical difference compared to control groups (A and C). It was observed an increase on myoglobin in groups C and D, differently of troponin, which did not show statistical difference ($P<0.05$) between groups. Mice from the group C showed an increase in lactate dehydrogenase (LDH) levels compared to other groups (A, B, and D). Histopathological evaluation of heart samples revealed necrosis, hemorrhagic regions and inflammatory infiltrates in mice from the Group C, differently from the group D where animals showed only inflammatory infiltrates. Based on these results we conclude that the $(PhSe)_2$ had a protective effect on the heart in experimental toxoplasmosis by modulating tissue and seric CK activity, and avoiding an increase on seric LDH levels, probably due to the antioxidant effect of this compound.

**Key-words:** *Toxoplasma gondii*, creatine kinase, adenylate kinase, lactate dehydrogenase, creatine kinase-MB, myoglobin, troponin.

## 1. Introduction

Due to the importance of toxoplasmosis to public health and the need to develop new drugs with less side effects that could. Thus, more natural products or even minerals have been used alone or associated with chemotherapy (Martins-Duarte and Souza, 2013). The organic compound diphenyl diselenide $(PhSe)_2$ is derived from selenium and has many pharmacological properties (Meotti et al., 2004; Nogueira et al., 2004) such as: antinociceptive, anti-inflammatory, antioxidant, anti-ulcer and neuroprotection (Ghisleni et al., 2003; Luchese et al., 2008; Meotti et al., 2004; Savegnago et al., 2007). Due to all these pharmacological properties, the $(PhSe)_2$ has become an interesting molecule in the management of cardiovascular diseases (Mancini, 2013).

*Toxoplasma gondii* is a protozoan that causes parasitic infection in various species of animals, and has felines as definitive host, and man and homoeothermic animals as intermediate hosts (Neves, 2000). The severity of systemic infection is related to the infective form acquired, host susceptibility, and parasite strain that may cause polysymptomatic disease (Tenter et al., 2000). In the acute phase of infection, tachyzoites are able to cross the intestinal wall, reaching other tissues through the bloodstream and/or lymphatic system (Tenter et al., 2000). It may compromise different organs, like the heart, where this parasite has been detected by PCR in sheep (Esteban-Redondo et al., 1999). In the chronic phase, bradyzoites can develop in any visceral organ, forming tissue cysts which represent another important source of infection (Dubey et al., 2004; Montoya and Liesenfeld, 2004). The tissue cysts often appear in the central nervous system and muscle due to the affinity of the parasite by these sites (Dubey, 1993; Dubey, 1998). In immunocompromised patients infection is usually fatal if not quickly diagnosed and treated (Amato Neto et al., 1995). The heart in cases of toxoplasmosis is the most common affected organ during the multivisceral course, showing myositis as secondary consequence of infection by the parasite (Gerberding, 1992). Cardiac biopsy in order to investigate myocardial toxoplasmosis is of little use as a diagnostic tool due to the difficulty in collection a sample of good size (Roldan et al., 1987; Sahasrabudhe et al., 2003). In a study of 18 patients with toxoplasmosis, the most common clinical signs were arrhythmia, atypical chest pain, and cardiac insufficiency (Leak et al., 1979).

The heart is a muscle with high demand of energy that must be provided by blood flow (Meyer et al., 2013), and it is known that 90% of the energy of the human body is from the generation of adenosine triphosphate (ATP) (Neubauer, 2007). The cardiac energy metabolism is comprised of three major components: fatty acids (β-oxidation or glycolysis); oxidative phosphorylation of adenosine diphosphate (ADP) resulting in ATP by the

respiratory chain, and lastly from the transfer and use of ATP by the enzyme-mediated myofibrils phosphotransfer network, preferably creatine kinase (CK) (Carvajal and Moreno Sanchez, 2003; Neubauer, 2007). The enzymes CK and AK are important enzymes for the energy metabolism (Rabinowitz and Vastag, 2012) since they are found in the cells of tissues with high energy demand, including heart, brain, and skeletal muscle (Gloria-Bottini, et al., 2011; Segal et al., 2007). The deficiency in the AK and CK activities can cause disturbances in cellular functions, compromising regions with increased enzymatic activity (Toren et al., 1994).

Biomarkers of cardiac function as CK, CK-MB, LDH, myoglobin, and troponin are important tools for the diagnosis of myocardial necrosis (Jaffe et al., 1996). The CKs constitute a group of different isoforms (CK-MM, CK-BB, and CK-MB) that are specific for each tissue. CK-MB isoform is found only in heart muscle (Wallimann et al., 1994) and it is associated to cardiac damage when its activity is increased (Aktas et al., 1993; Schober et al., 1999). The myoglobin is a molecule released into circulation about an hour after the death of myocardial cells (Lee et al., 1990; Mair et al., 1995; Newby et al., 1998), differently of troponins that are considered the most sensitive biochemical markers of myocardial injury (Hamm and Braunwald, 2000). Although these markers (CK-MB, troponin, and myoglobin) are considered important prognostic tools, they do not have to be measured in conjunction to indicate acute myocardial ischemic syndrome (Cannon et al., 2001).

Recently, it was observed alterations on energy metabolism and increased biomarkers of cardiac function in heart tissue in other protozoan infections, such as *Trypanosoma evansi,* contribuiting directly to disease pathogenesis (Baldissera et al., 2015). Based on this evidence, and due to complications caused by *T. gondii* with great impact on public health, and the lack of drugs or other components that act on both stages of infection, our study aims to evaluate the activities of AK and CK in heart tissue samples, as well as to evaluate seric biomarkers of cardiac function such as CK, CK-MB, LDH, myoglobin, and troponin in mice infected by *T. gondii* and treated with $(PhSe)_2$.

## 2. Materials and Methods

### 2.1 *Toxoplasma gondii* strain

The ME-49 strain, which is considered a cystogenics strain, was used in this study. Initially, samples of ME-49 kept in liquid nitrogen were used to inoculate one Swiss female mouse (M1). Thirty-two days later, M1 was humanely euthanized for brain collection. Brain

homogenate was obtained using saline solution and contained cysts with bradyzoites. Cysts were orally inoculated in other three female mice (M2, M3, and M4) in order to reactivate *T. gondii* virulence. Mice M2, M3 and M4 were euthanized 25 days PI and their brain collected. Cysts were counted and separated for further the experimental inoculation.

**2.2 Diphenyl diselenide**

Diphenyl diselenide $(PhSe)_2$ (99.9%) was purchased from Sigma (St. Louis, MO, USA) and dissolved in dimethyl sulfoxide (DMSO, Sigma-Aldrich, USA) at final concentration of 0.1%.

**2.3 Animal and experimental design**

The experimental design was composed of forty female Swiss mice, 60-day-old, weighing an average of 25 ± 5g. They were kept in cages, housed on a light/dark cycle of 12 hours in an experimental room under controlled temperature and humidity (23±1 ºC; 70 % respectively), fed with commercial ration, and received water *ad libitum*. All the animals were subject to a period of 15 days for adaptation.

Four groups (A, B, C, and D) with ten mice each were formed. Groups A and B were composed of uninfected mice used as control, while mice of groups C and D were orally inoculated with 0.25 mL of brain homogenate containing 50 cysts of *T. gondii* (ME-49 strain) bradyzoites. Groups B and D received $(PhSe)_2$ subcutaneously at dose of 5 μmol $kg^{-1}$ on days 1 and 20 post-infection (PI), as suggested by Barbosa et al. (2014). Mice were clinically observed to check on clinical signs of toxoplasmosis.

**2.4 Sample collection**

For sampling, mice were anesthetized using isoflurane and blood was drawn by cardiac puncture. These samples were stored in tubes without anticoagulant, and centrifuged at 3.500g for 10 minutes for sera separation. Mice were euthanized by decapitation and hearts were collected for biochemical and histopathological analyzes, as described below. Heart samples were stored at -80 °C during two weeks for later assessment of enzymatic activities. Additionally, heart samples were conserved in formalin (10%) to perform histological analyses. A heart fragment of each mice was used for molecular analysis (PCR) to detect the presence of *T. gondii* DNA. Brain fragment was collected mainly to confirm the infection by the presence of parasitic cysts of *T. gondii*.

**2.5 Tissue preparation**

Immediately after euthanasia, hearts were removed and dissected on a Petri dish on ice. In order to measure enzymatic activity of enzymes of the phosphoryltransfer network, they were washed in SET (Tris Sacarose EGTA) buffer (0.32 M sucrose, 1 mM EGTA, 10 mM Tris-HCl, pH 7.4) and homogenized (1:10 v/v) in the same SET buffer with a Potter-Elvehjem glass homogenizer. Centrifugation of homogenate was done at 800*g* for 10 min at 4 °C. Part of this supernatant was used to evaluate AK activity; the pellet was discarded and the supernatant was once again centrifuged at 10.000 g for 15 min at 4 °C. The supernatant containing cytosol and other cellular components such as endoplasmatic reticulum were collected for determination of cytosolic CK activities. The pellet containing mitochondria was washed twice with the same SET buffer, re-suspended in 100 mM Trizma and 15 mM $MgSO_4$ buffer (pH 7.5) to evaluate mitochondrial CK activity. The supernatants were stored for two weeks at -80 °C when the assay was performed.

**2.6 Creatine kinase and adenylate kinase activities**

Creatine kinase activity was assayed in the reaction mixture containing the following final concentrations: 65 mM Tris-HCl buffer, pH 7.5, 7 mM phosphocreatine, 9 mM $MgSO_4$, and 1 μg of protein in a final volume of 100 μL. After 10 min of pre-incubation at 37 °C, the reaction was started by the addition of 0.3 μmol of ADP and stopped after 10 min by the addition of 1 μmol of ρ-hydroxymercuribenzoic acid. The concentrations of reagents and the incubation time were chosen to assure linearity of the enzymatic reaction. Appropriate controls were carried out to measure chemical hydrolysis of phosphocreatine. The creatine was estimated according to the colorimetric method of Hughes (1962). The color was developed by the addition of 0.1 mL of 2 % α-naphthol and 0.1 mL of 0.05 % diacetyl in a final volume of 1 mL and read after 20 min at 540 nm. Results were expressed as nmol of creatine formed per min per mg of protein.

Adenylate kinase activity was measured with a coupled enzymatic assay using hexokinase (HK) and glucose 6-phosphate dehydrogenase (G6PD) according to Dzeja et al. (1999). The reaction mixture contained 100 mM KCl, 20 mM HEPES, 20 mM glucose, 4 mM $MgCl_2$, 2 mM $NADP^+$, 1 mM EDTA, 4.5 U/mL of HK, 2 U/mL of G6PD and 20 μL of homogenate. The reaction was initiated by the addition of 2 mM ADP and the reduction of $NADP^+$ was followed at 340 nm for 3 min in a spectrophotometer. ADP, $NADP^+$, G6PD, and HK were dissolved in milli-Q water. The reagents concentration and assay time (3 min) were

chosen to assure the linearity of the reaction. The results were expressed in pmol of ATP formed per min per mg of protein.

Protein content in heart homogenate was determined by the method of Lowry et al. (1951) using serum bovine albumin as the standard. This analysis was used to express the results of enzymes in heart homogenate.

**2.7 Biomarkers of cardiac function**

Whole blood (WB) and plasma from mice were used for analyses of biomarkers of cardiac function. Samples were stored in tubes containing ethylenediaminetetraacetic acid (EDTA). WB samples were centrifuged at 3000 rpm for 15 minutes, separating plasma, which was stored at -20 °C until analysis. The seric levels of creatine kinase (CK), MB-isoenzyme of creatine kinase (CK-MB), and lactate dehydrogenase (LDH) were evaluated in an automated SBA200 (Celm) by the wet chemistry method. Myoglobin and troponin were evaluated in an automated Alere (Triage®MeterPro) by immunofluorescence method. Tests were carried out in duplicate.

**2.8 *Toxoplasma gondii* DNA extraction**

Heart samples frozen at -80ºC were processed following the protocol described by Sambrook et al. (1989). First, cardiac tissue were macerated with 500µL of lysis buffer (10 mM Tris-HCl pH8.0; 25 mM EDTA pH 8.0; 100 mM NaCl; 1% SDS). After, adding 20µL of proteinase K (20 mg mL), incubating in double dry at 56ºC for 2 h or overnight at 37ºC. After this period, 500µL of phenol-chloroform (1:1) was added and mixed, followed by centrifugation at 12.000 g for 10 minutes. The aqueous phase was transferred to another tube (approximately 400µL) to add the same volume of absolute isopropanol. Then, the mixture was left to precipitate for 2 hours or overnight, and centrifuged at 12.000 g for 30 min. Supernatant was discarded by inverting the tubes, and the pellet re-suspend in 1 mL of cold ethanol 70%, followed by centrifugation at 12.000g for 10 min. Again, supernatant was discarded by inverting tubes, allowing to dry at room temperature, and the pellet was re-suspended in 30µL de TE (10 mM Tris-HCl pH 8.0; 1mM EDTA pH 8.0) and incubated at 56ºC for 10 min, followed by a last centrifugation for final storage at -20ºC.

**2.9 Nested PCR B1 - *Toxoplasma gondii***

PCR was carried out in order to determine the presence of *T. gondii* DNA. The reaction was performed as described by Burg et al. (1989), with modifications, using the gene target

B1. The pairs of primers used in the first reaction were: T1 (5'-AGCGTCTCTCTTCAAGCAGCGTA-3') and T2 (5'-TCCGCAGCGACTTCTATCTCTGT-3') used in PCR and the primer pair T3 (5'-TGGGAATGAAAGAGACGCTAATGTG-3') and T4 (5'-TTAAAGCGTTCGTGGTCAACTATCG-3') at nested PCR in the second reaction, having an amplified fragment of 300pb and 155pb of length respectively, as described by Yai (2000). PCR reaction was performed with a final volume of 25 µL containing 50ng of DNA template, 6 µM of each primers, 100 µM of dNTPs (Invitrogen®), 2 mM $MgCl_2$, 2.5U Taq DNA polymerase (Invitrogen®), and 2.5µL of enzyme-buffer. PCR products were subsequently used in a second stage (nested PCR), using the same reaction mixture previously described, except for the primer pairs T3-T4. For this pourpose, samples were diluted in 24.0 µL of the mixture and 1 µL of the PCR product was used. All the amplifications were executed in an automatic thermocycler (PTC-100 Programmable Thermal Controller (MJ Research). The amplifications followed the cycling profile: initial denaturation: 94 ºC for 3 min; denaturation: 94 ºC for 45 second; hybridization: 55 ºC for 1 min; extension: 72 ºC for 90 second. The cycle was repeated 25 more times from denaturation in conventional PCR and 35 times for nested PCR. Finally, the final extension was done at 72 ºC for 10 min. A positive control (DNA of strain ME-49 of *T. gondii* extracted from sheep heart), and a negative control (PCR DNA free) were used for each reaction. The product of nested PCR was stained by 2% with ethidium bromide and subjected to electrophoresis.

### 2.10 Histology

Heart and brain fragments of uninfected and infected mice were collected and stored in 10 % formalin solution. For histopathology, sagittal sections were obtained (interval of 3 mm) and stained with hematoxylin and eosin (H&E).

### 2.11 Statistical analyses

First, the data of AK, CK and biomarkers of cardiac function were subjected to normality test, which showed normal distribution. Therefore, these data were analyzed by the Duncan test ($P<0.05$). The results were shown as mean and standard deviation.

## 3. Results

### 3.1 Disease course and histopathology

Mice of groups A and B (uninfected) did not show any clinical manifestation of disease throughout the experiment. Animals infected with the strain ME-49 showed bristling and weight loss (groups C and D), and had infection confirmed by histopathology, i.e., the presence of parasitic cysts in the brain of all infected animals in both groups (Figure 1-A). Mice of groups A and B did not show histological lesions in the heart. It is important to emphasize that all mice of the group C showed moderate to severe focally and extensive necrosis with hemorrhage (Figure 1-B, and D); additionally in the group C, it was observed moderate focally to extensive lymphoplasmacytic inflammatory infiltrate. Nine (9) mice of the group D showed moderate lymphoplasmacytic infiltrate in the heart (Figure 1-C).

### 3.2 AK and CK activities in the heart

Results of AK and CK activities are shown in Figure 2. Mice of the group C (infected and untreated) showed decreased activity of AK compared to the group A. Similar results were obtained for mice of the group D. Mice of the group B (uninfected and treated) did not show statistical difference regarding AK activity compared to the group D. Mice of the group C showed an increase in CK activity compared to negative controls (groups A and B).

### 3.3 Seric biomarkers of cardiac function

Seric activities of CK and CK-MB, levels of myoglobin, troponin and LDH are shown in Table 1. Mice infected by *T. gondii* (the group C) showed an increase in the CK activity ($P<0.05$) compared to groups A and D. The mice infected by *T. gondii* and treated with $(PhSe)_2$ (the group D) not show statistical difference in CK-MB levels ($P<0.05$) compared to groups A and C. Mice infected by *T. gondii* (groups C and D) showed an increased level of mioglobin ($P<0.05$) compared to groups A and B. The troponin levels did not differ statistically between groups ($P>0.05$). Mice of the group C showed an increase in LDH levels compared to groups A and B. The treatment with $(PhSe)_2$ in the group D was able to prevent the increase of LDH levels, since the results were quite similar to the ones obtained to groups A and B, and it was lower than the one reached in the group C.

### 3.4. Nested PCR B1 - *Toxoplasma gondii*

Amplification occurred on all reaction of positive controls, and amplicons were not visualized in negative controls. The samples of DNA extracted from the heart of mice infected by *T. gondii* (group C and D) were not amplified in both reactions of PCR.

## 4. Discussion

*T. gondii* is a parasite capable of infecting any nucleated host cell (Montoya and Liesenfeld, 2004). In the acute phase of infection, the parasite is able to rapidly cross the intestinal cells and reach other cell types in different tissues, through the bloodstream and/or lymphatic circulation (Tenter et al., 2000). Cystic forms are predominant during chronic infection and are most often found in the central nervous system, retina, cardiac and skeletal muscle (Dubey and Jones, 2008). This situation might explain the reason why the DNA of the parasite was not found in the heart, even with forms of parasitic cysts histologically observed in brain samples. However, *T. gondii* has a greater predilection for the CNS, only passing through the heart during the acute phase, or during eventual parasitemia in the chronic phase. Other authors reported that *T. gondii* may be more probably found in the heart of infected people, due to little knowledge about the frequency of *T. gondii* in different organs and tissues (Alvarado-Esquivel et al., 2011; Chandenier et al., 2000; Falangola, 1993; Kean and Grocott 1945; Lanjewar et al., 2006; Montoya et al. 2004). Toxoplasmosis is very common in humans, but the clinical manifestations are confined to risk groups (immunocompromised patients). The disease in immunocompetent individuals has mild clinical manifestations and lymphadenopathy can be observed; more severe clinical manifestations such as encephalitis, myocarditis or hepatitis may occur but are very rare in immunocompetent patients (Bowie et al., 1997; Dubey and Beattie, 1988).

This study evaluated the AK and CK activity in the heart of mice infected by *T. gondii* and treated with $(PhSe)_2$, important enzymes in modulating ATP. The CK showed increased activity in mice of the group D, unlike the AK that showed reduced activity in this group. This CK increase may be justified by the mechanism of inter-relationship between these enzymes. We also believe that the *T. gondii* causes an increase in the consumption of ATP, decreasing the levels of these nucleotides. It can be reflected in the reduction of AK activity, catalyzing the reaction of 2 ADP (adenosine diphosphate) to form ATP, plus AMP (adenosine monophosphate) (Carrasco et al., 2001). CK and AK are important eznymes promoting energy metabolism and acting in the production and delivery of ATP (adenosine triphosphate) (Janssen, 1971; Segal et al., 2007). Both enzymes are usually found in organs that require large amounts of ATP as brain, heart, and skeletal muscles (Gloria-Bottini, et al., 2011; Janssen, 1971; Segal et al., 2007). In this context, our study with mice infected by *T. gondii*, showed that AK and CK are part of the same groups of phosphoryl transfer system, as well as when in metabolic stress situations, the energy transmission is compromised when one of

these enzymes reduce or interrupt their activity (Pucar et al, 2000), like our results on AK activity.

Seric biomarkers of cardiac function were also analyzed. These biomarkers serve as an indicator of normal biological processes, pathogenic processes, or pharmacologic intervention that provide information based on the exposure of the disease, lesion length, and prognosis (Oyama and Sisson, 2004). For this reason, we evaluated CK, CK-MB, LDH, myoglobin, and troponin. It was observed an increase in CK activity in infected animals and a decrease in CK activity in mice infected and treated with $(PhSe)_2$. The CK-MB decreased in infected mice treated with $(PhSe)_2$. Also, LDH activity decreased in treated animals, emphasizing that, mice of the group D had no hemorrhages or necrosis, but six (6) mice showed inflammatory infiltrates. The CK-MB has emerged as an early marker (less than 6 hours) for cardiac injury, even being less specific and technically difficult to obtain its results (Puleo et al., 1994; Zimmerman et al., 1999). These enzymes act as regulators of high energy phosphate production, as well as in the regulation of contractile tissues such as the cardiac muscle. In this tissues CK-MB is primarily found, responding for 15 to 40% of the total CK activity (the remaining is CK-MM) (Kemp et al., 2004). Thus, an increase in CK-MB might be associated with cardiac damage (Aktas et al., 1993; Schober et al. 1999).

Due to limitations of seric markers of cardiac function, it was necessary to use other methods or novel diagnostic markers, in order to obtain greater specificity (Godoy et al., 1998). Therefore, the troponins have very similar structure between species (O'brien et al., 1997), receiving increasing attention as highly specific markers of cellular injury (Godoy et al., 1998) and being regarded as preferable cardiac injury biomarker in mammalian (O'brien, 2008). In this study troponin showed no statistical difference between groups, despite the histopathological lesions observed in heart samples. In immunoassays developed by for Gupta and de Lemos (2007), it was demonstrated that high concentrations of troponin I can be measured in the peripheral blood, and it may reflect minimal lesions in the myocardium, even in the absence of increased levels of CK or CK-MB. In humans, the troponin I can be detected about 4 to 6 hours after the onset of ischemia, peaking within 24 hours and remained elevated in the bloodstream for at least five days after the episode of myocardial infarction. Although it presents a biological half-life of 120 minutes it has a good diagnostic window due to the continued release of this protein myofilament disintegrating (Gupta and de Lemos, 2007). Thus, we believe that non-elevated levels of troponin observed in our results may be a consequence of a peak release of this protein.

Myoglobin is also widely used as a seric marker for detecting cardiac injury. However, myoglobin is a small protein that is rapidly released into the bloodstream after heart or muscle injury; thus, myoglobin has the ability to be detected earlier in the course of an acute myocardial infarction (AMI). The importance of myoglobin is related to the release duplication, being released in the circulation prematurely after 2 to 3 hours, with a doubling time of 2 hours, reaching a peak plasma level of 6 to 9 hours, normalizing after 24 hours (Puleo et al., 1994, Zimmernam et al., 1999). We observed an increase of myoglobin in infected animals and treated animals (groups C and D) compared to the negative control group. The presence of inflammation and tissue damage in heart samples of animals infected by *T. gondii* allowed the elevation of seric myoglobin 30 days after infection.

Due to the medical importance of toxoplasmosis, in this study we evaluated the effect of $(PhSe)_2$ in the experimental infection by *T. gondii*. $(PhSe)_2$ derived from selenium has been widely studied by presenting lipophilic character and numerous pharmacological properties (Ghisleni et al., 2003; Nogueira et al., 2003; Savegnago et al., 2008), besides having antioxidant effects in toxicity induced by oxidative stress (Luchese et al., 2008; Meotti et al., 2004; Santos et al., 2005). In summary, this study demonstrated that infection by *T. gondii* causes cardiac changes due to the course of infection as showed in histophatology, and changes important parameters responsible for energy homeostasis and cardiac function (CK, CK-MB, and LDH).

Based on these results, we conclude that the increased activities of CK either in the heart and sera, as well as increased myoglobin and LDH levels, are related to the damage caused by the parasite. It is assumed that the decrease in CK-MB can be related to its seric peak that appeared hours after infection; and a reduction in AK in the tissue might be due to the interrelation mechanism between the AK and CK. However, animals treated with $(PhSe)_2$ showed a decrease in tissue damage caused by the parasite, and reduced seric LDH; thus, reinforcing the concept of $(PhSe)_2$ as antioxidant.

**Ethics Committee.** These procedures were approved by the Animal Welfare Committee of the Universidade do Estado de Santa Catarina under protocol number 025/2015.

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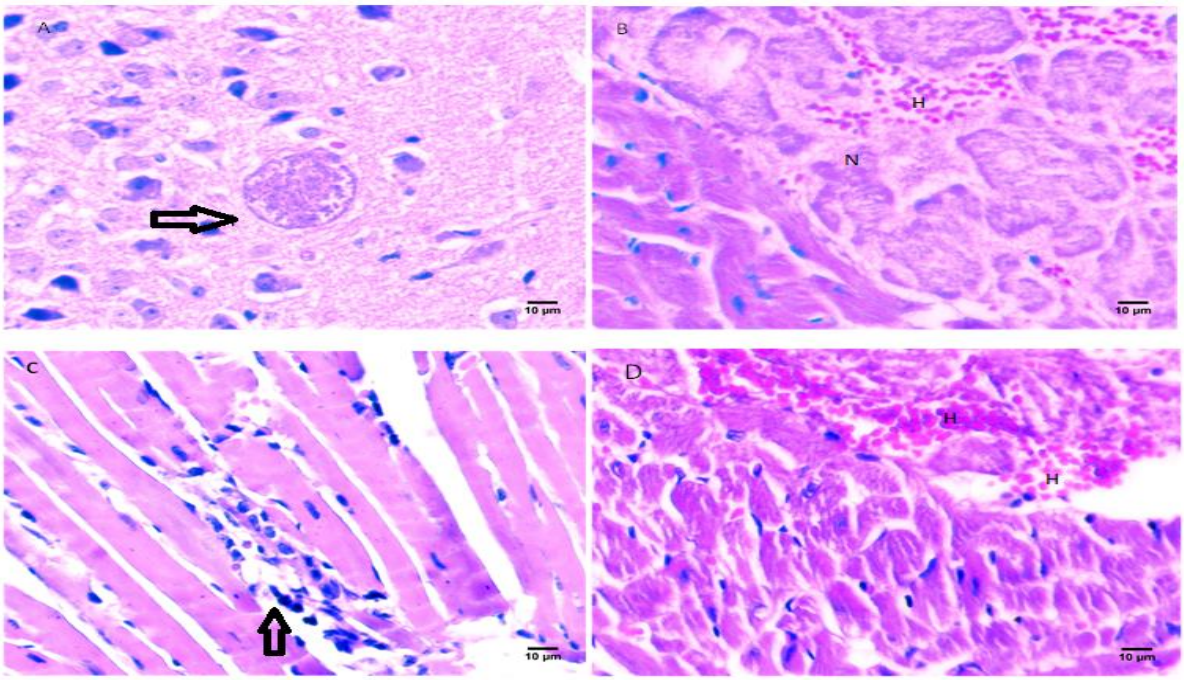


Fig. 1. Histopathological findings in heart and brain of mice infected by Toxoplasma gondii (ME-49 strain) and treated with (PhSe)2. [A] Brain. Parasitic cyst of T. gondii containing bradyzoites (arrow) in all mice of groups C and D; [B] Severe and extensive necrosis (N) and hemorrhage (H) in heart of six (6/10) mice of the group C; [C] Moderate and extensive lymphoplasmacytic inflammatory infiltrate in heart of nine (9/10) mice from the group D. [D] Moderate extensive necrosis (N) and hemorrhage (H) in heart of four (4/10) mice of the group C .

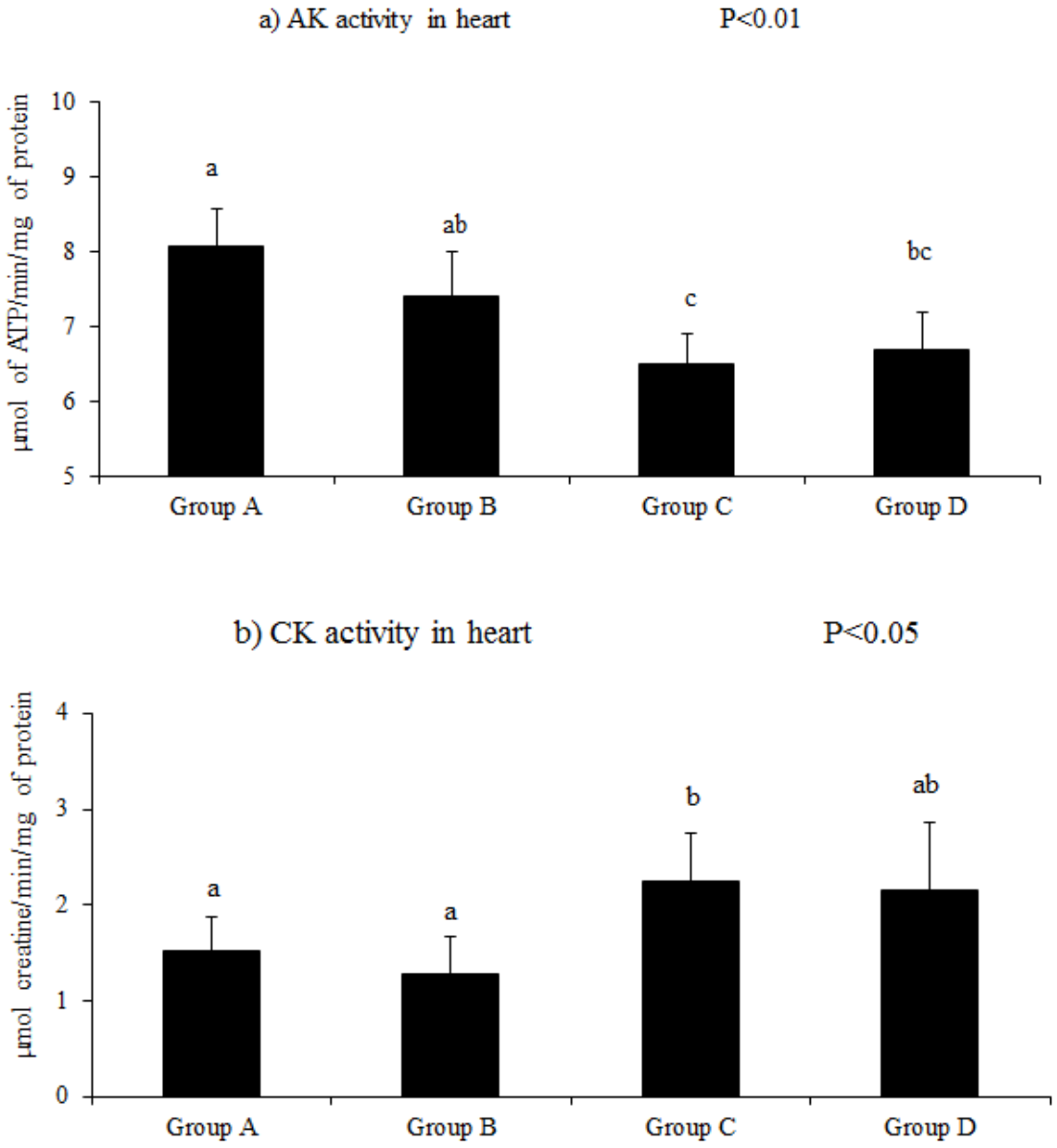


Fig 2. Adenylate kinase (AK) and creatine kinase (CK) activities in heart of mice experimentally infected by *T. gondii* (30 days PI) and treated with $(PhSe)_2$ (5µmol.kg$^{-1}$). Note: average and standard deviation with different letters in the same graph differ statistically [P<0.05] according to statistical analysis performed by Duncan test. The group A (uninfected), the group B (uninfected and treated $(PhSe)_2$), the group C (infected and untreated) and the group D (infected and treated with $(PhSe)_2$.

Table 1. Effects of *T. gondii* in mice on seric biomarkers (CK, CK-MB, LDH), and cardiac function (myoglobin and troponin) treated with diphenyl diselenide, subcutaneously.

| **Groups** | **Biomarkers of cardiac function** | | | | |
|---|---|---|---|---|---|
| | CK (U/L) | CK-MB(U/L) | Myoglobin (ng/mL) | Troponin (ng/mL) | LDH (U/L) |
| **A** | $152.6 \pm 3.9^{b}$ | $41.5 \pm 17.5^{ab}$ | $18.2 \pm 4.1^{b}$ | $0.16 \pm 0.09^{a}$ | $978.5 \pm 186.7^{b}$ |
| **B** | $176.6 \pm 67.6^{ab}$ | $42.1 \pm 7.3^{a}$ | $16.2 \pm 6.3^{b}$ | $0.16 \pm 0.12^{a}$ | $1193.1 \pm 236.8^{b}$ |
| **C** | $223.0 \pm 81.9^{a}$ | $39.7 \pm 17.8^{ab}$ | $23.2 \pm 3.7^{a}$ | $0.20 \pm 0.07^{a}$ | $1533.0 \pm 323.7^{a}$ |
| **D** | $134.6 \pm 24.1^{b}$ | $31.7 \pm 6.0^{b}$ | $23.7 \pm 4.9^{a}$ | $0.20 \pm 0.12^{a}$ | $953.7 \pm 178.4^{b}$ |

Data are expressed as average and standard deviation. Note: Averages followed by the same letter in the same column are not statistically different by analysis of Duncan test ($P<0.05$). Group A (uninfected); group B (uninfected and treated $(PhSe)_2$); group C (infected and untreated) and group D (infected and treated with $(PhSe)_2$).

# 5 PUBLICAÇÕES CIENTÍFICAS

## 5.2 ARTIGO II:

Diphenyl diselenide supplementation in infected mice by *Toxoplasma gondii*: protective effect on behavior, neuromodulation and oxidative stress caused by disease

**Artigo Publicado na Revista Experimental Parasitology**

# Diphenyl diselenide supplementation in infected mice by *Toxoplasma gondii*: protective effect on behavior, neuromodulation and oxidative stress caused by disease

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**ABSTRACT**

The aim of this study was to evaluate the effect of subcutaneous administration of diphenyl diselenide $(PhSe)_2$ on animal behavior and activities of acetylcholinesterase (AChE), adenylate kinase (AK), and creatine kinase (CK) in the brain of mice infected by *T. gondii*. In addition, thiobarbituric acid reactive species (TBARS) levels and glutathione (GR, GPx and GST) activity were also evaluated. For the study, 40 female mice were divided into four groups of 10 animals each: group A (uninfected and untreated), group B (uninfected and treated with $(PhSe)_2$), group C (infected and untreated) and group D (infected and treated with $(PhSe)_2$). The mice were inoculated with 50 cysts of the ME49 strain of *T. gondii*. After infection the animals of the groups B and D were treated on days 1 and 20 post-infection (PI) with 5.0 µmol/kg of $(PhSe)_2$ subcutaneously. Behavioral tests were conducted on days 29 PI to assess memory loss (object recognition), anxiety (elevated plus maze), locomotor and exploratory activity (Open Field) and it was found out that infected and untreated animals (group C) had developed anxiety and memory impairment, and the $(PhSe)_2$ treatment did not reverse these behavioral changes on infected animals treated with $(PhSe)_2$ (group D). The results showed an increase on AChE activity ($P<0.01$) in the brain of infected and untreated animals (group C) compared to the uninfected and untreated animals (group A). The AK and CK activities decreased in infected and untreated animals (group C) compared to the uninfected and untreated animals (group A) ($P<0.01$), however the $(PhSe)_2$ treatment did not reverse these alterations. Infected and untreated animals (group C) showed increased TBARS levels and GR activity, and decreased GPx and GST activities when compared to uninfected and untreated animals (group A). Infected animals treated with $(PhSe)_2$ (group D) decreased TBARS levels and GR activity, while increased GST activity when compared to infected and untreated animals (group C). It was concluded that $(PhSe)_2$ showed antioxidant activity, but the dose used had no anti-inflammatory effect and failed to reverse the behavioral changes caused by the parasite.

**Keywords:** Toxoplasmosis, Energy metabolism, Acetylcholine, ATP, $(PhSe)_2$

## 1. Introduction

*Toxoplasma gondii* represents one of the most common parasitic infections in the world (Hunter and Sibley, 2012). The asexual cycle can occur within any warm-blooded animal, but the sexual cycle is restricted to the feline intestinal epithelium. *T. gondii* is acquired through consumption of tissue cysts in undercooked meat, as well as food and water contaminated with oocysts. Once ingested, it differentiates into a rapidly replicating asexual form and disseminates throughout the body during acute infection (Dubey, 2007).

After stimulation of the host immune response, *T. gondii* differentiates into a slow-growing, asexual cyst form that is the hallmark of chronic infection (Dubey, 1988). During chronic infection, the parasite forms tissue cysts predominantly in the brain and muscles of the host (Hutchinson, 1987). The encysted form of *T. gondii* has been associated with behavioral changes in rodents, such as higher activity and decreased predator vigilance (Webster et al., 1993).

According to the scientific literature, it is speculated that the behavioral alterations may be related to changes in the concentrations of neurotransmitters in the brain, mainly acetylcholine (ACh). According to Tonin et al. (2014), the acetylcholinesterase (AChE) activity in the brain was increased in animals infected by *T. gondii.* This increase may be directly related to the increase on enzyme expression in the brain of animals infected by *T. gondii,* along with the inflammatory response against parasite infection. It has been reported that infection by *T. gondii* is related to neurodegenerative diseases and these disorders have been associated to changes in the energy metabolism of the brain (Prandota, 2014).

In this study, important enzymes of the cerebral energy metabolism, such as creatine kinase (CK) and adenilate kinase (AK) were analyzed. These enzymes are found in cells with high energy demand, including heart, brain and skeletal muscle (Segal et al., 2007; Gloria-Bottini et al., 2011). The CK has cellular energy buffer functions in periods of high consumption of adenosine triphosphate (ATP) by the cell. AK also acts in cellular signaling processes with the use of ATP (Dzeja and Terzic, 1998). The deficiency in AK and CK activities may lead to disturbances in cellular functions (Toren et al., 1994), compromising regions that need increased demand for ATP, such as the brain, as it was observed in rats experimentally infected by *Trypanosoma evansi* (Baldissera et al., 2015).

One-third of the human population is chronically infected with parasitic cysts of *T. gondii,* which can be reactivated causing severe disease especially to individuals with reduced immune surveillance. Nowadays, there are no drugs available to clear the cysted form *T.*

*gondii* formed during the chronic stages of the infection. This therapeutic gap is due in part to an incomplete understanding of both host and pathogen responses during the progression of *T. gondii* infection (Martins-Duarte et al., 2013) For the purpose of enhancing treatment, natural products and minerals have been used alone or associated with chemotherapy. Compounds of selenium are some examples (Barbosa et al., 2014) and $(PhSe)_2$ is a simple and stable organic selenium (OS) compound. It is an electrophilic reagent used in the synthesis of a variety of pharmacologically active OS compounds (Ghisleni et al., 2003). Recently, the biological activities of $(PhSe)_2$ have been studied and this compound has become a good candidate for therapeutic treatment of toxoplasmosis (Barbosa et al., 2014). According to Nogueira et al. (2001), the $(PhSe)_2$ may cause minimal toxicity when administrated to mice and rats acutely infected in doses that have anti-inflammatory properties. However, chronic exposure to high doses of $(PhSe)_2$ caused nervous effects in mice, indicating that the brain is a potential target for the toxic effect of OS compounds (Maciel et al., 2000; Jacques-Silva et al., 2001). The organic forms of selenium like $(PhSe)_2$ act increasing thiol oxidation by accelerating the electron transfer from thiols to oxidized acceptors such as cytochrome C (Engin et al., 2012). In addition, the reaction catalyzed by OS compounds is similar to that catalyzed by glutathione peroxidase (GPx). The concept that selenium-containing molecules may be better nucleophiles than classical antioxidants has led to the design of synthetic OS drugs.

Due to the important of $(PhSe)_2$ mainly related to antioxidant and anti-inflammatory, it was suggested that $(PhSe)_2$ is able to modulate directly or indirectly the activity of regulatory enzymes of ATP and acetylcholine levels, minimizing the pathogenic effects of the disease (Ghisleni et al., 2003; Nogueira et al., 2003; Savegnago et al., 2007). Therefore, the main objective of this study was to evaluate the effect of treatment with $(PhSe)_2$ on animal behavior, and enzymatic activities involved in the metabolism of neurotransmitters (AChE, AK, and CK) in the brain of mice experimentally infected by *T. gondii*, as well as to investigate its oxidative/antioxidant status.

## 2. Materials and Methods

### 2.1. *Toxoplasma gondii* strain

This study used one strain considered as cystogenic (ME49), kept in liquid nitrogen 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 parasite´s virulence. The three mice were

euthanized for collection of brain on 30 days PI, and parasitic cysts were counted and separated for further animal inoculation.

**2.2. Diphenyl diselenide**

Diphenyl diselenide $(PhSe)_2$ (99.9%) was purchased from SIGMA (St. Louis, MO, USA). $(PhSe)_2$ was dissolved in dimethyl sulfoxide (DMSO, Sigma-Aldrich, USA) at the final concentration of 0.1%.

**2.3. Animal and experimental design**

Forty 60-day-old female mice weighing an average of 25 ± 5g were used in this study. They were housed in cages on a light/dark cycle of 12 h in an experimental room with controlled temperature and humidity (23±1 ºC; 70 %, respectively). Animals were fed with commercial feed, and received water *ad libitum*. All animals were subject to a period of 15 days for adaptation. The animals were divided into four groups (A, B, C and D) with ten animals each. The Groups A (uninfected and untreated), group B (uninfected and treated $(PhSe)_2$), group C (infected and untreated) and group D (infected and treated $(PhSe)_2$). The groups C and D were infected orally with 0.25 mL of brain homogenate containing 50 cysts with bradyzoites of *T. gondii* (ME49 strain). Treatment with $(PhSe)_2$ was administered subcutaneously on days 1 and 20 post-infection (PI) at 5.0 μmol $kg^{-1}$ (Barbosa et al., 2014). Animals were checked daily for clinical signs of the disease.

**2.4. Tests on animal behavior**

*2.4.1. Elevated plus maze (EPM)*

On day 29 PI, the animals were submitted to anxiolytic-like behavior using the elevated plus maze as previously described (Pellow et al., 1985). Initially, the animals were placed on the central platform of the maze in front of an open arm. The animal had 5 min to explore the apparatus. The time spent and the number of entries into the open and closed arms were recorded. The anxiolytic behavior was recorded by a significant increase in the open arms (Clenet et al., 2006).

*2.4.2. Open field test*

The open field test was performed immediately after the EPM test. Each animal was placed individually at the center of the apparatus and observed for 4 min to record locomotor (number of segments crossed with all four paws) and exploratory activities (expressed by the

number of times the animal was rearing on its hind limbs) (Walsh and Cummins, 1976). The open field session lasted for 5 min and during this time an observer that was not aware of the pharmacological treatments, recorded the number of crossing and rearing responses manually. This test was carried out to identify motor disabilities in the animal.

*2.4.3. Novel object recognition task*

A novel object recognition task was carried out as described previously by Gomes et al. (2014). The task was performed in a 30×30×30 cm wooden chamber with dark walls, front wall made of Plexiglas, and floors covered with ethyl vinyl acetate sheet. A light bulb hanging 60 cm above the behavioral apparatus provided constant illumination of about 40 lux, and an air-conditioner provided constant background sound isolation. Plastic mounting bricks of different shapes and colors with the same size were used in a counterbalanced manner. Animals had shown no-preference previously for any of the objects. Chambers and objects were cleaned with 30% ethanol immediately before and after each behavioral evaluation. The task consisted of adaptation, training, and testing sessions, each lasting 8 min. In the first session, mice were individually placed into the behavioral apparatus for adaptation and then returned to their cages. Twenty-four hours later, the animals were subjected to a training session in which the animals were exposed to two of the same objects (object A), and the exploration time was recorded with two stopwatches. Exploration was recorded when the animal touched or reached the object with the nose at a distance of less than 2 cm. Climbing or sitting on the object was not considered exploration. The test session was carried out 24 hours after training. Mice were placed back in the behavioral chamber and one of the familiar objects (object A) was replaced by a new object (object B). The time spent exploring the familiar and the new object was recorded. The discrimination index was calculated taking into account the difference of the time spent exploring the new and familiar objects, using the formula:

$([(T_{novel}-T_{familiar}) / (T_{novel} + T_{familiar})] \times 100(\%))$

**2.5. Sample collection and tissue preparation**

One day after behavior tests (day 30 PI), the animals were anesthetized with isoflurane, and euthanized by decapitation. The brain was removed, and separated into right and left hemispheres. The right hemisphere of animals from the groups A, B, C and D were homogenized with Tris-HCl 10 mM, pH 7.4 on ice. The homogenates were centrifuged at 1.800 rpm at 4 ºC for 10 min to yield a supernatant 1 (S1) that was used for AChE, AK, CK,

glutathione activities, and TBARS levels. Aliquots of resulting brain structure homogenates were stored at −20 °C until utilization. Protein was determined previously in a strip 0.8 mg/mL and determined by Coomassie blue method as previously described (Bradford, 1976), using bovine serum albumin as standard solution.

**2.6. Adenylate kinase and creatine kinase activities**

The brain after removed and dissected on a glass dish over ice. For the assay of the enzymes, total brain were washed in SET buffer (0.32 M sucrose, 1 mM EGTA, 10 mM Tris-HCl, pH 7.4) and homogenized (1:10 w/v) in the same SET buffer with a Potter glass homogenizer. The homogenate was centrifuged at 800 *g* for 10 min at 4 ºC. The supernatant was centrifuged and used for the determination of AK activities. AK activity was measured with a coupled enzyme assay with hexokinase (HK) and glucose 6-phosphate dehydrogenase (G6PD), according to Dzeja et al. (1999). The reaction mixture contained 100 mM KCl, 20 mM HEPES, 20 mM glucose, 4 mM $MgCl_2$, $NADP^+$, 1 mM EDTA, 4.5 U/mL of HK, 2 U/mL of G6PD, and 20 μL of homogenate. The reaction was initiated by the addition of 2 mM ADP and the reduction of $NADP^+$ was followed at 340 nm for 3 min in a spectrophotometer. Reagent concentration and assay time (3 min) were chosen to assure the linearity of the reaction. Results were expressed in μmol of ATP formed per min per mg of protein. PK activity was assayed essentially as described by Leong et al. (1981). The incubation medium consisted of 0.1 M Tris/HCl buffer, pH 7.5, 10 mM $MgCl_2$, 0.16 mM NADH, 75 mM KCl, 5.0 mM ADP, 7 U of lactate dehydrogenase, 0.1 % (v/v) Triton X-100, and 10 μL of homogenize. After 10 min of pre-incubation at 37 ºC, the reaction was started by the addition of 1 mM phosphoenolpyruvate. All assays were performed in duplicate at 25 °C. The results were expressed as μmol of pyruvate formed per min per mg of protein. CK activity was assayed as a method described by Hughes (1962). The reaction mixture contained the following final concentrations: 65 mM Tris-HCl buffer, pH 7.5, 7 mM phosphocreatine, 9 mM $MgSO_4$ and 20 μL of homogenate. The reaction was started by the addition of 0.3 μmol of ADP after 10 min of pre-incubation at 37 ºC. The color was developed by the addition of 0.1 mL 2 2% α-naphtol and 0.1 mL 0.05 % diacetyl in a final volume of 1 mL. Reading was performed after 20 min on wavelength of 540 nm. The results were expressed as μmol of creatine formed per min per mg of protein. The protein content of brain homogenates were determined by the method of Lowry et al. (1951), using serum bovine album as the standard.

**2.7. Determination of acetylcholinesterase activity**

Acetylcholinesterase activity (AChE) was determined by the methodology of Ellman et al. (1961) modified by Rocha et al. (1993). For analysis of AChE enzyme we use brain, the reaction mixture contained 100 mM $K^+$ phosphate buffer, pH 7.5 and 1 mM 5,5′-dithiobisnitrobenzoic acid (DTNB). The method is based on the formation of the yellow anion, 5.5′-dithio-bis-acid-nitrobenzoic, measured by absorbance of 412 nm during 2 min incubation at 25 °C. The enzyme (40–50 μg of protein) was pre-incubated for 2 min. The reaction was initiated by adding 0.8 mM acetylthiocholine iodide (AcSCh) as substrate. All samples were run in triplicate and the enzyme activity was expressed in μmol AcSCh/h/mg of protein.

**2.8. Determination of thiobarbituric acid reactive species (TBARS) levels**

Lipid peroxidation was estimated by measuring TBARS and expressed in terms of malondialdehyde (MDA) content, according to the method of Ohkawa et al. (1978). In this method, MDA, an end product of fatty acid peroxidation, reacts with thiobarbituric acid (TBA) to form a colored complex. Briefly, the supernatant fraction of the brain was incubated at 95 ºC for 60 min in acid medium containing 8.1% sodium dodecyl sulfate, 0.5 mL of acetic acid buffer (500 mM, pH 3.4) and 0.6% TBA. TBARS levels were measured at 532 nm, and the absorbance was compared with the standard curve using malondialdehyde. The results were expressed in nanomoles of malondialdehyde/mg of protein.

**2.9. Glutathione activity in brain**

*2.9.1. Glutathione S-transferase (GST) activity*

GST activity was assayed spectrophotometrically at 340 nm by the method of Habig et al. (1974). The mixture contained an aliquot of brain supernatant or test with 0.1 M potassium phosphate buffer (pH 7.4), 100 mM GSH and 100 mM 1-chloro-2,4-dinitrobenzene (CDNB), which was used as substrate. The enzymatic activity was expressed as ηmol CDNB/h/mg of protein.

*2.9.2. Assay of reductase glutathione (GR)*

For the measurement of GR activity, we used a method previously described by Carlberg and Mannervik (1985). The method is based on the utilization of the enzyme-oxidized glutathione (GSSG), to convert GSSG to GSH in the presence of the cofactor NADPH. Briefly, brain supernatant (50 μL) was added to medium containing 0.2 M

phosphate buffer (0.2 M $K_2HPO_4$ and 2 mM EDTA, pH 7.0) and NADPH (2 mM). The reaction was initiated by adding GSSG (20 mM) substrate. GR levels were measured by absorbance at 340 nm during 2min of incubation. GR activity was determined using the molar extinction coefficient 6220 $M^{-1}\,cm^{-1}$ and expressed as ηmol NADPH oxidized/h/mg of protein.

*2.9.3. Glutathione peroxidase (GPx) activity*

GPx activity was determined using glutathione reductase and NADPH. The method is based on the oxidation of NADPH, which is indicated by a decrease in the absorbance at 340 nm (Paglia et al., 1967). The enzymatic activity was expressed as ηmol NADPH oxidized/h/mg of protein.

**2.10. Histopathology**

For histopathology, the left hemisphere of the brain was used. Sections of 3 mm were cut and stained with hematoxylin and eosin. Slides were observed by a pathologist in a blind way.

**2.11. Statistical analyses**

First, the data of AK, CK, AChE, TBARS, GR, GPx and GST were subjected to normality test, which showed regular distribution. Therefore, these data were analyzed by the Duncan test. Statistical analyses to behavioral tests were performed using one-way-ANOVA followed by the Bonferroni post hoc analysis. A value of $P<0.05$ was considered significant. Results were presented in mean and standard deviation.

**3. Results**

**3.1. Disease course and histopathology**

During the course of the experiment animals from groups A and B (uninfected) did not show any clinical manifestation of the disease. However, infected mice (groups C and D) showed clinical signs such as bristly, apathy and tremors. Histopathological analysis of the brain of *T. gondii* infected animals (groups C and D) showed similar changes, which were described as moderate inflammatory infiltrate in the meninges, gliosis, presence of perivascular cuffs, malacia, as well as the presence of mild to moderate parasitic cysts (Figure 1).

### 3.2. Behavioral changes

On day 29 PI, *T. gondii* infected animals had an increased ($P<0.05$) percentage of time spent in the open arms when compared to the uninfected and untreated animals (group A) (Figure 2A). Uninfected animals treated with $(PhSe)_2$ did not altered the time spent in the open arms when compared to the uninfected and untreated animals (group A), as well as observed for infected animals treated with $(PhSe)_2$ (group D) when compared to infected and untreated animals (group C).

The object recognition test showed a reduction in the percentage of the discrimination index on infected and untreated animals (groups C) when compared the uninfected and untreated animals (groups A) (Figure 2B). Uninfected animals treated with $(PhSe)_2$ (group B) showed a reduction in the percentage of index when compared to the uninfected and untreated animals (group A), as well as observed in infected animals treated with $(PhSe)_2$ (group D), when compared to the infected and untreated animals (group C). Statistical analysis (one-way ANOVA) in open field test showed no significative differences in the number of crossings and rearing between the groups (data not show).

### 3.3. AK and CK activities

The CK and AK activities in the brain homogenate were shown in Figure 3. *T. gondii* infected and untreated animals (group C) showed a decreased on enzymatic activities when compared to the uninfected and untreated animals (group A). No was observed difference between uninfected animals treated with $(PhSe)_2$ (group B) on AK and CK activities when compared to the uninfected and untreated animals (group A), as well as observed in infected animals treated with $(PhSe)_2$ (group D) when compared to the infected and untreated animals (group C).

### 3.4. Acetylcholinesterase activity

AChE activity in brain of *T. gondii* infected mice and treated with $(PhSe)_2$ are shown in Figure 4. Infected animals and untreated (group C) by *T. gondii* showed an increased AChE activity when compared to the uninfected and untreated animals (group A). Uninfected animals treated with $(PhSe)_2$ (group B) showed an increased AChE activity when compared to uninfected and treated animals (group A), similarly observed between infected animals treated with $(PhSe)_2$ (group D) when compared to infected and untreated animals (group C).

### 3.5. TBARS levels

The TBARS levels in the brain of infected mice by *T. gondii* treated with $(PhSe)_2$ are shown in Table 1. TBARS levels of infected and untreated animals (group C) had a significantly increased ($P<0.05$) in the brain when compared to the uninfected and untreated animals (group A). Uninfected and treated animals with $(PhSe)_2$ (group B) were not different statistically compared to the uninfected and untreated animals (group A). The treatment with $(PhSe)_2$ reduced TBARS levels in the brain of infected and treated animals with $(PhSe)_2$ (group D) when compared to infected and untreated animals (group C).

### 3.6. Glutathione's activity

The glutathione's activities in the brain on infected mice by *T. gondii* treated with $(PhSe)_2$ are show in Table 1. GPx and GST activities decreased in infected and untreated animals (group C) when compared to uninfected and untreated animals (group A), while GR activity increased in infected animals (group C) when compared to uninfected and untreated animals (group A). No was observed difference on GR, GPx and GST activities on uninfected animals treated $(PhSe)_2$ (group B) when compared to uninfected and untreated animals (group A). GR activity decreased on infected animals treated with $(PhSe)_2$ (group D) when compared to infected and untreated (group C), while GST activity increased on infected animals treated with $(PhSe)_2$ (group D) when compared to infected and untreated animals (group C). No was observed difference regarding GPx activity on infected animals treated with $(PhSe)_2$ (group D) when compared to infected and untreated animals (group C) ($p>0.05$)

## 4. Discussion

In this study, we have demonstrated that subcutaneous administration of $(PhSe)_2$, was not able to reverse the behavioral changes caused by *T. gondii* in mice. The animals were submitted to tests to recognize objects (long and short-term memory), elevated plus maze (anxiety), and open field (locomotor and exploratory activity).

Parasite's location has been proposed as an important factor in the behavioral changes observed in rodents infected by this protozoan (Cox and Holland, 2001). Furthermore, mice infected by *T. gondii* showed decrease anxiety levels, that increased their inhibition, making them more susceptible to predation and transmission of the zoonosis to felids (Cox and Holland, 2001). In this study, *T. gondii* infected mice showed memory loss, and anxiolytic behavior. Savegnago et al. (2008) reported that $(PhSe)_2$ had anxiolytic and antidepressant effects in mice, effects involved at least in part, by an interaction with L-arginine/nitric

oxide/cyclic guanosine monophosphate. On the other hand, Nogueira et al. (2001) reported that $(PhSe)_2$ had inhibitory effect on glutamatergic neurotransmitter in the hippocampus hindering the formation of long-term object recognition memory. However, the $(PhSe)_2$ was not able to reverse the behavioral findings in this study.

$(PhSe)_2$ is an organic compound selenium derivative that deserves special attention because of its numerous pharmacological properties: neuroprotective (Ghisleni et al., 2003), anti-inflammatory (Nogueira et al., 2003), antidepressant, and anxiolytic (Savegnago et al., 2008), besides antioxidant effects in cases of toxicity induced by oxidative stress (Meotti et al., 2004; Santos et al., 2005; Luchese et al., 2008).

The enzyme AChE is found mainly in the brain and is involved in the hydrolysis of ACh and thus it can control the levels of this molecule in the synaptic cleft (Schetinger et al., 2000; Kawashima and Fujii, 2003). Increased AChE activity showed in this study in infected animals may be associated with the ACh hydrolysis, leading to memory loss. This work is according to Tonin et al. (2014), which reported that mice experimentally infected by *T. gondii* had an increase on AChE activity. According to the literature, increased AChE activity in the brain leads to higher hydrolysis of ACh in the synaptic cleft (Kawashima and Fujii, 2003; Anglister et al., 2008), which may cause damage to the central nervous system (CNS) leading to changes in behavior, memory, movement, and balance (Silva, 1998). The increase in AChE is also associated with anti-inflammatory effect since it rapidly reduces the levels of ACh, considered an anti-inflammatory molecule (Engin et al., 2012). In this study, the treatment with $(PhSe)_2$ of mice infected by *T. gondii* was not able to decrease AChE activity in the brain, but increased the activity of this enzyme, contributing to the inflammatory process shown in histopathological studies (Anglister et al., 2008; Engin et al., 2012). The mechanism by which selenium activates AChE activity is not known, requiring more studies.

Histopathological findings such as moderate focal malacia associated to parasitic cysts of *T. gondii,* containing bradyzoites and moderate meningeal nonsuppurative (lymphoplasmacytic) and inflammatory infiltrate may influence the pathogenesis of toxoplasmosis regarding the neurotransmission and inflammatory responses against the protozoan. Kawashima and Fujii (2003), as well as Duncan (2003), have reported that the acute phase of *T. gondii* infection is caused by weight increase in the brain of infected animals, and a decrease in the chronic phase. This increase is probably related to the influx of inflammatory cells into the brain, and its reduction is related to cell death resulted from inflammation (Ferguson and Hutchison, 1987; Carruthers and Suzuki, 2007) as observed on histology analyses. Evidence has shown that inflammation induced by the pathogen is

associated to an increased production of ROS and RSN (reactive nitrogen species). Several studies have reported associations between local or systemic infection and oxidative damage (Titheradge, 1999; Sebbane et al., 2006). Park et al. (2009) described oxidative stress as an imbalance between the production of reactive oxygen species (ROS) and antioxidant capacity. In the present study, we evaluated the TBARS and glutathione levels in the brain. The substances reactive to thiobarbituric acid (TBARS) are usually used for evaluation of oxidative stress, as well as the analysis of glutathione levels that play a key role in biotransformation and elimination of xenobiotics in defense cells against oxidative stress neutralizing ROS (Mieyal et al., 1995; Joseph et al., 1997; Alexi et al., 1998; Gianni et al., 2004). The increase in TBARS levels in *T. gondii* infected mice occurs probably as a result of the interaction between the parasitic infection, leading to increased ROS production (Van Gisbergen et al. 2005), as well observed by Barbosa et al. (2014). Probably, the presence of parasitic cysts in the brain increased tissue damage and decreased antioxidant enzymes. Researchers have shown that activation of the inflammatory response reduce antioxidant defenses, and exposes the host to an increased risk of oxidative stress (Alonso-Alvarez et al., 2006; Sorci and Faivre, 2009).

The interesting finding in the assessment of this parameter was that $(PhSe)_2$ had a protective action as an antioxidant, decreasing TBARS levels in infected mice and increasing the GST activity in the brain. A possible explanation for this, lies on two aspects; first, the oxidation pathway; and second, the chemical distribution of $(PhSe)_2$. The enzyme GST reacting with carbonyl compounds during lipid peroxidation and the glycoxidation of carbohydrates, accumulating during chronic disease (Dalle-Donne et al., 2003). The $(PhSe)_2$ decreased GPx activity in the brain once strengthened the antioxidant effect of this compound, due to $(PhSe)_2$ similar activity of the enzyme glutathione peroxidase (GPx) (Nogueira et al., 2004).

Oxidative stress is also associated with the cascade of mitochondrial dysfunction, and energy metabolism deficiency (Lester-Coll et al., 2006). Prandota (2014) and Baldissera et al. (2015) reported that infection by *T. gondii* and *T. evansi*, respectively, is related to neurodegenerative diseases and disorders of energy metabolism. The enzyme CK and AK are related to energy metabolism and have fundamental importance in the production and delivery of ATP (Janssen, 2003). In this work, AK and CK activities were decreased in infected and untreated animals with *T. gondii.* In this context, a recent study of mice infected by *T. gondii*, demonstrated that ATP levels in the brain are also related to the stage of the disease, i.e., peaks of ATP in the acute phase and reduction during the chronic phase of the disease (Tonin

et al., 2014). However, during metabolic stress, the energy transmission is compromised when an enzyme with this type of activity is decreased or absent (Carrasco et al., 2001).

This study demonstrated that *T. gondii* causes pathological modifications associated with biochemical and behavioral changes in the central nervous system. This work demonstrated that the treatment with $(PhSe)_2$ was able to increase the activity of AChE in the brain of infected mice. Moreover, the treatment with $(PhSe)_2$ showed antioxidant effect by increasing the activities of GST and reducing TBARS levels. It was concluded that $(PhSe)_2$ showed antioxidant activity, but the dose used had no anti-inflammatory effect and failed to reverse the behavioral changes caused by the parasite.

**Ethics Committee**

The procedures were approved by the Animal Welfare Committee of Universidade do Estado de Santa Catarina under protocol number 025/2015.

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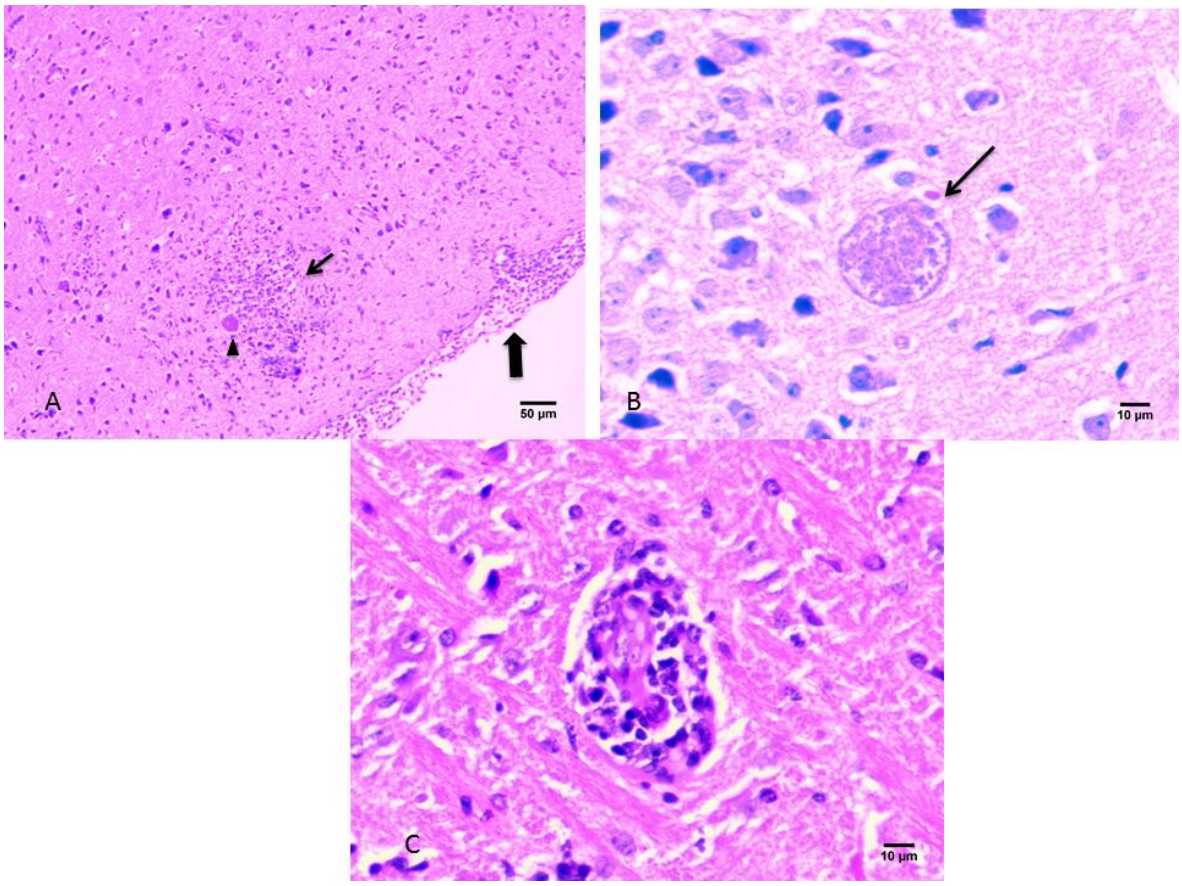


**Fig. 1:** Histopathological findings in the brain of mice infected by *T. gondii* and treated with $(PhSe)_2$. (A) Moderate focal malacia (large arrow) associated with cysts of *T. gondii* containing bradyzoites (arrowhead) and meningeal moderate nonsuppurative (lymphoplasmacytic) inflammatory infiltrate (small arrow). (B) One cyst of *T. gondii* containing bradyzoites (arrow). (C) Mild to moderate perivascular nonsuppurative (lymphoplasmacytic) inflammatory infiltrate.

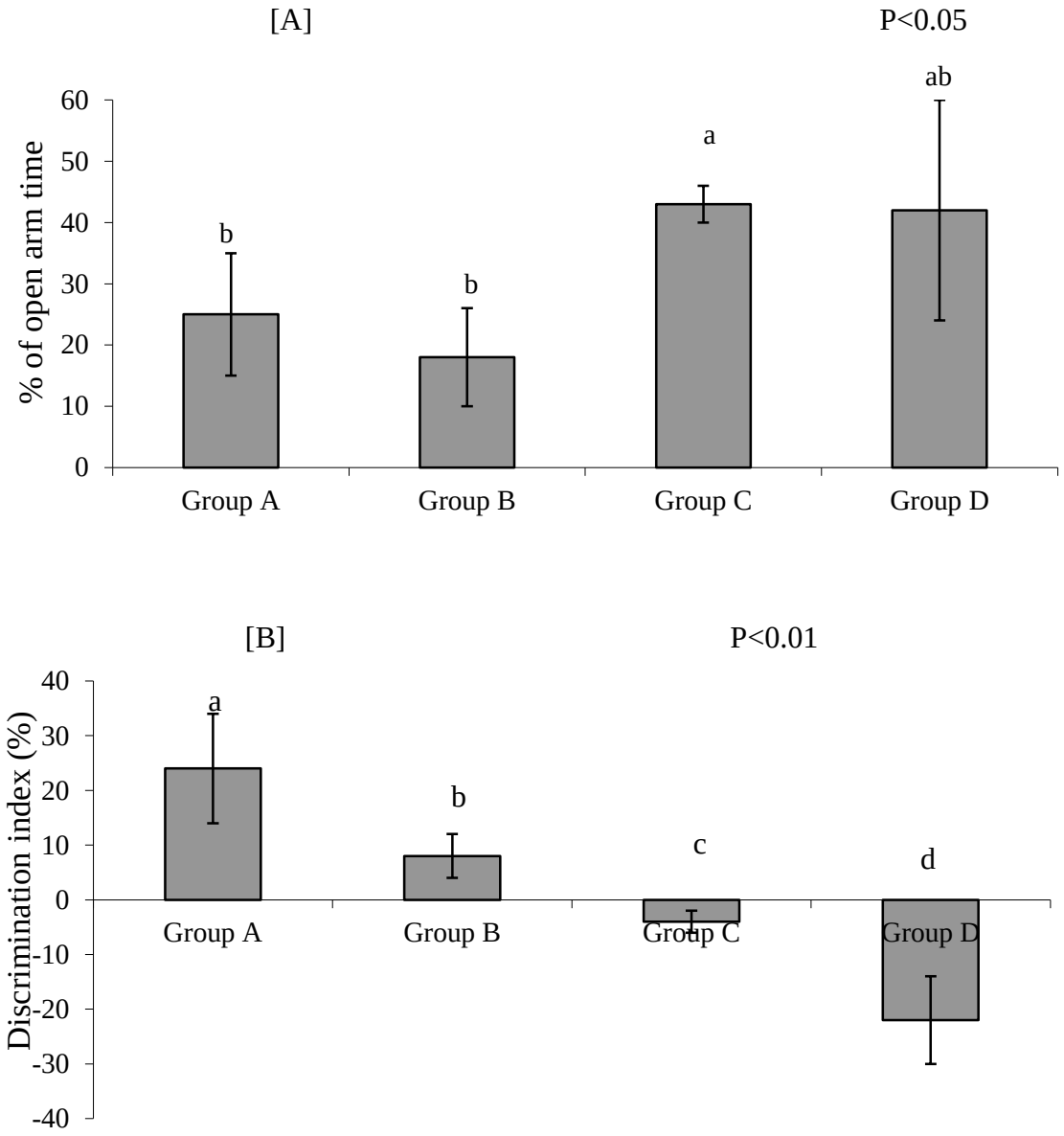


**Fig. 2:** Effect of $(PhSe)_2$ in infected animals by *T. gondii*. (A) Percentage of open arm time in elevated plus-maze task. (B) Discrimination index (%) on test trial (memory test) in object recognition memory task. One-way ANOVA – Bonferroni post-hoc analyses. Data are mean ± SEM for 10 animals in each groups (P<0.05 and 0.01) compared to the control group. The group A (uninfected and untreated), group B (uninfected and treated $(PhSe)_2$), group C (infected and untreated) and group D (infected and treated with $(PhSe)_2$). Columns followed by the same letters that there are no statistical difference between groups (P>0.05).

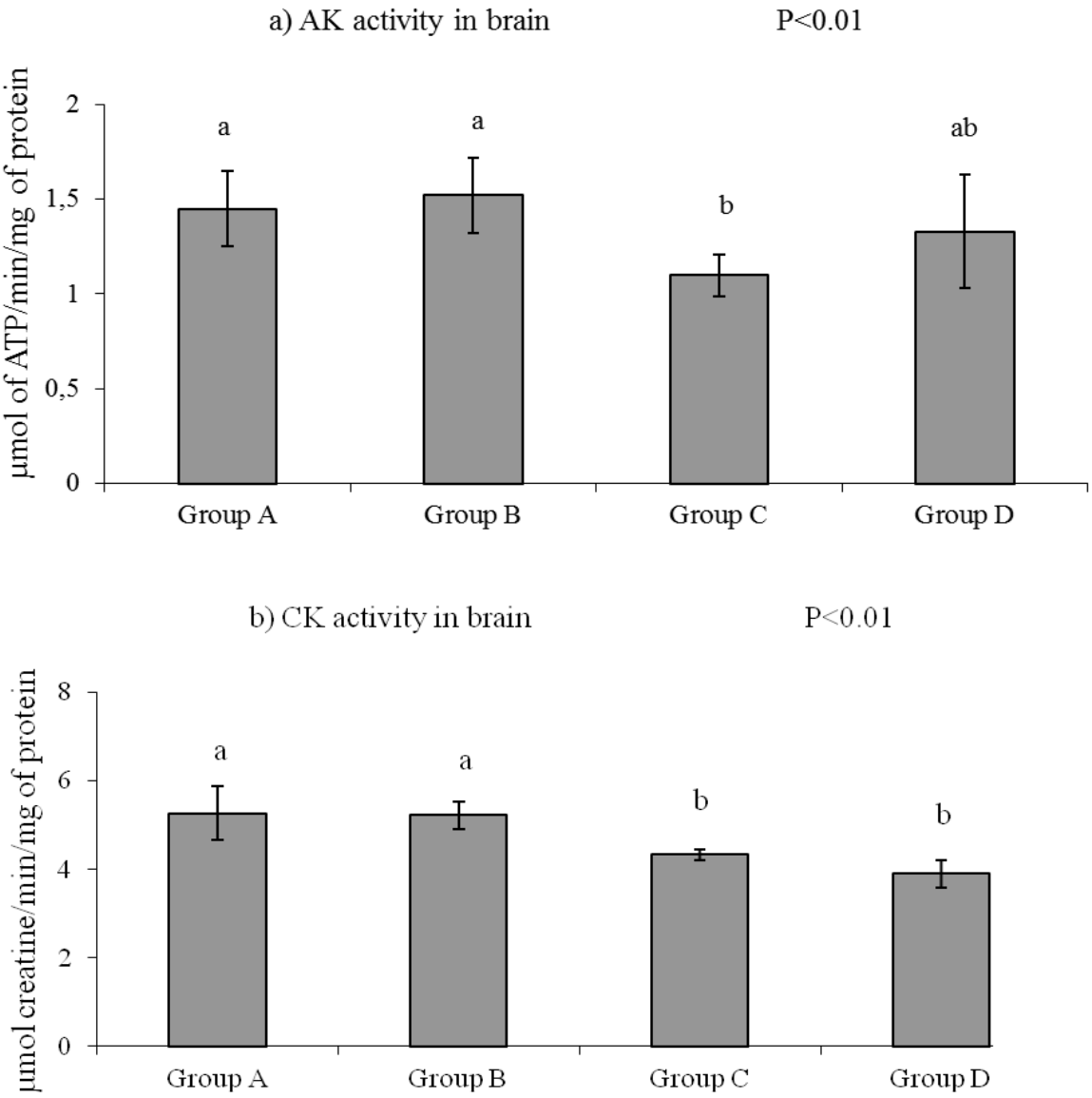


**Fig. 3:** Adenylate kinase (AK) (Fig. 3A) and creatine kinase (CK) (Fig. 3B) activities in the brain of mice experimentally infected by *T. gondii* treated with $(PhSe)_2$. The group A (uninfected and untreated), group B (uninfected and treated $(PhSe)_2$), group C (infected and untreated) and group D (infected and treated with $(PhSe)_2$). Columns followed by the same letters that there are no statistical difference between groups ($P>0.05$).

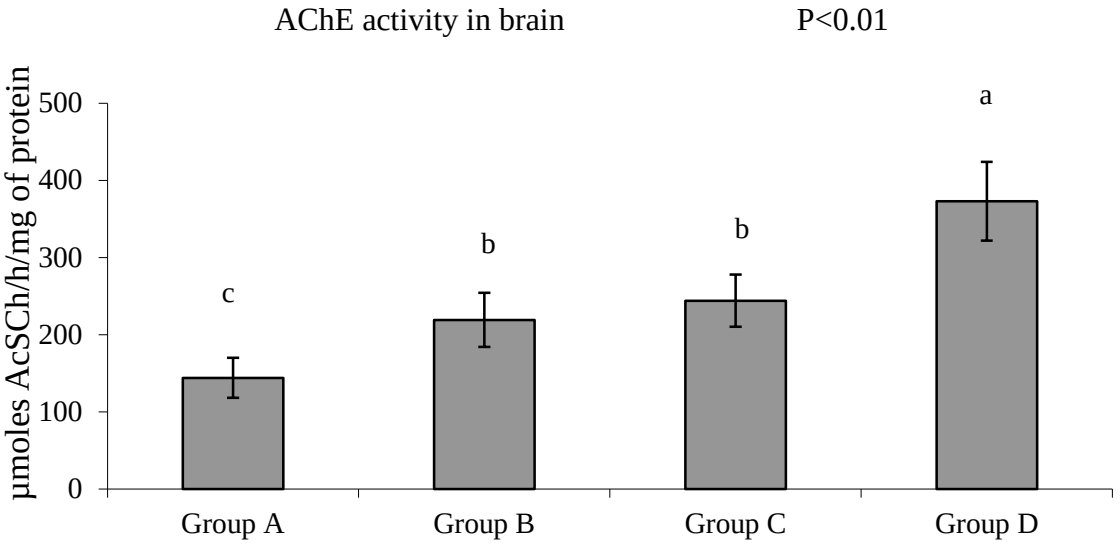


**Fig. 4:** Acetylcholinesterase activity in the brain of mice infected by *T. gondii* and treated with $(PhSe)_2$. The group A (uninfected and untreated), group B (uninfected and treated $(PhSe)_2$), group C (infected and untreated) and group D (infected and treated with $(PhSe)_2$). Columns followed by the same letters that there are no statistical difference between groups (P>0.05).

**Table 1:** Thiobarbituric acid reactive species (TBARS) levels, glutathione reductase (GR), glutathione peroxidase (GPx) and glutathione S-transferase (GST) activities in the brain of mice infected by *Toxoplasma gondii*.

| Variables | Group A | Group B | Group C | Group D |
|---|---|---|---|---|
| TBARS (nmol MDA/mg protein) | $18.7\pm2.8^{b}$ | $20.6\pm3.1^{ab}$ | $22.1\pm2.7^{a}$ | $15.0\pm0.5^{c}$ |
| GR (nmol NADPH oxidized/h/mg protein) | $39.6\pm10.4^{b}$ | $32.3\pm7.3^{b}$ | $52.5\pm14.7^{a}$ | $34.4\pm5.9^{b}$ |
| GPx (nmol NADPH oxidized/h/mg protein) | $27.9\pm8.4^{a}$ | $24.0\pm5.6^{ab}$ | $19.9\pm5.7^{b}$ | $20.5\pm5.2^{b}$ |
| GST (µmol CDNB oxidized/min/mg protein) | $232.4\pm20.1^{a}$ | $221.9\pm29.6^{ab}$ | $200.3\pm14.5^{b}$ | $223.0\pm11.5^{a}$ |

Note: mean and standard deviation with the same letters in the same line means that there are no statistical difference between groups (P>0.05).

# 6 DISCUSSÃO

Estudos relatam que o *T. gondii* é capaz de alterar o comportamento de roedores, aumentando a chance de serem predados por felinos (QUEIROZ et al., 2013; WEBSTER et al., 1993). O mesmo foi observado neste estudo com o uso do teste de labirinto em cruz elevada onde os animais infectados com *T. gondii* exploraram mais vezes os braços abertos demostrando-se menos ansiosos o que aumentou o fator de inibição destes animais deixando-os mais susceptíveis a predação e à transmissão da zoonose aos felídeos (COX e HOLLAND, 2001). No entanto, no teste de reconhecimento de objeto que avalia a memória dos animais foi possível observar uma redução significativa na memória dos animais infectados com *T. gondii* demostrando que a administração subcutânea de $(PhSe)_2$ não foi capaz de reverter as alterações comportamentais provocada pelo parasito.

Os cistos presentes no encéfalo dos animais infectados com *T. gondii* aumentaram o dano tecidual e ativaram a resposta inflamatória reduzindo as defesas antioxidantes e expondo o hospedeiro a um risco aumentado de estresse oxidativo. Considerando as propriedades anti-inflamatórias e antioxidantes do $(PhSe)_2$, pôde-se verificar neste estudo que o $(PhSe)_2$, mesmo em dose muito baixa (5 μmol/kg), foi capaz de modular o perfil oxidativo e aumentar o *status* antioxidante em camundongos infectados com *T. gondii*. Os resultados demonstraram que $(PhSe)_2$ foi capaz de diminuir os níveis das espécies reativas ao ácido tiobarbitúrico (TBARS) e aumentar a atividade da GST no encéfalo, porém o $(PhSe)_2$ não foi capaz de diminuir a atividade da AChE em animais infectados com *T. gondii*, corroborando com os achados comportamentais.

De acordo com a literatura, o estresse oxidativo está associado também com as disfunções da cascata mitocondrial e deficiência no metabolismo energético. Prandota (2014) e Baldissera et al. (2015) relataram que a infecção por *T. gondii* e *Trypanosoma evansi*, respectivamente, estão relacionadas com doenças neurodegenerativas e desordens do metabolismo energético. As enzimas CK, PK e AK são importantes enzimas do metabolismo energético e exercem fundamental importância na produção e liberação do ATP (JANSSEN, 2003). Neste estudo, a atividade das enzimas AK e CK no encéfalo apresentou redução em animais infectados e não tratados, quando comparados com o controle negativo (grupo A), mas o $(PhSe)_2$ não foi capaz de reverter esses achados. Já no tecido cardíaco, a atividade sérica da AK diminuiu e a CK aumentou em animais infectados e tratados com $(PhSe)_2$, quando comparado com o controle negativo (grupo A) e uma possível explicação seria devido às enzimas AK, PK e CK apresentarem uma inter-relação funcional entre elas, em

experimentos onde uma dessas enzimas tem sua atividade diminuída por uso de inibidores enzimáticos, por exemplo, a outra enzima acaba elevando sua atividade para suprir a demanda deixada pela primeira (DZEJA et al., 1996). Neste contexto, um estudo realizado por Tonin et al. (2014a) demonstrou que os níveis de ATP em cérebro estão relacionados com o estágio da doença, por exemplo, níveis de ATP aumentam na fase aguda e reduzem na fase crônica da infecção. No entanto, durante o estresse metabólico, a transmissão de energia é comprometida 2001), portanto, acreditava-se que o selênio pudesse modular a atividade das enzimas envolvidas no metabolismo enérgico, reduzido os efeitos negativos da doença.

Os biomarcadores séricos de função cardíaca são utilizados como indicadores de processos fisiológicos, patogênicos e de intervenções farmacológicas que fornecem informações baseadas na exposição à doença, tamanho da lesão e prognóstico (OYAMA e SISSON, 2004). Estudos demonstram que biomarcadores de função cardíaca, como a CK, principalmente a isoforma MB (CK-MB) e lactato desidrogenase (LDH) são ferramentas importantes para o diagnóstico de necrose miocárdica (JAFFE et al., 2006). A análise histopatológica de coração revelou necrose, regiões hemorrágicas e infiltrados inflamatórios em animais infectados com T. *gondii*, diferentemente do que foi encontrado nos animais tratados com $(PhSe)_2$ onde foi observado apenas infiltrado inflamatório. Neste estudo, ocorreu uma redução na atividade da CK, CK-MB e LDH em animais infectados e tratados com $(PhSe)_2$ quando comparado com os animais apenas infectados (grupo C), porém o $(PhSe)_2$ não reverteu o aumento da mioglobina quando comparado com os animais infectados e não tratados. Em um estudo realizado por Baldissera et al. (2015) foi observado aumento dos biomarcadores de função cardíaca no tecido cardíaco de ratos infectados experimentalmente com *T. evansi*, um protozoário que assim como *T. gondii* causa complicações cardíacas. O composto $(PhSe)_2$ mesmo em doses muito baixa apresentou efeito antioxidante e anti-inflamatórios conforme achados da analise histopatológica, assim, a associação do $(PhSe)_2$ com medicamentos utilizados para a toxoplasmose poderia ser uma opção mais eficaz e com menos efeitos colaterais para o tratamento desta doença.

# 7 CONCLUSÃO

Considerando todas as variáveis investigadas e os resultados obtidos, podemos concluir que a infecção pelo *T. gondii*, pode induzir alterações no tecido cerebral, processos inflamatórios, além de induzir ao estresse oxidativo e alterações no coração de camundongos infectados. A administração subcutânea do composto $(PhSe)_2$ apresentou efeito antioxidante, porém não foi capaz de reverter os achados comportamentais relacionados a perda de memória e comportamento ansiolítico causados pelo parasito. Os tecidos dos animais tratados com essa forma de selênio tiveram intensidade menor de lesões, o que sugere que de forma direta ou indireta o tratamento teve efeito protetor para órgão, principalmente coração. Com base nesses dados, estudos futuros associando $(PhSe)_2$ com quimioterápicos tradicionais pode-se aumentar a eficácia do tratamento e reduzir efeitos colaterais de drogas tradicionalmente usadas.

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# 9 ANEXOS

## 9.1 ANEXO 1


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

**CERTIFICADO**

Certificamos que a proposta intitulada "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.", protocolada 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 **aprovada** 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 (Acadêmica)

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

| | | | | | |
|---|---|---|---|---|---|
| Origem: | 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

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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, 12 de dezembro de 2016

Profa. Dra. Daniela Bitencourt Rosa Leal
Coordenadora da Comissão de Ética no Uso de Animais
Universidade Federal de Santa Maria

Prof. Dr. Denis Broock Rosemberg
Vice-Coordenador da Comissão de Ética no Uso de Animais
Universidade Federal de Santa Maria

# 9.2 ANEXO 2

Cetea UDESC
Comitê de Ética em Experimentação Animal
Centro de Ciências Agroveterinárias

**CARTA DE APROVAÇÃO**

O(s) projeto(s) abaixo relacionado(s):

**Protocolo: 01.67.14**
**Título:** Efeitos da suplementação de selênio sobre enzimas do metabolismo energético em coração,cérebro e fígado de camundongos infectados com *Toxoplasma gondii*.
**Coordenador/Pesquisador:** Aleksandro S. da Silva

**Protocolo: 01.73.14**
**Título:** Indução hormonal da lactação em ovinos leiteiros: influencia sobre a composição do leite, perfil oxidativo e parâmetros bioquímicos no sangue e no leite.
**Coordenador/Pesquisador:** Aleksandro S. da Silva

Foi(ram) analisado(s) pelo Comitê de Ética em Experimentação Animal da UDESC (CETEA/UDESC) tendo sido **APROVADO(S)** em seus aspectos éticos e metodológicos, para utilização de animais em pesquisa, de acordo com as diretrizes e normas nacionais e internacionais, especialmente a Lei 11.794 de 08 de novembro de 2008 que disciplina a criação e utilização de animais em atividades de ensino e pesquisa no Brasil.

Lages, 12 de dezembro de 2014.

Prof. Ubirajara Maciel da Costa
Coordenador do CETEA/UDESC



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# 9.3 ANEXO 3

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Full length article

## *Toxoplasma gondii*: Effects of diphenyl diselenide in experimental toxoplasmosis on biomarkers of cardiac function

Vanessa S. Machado [a, b], Nathieli B. Bottari [b], Matheus D. Baldissera [a], Maria Isabel de Azevedo [a], Virginia C. Rech [c], Francine R. Ianiski [c], Rodrigo A. Vaucher [d], Ricardo E. Mendes [e], Giovana Camillo [f], Fernanda F. Vogel [f], Mario L. de la Rue [a], Guilherme M. Carmo [b], Alexandre A. Tonin [g], Aleksandro S. Da Silva [b, h, *]

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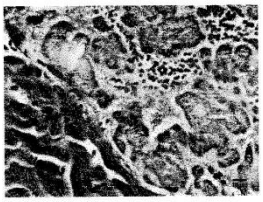
### HIGHLIGHTS

- *Toxoplasma gondii* cause an important infectious disease in humans and animals.
- *T. gondii* infection causes pathological in the heart.
- Diphenyl diselenide $(PhSe)_2$ was able to modulate enzymes related to energy metabolism in the heart.
- The $(PhSe)_2$ treatment reduced the degree and number of lesions in the heart of mice infected with *T. gondii*.

### GRAPHICAL ABSTRACT



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### ABSTRACT

This study aimed to investigate the effects of diphenyl diselenide $(PhSe)_2$ to treat mice experimentally infected by *Toxoplasma gondii* on seric biomarkers of cardiac function (creatine kinase, creatine kinase MB, troponin, and myoglobin), and lactate dehydrogenase, as well as to evaluate the enzymatic activity of creatine kinase (CK) and adenylate kinase (AK) in heart tissue. For the study, 40 female mice were divided into four groups of 10 animals each: the group A (uninfected and untreated), the group B (uninfected and treated), the group C (infected and untreated) and the group D (infected and treated). The inoculation was performed with 50 cysts of *T. gondii* (ME-49 strain). Mice from groups B and D were treated at days 1 and 20 post-infection (PI) with 5 μmol $kg^{-1}$ of $(PhSe)_2$ subcutaneously. On day 30 PI, the mice were anesthetized and euthanized for blood and heart collection. As a result, it was observed a decrease in AK activity ($P < 0.01$) in the heart samples of groups C and D compared to the group A. Cardiac CK increased in the group C compared to the group A ($P < 0.01$). CK levels increased in infected mice (the group C) compared to other groups (A and D). Regarding CK-MB level, there was a decrease in the group D compared to the group B, without statistical difference compared to control groups (A and C). It was observed an increase on myoglobin in groups C and D, differently of troponin, which did not show

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# 9.4 ANEXO 4

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Full length article

## Diphenyl diselenide supplementation in infected mice by *Toxoplasma gondii*: Protective effect on behavior, neuromodulation and oxidative stress caused by disease

Vanessa Schopf Machado [a, b], Nathieli B. Bottari [b], Matheus D. Baldissera [a], Virginia C. Rech [c], Francine R. Ianiski [c], Cristiane Signor [b], Maribel A. Rubin [b], Emily P. Waczuk [f], Claiton I. Schwertz [e], Ricardo E. Mendes [e], Giovana Camillo [g], Fernanda F. Vogel [g], Mario L. de la Rue [a], Vera M. Morsch [b], Maria Rosa C. Schetinger [b], Pâmella K.S. Frühauf [h], Aleksandro S. Da Silva [b, d, *]

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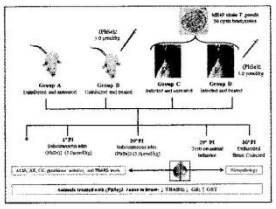
### HIGHLIGHTS

- Toxoplasmosis is an important infectious disease in humans and animals.
- *T. gondii* infection causes pathological and behavioral changes in the brain.
- $(PhSe)_2$ was able to modulate enzymes related to energy metabolism in the brain.
- $(PhSe)_2$ modulate AK, CK and AChE in the brain.
- The treatment with $(PhSe)_2$ showed antioxidant effect.

### GRAPHICAL ABSTRACT



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### ABSTRACT

The aim of this study was to evaluate the effect of subcutaneous administration of diphenyl diselenide $(PhSe)_2$ on animal behavior and activities of acetylcholinesterase (AChE), adenylate kinase (AK), and creatine kinase (CK) in the brain of mice infected by *Toxoplasma gondii*. In addition, thiobarbituric acid reactive species (TBARS) levels and glutathione (GR, GPx and GST) activity were also evaluated. For the study, 40 female mice were divided into four groups of 10 animals each: group A (uninfected and untreated), group B (uninfected and treated with $(PhSe)_2$), group C (infected and untreated) and group D (infected and treated with $(PhSe)_2$). The mice were inoculated with 50 cysts of the ME49 strain of *T. gondii*. After infection the animals of the groups B and D were treated on days 1 and 20 post-infection (PI) with 5.0 μmol/kg of $(PhSe)_2$ subcutaneously. Behavioral tests were conducted on days 29 PI to assess memory loss (object recognition), anxiety (elevated plus maze), locomotor and exploratory activity (Open Field) and it was found out that infected and untreated animals (group C) had developed anxiety and memory impairment, and the $(PhSe)_2$ treatment did not reverse these behavioral changes on

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