UNIVERSIDADE FEDERAL DE SANTA MARIA
CENTRO DE CIÊNCIAS NATURAIS E EXATAS
PROGRAMA DE PÓS-GRADUAÇÃO EM CIÊNCIAS BIOLÓGICAS:
BIOQUÍMICA TOXICOLÓGICA

Gláubia da Silva Sartori

DISSELENETO DE DIFENILA EM MODELOS DE INFECÇÃO VIRAL CAUSADA POR HERPES *SIMPLEX* VÍRUS DOS TIPOS 1 E 2

Santa Maria, RS

2017

Gláubia da Silva Sartori

DISSELENETO DE DIFENILA EM MODELOS DE INFECÇÃO VIRAL CAUSADA POR HERPES *SIMPLEX* VÍRUS DOS TIPOS 1 E 2

Tese apresentada ao Programa de Pós-Graduação em Ciências Biológicas: Bioquímica Toxicológica, da Universidade Federal de Santa Maria (UFSM, RS), como requisito para obtenção do título de **Doutora em Bioquímica Toxicológica**.

Orientadora: Prof.ª Dr.ª Cristina Wayne Nogueira
Co-orientadora: Prof.ª Dr.ª Marina Prigol

Santa Maria, RS
2017

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

DA SILVA SARTORI, GLÁUBIA
DISSELENETO DE DIFENILA EM MODELOS DE INFECÇÃO VIRAL CAUSADA POR HERPES SIMPLEX VÍRUS DOS TIPOS 1 E 2 / GLÁUBIA DA SILVA SARTORI.- 2017.
96 f.; 30 cm

Orientadora: CRISTINA WAYNE NOGUEIRA
Coorientadora: MARINA PRIGOL
Tese (doutorado) - Universidade Federal de Santa Maria, Centro de Ciências Naturais e Exatas, Programa de Pós-Graduação em Ciências Biológicas: Bioquímica Toxicológica, RS, 2017

1. HSV 2. SELÊNIO 3. ANTIVIRAL 4. ESTRESSE OXIDATIVO 5. INFLAMAÇÃO I. WAYNE NOGUEIRA, CRISTINA II. PRIGOL, MARINA III. Título.

Gláubia da Silva Sartori

DISSELENETO DE DIFENILA EM MODELOS DE INFECÇÃO VIRAL CAUSADA POR HERPES SIMPLEX VÍRUS DOS TIPOS 1 E 2

Tese apresentada ao Curso de Pós-Graduação em Ciências Biológicas: Bioquímica Toxicológica, da Universidade Federal de Santa Maria (UFSM, RS), como requisito para obtenção do título de **Doutor em Bioquímica Toxicológica**.

Aprovado em 02 de março de 2017:

Cristina Wayne Nogueira, Drª (UFSM)
(Presidente/Orientador)

Marina Prigol, Drª (Unipampa).
(Coorientador)

Ana Cláudia Franco, Drª. (UFRGS)

Cinthia Melazzo de Andrade, Dr.ª (UFSM)

Claudia Maria Oliveira Simões, Dr.ª (UFSC)

André Passaglia Schuch, Dr. (UFSM)

Santa Maria, RS
2017

Dedico esta tese a minha família,
a qual sempre me apoiou em todas as minhas escolhas.

AGRADECIMENTOS

Agradeço acima de tudo a Deus por abençoar a minha vida, a minha família e pelas oportunidades oferecidas ao longo desta caminhada.

À minha mãe, Lídia, por ser uma mãe maravilhosa, amorosa, amiga e acima de tudo uma mulher guerreira, sempre presente. Ao meu pai, Sérgio, pelo apoio incondicional. Ao meu irmão, Gláuker, pelo companheirismo e amizade. Aos meus queridos e amados avós paternos Clarindo e Dulce, avós maternos Vilma e Miguel, por todo o carinho e dedicação. Aos meus queridos tios Artidor e Élida, eu morando longe de casa foram como meus segundos pais, sempre por perto e me ajudando no que for preciso. A minha “filhota” beagle Laila, minha alegria de viver, me faz sentir especial a qualquer momento com tanto amor, brincadeiras e lambidas.

Em especial ao meu amor Fernando, por tudo que me ensinou e continua ensinando, me apresentou o “mundo científico”, assim fica até mais fácil discutirmos nossas ideias e resultados, um grande amigo, amante e companheiro, sempre presente nas horas mais difíceis com bons conselhos, uma pessoa que admiro muito. Também agradeço à sua família, sempre carinhosa comigo, outra família que ganhei.

À professora Marina, por me acompanhar e me orientar desde o princípio, pela dedicação, parceria, amizade e carinho durante estes anos. Agradeço muito mesmo.

À professora Cristina, um exemplo de profissional, agradeço pela oportunidade, dedicação e grande amizade que marcará para sempre minha vida. A sua visão de mundo e de trabalho em equipe servem de incentivo para buscar o conhecimento e seguir em frente.

Ao professor GZ, pela orientação, apoio e muitos momentos de diversão, muito obrigada.

Ao professor Luiz Rodrigues Jr., pela parceria e apoio desde os tempos de UNIFRA, muito obrigada.

Ao professor Eduardo Flores, pela parceria e apoio no laboratório de virologia da UFSM, muito obrigada.

À Juliana Cargnelutti, que me “salvou” em meio a um doutorado cheio de percalços e surpresas, uma pessoa incrível e parceira, sempre me ajudando em tudo que precisava no laboratório de virologia.

Ao César, por dividir comigo as angústias e alegrias de se fazer um doutorado sanduiche no exterior, pelas viagens e parceria de sempre.

Ao Marcel, pela sinceridade e dedicação, não só comigo mas com todos a sua volta, sempre muito gentil, um grande amigo do coração que levarei comigo sempre.

À minha aluna de iniciação científica Natália, obrigado por ter me acompanhado durante o doutorado, espero que eu possa ter contribuído um

pouco também para sua formação e que tenhas um ótimo futuro pela frente, que seja uma grande doutora.

Aos grandes e especiais amigos que fiz no Lab. Cris e GZ, César, Marcel, Natália, Marina, Ana Cristina, Crisinha, Bibiana, Zé Neto, obrigada pela amizade, que sigamos sempre firmes e fortes.

Ao Simone Agostini e Roberta Mancuso, pois não foram somente importantes na minha vida cientifica quando fiz o doutorado sanduiche em Milão mas também foram muito amáveis, receptivos e até hoje nossa amizade me faz sentir muitas saudades assim como foram os demais do laboratório, sempre muito gentis. Ao Prof. Mario Clerici e a Don Gnocchi Foundation – ONLUS (Milão) por me receberem e confiarem em nosso trabalho.

Ao pessoal do Lab. Cris e Lab. GZ, agraço pelo companheirismo e amizade.

À UFSM, por ter me dado oportunidade de estudar e realizar minha tese, um lugar incrível e especial que contribuiu tanto em minha vida.

À banca, Professores Cinthia Melazzo, André Schuch, Ana Claudia Franco e Cláudia Simões pela disposição em avaliar meu trabalho.

Aos professores e funcionários do PPGBTOX – UFSM.

À CAPES pelo auxílio financeiro durante o doutorado.

A todos que de alguma forma contribuíram para a realização deste trabalho.

"A verdadeira viagem de descobrimento não consiste em procurar novas paisagens, e sim em ter novos olhos. "

Marcel Proust

RESUMO

DISSELENETO DE DIFENILA EM MODELOS DE INFECÇÃO VIRAL CAUSADA POR HERPES *SIMPLEX* VÍRUS DOS TIPOS 1 E 2

Autora: Gláubia da Silva Sartori
Orientadora: Dr.ª Cristina Wayne Nogueira
Co-orientadora: Dr.ª Marina Prigol

Doenças infecciosas ocasionadas pelo herpes *simplex* vírus do tipo 1 e 2 (HSV-1 e HSV-2) estão entre as patologias que mais acometem a população. A busca por novas alternativas terapêuticas para o seu tratamento torna-se relevante devido ao crescimento de casos resistentes ao Aciclovir, referência no tratamento de infecções herpéticas. Desta forma, esta tese investigou o efeito do disseleneto de difenila $(PhSe)_2$, uma molécula orgânica de selênio, no tratamento de infecções geradas pelo herpes *simplex* vírus humano 1 (HSV-1) e 2 (HSV-2) *in vitro* e *in vivo*. No protocolo 1 referente aos artigos 1 e 2, foi realizado incialmente o teste de viabilidade celular (MTT) para verificar se há toxicidade do composto (1-100μM) e posteriormente verificou-se o seu efeito sobre o HSV-2 através do ensaio de redução de placa viral, ambos os testes realizados em cultura de células VERO. Após, foram realizados os experimentos *in vivo*. O protocolo consistiu de 5 dias de pré-tratamento com $(PhSe)_2$ (5 mg/kg, via intragástrica, i.g.), infecção intravaginal no dia 6 e pós-tratamento com o composto por mais 5 dias. Ao final dos tratamentos, os animais foram mortos e análises *ex vivo* dos tecidos genital, renal, hepático e plasma foram realizadas. A evolução da lesão extravaginal foi analisada a cada dia pós-infecção. Outros parâmetros também foram investigados como carga viral, análise histológica, estresse oxidativo, inflamação, resposta imune, atividades de enzimas antioxidantes e anti-inflamatórias, parâmetros de toxicidade renal e hepática. No protocolo 2 referente ao manuscrito 1, as análises do efeito antiviral do $(PhSe)_2$ contra a infecção por HSV-1 foram realizadas em dois tempos diferentes após a infecção (4 e 24 horas) em culturas de células gliais. Foi quantificada a carga viral através dos ensaios de redução de placa, inibição da síntese de DNA viral e inibição de genes envolvidos no ciclo replicativo (ICP27, ICP0, ICP8 e DNA polimerase viral). Também foi investigada a ativação do sistema imunológico (NF-κB e TNF-α) nas células infectadas por HSV-1. Os resultados referentes aos artigos 1 e 2 demonstraram um efeito antiviral do $(PhSe)_2$ *in vitro* e *in vivo* bem como a redução do estresse oxidativo, inflamação, restauração da capacidade antioxidante como o conteúdo de tióis não-proteicos e da atividade de enzimas antioxidantes. Ainda, o composto protegeu contra efeitos tóxicos gerados pela infecção a nível renal e hepático. Os achados obtidos no manuscrito 1 também foram positivos, uma vez que foi confirmado o efeito antiviral do $(PhSe)_2$ contra a infecção por HSV-1 nas células gliais bem como a redução da síntese de DNA viral e regulação dos genes do ciclo replicativo. O composto reduziu a expressão da maioria dos genes virais estudados nos diferentes tempos de análise após a infecção. E de certa forma apresentou efeito imunomodulador através da redução da expressão da citocina TNF-α. A partir dos dados obtidos pode-se considerar o $(PhSe)_2$ uma importante alternativa terapêutica frente as infecções causadas pelo HSV-1 e HSV-2.

Palavras-chave: HSV. Selênio. Antiviral. Estresse oxidativo. Inflamação. Citocinas.

ABSTRACT

DIPHENYL DISSELENIDE ON VIRAL INFECTION MODELS CAUSED BY HERPES SIMPLEX VIRUSES TYPES 1 AND 2

Author: Gláubia da Silva Sartori
Advisor: Dr.ª Cristina Wayne Nogueira
Co-advisor: Dr.ª Marina Prigol

Infectious diseases caused by herpes simplex virus types 1 and 2 (HSV-1 and HSV-2) are among the pathologies that most affect the population. The search for new therapeutic alternatives for its treatment becomes relevant due to the growth of cases resistant to Aciclovir, a reference in the treatment of herpetic infections. Thus, this thesis investigated the effect of diphenyl diselenide $(PhSe)_2$, an organic molecule of selenium, for the treatment of infections caused by herpes simplex virus 1 (HSV-1) and 2 (HSV-2) *in vitro* and *in vivo*. In the protocol 1 related to articles 1 and 2, the cell viability test (MTT) was initially performed to check the toxicity of the compound (1-100 μM) and later its effect on the virus was verified by the plaque reduction assay, both tests performed on VERO cell culture. After, the experiments were carried out *in vivo*. The protocol consisted of treatment with $(PhSe)_2$ (5 mg/kg/day, intragastric, i.g.) 5 days before and 5 days after infection; mice were infected at day 6. The extravaginal lesion score was evaluated from days 6 to 10. At the end of treatments, the animals were killed and *ex vivo* analyses of the genital, renal, hepatic and plasma tissues were performed. Other parameters were also investigated such as viral load, histological analysis, oxidative stress, inflammation, immune response, antioxidant and anti-inflammatory enzyme activities, renal and hepatic toxicity parameters. In the protocol 2 related to the manuscript 1, the antiviral effect of $(PhSe)_2$ against HSV-1 infection was investigated at different times post-infection (4 and 24 hours) in glial culture cells. The viral load was quantified through plaque reduction assay, inhibition of viral DNA synthesis and inhibition of genes involved in the replicative cycle (ICP27, ICP0, ICP8 and viral DNA polymerase). The activation of the immune system (NF-κB and TNF-α) in HSV-1 infected cells was also investigated. The results of articles 1 and 2 demonstrated an antiviral effect of $(PhSe)_2$ *in vitro* and *in vivo* as well as the reduction of oxidative stress, inflammation and restoration of antioxidant capacity, such as the content of non-protein thiols and the activity of antioxidant enzymes. Furthermore, the compound protected against toxic effects generated by infection at the renal and hepatic levels. The results obtained in manuscript 1 were also positive because the antiviral effect of $(PhSe)_2$ against HSV-1 infection in glial cells was confirmed as well as the reduction of viral DNA synthesis and regulation of the replicative cycle genes. The compound reduced the expression of most viral genes studied at different time points after infection. $(PhSe)_2$ showed an immunomodulatory effect by reducing the expression of the cytokine TNF-α. From these data, $(PhSe)_2$ can be considered an important therapeutic alternative to treat HSV-1 and HSV-2 infections.

Keywords: HSV. Selenium. Antiviral. Oxidative stress. Inflammation. Cytokines.

LISTA DE FIGURAS

LISTA DE ABREVIATURAS

$(PhSe)_2$ - Disseleneto de Difenila

Aβ - Beta Amilóide

Ca^{2+} - Cálcio

CAT - Catalase

CMV - Citomegalovírus

DA - Doença de Alzheimer

ERN - Espécies Reativas De Nitrogênio

ERO - Espécies Reativas De Oxigênio

g - Glicoproteína

GPx - Glutationa Peroxidase

GSH - Glutationa

HSV - Herpes simplex vírus

HSV-1 - Herpes simplex vírus 1

HSV-2 - Herpes simplex vírus 2

I.g - Intragástrico

IE - *Immediate Early Gene*

IFN - Interferon

IFN-γ - Interferon Gama

MAPKs - Proteínas Quinases Ativadas Por Mitógenos

MPO - Mieloperoxidase

NF-κB - Fator Nuclear kappa B

NK - Natural Killer

NPSH - Tióis Não Proteicos

PCR - Reação em cadeia da Polimerase

PPA - Proteína Precursora Amilóide

PPRs - Receptores De Reconhecimento De Padrões

RNAm - RNA mensageiro

Se - Selênio

SNC - Sistema Nervoso Central

SNP - Sistema Nervoso Periférico

SOD - Superóxido Dismutase

TLR - Receptores tipo Toll

TLR2 - Receptores tipo Toll 2

TNF- α - Fator De Necrose Tumoral Alfa

TR - Tioredoxina Redutase

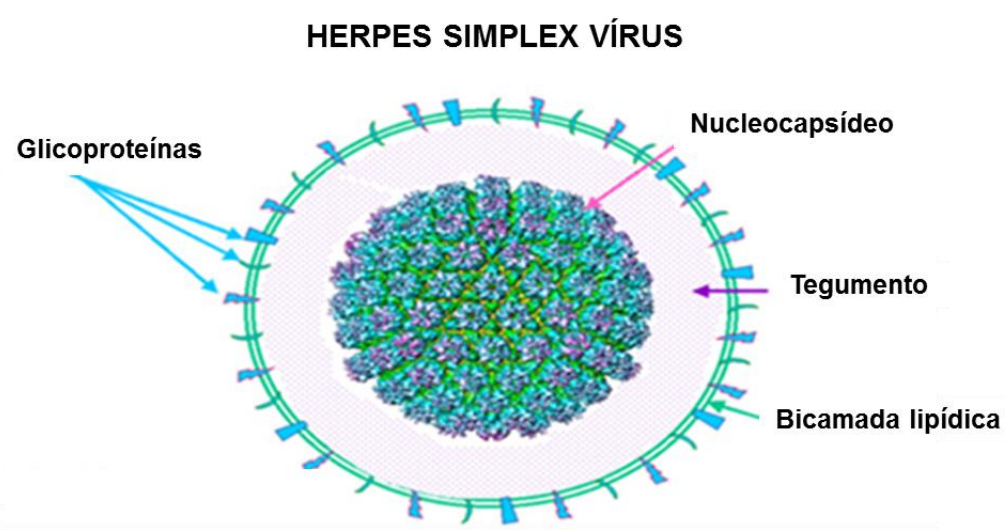
SUMÁRIO

1. INTRODUÇÃO

1.1 Infecção Viral

O herpes *simplex* vírus (HSV) pertence à família *Herpesviridae*, cujos membros são constituídos por um filamento linear de DNA de dupla fita envolvido em uma capa proteica icosaédrica composta de 162 capsômeros (nucleocapsídeo). Ao seu redor, há um tegumento amorfo e um envelope lipídico externo (MORIARTY et al., 2009) conforme mostra a Figura 1.

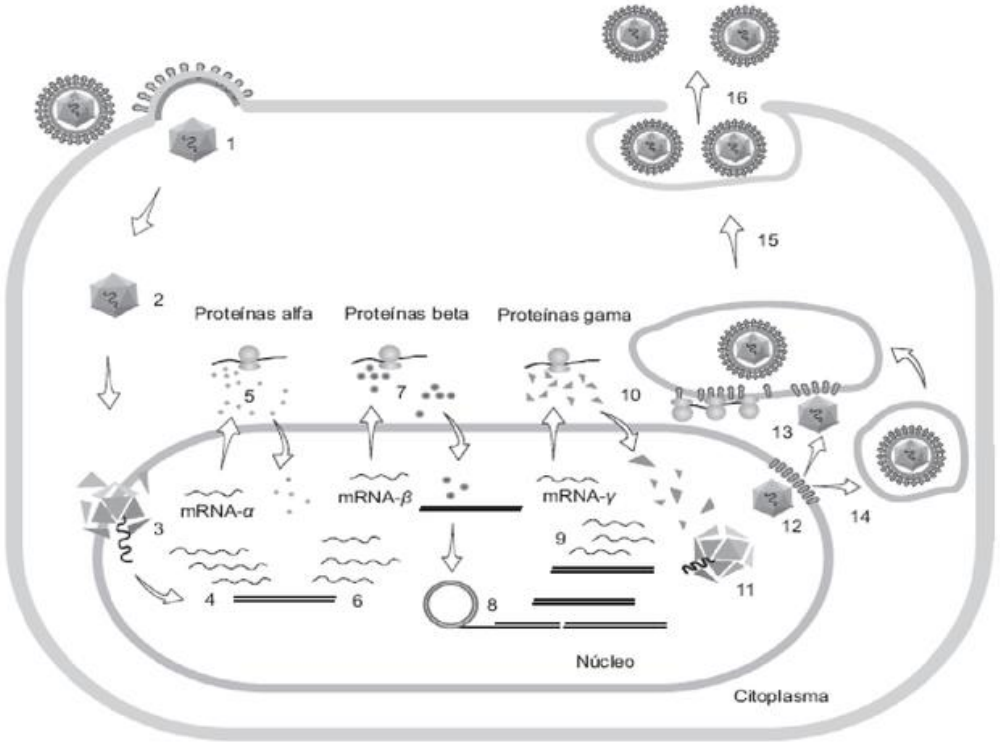
Figura 1 - Estrutura do herpes *simplex* vírus. Adaptado de (CITIZENDIUM, 2008).



Infecções causadas pelo herpes *simplex* vírus tipos 1 e 2 (HSV- 1 e HSV-2) são mais frequentes do que se pode imaginar. Estes vírus possuem características biológicas particulares, tais como a capacidade de causar diferentes tipos de doenças como ceratites e encefalites, assim como estabelecer infecções latentes ou persistentes por toda a vida dos hospedeiros. O contágio ocorre geralmente por contato íntimo da pessoa portadora do vírus a partir de uma superfície de mucosa ou de lesão infectante (LAZARINI et al., 2006; PATEL et al., 2011).

O ciclo replicativo inicia-se com a ligação do vírion ao receptor de superfície celular (heparansulfato), através das glicoproteínas do envelope externo. Pelo menos, sete glicoproteínas já foram identificadas, no entanto, apenas três (gB, gD e gH) são essenciais para a infectividade viral (MCCORMACK et al., 2006). O vírus se adere a três diferentes tipos de receptores de superfície celular e logo após funde-se com a membrana plasmática. O capsídeo, menos o seu envelope, é transportado para o poro nuclear através do qual libera o seu DNA viral para o núcleo. O HSV, por sua vez, replica-se em um ciclo de três transcrições: gene α (proteína inicial que regula principalmente a replicação viral denominada *immediate early gene* – IE); gene β (proteína inicial da síntese e empacotamento do DNA viral, denominado *early gene*); e o gene γ (proteína tardia, os quais constituem a forma infectiva do vírus, denominado *late gene*) (WARD et al., 1994) (Figura 2).

Figura 2 - Etapas do ciclo replicativo viral. Fonte: (HSV-2, 2017).



Os genes da fase α codificam as proteínas que são responsáveis pela regulação gênica da próxima etapa de transcrição (expressão dos genes β), dentre elas, as proteínas ICP0, ICP4, ICP22, ICP27 e ICP47. A proteína ICP4

reprime a expressão dos genes imediatamente iniciais (GU et al., 1994; CARROZZA et al., 1996) já a ICP27 inibe o processamento do RNAm (SANDRI-GOLDIN et al., 1992; LINDBERG et al., 2002) e a ICP47 impede o transporte de peptídeos antigênicos para o retículo endoplasmático (HILL et al., 1995). A síntese das proteínas α ocorre entre 2-4 horas após a infecção da célula (ROIZMAN, 1996).

Na fase β, são expressas as proteínas responsáveis pela replicação do DNA viral e produção de substratos para a síntese de DNA. Dentre elas, destaca-se a timidina quinase viral (TK) e a DNA polimerase viral (UL30) (GONG et al., 2002). O pico da síntese dessas proteínas ocorre entre 5-7 horas após a infecção (LEHMAN et al., 1999). Após o fim da fase β, inicia-se a expressão dos genes responsáveis pela síntese das proteínas estruturais do HSV, os genes da fase γ. A fase γ, ou tardia, se caracteriza pela produção de proteínas estruturais do vírion, (como proteínas presentes no tegumento e no envelope) e a montagem da partícula viral (ROIZMAN, 1996; BOEHMER et al., 1997). Essas proteínas estruturais serão responsáveis pela formação e montagem do capsídeo. A replicação completa dura em média de 18-20 horas (JACOBS et al., 1999).

Após o vírus infectar o epitélio, este sofre replicação e pode ser transmitido ao sistema nervoso através do transporte retrógrado nos neurônios sensoriais. O vírion perde o seu envoltório, penetrando nas terminações nervosas cutâneas, depois o nucleocapsídeo migra centralmente para o gânglio nervoso sensitivo, onde estabelece latência. A replicação no núcleo e a migração periférica dos virions levam ao surgimento de novas lesões, podendo ser distantes do sítio inicial de inoculação, geralmente na segunda semana após o episódio primário (WHITLEY et al., 2001).

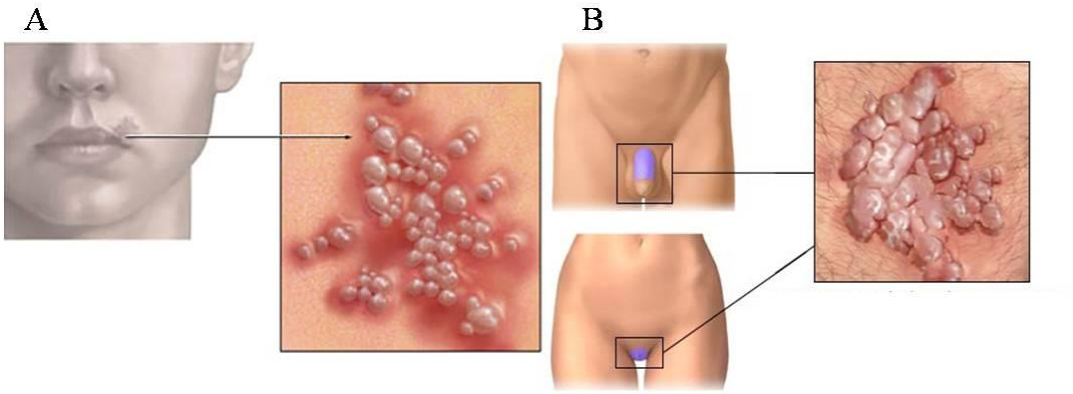
A célula hospedeira transforma-se em uma verdadeira “fábrica” de HSV, onde todas as funções celulares são completamente subordinadas aos processos replicativos virais. A menos que a latência seja estabelecida, ocorre uma parada imediata na produção proteica celular, além de ocorrer uma degradação do RNA mensageiro (RNAm) celular, ocasionando a morte da célula infectada. O HSV torna-se latente nos gânglios nervosos sensitivos, preferencialmente nas raízes dorsos sacrais para o HSV-2 e no nervo trigêmeo para o HSV-1 (WHITLEY, 2002).

As lesões herpéticas são caracterizadas por pápulas eritematosas, úlceras geralmente superficiais e intensamente dolorosas, principalmente durante a infecção primária. Também podem ocorrer outros sintomas como prurido, disúria, febre e mal-estar (PATEL et al., 2011).

O HSV-1 é o principal causador do herpes labial na região orofacial, estas infecções primárias ocorrem em torno dos 20 anos de idade e são normalmente assintomáticas (Figura 3A). Dados revelam que anticorpos contra o vírus são encontrados em cerca de 80% dos adolescentes (WEISS, 2004). A prevalência de infecção pelo HSV-1 é de 60% a 80% na população mundial, o que pode significar uma ampla transmissão viral. Este fato pode ser influenciado por fatores como idade (a prevalência de infecção é de > 40% aos 15 anos de idade, e de 60 a 90% em adultos; em populações não expostas a fatores de risco, esta prevalência tende a se elevar linearmente com a idade, com picos durante a infância e a adolescência); etnia (prevalência de 35% em afro-americanos e de 18% em caucasianos americanos) e localização geográfica (nos EUA, 90% da população são carreadores do vírus; no Brasil a soroprevalência é classificada por faixa etária, sendo a maior incidência de casos entre 35 - 44 anos, aproximadamente 96% dos homens e mulheres) (SMITH et al., 2002; COWAN et al., 2003).

Já o herpes genital, causado pelo HSV-2, é uma das doenças sexualmente transmissíveis mais prevalentes no mundo e é a forma mais comum de úlcera na região genital (Figura 3B). Em geral, seu quadro clínico costuma ser menos grave do que o do HSV-1. O herpes genital primário aparece como máculas e pápulas, seguidas de vesículas, pústulas e ulceração (eliminação assintomática do vírus ocorre em 12% dos casos). Já os casos de recorrências surgem mais brandos, que se resolvem em 2 semanas (três a quatro vesículas penianas ou lesões ulcerosas vulvares). Um terço dos pacientes apresenta mais de seis episódios de herpes genital recorrente ao ano, um terço, dois episódios, e o restante raramente apresenta recorrência. Um estudo realizado na cidade do Rio de Janeiro demonstrou que em uma população de doadores de sangue voluntários, a soroprevalência para o HSV-2 foi de 29,1%, mas, desse total, apenas 7% referiram história prévia de herpes genital. Em outro estudo americano, avaliações similares detectaram prevalência variável de 1% a 20% para o HSV-2 (LUPI, 2011; PATEL et al., 2011).

Figura 3 - Lesões herpéticas causadas pelo HSV-1 (A) e HSV-2 (B). FONTE: (CLINICACISO.NO.COMUNIDADES.NET, 2013).



Há relatos de que apenas 10 a 20% dos pacientes infectados se definam como portadores de herpes simples; até 60% dos indivíduos soropositivos para o HSV apresentam quadros assintomáticos não reconhecidos pelos próprios pacientes como herpes simples (WALD et al., 1996). Esta problemática se correlaciona ao modo de disseminação do vírus na população, pois recidivas são interpretadas como estados gripais, aftas ou infecções genitais inespecíficas.

O HSV-1 e o HSV-2 diferem geneticamente assim como seus efeitos citopáticos, porém ambos podem gerar um quadro de encefalite necrosante aguda durante a infância e idade adulta. A apresentação clínica desta doença é similar a casos de encefalites causadas por outros tipos de vírus endêmicos como enterovírus, varicela zoster e citomegalovírus. Geralmente os pacientes apresentam dor de cabeça, febre, alterações comportamentais e até convulsões generalizadas (SHANKAR et al., 2008).

O diagnóstico pode ser confirmado através do isolamento viral em cultura de células ou detecção do seu DNA por Reação em Cadeia da Polimerase (PCR). As amostras devem ser coletadas de vesículas e lesões presentes na pele, armazenadas corretamente e encaminhadas para análise laboratorial. O diagnóstico sorológico da infecção por HSV ajuda somente para determinar se houve uma prévia exposição ao vírus (RECHENCHOSKI et al., 2016).

1.2 O Processo infeccioso no sistema nervoso central (SNC)

Como já citado anteriormente, as infecções virais por HSV-1 e por HSV-2 quando atingem latência nos gânglios nervosos podem chegar a causar um quadro preocupante de encefalite. Esta inflamação no SNC resulta na ativação do sistema imunológico inato local, o que pode gerar um dano cerebral considerável e consequentemente doenças neurodegenerativas (MARTIN et al., 2014). Dados recentes da literatura têm associado a presença de anticorpos contra o HSV-1 ao desenvolvimento de doenças neurodegenerativas em pacientes mais idosos, como, por exemplo, a doença de Alzheimer (DA), uma demência multifatorial caracterizada por grave mudança de personalidade e prejuízo cognitivo (MANCUSO et al., 2014a; MANCUSO et al., 2014b).

Uma das hipóteses que explica a patogênese molecular da DA baseia-se em uma superprodução de peptídeos beta amilóides (Aβ) nos neurônios. Este acúmulo no espaço extracelular, por sua vez, acarreta na formação das placas amilóides, uma caracteristica marcante deste tipo de demência. O peptideo Aβ é produzido por uma glicoproteína transmembrana chamada proteína precursora amilóide (PPA) através de um processo de endoproteólise (SINHA et al., 1999; VASSAR, 2001). Outros estudiosos defendem a hipótese de que novas partículas virais produzidas no sistema nervoso periférico (SNP) podem recrutar células que contenham a PPA em suas membranas, possivelmente secretadas pelo aparelho de Golgi (BEARER, 2004). O transporte do vírus ao cérebro pode ocorrer pela liberação e hidrólise da PPA, o que pode contribuir de alguma forma para o acúmulo das placas amilóides. Alguns dados da literatura sugerem ainda que a PPA em si pode participar do transporte do vírus entre os neurônios (SATPUTE-KRISHNAN et al., 2006). Desta forma, estes fatores estimulam uma investigação mais detalhada sobre o papel do HSV-1 na propagação e formação de fragmentos neurotóxicos, tais como a PPA, que podem estar associados ao aparecimento da DA.

Evidências moleculares demonstraram que infecções causadas pelo HSV-1 em células neuronais e gliais geram um aumento nos níveis intracelulares da Aβ, uma redução da PPA e a fosforilação da proteina Tau, principal componente dos emaranhados neurofibrilares. Estes eventos, por sua vez, são os mesmos encontrados na DA. Portanto, considera-se que a infecção por HSV-1 pode estar interligada a formação de placas amilóides e emaranhados

neurofibrilares promovidos por uma inflamação exacerbada (WOZNIAK et al., 2009). Desta maneira, destaca-se a relevância de se estudar o efeito do composto não somente sobre a infecção viral como também sobre as consequências geradas pelo mesmo.

1.3 Herpes simplex vírus e pacientes imunodeprimidos

As complicações oriundas da infecção herpética recorrente são raras mas ganham atenção quando se referem a pacientes imunodeprimidos, pois envolvem casos de encefalites, hepatites, pneumonia, esofagites e queratites (KOELLE et al., 2003) . Nestes pacientes as infecções recorrentes são a maior causa de morbidade e mortalidade ocasional uma vez que o aparecimento das lesões são mais frequentes e persistentes (FATAHZADEH et al., 2007). Deste modo, trata-se de um vírus bastante frequente em indivíduos com infecção pelo HIV, em que, numa fase inicial, a doença tem um comportamento idêntico aos doentes não imunodeprimidos, com a característica erupção vesicular, sendo, na maioria dos casos, seguida de erupções ou úlceras orais ou urogenitais por vezes de grandes dimensões e bastante dolorosas, ou ainda, pneumopatias e meningoencefalites, que requerem um tratamento mais rigoroso ou internação hospitalar (DESAI et al., 2015).

Estudos *in vitro* demonstraram que células de linhagem de linfócitos CD4+ (CEM), quando co-infectadas com HIV-1 e HSV-1 em altos MOIs, produzia uma quantidade abundante do retrovírus (ALBRECHT et al., 1989). Quando essas células eram infectadas com cepas de HSV-1 mutantes para proteínas regulatórias de fase, foi observado que a proteína ICP4 poderia estar envolvida no aumento da replicação do HIV-1 na co-infecção. Entre as proteínas do HIV-1 críticas na indução da replicação estimuladas por HSV-1, a Tat desempenha uma função importante. A indução da replicação do HIV-1 por HSV-1 só é possível na presença de Tat, sugerindo que o HSV-1 não é capaz de induzir o ciclo replicativo completo do HIV- 1 (POPIK et al., 1994). Estima-se que dois terços dos indivíduos com HIV-1 são co-infectados com HSV (CHENTOUFI et al., 2012), além disso, estudos com mulheres grávidas HIV positivas mostram esta co-infecção aumenta significativamente o risco de transmissão perinatal do HIV (CHEN et al., 2005; ANZIVINO et al., 2009).

O Herpes simples visceral também é mais comum nos pacientes com AIDS gravemente imunodeprimidos. Os órgãos mais acometidos são o pulmão, esôfago, fígado e as glândulas suprarrenais (LUPI, 2011). Já a Hepatite pode ser causada tanto por infecções por HSV-1 como por HSV-2 e pode resultar em falência aguda do fígado ou até a morte. Geralmente, o diagnóstico precoce é decisivo para que o quadro clínico seja tratado e recuperado com sucesso (RIEDIGER et al., 2009). Além disso, as infecções virais também acometem pacientes imunodeprimidos que possuem falência renal e pacientes que fizeram transplante renal e uso de drogas imunossupressoras (ANDRES, 2005; KHAMENEH et al., 2008).

1.4 O papel do sistema imunológico nas infecções por HSV

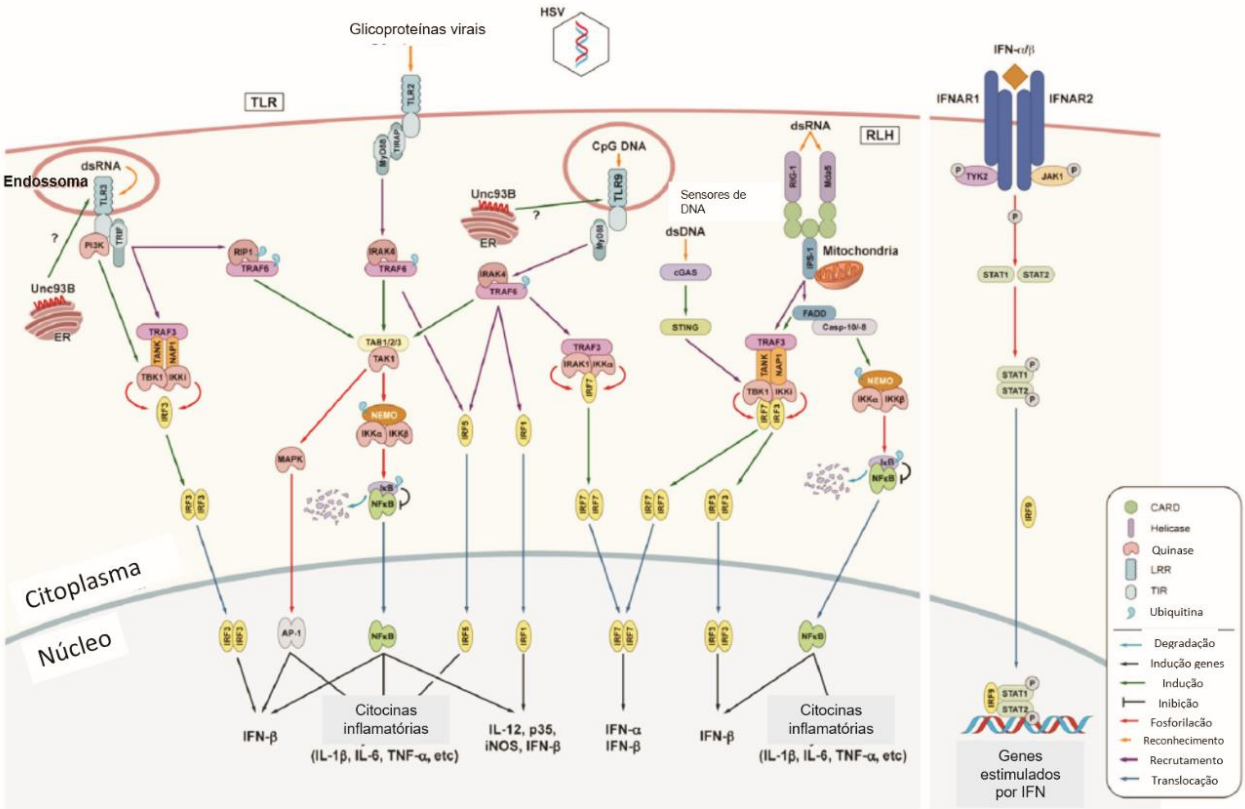
O sistema imunológico desempenha um papel importante na limitação da replicação viral durante a infecção primária e na manutenção do estado latente viral durante períodos prolongados com ausência de infectividade e sintomas clínicos. Ao invadir a célula hospedeira o vírus influencia processos celulares ativando cascatas oxidativas, inflamatórias e imunológicas, as quais podem estar direta ou indiretamente correlacionadas (STANFIELD et al., 2015). Durante a resposta imunológica inata ocorrem as seguintes etapas: secreção de proteínas, como as do complemento e as defensinas; rápida resposta inicial das células epitelias e dentríticas induzidas pelo vírus, caracterizada predominantemente pela produção de interferon; e recrutamento de células de defesa como neutrófilos, macrófagos e natural killer (NK) (DUERST et al., 2003).

Modelos de infecção *in vitro* têm explorado quais respostas celulares podem ser limitantes no ciclo de replicação viral e de que forma elas podem propagar o vírus uma vez que o mesmo se estabelece. Respostas que podem incluir: produção de interferon (IFN) tipo 1, os quais são gerados nas primeiras horas após a infecção; neutrófilos, os quais se acumulam na mucosa dentro de 24 h após a infecção viral e secretam uma grande quantidade do fator de necrose tumoral α (TNF- α); macrófagos, os quais são responsáveis pela fagocitose; células NK, que são recrutadas ao local da infecção, produzem IFN e participam diretamente na lise da célula infectada; e as células dentríticas, que vinculam a reposta imune inata e adaptativa (CHAN et al., 2011).

A cascata de sinalização do IFN tipo 1 é a primeira linha de defesa e medeia uma ampla variedade de respostas do sistema imune inato contra o estímulo infeccioso. Geralmente é ativada através do reconhecimento dos constituintes virais pelos receptores de reconhecimento de padrões (PPRs), os quais ativam a expressão de múltiplos genes estimulados por IFN. Os PPRs citosólicos incluem muitos membros da família dos receptores do tipo Toll (TRLs ou Toll-like receptors em inglês) e determinados sensores de DNA e RNA (WHITLEY, 2006; SU et al., 2016).

A ativação da maioria dos PPRs, por exemplo, os TLRs, conduzem a sinalização de ativação do fator nuclear kappa B (NF-κB), o qual ativa a expressão de um vasto número de citocinas que estão envolvidas na resposta imune inata, especialmente na cascata de sinalização do IFN. A subunidade p65/RelA do NF-κB é crucial no fator de transcrição do mesmo principalmente durante a resposta imune inata da célula hospedeira (PIRET et al., 2015) (Figura 4).

Figura 4 - Detecção do HSV por receptores de reconhecimento de patógenos. Adaptado de PIRET e BOIVIN, 2015.



Muitos vírus, como os de RNA e DNA, conseguem interferir na resposta inflamatória do hospedeiro através da interação com a subunidade p65/RelA. Estudos sugerem que a US3, uma serina/treonina proteína quinase viral, é responsável por hiperfosforilar a subunidade p65/RelA, evitando assim a translocação nuclear do NF-κB de forma que não ocorra a sua ativação e ainda a redução da expressão de várias citocinas inflamatórias (WANG et al., 2014). Através destes recentes relatos sobre a interação vírus e resposta imune inata da célula hospedeira, pode-se imaginar a complexidade deste entendimento, pois há muitas etapas da via de sinalização envolvidas na resposta de defesa do hospedeiro.

1.5 O papel do estresse oxidativo no processo infeccioso

A entrada e subsequente replicação viral nas células eucarióticas pode desencadear rotas de estresse oxidativo, como a de produção de radicais livres (SCHWARZ, 1996; NENCIONI et al., 2011).

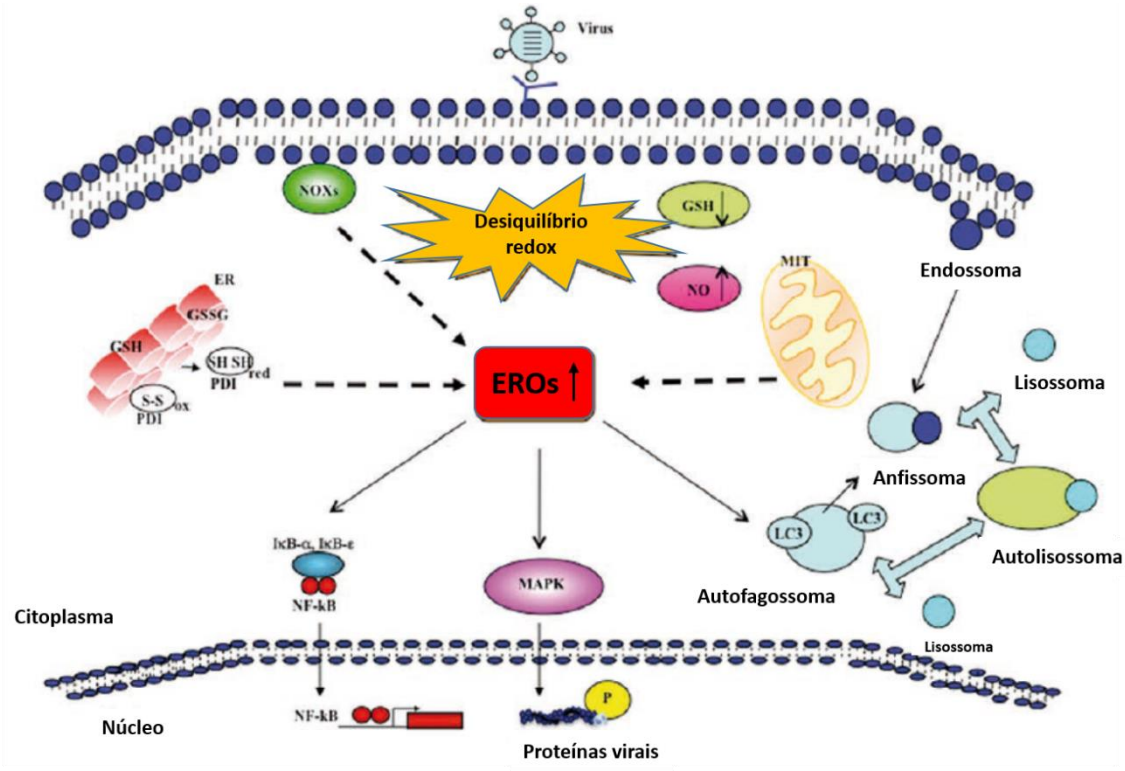
Estudos têm demonstrado que ambos os vírus da herpes (HSV-1 e HSV-2) podem induzir a produção de mediadores pró-inflamatórios, recrutamento e ativação de macrófagos e neutrófilos, uma rápida produção de espécies reativas de oxigênio e eventos apoptóticos, de forma a combater os patógenos invasores responsáveis pelo desenvolvimento das lesões (NOVELLI et al., 2004; SCHACHTELE et al., 2010). Uma superprodução destas espécies ou um sistema antioxidante deficiente pode resultar em um processo de estresse oxidativo e desencadear um metabolismo celular alterado, transdução de sinais irregulares e mudanças funcionais entre as células e tecidos (ARRIGO et al., 2005).

Quando o equilíbrio oxidativo intracelular é sobrecarregado, as espécies reativas de oxigênio (ERO) e de nitrogênio (ERN) se acumulam constituindo um meio altamente oxidante e conduzem assim ao dano celular. De fato a regulação deste estado redox é extremamente importante para o bom funcionamento da célula, inclusive suas funções metabólicas (KAVOURAS et al., 2007). As ERO podem derivar especies pro-oxidantes que são subdivididas em dois grupos: radicais e não radicais. O grupo radical inclui componentes como o radical hidroxil (OH), o óxido nítrico (NO), o superóxido (O2), o peroxil (ROO) e alkoxil

(RO) e o oxigênio singlet (O). O grupo não radical inclue uma variadade de substâncias, algumas extremamente reativas. Alguns deles são o ácido hipocloroso (HClO), peróxido de hidrogênio (H2O2), peróxidos orgânicos, e o peroxinitrito (ONOO) (BURCH et al., 2005).

Além disso, a superprodução das EROs ocasionada pelas infecções virais pode interferir no equilibrio do sistema da glutationa (GSH), que afeta também o estado redox da célula hospedeira e favorece mais ainda a replicação viral. Estudos realizados em diferentes culturas celulares mostram que um aumento no conteúdo de GSH resulta em uma inibição concentração-dependente da replicação viral (PALAMARA et al., 1995; VOGEL et al., 2005). Estas alterações no estado redox celular estão representadas na Figura 5.

Figura 5 - Principais vias do estresse oxidativo celular ativadas pela infecção viral. FONTE: Adaptado de NENCIONI et al., 2011.



Infecções causadas por HSV-1 ou citomegalovírus humano (HCMV) podem gerar o acúmulo de ERO e ERN *in vitro* (KAVOURAS et al., 2007). Outras

pesquisas demonstram que culturas celulares de micróglia reconhecem o vírus através de receptores TLR2 e assim desencadeiam uma resposta inflamatória incluindo a produção de citocinas, quimiocinas, indução de apoptose (ARAVALLI et al., 2005; ARAVALLI et al., 2006; ARAVALLI et al., 2008) e geração de EROs (LEE et al., 2004; YANG et al., 2008).

1.6 Agentes antivirais e o desenvolvimento de vacinas

Os agentes antivirais, como o tradicional Aciclovir, Valaciclovir, e Famciclovir são geralmente usados no tratamento do HSV. Estes antivirais orais penetram nas células infectadas e agem como análogos de nucleosídeos; eles se ligam e são fosforilados pela timidina quinase viral. Por consequência, os antivirais são fosforilados novamente pelas enzimas celulares e começam a competir com os nucleosídeos para se ligar a enzima DNA polimerase viral, assim inativando-a de forma a reduzir a replicação do vírus (JIANG et al., 2016).

O Aciclovir, por sua vez, é um análogo acíclico de guanosina que atua na inibição da enzima viral DNA polimerase, tornando-se um inibidor competitivo da ligação da enzima com a guanosina trifosfato. Contudo, a principal limitação do uso deste medicamento é a sua baixa biodisponibilidade (15-20%) sendo necessário a administração de várias doses diárias, normalmente 5 vezes ao dia (MCKENDRICK et al., 1986; NOLKEMPER et al., 2010), além do aparecimento de efeitos adversos, tais como dor de cabeça, náuseas, diarréia, toxicidade renal. O desenvolvimento de resistência ao tratamento devido a mutações no DNA viral, as quais podem desencadear ausência da timidina quinase ou a síntese alterada da enzima DNA polimerase (FERRÁN et al., 2006; BISWAS et al., 2008) também é uma caracteristica limitante. Já o Valaciclovir funciona como uma pró-droga do Aciclovir, convertida a esta segunda através da metabolização por hidroxilases intestinais e hepáticas sendo eficaz na redução da duração de um surto. Ainda, possui maior biodisponibilidade, três a cinco vezes superior que a do Aciclovir, necessitando de menor frequência posológica (uma vez ao dia). Por serem altamente nefrotóxicos, o Foscarnet e o Cidofovir são reservados para infecções mucocutâneas Aciclovir-resistentes, em indivíduos imunocomprometidos e administrados por via intravenosa (FATAHZADEH et al.,

2007).

Estes medicamentos são indicados principalmente em indivíduos que experimentam surtos frequentes e em pacientes imunocomprometidos. Se levar em consideração a extensão de replicação viral nos primeiros 2 dias de recorrência, a intervenção precoce torna-se essencial (WHITLEY et al., 2001). Não existe cura para a infecção herpética, apenas poucos tratamentos reduzem os sintomas da expressão clínica. Em razão disso, existe uma grande demanda de pesquisas de antivirais para o tratamento das infecções causadas pelo HSV.

Atualmente, o desenvolvimento de outros métodos que possam resolver e tratar infecções causadas pelo HSV são de grande relevância, como o caso das vacinas. A criação de uma vacina profilática eficiente torna-se complicada devido a um complexo ciclo de replicação viral latente e também pela falta de conhecimento suficiente ainda sobre a função imune durante os estágios primários e latentes da infecção (GOTTLIEB et al., 2017). O estudo de novos candidatos a vacina para o HSV ainda tem um longo caminho a seguir, porém ensaios clínicos já estão nas fases I e II. Duas estratégias estão sendo testadas para vacinação contra o HSV-2: primeiro uma abordagem clássica que utiliza uma vacina profilática dirigida a pessoas que não estão infectadas para prevenir a aquisição do vírus e segundo, uma vacina terapêutica direcionada aos que já estão infectados para reduzir a transmissão e a latência viral (GOTTLIEB et al., 2017). Devido à complexa resposta imune viral, ainda não está claro quais são os tipos de reações imunológicas mais importantes. Provavelmente a resposta mediada por anticorpos neutralizados e a resposta mediada por imunidade celular podem ser relevantes no desenvolvimento de vacinas profiláticas (CAIRNS et al., 2015).

Evidências recente indicam que a estimulação de células T de memória residentes no tecido é essencial para este tipo de vacinação terapêutica (ZHU et al., 2013). O aumento de anticorpos contra gD2 viral estão associados a maior eficácia da vacina contra o HSV-1, revelando uma primeira proteção imunológica (BELSHE et al., 2014). Os atuais candidatos estudados estão voltados para produção da vacina contra HSV-2, porém a identificação de epítopos reativos cruzados contra HSV-1 e HSV-2 aumenta a possibilidade do desenvolvimento de uma vacina mais completa que possa exercer efeitos sobre ambos os tipos do vírus (JING et al., 2016). Outro estudo em camundongos e porcos da índia,

revela a possibilidade do desenvolvimento de um vírus atenuado, o R7020, que promove uma imunogenicidade contra infecções causadas tanto por HSV-1 quanto por HSV-2 (CHUNG et al., 2002). Entretanto, estas pesquisas ainda precisam ser desenvolvidas com maior profundidade.

1.7 Selênio e o Disseleneto de Difenila

O estudo de novas substâncias que contribuam de alguma forma para o tratamento de infecções geradas pelo HSV é bastante relevante. Sabe-se que o elemento Selênio (Se) está presente em diversos alimentos de importante valor nutricional, sendo assim fundamental para a manutenção da saúde e do sistema imunológico. De maneira geral, o Se é raramente encontrado em seu estado natural, podendo combinar-se com metais ou não metais para formar compostos inorgânicos ou apresentar-se sob a forma de compostos orgânicos (HOFFMANN et al., 2008; ZENG, 2009).

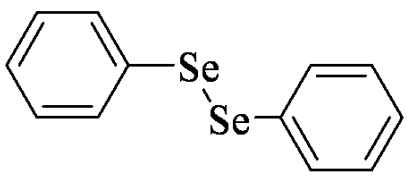
Muitos estudos relatam o Se como constituinte essencial no SNC, não somente pelo seu efeito antioxidante mas também por manter o estado redox celular, melhorar a dinâmica mitocondrial, participar na regulação dos canais de cálcio (Ca^{2+}) e na modulação da neurogênese. A relevância do Se na patogênese de diferentes doenças neurológicas tem sido demonstrada em pesquisas com humanos, em modelos animais e ensaios de cultura celular, sendo à sua deficiência associada a uma perturbação no metabolismo destas células nervosas (PAPP et al., 2010).

Cardoso e cols. revelaram uma correlação negativa entre o declínio cognitivo e níveis de Se (RITA CARDOSO et al., 2014), visto que pacientes que apresentavam prejuízo cognitivo leve e pacientes portadores da DA demonstraram uma redução dos níveis de Se presentes nos eritrócitos em comparação a pacientes saudáveis (sem prejuízo cognitivo), correlacionado a redução da função cognitiva. Estes resultados corroboram com outros estudos que relacionam a deficiência de Se ao maior risco de aparecimento da demência. Ainda, acredita-se que a suplementação com Se pode ser muito importante para o desenvolvimento cognitivo. Um outro estudo realizado por Cardoso e cols. demonstrou que uma dieta com ingesta diária de castanha do Pará por 6 meses

(aproximadamente 288 mg de Se) melhorou o desempenho cognitivo em animais experimentais (RITA CARDOSO et al., 2016). Pesquisas *in vitro* também revelam que uma suplementação com selenito de sódio reduziu a hiperfosforilação da proteína tau, outra proteína responsável pela perda da função neuronal (HARATAKE et al., 2013). Além disso, o Se participa de várias rotas metabólicas e do sistema de enzimas antioxidantes, tais como, o da glutationa peroxidase (GPx) e tioredoxina redutase (TR), desempenhando um papel de proteção nestas células contra o estresse oxidativo (PAPP et al., 2007; HOFFMANN et al., 2008). Também já foi demonstrado efeito imunomodulador do Se (RAYMAN, 2012) bem como efeito antiviral contra diferentes doenças infecciosas (SCHRAUZER et al., 1994; YU et al., 1997).

Compostos orgânicos de Se destacam-se pelos seus efeitos promissores relacionados a patologia da DA, principalmente em estudos com animais experimentais, como por exemplo o disseleneto de *p*-metoxi-difenila $(MeOPhSe)_2$, que reduziu o estresse oxidativo e o declínio cognitivo induzido por estreptozotocina em roedores (PINTON et al., 2013). Entre outros compostos orgânicos de selênio, que vêm sendo estudados, destaca-se o disseleneto de difenila $(PhSe)_2$ (Figura 6), um composto com diversas propriedades farmacológicas descritas tanto em ensaios *in vitro* como *in vivo.* Dentre algumas atividades dos derivados do Se podemos citar ação neuroprotetora (GHISLENI et al., 2003; NOGUEIRA et al., 2004; DOBRACHINSKI et al., 2014) , antiviral (SARTORI et al., 2016a), antifúngica (WOJTOWICZ et al., 2004; LORETO et al., 2011; CHASSOT et al., 2016; VENTURINI et al., 2016) antioxidante e anti-inflamatória (NOGUEIRA et al., 2003; PETRONILHO et al., 2016; SARTORI et al., 2016b) estas últimas por sua vez, características bem marcantes do composto.

Figura 6 - Estrutura química do $(PhSe)_2$.



Com base nas propriedades descritas dos compostos orgânicos de selênio e das poucas alternativas terapêuticas disponíveis para o tratamento das patologias causadas pelo HSV, a avaliação dos efeitos do $(PhSe)_2$ em diferentes modelos de infecção viral tornam-se relevantes.

2. OBJETIVOS

2.1 Objetivo geral

Esta tese teve como objetivo geral avaliar os efeitos do composto orgânico de selênio, disseleneto de difenila, sobre o processo de infecção causado pelos vírus HSV-1 e HSV-2 tanto em testes *in vitro* como *in vivo*.

2.2 Objetivos específicos

Considerando os aspectos mencionados, os objetivos específicos desta tese compreendem:

- Em experimentos *in vitro* determinar:

- o efeito citotóxico do $(PhSe)_2$ em células VERO (células de rim de macaco verde africano) e astrocitomas;

- o efeito antiviral do $(PhSe)_2$ em células infectadas por HSV-1 e HSV-2;

- o possível mecanismo de ação antiviral do $(PhSe)_2$ através da inibição da expressão de genes envolvidos no ciclo de replicação viral do HSV-1 em astrocitomas.

- a produção de citocinas no modelo de infecção por HSV-1 em astrocitomas;

- Em experimentos *in vivo* :

- Avaliar o desenvolvimento das lesões extravaginais causadas pelo modelo de infecção por HSV-2 em camundongos;

- Em experimentos *ex vivo* investigar:

- o efeito antioxidante do composto nos diferentes tecidos dos animais infectados por HSV-2;

- o efeito anti-inflamatório do composto nos diferentes tecidos dos animais infectados por HSV-2;

- a produção de citocinas no tecido genital dos animais infectados por HSV-2;

- o efeito antiviral do $(PhSe)_2$ no tecido genital dos animais infectados por HSV-2;

- o efeito do tratamento com $(PhSe)_2$ sobre as alterações histológicas no tecido genital dos animais infectados por HSV-2.

- a toxicidade hepática e renal causada pela infecção por HSV-2.

3. DESENVOLVIMENTO

O desenvolvimento desta tese está apresentado sob a forma de dois artigos científicos e um manuscrito. Os itens Materiais e Métodos, Resultados, Discussão e Referências Bibliográficas encontram-se nos próprios artigos ou manuscrito, os quais estão estruturados de acordo com as normas de cada revista onde foram publicados ou submetido, respectivamente.

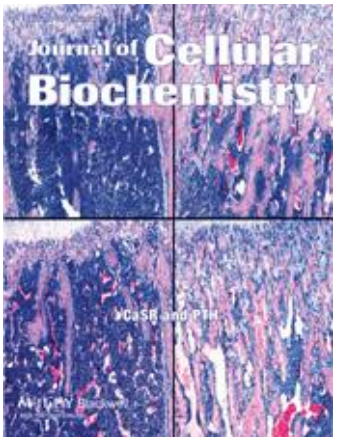
Em anexo a esta tese encontram-se as autorizações da editora para reprodução dos artigos científicos, bem como a aprovação do projeto de pesquisa pela Comissão de Ética no Uso de Animais (CEUA) da Universidade Federal de Santa Maria.

3.1 Artigo 1

Efeito Antiviral do Disseleneto de difenila na infecção por herpes simplex vírus 2 em camundongos fêmeas BALB/C

ANTIVIRAL ACTION OF DIPHENYL DISELENIDE ON HERPES SIMPLEX VIRUS 2 INFECTION IN FEMALE BALB/C MICE

Gláubia Sartori, Natália Silva Jardim, Marcel Henrique Marcondes Sari, Fernando Dobrachinski, Ana Paula Pesarico, Luiz Carlos Rodrigues Jr., Juliana Cargnelutti, Eduardo F. Flores, Marina Prigol, Cristina W. Nogueira



Journal of Cellular Biochemistry v.117, 1638–1648, 2016
DOI 10.1002/jcb.25457

ARTICLE
Journal of Cellular Biochemistry 117:1638–1648 (2016)

Journal of Cellular Biochemistry

Antiviral Action of Diphenyl Diselenide on Herpes Simplex Virus 2 Infection in Female BALB/c Mice

Gláubia Sartori,[1,2] Natália Silva Jardim,[1] Marcel Henrique Marcondes Sari,[1] Fernando Dobrachinski,[1] Ana Paula Pesarico,[1] Luiz Carlos Rodrigues Jr.,[3] Juliana Cargnelutti,[4] Eduardo F. Flores,[4] Marina Prigol,[5] and Cristina W. Nogueira[1]*

[1]*Laboratório de Síntese, Reatividade e Avaliação Farmacológica e Toxicológica de Organocalcogênios, Departamento de Bioquímica e Biologia Molecular, Centro de Ciências Naturais e Exatas, Universidade Federal de Santa Maria, Santa Maria CEP 97105-900, Rio Grande do Sul, Brazil*

[2]*Don C. Gnocchi Foundation, ONLUS, Piazza Morandi 3, Milan 20100, Italy*

[3]*Laboratório de Biologia Molecular e Cultivo de Células, Centro Universitário Franciscano, Conjunto I, UNIFRA, Santa Maria CEP 97010-032, Rio Grande do Sul, Brazil*

[4]*Setor de Virologia, Departamento de Medicina Veterinária Preventiva e Departamento de Microbiologia e Parasitologia, Centro de Ciências Rurais, Universidade Federal de Santa Maria, UFSM, Av. Roraima, No. 1000, Santa Maria 97105-900, Rio Grande do Sul, Brazil*

[5]*Universidade Federal do Pampa, Campus Itaqui, Itaqui CEP 97650-000, Rio Grande do Sul, Brazil*

ABSTRACT

Diphenyl diselenide, $(PhSe)_2$, is an organoselenium compound with pharmacological actions mostly related to antioxidant and anti-inflammatory properties. The study investigated its antiviral and virucidal actions against herpes simplex virus 2 (HSV-2) infection in vitro and in a vaginal infection model in mice. The plaque reduction assay indicated that $(PhSe)_2$ showed virucidal and antiviral actions reducing infectivity in 70.8% and 47%, respectively. The antiviral action of $(PhSe)_2$ against HSV-2 vaginal infection was performed by infecting mice (10^5 PFU/ml^{-1}) at day 6. The treatment with $(PhSe)_2$ (5 mg/kg/day, intragastric [i.g.]) followed 5 days before and for more 5 days after infection. The extravaginal lesion score was evaluated from days 6 to 10. At day 11, animals were killed, and histological evaluation, determination of viral load, and TNF-α and IFN-γ levels were performed in supernatants of homogenized vaginal tissue. The levels of reactive species (RS), protein carbonyl, non-protein thiols (NPSH), nitrate/nitrite (NOx), and malondialdehyde (MDA), and the activities of myeloperoxidase (MPO), superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GPx), and glutathione reductase (GR) were determined. $(PhSe)_2$ reduced the histological damage, extravaginal lesion scores, the viral load of vaginal tissue, and the activity of MPO, but increased the levels of TNF-α, IFN-γ. $(PhSe)_2$ attenuated the increase of RS, MDA, NOx levels and the activity of GR caused by infection. $(PhSe)_2$ also attenuated the reduction of NPSH content and the inhibition of CAT, SOD, and GPx activities. The antiviral action of $(PhSe)_2$ against HSV-2 infection was related to its immunomodulatory, antioxidant, and anti-inflammatory properties. J. Cell. Biochem. 117: 1638–1648, 2016.

KEY WORDS: HSV-2; SELENIUM; IMMUNE SYSTEM; OXIDATIVE STRESS; INFLAMMATION

Infection by herpes simplex virus 2 (HSV-2) is the most common cause of genital ulcer disease. The virus displays lytic and latent cycles that allow the pathogen to increase lifelong infections in humans [Ahmed et al., 2003; Moriarty et al., 2008]. Clinical manifestations are influenced by the site of infection, immunological status of the host, and the immunity developed by previous infections with this virus. In general, herpetic lesions are characterized by erythematous papules, ulcers typically superficial with an erythematous outline and a grayish base, which are intensely painful, mainly during primary infection [Patel et al., 2011]. The replicative activity of the virus can trigger stress pathways, including those induced by oxidative stress. It has been reported an increase in inflammatory mediators and reactive oxygen species (ROS) formation during HSV-2 genital infection and HSV-1 brain infection [Sartori et al., 2012;

Grant sponsor: FAPERGS; Grant sponsor: CAPES; Grant sponsor: CNPq.

*Correspondence to: Cristina W. Nogueira, Laboratório de Síntese, Reatividade e Avaliação Farmacológica e Toxicológica de Organocalcogênios, Centro de Ciências Naturais e Exatas, Universidade Federal de Santa Maria, Santa Maria CEP 97105-900, Rio Grande do Sul. Brazil. E-mail:criswn@ufsm.br

Manuscript Received: 11 September 2015; Manuscript Accepted: 4 December 2015

Accepted manuscript online in Wiley Online Library (wileyonlinelibrary.com): 7 December 2015

DOI 10.1002/jcb.25457 • © 2015 Wiley Periodicals, Inc.

Schachtele et al., 2012]. Herpes virus infections have been reported to damage the major antioxidant defense glutathione (GSH) and some antioxidant enzymes. Moreover, it triggers the accumulation of ROS and reactive nitrogen species (RNS) that can disrupt the redox state of infected cells and facilitate the viral replication [Kavouras et al., 2007; Nencioni et al., 2011].

Antiviral agents, such as acyclovir, are normally used in the treatment of HSV. These oral drugs penetrate in infected cells and act as nucleoside analogs in order to reduce virus replication [Nolkemper et al., 2010]. The occurrence of mutations in viral DNA polymerase and thymidine kinase has led to an increase in drug resistance mainly in immunocompromised patients [Biswas and Field, 2008]. Because of that, there are intense researches in molecules and cellular strategies to improve the immune system to prevent and treat HSV; some therapies can work to strengthen and activate the innate and specific immune system components against HSV infections [Ivec et al., 2007].

Diphenyl diselenide, $(PhSe)_2$, is an organoselenium compound (see Supplementary Fig. S1), and the interest in studying this molecule is due to its pharmacological actions, mostly related to antioxidant and anti-inflammatory properties [Nogueira and Rocha, 2010]. This way, it has been demonstrated that selenium is an immunomodulatory element [Rayman, 2012] and shows benefits in infection diseases, such as HIV [Schrauzer and Sacher, 1994], *Coxsackie B* virus (CBV) [Ge et al., 1987] and *Hepatitis B* virus (HBV) [Yu et al., 1997].

Considering the need for new strategies to prevent or treat infections caused by HSV-2, the aim of this study was to investigate antiviral and virucidal actions of $(PhSe)_2$ against HSV-2 infection in vitro and in a vaginal infection model in mice.

MATERIALS AND METHODS

CHEMICALS

Reagents, such as dicloroflouresceine diacetate (DCFH-DA), N,N,N′, N′-tetramethylbenzidine, hexadecyl trimethylammonium bromide, *p*-dimethylamino benzaldehyde, epinephrine, dinitrophenyl hydrazine, Ellman's reagent (dithiobisnitrobenzoic acid—DTNB), were purchased from Sigma (St. Louis, MO).

$(PhSe)_2$ was prepared and characterized based on a previous study carried out by Paulmier [1986]. ^1H and ^{13}C nuclear magnetic resonance spectroscopy analysis showed analytical and spectroscopic agreement with the assigned structures. The chemical purity of this organoselenium compound (99.9%) was determined by gas chromatography-mass spectrometry (Shimadzu QP2010PLUS GC/MS combination). This compound is stable under storage conditions at room temperature, humidity, and light. All other chemicals were obtained of analytical grade or from standard commercial suppliers.

In in vivo experiments, $(PhSe)_2$ and acyclovir were dissolved in canola oil and 1% (v/v) dimethyl sulfoxide (DMSO), and diluted in distilled water, respectively.

In in vitro experiments, $(PhSe)_2$ was diluted in 0.6% (v/v) DMSO in culture medium at different concentrations: 0.1 μM; 1 μM; 5 μM; 15 μM; 30 μM; 50 μM, and 100 μM. Acyclovir was also diluted in 0.6% (v/v) DMSO in culture medium at 10 μM [Cheng et al., 2002].

ANIMALS

The experiments were carried out using female adult BALB/c mice (20–22 g, 8 weeks of age) from our own breeding colony. The animals were kept on a separate animal room, on a 12 h light/dark cycle, at temperature of 22 ± 2°C with free access to food (Guabi, RS, Brazil) and water. The present experimental study was approved by the Committee on Care and Use of Experimental Animal Resources from the Federal University of Santa Maria, Brazil, and registered under the number of 006/2013. All efforts were made to minimize animal suffering and to reduce the number of animals used.

VIRUS PRODUCTION AND TITRATION

HSV-2 strain 333 was amplified and quantitated in Vero cells (African Green monkey kidney cells, ATCC CCL81). Vero cells were grown in Roswell Park Memorial Institute medium (RPMI) containing ampicillin (1.6 mg/L), streptomycin (0.4 mg/L), and amphotericin B (2.25 mg/L), supplemented with 10% fetal bovine serum (FBS) and maintained in an incubator with CO_2 at 5% and 37°C. For virus amplification, Vero cells were infected with multiplicity of infection of 0.01 (one plaque formation units per 100 cells) of HSV-2, and incubated at 37°C with CO_2 at 5%. After 72 h, the infected cells were submitted to freeze-thaw and the viral suspension was aliquotted and stored at −80°C. Virus quantitation was performed in duplicate using plates of six wells. For this, the original virus was submitted to dilution (10^{-1} to 10^{-6}), 400 μL of each dilution were inoculated on Vero cells and incubated at 37°C in 5% CO_2 for 1 h and 30 min. After that, the inoculum was removed and 3 ml of RPMI plus 2% low-melting point agarose was added to each well. After 72 h, viral titres were calculated using the PFU/ml method [Spear and Roizman, 1972].

EXPERIMENTAL PROCEDURES

In vitro experiments. *Cytotoxicity, virucidal, and antiviral assays.* The viability of Vero cells treated with $(PhSe)_2$ was determined by MTS (3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H- tetrazolium) assay (Promega kit, Madison, WI) [Riss and Moravec, 1992]. Briefly, cells were grown in 96-well microplates (2.5×10^4 cells per well) till confluence, and the media was replaced by RPMI containing (FBS) 10% supplemented with $(PhSe)_2$ at different concentrations (0.1–100 μM). Cells were incubated at 37°C with CO_2 at 5% for 48 h. After this time, the old medium was carefully aspirated, the cells were washed with RPMI, and 100 μl of new medium containing FBS 10% was added. After that, 20 μl of ready MTS dye was added and carefully mixed. The plate was incubated at 37°C for 1 h. The optical density was determined at 490 nm. Results were expressed by percentage of viable cells calculated in comparison to control cells.

The antiviral activity of $(PhSe)_2$ was measured by plaque reduction assay. $(PhSe)_2$ action against HSV-2 infection was evaluated by three different schemes of treatments, which can contribute to elucidate the mechanisms of action of this compound in vitro. $(PhSe)_2$ was applied at different time points: (i) 1 h of pre-treatment of the virus (virucidal assay); (ii) pre-treatment of cells before infection; or (iii) post-treatment of cells after infection, as described by Astani et al., [2010] with some modifications [Astani et al., 2010]. Additional positive control included acyclovir (10 μM).

The virucidal assay was performed using a constant virus titer (HSV-2–1.8×10^4 PFU/ml) and the non-cytotoxic concentration of compound (5 μM). The suspensions (test: virus and compound; and control: virus and RPMI) were incubated in microtubes for 1 h at 37°C. Following the incubation period, aliquots of each suspension were inoculated on Vero cells, and viral adsorption was performed for 1 h at 37°C. Residual inoculum was removed and the infected cells were overlaid with 3 ml of RPMI containing 2% low-melting point agarose and 10% of FBS, and incubated for 48 h at 37°C. After this time, infected cells were submitted to fixation, staining using formaldehyde 10%, and crystal violet 0.05% for 2 h at room temperature. Finally, the crystal violet was removed and plaque counting was performed (microscope). The percentage of virus inhibition was calculated based on the plaque counts observed in treated cells compared to the virus control (untreated cultures).

The procedures used to perform the pre- and post-treatment assays were similar to those described for the virucidal assay. For the pre-treatment assay, cells were incubated with the compound for 1 h prior to virus inoculation. After the incubation period, the compound was removed and viral inoculation (1×10^2 PFU/well) was performed as described above. In the post-treatment assay, after 1 h of virus adsorption, the plates were washed and the medium replaced with RPMI containing 2% low-melting point agarose, 10% of FBS, and $(PhSe)_2$ at a concentration of 5 μM were added to the cells for a period of 48 h at 37°C with CO_2 at 5%. Negative controls (composed by RPMI and vehicle 0.6% DMSO) were included in all experiments. All results were calculated as the average of four independent experiments.

In vivo experiments. *Genital infection in mice.* Mice were anesthetized with ketamine (150 mg/kg) and xylazine (10 mg/kg) injected intraperitoneally (i.p), and inoculated intravaginally by scratching small areas of the skin with the needle of a syringe containing 10^5 PFU/ml^{-1} of HSV-2 in a 10 μl volume. The concentration used to induce lesions with HSV-2 333 was based on a pilot study.

Experimental protocol. Animals were divided in six groups (n = 5–7). Group I: control (vehicle); group II: HSV-2-infected mice; group III: $(PhSe)_2$ (5 mg/kg) treated mice; group IV: acyclovir (5 mg/kg) treated mice; group V: $(PhSe)_2$ treated and HSV-2-infected mice; and group VI: acyclovir-treated and HSV-2 infected mice. Five days before infection with HSV-2, groups I and II received canola oil vehicle (10 ml/kg), groups III and V received $(PhSe)_2$ at a dose of 5 mg/kg by gavage once a day. At day 5, mice from groups II, V, and VI were submitted to HSV-2 infection as previously described. Groups I, III, and IV were submitted to the same procedure applied for genital infection, however, without any viral particle. Animals were treated with vehicle, $(PhSe)_2$, or acyclovir (5 mg/kg) for more 5 days (once a day by gavage) after HSV-2 infection. The dose of $(PhSe)_2$ was chosen based on a previous pilot study accomplished to verify toxic effects in mice and the dose of acyclovir was chosen based on a previous pilot study which confirmed the minimum dose with significant antiviral effects [Quenelle et al., 2011]. Supplementary Figure S3 shows the experimental protocol.

Extravaginal lesions were recorded every day for each animal and scored according to a six-point scale as follows: 0: no sign of infection; 1: slight genital erythema and edema; 2: moderate genital inflammation; 3: severe exudative genital lesions; 4: hind limb paralysis; and 5: death [Hayashi et al., 2010]. Then, they were killed on the 11th day by cervical dislocation, and vaginal tissues were removed for ex vivo experiments (histology and biochemical analyses).

Ex vivo experiments. *Tissue preparations (S1) and (P2).* For viral titration, vaginal tissue (n = 4) was immediately collected and homogenized (1/10, w/v) in RPMI, then it was centrifuged for 10 min at 3,000*g* at 4°C to yield a pellet, that was discarded, and a low-speed supernatant (S1) was obtained.

For antioxidant assays, vaginal tissue (n = 5–7) was immediately homogenized in cold 50 mM Tris–HCl, pH 7.4 (1/10, w/v). The homogenate was centrifuged for 10 min at 3,000*g* to yield a pellet, that was discarded, and a low-speed supernatant (S1) was obtained and used to determine the levels of reactive species, non-protein thiols, and malondialdehyde and the activities of glutathione reductase, glutathione peroxidase, superoxide dismutase, and catalase.

Protein carbonyl content was determined in vaginal tissues without centrifugation.

Samples used for myeloperoxidase activity (P2) and nitrite/nitrate levels assays were prepared under different conditions detailed in myeloperoxidase (MPO) activity and nitrate/nitrite (NOx) content sections, respectively.

HISTOLOGICAL ANALYSES

Three mice per group were submitted to detailed necropsy evaluation. Small pieces of vaginal tissue from individual mouse were fixed in 10% buffered formalin solution for 24 h. After that, the samples were dehydrated in ethanol, cleared in xylene, and embedded in paraffin. Serial sections of 4 μm in thickness were cut and stained with hematoxylin and eosin, and evaluated under light microscopy. A histologist who was not aware of sample assignment to experimental groups analyzed the sections. Only qualitative histology was performed. Morphological changes in the layers of epidermis and the loose and dense connective tissue in dermis were observed. The histological features in skin injuries were as follows: granulation tissue, vascular congestion, hemorrhage, neutrophil leukocyte infiltration. The tissue morphology was assessed by light microscopy.

VIRAL TITRATION

Vaginal tissue fragments were collected during the necropsy, homogenized in RPMI (1/10, w/v) and submitted to virus isolation and titration in Vero cells. Infectivity in positive samples was quantitated by serial dilutions and plaque reduction assay using semi-solid medium (RPMI, 10% of FBS and 2% of agarose). After 72 h of incubation at 37°C CO_2 5%, the viral titers were calculated using the PFU/ml method [Spear and Roizman, 1972].

IFN-γ AND TNF-α ANALYSIS

Interferon gamma (INF-γ) and tumor necrosis factor alpha (TNF-α) production were analyzed in the supernatant of vaginal cells extracts by Cytometric Bead Array (CBA) kit (Dickinson, San Jose, CA). The vaginal tissue samples were homogenized 1/2 (w/v) in cold 50 mM

Tris–HCl, pH 7.4 and used to combine with the beads of the CBA. Analyses were performed in FACS Canto using Flowjo software.

MYELOPEROXIDASE (MPO) ACTIVITY

The samples were homogenized in potassium phosphate buffer (20 mM, pH 7.4) containing EDTA (0.1 mM). After homogenization, samples were centrifuged at 2,000*g* at 4°C for 10 min to yield a low-speed supernatant fraction (S1). Then, S1 was centrifuged again at 20,000*g* at 4°C for 15 min to yield a final pellet that was resuspended in potassium phosphate buffer (50 mM, pH 6.0) containing hexadecyltrimethylammonium bromide (0.5%) (P2). Samples were finally frozen thawed three times for posterior MPO assay. In addition, aliquots of vaginal tissue preparations were frozen (−20°C) for 1 week for posterior analysis [Grisham et al., 1986].

An aliquot of P2 (20 μl) was added to a medium containing potassium phosphate buffer (50 mM; pH 6.0), hexadecyltrimethyl ammonium bromide (0.5%), and N,N,N′,N′-tetramethylbenzidine (1.5 mM) to determine the MPO activity. Kinetic analysis was started after H_2O_2 (0.01%) addition, and color reaction was measured at OD 655 nm at 37°C. Results are expressed as mmol MPO/mg of protein.

OXIDATIVE STRESS PARAMETERS

Reactive species (RS) levels. An aliquot of S1 (10 μl) was incubated with 10 μl of 2′,7′-dichlorofluorescein diacetate (DCHF-DA; 1 mM) to determine the levels of RS production in tissue. The oxidation of DCHF-DA to fluorescent dichlorofluorescein (DCF) occurs through intracellular RS detection. The DCF fluorescence intensity emission was recorded at 520 nm (with 480 nm excitation) 30 min after the addition of DCHF-DA to the medium (spectrofluorimetric method). The RS levels were expressed as μmol/mg of protein [Loetchutinat et al., 2005].

Malondialdehyde (MDA) levels. The determination of MDA levels was performed by the high-performance liquid chromatography (HPLC)-UV detection method with slight modifications [Grotto et al., 2007]. Briefly, an aliquot of 75 μl of S1 or malondialdehyde bis (dimethylacetal) solution (as standard, dissolved in distilled water) was added to 25 μl of 3 N NaOH and incubated at 60°C for 30 min. After, 125 μl of 6% H3PO4 and 125 μl of 0.8% 2-thiobarbituric acid (TBA) were added and the mixture was heated at 90°C for 2 h. Then, the mixture was cooled and kept at −20°C until extraction with *n*-butanol. For this, samples were added to 50 μl of 10% sodiumdodecyl sulfate (SDS) and 300 μl of *n*-butanol, vortex-mixed for 1 min and centrifuged at 3,000*g* for 10 min. The TBA-MDA adduct of butanol layer was analyzed on a Shimadzu HPLC apparatus. The analytical column was 5 μm particles and 100 A° pore size, Phenomenex ODS-2 C18 reverse-phase column (4.6 9 150 mm, Allcrom, BR). The mobile phase was a mixture of ultrapure water and methanol (50:50; v/v). The HPLC analysis was performed under isocratic conditions at a flow rate of 0.6 ml/min and UV detector set at 532 nm, with a sample volume injection of 20 μl. The lipid peroxidation was expressed as nmol MDA/mg of protein.

Protein carbonyl content. The samples were diluted 1:5 (v/v) and an aliquot of 1 ml was added to the reaction mixture containing 200 μl of 10 mM dinitrophenyl hydrazine (prepared in 2 MHCl). After that, the samples were kept in the dark for 1 h and the tubes were mixed with vortex agitation for for 15 min. After, 500 μl of denaturation buffer, 1,500 μl of ethanol and 1,500 μl of hexane were added to each tube. The tubes were mixed with vortex agitation for 40 s and centrifuged for 15 min. The supernatants obtained were discarded. Then, the pellets were washed two times with 1 ml ethanol: ethyl acetate (1:1, v/v) and ressuspended in 1 ml of denaturation buffer. The sample tubes were mixed with vortex agitation for 5 min and the absorbance was measured at 370 nm (UV). Results were reported as carbonyl content (nmol/mg of protein) [Reznick and Packer, 1994].

Nitrate/nitrite (NOx) content. The samples were homogenized with $ZnSO_4$ (200 mM) and acetonitrile (96%), centrifuged at 16,000*g* at 4 °C for 30 min, and the supernatant was collected for the nitrate/nitrite assay [Miranda et al., 2001]. The resulting pellet was suspended in NaOH (6 M) for protein determination. NOx content was estimated in a medium containing 300 μl of 2% VCl_3 (in 5% HCl), 200 μl of 0.1% N-(1-naphthyl) ethylenediamine dihydrochloride, and 200 μl of 2% sulfanilamide (in 5% HCl). After incubation at 37°C for 60 min, nitrite levels were determined at 540 nm (UV). Results were reported as NOx/mg of protein.

Superoxide dismutase (SOD) activity. SOD activity was based on the capacity of SOD to inhibit autooxidation of epinephrine to adrenochrome. The enzymatic reaction was initiated by adding an S1 aliquot (150 μl) of the homogenized tissue and the substrate (epinephrine) to a concentration of 4 mM in a medium containing 50 mM bicarbonate buffer, pH 10.3. The color reaction was measured at 480 nm. One unit of enzyme was defined as the amount of enzyme required to inhibit the rate of epinephrine autooxidation by 50% at 26°C [Misra and Fridovich, 1972]. The enzymatic activity was expressed as U/mg of protein.

Catalase (CAT) activity. CAT activity monitors the disappearance of H_2O_2 in the homogenate at 240 nm. The enzymatic reaction was initiated by adding an aliquot of 20 μl of S1 and the substrate (H_2O_2) to a concentration of 0.3 mM in a medium containing 50 mM phosphate buffer, pH 7.0. The enzymatic activity was expressed in U/mg of protein (one Unit decomposes 1 μM of H_2O_2 per min at pH 7 at 25°C/mg protein [Aebi, 1984].

Glutathione system (GPx and GR activities). The GPx activity in S1 was determined based on the method described by Wendel [Wendel, 1981], through the GSH/NADPH/glutathione reductase (GR) system and dismutation of H_2O_2 at 340 nm. The enzyme activity is measured indirectly by means of NADPH decay. H_2O_2 is decomposed, generating oxidized glutathione (GSSG) from reduced GSH. GSSG is regenerated back to GSH by the GR present in the assay media, at the expense of NADPH. The enzymatic activity was expressed in nmol NADPH/min/mg of protein.

The GR activity in S1 was determined as described by Calberg & Mannervik [Carlberg and Mannervik, 1975]. GSSG is reduced by GR at the expense of NADPH consumption that is followed at 340 nm. The GR activity is proportional to NADPH decay and was expressed as nmol NADPH/min/mg of protein.

Non-protein thiols (NPSH) levels. S1 was mixed (1:1) with 10% trichloroacetic acid with the aim to determine NPSH levels. After centrifugation, the protein pellet was discarded and free -SH groups were determined in the clear supernatant. An aliquot of supernatant was added in 1 M potassium phosphate buffer pH 7.4 and 10 mM DTNB [Ellman, 1959]. The color reaction was measured at 412 nm. NPSH levels were expressed as μmol/ mg of protein.

PROTEIN QUANTIFICATION

Protein concentration was measured by the method of Bradford [Bradford, 1976] using bovine serum albumin as a standard.

STATISTICAL ANALYSIS

For in vitro assays, one-way analysis of variance (ANOVA) was performed, followed by post-hoc comparisons using the Newman–Keuls's multiple comparison test when appropriate. The lesion score results were analyzed by the D'Agostino and Pearson omnibus normality test and the non-parametric Scheirer–Ray–Hare-test followed by the Dunns's test was applied. Ex vivo data were analyzed using a two-way analysis ($(PhSe)_2 \times$ HSV-2 and acyclovir $\times$ HSV-2) of variance (ANOVA), followed by post-hoc comparisons using the Duncan's multiple range test when appropriate. Main effects are presented only when the second order interaction was not significant. $(PhSe)_2$ and acyclovir treatments were compared using the Student's t-test. Data are expressed as the mean(s) $\pm$ S.E.M and analyzed by GraphPad Prism (version 6). Differences between groups were considered statistically significant when $P < 0.05$.

RESULTS

$(PhSe)_2$ HAD ANTIVIRAL AND VIRUCIDAL ACTIONS AGAINST HSV-2 REPLICATION

The viability of cells treated with $(PhSe)_2$ at concentrations of 0.1–5 μM was greater than 90%, indicating no toxicity of this compound to Vero cells (mean absorbance of control cells was 0.608). By contrast, concentrations equal or higher than 15 μM were cytotoxic (Supplementary Fig. S2). Therefore, a non-cytotoxic concentration of 5 μM $(PhSe)_2$ was chosen for further antiviral studies.

As shown in Figure 1, both pre- and post-treatment with $(PhSe)_2$ at a concentration of 5 μM significantly reduced viral replication of HSV-2 (47.7 % $P < 0.01$ and 47.3% $P < 0.0001$, respectively) when compared with infected control cells. As expected, acyclovir achieved the highest antiviral effect when applied after the adsorption period (post-treatment), reducing by 82.6% the viral plaques ($P < 0.0001$) (Fig. 1A). Mean replication rate of HSV-2 infected cells were 9×10^1 PFU/ml.

The virucidal action of $(PhSe)_2$ was also demonstrated in this experiment. The viral replication was reduced 70% ($P < 0.0001$) when a pre-treatment of virus with $(PhSe)_2$ occurred before the infection of cells (Fig. 1B). Mean replication rate of HSV-2 infected cells were 2.9×10^2 PFU/ml. In all experiments, infected cells with untreated virus were used as control.

$(PhSe)_2$ TREATMENT REDUCED EXTRAVAGINAL LESIONS SCORE AND CELLULAR INFILTRATION IN VAGINAL TISSUES OF MICE

The score results revealed a significant effect of HSV-2 infection ($H(1) = 111.73$; $P < 0.0001$) in vaginal tissues of mice (Fig. 2). HSV-2 infection caused an increase in the lesion score means from the second day. The results showed that treatment with $(PhSe)_2$ delayed the progression of lesions caused by infection from the second to the fourth day ($H(1) = 10.68$; $P < 0.01$). $(PhSe)_2$ was not

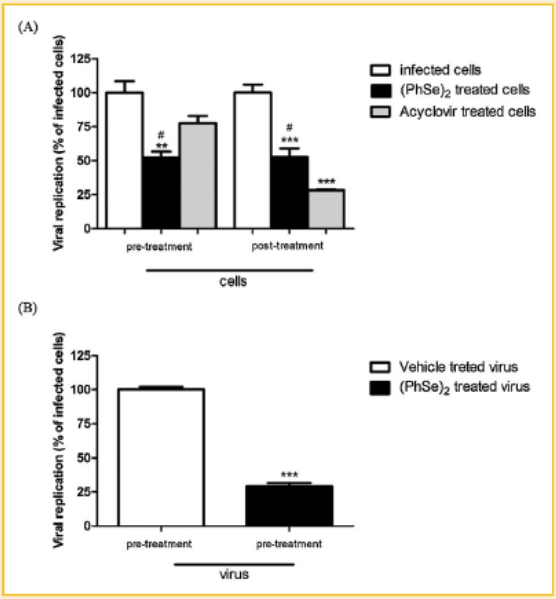


Fig. 1. Antiviral (A) and virucidal (B) actions of $(PhSe)_2$ at a concentration of 5 μM against HSV-2 infection determined by plaque reduction assay. Acyclovir at a concentration of 10 μM was used as positive control. Results (PFU/ml) are expressed as the mean $\pm$ S.E.M. ($n = 4$) and were analyzed by ANOVA, followed by the Newman–Keuls's multiple comparison test when appropriate. $^{**}P < 0.001$, $^{***}P < 0.0001$ versus untreated infected cells; $^{\#}P < 0.01$ versus acyclovir-treated infected cells.

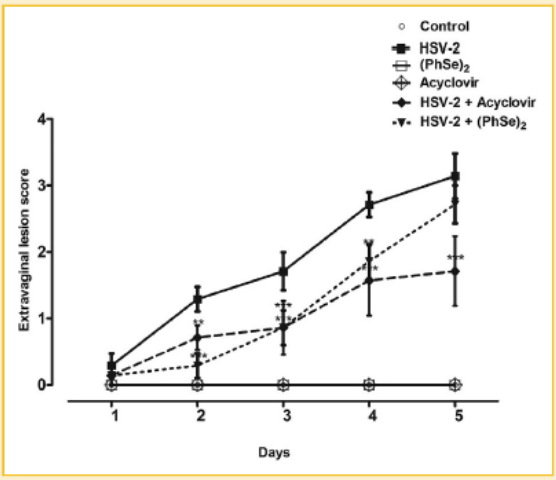


Fig. 2. Effect of treatment with $(PhSe)_2$ at a dose of 5 mg/kg on extravaginal lesion scores of mice infected with HSV-2. Data are expressed as the mean $\pm$ S.E.M. ($n = 5$–7) and were analyzed by the non-parametric Scheirer–Ray–Hare-test followed by the Dunns's test when appropriate. $^{**}P < 0.01$; $^{***}P < 0.001$ versus HSV-2 group. Control, control group; HSV-2, HSV-2-infected group; $(PhSe)_2$, $(PhSe)_2$-treated group; Acyclovir, acyclovir-treated group; HSV-2 + $(PhSe)_2$, HSV-2 infected and $(PhSe)_2$-treated group; HSV-2 + Acyclovir, HSV-2 infected and acyclovir-treated group.

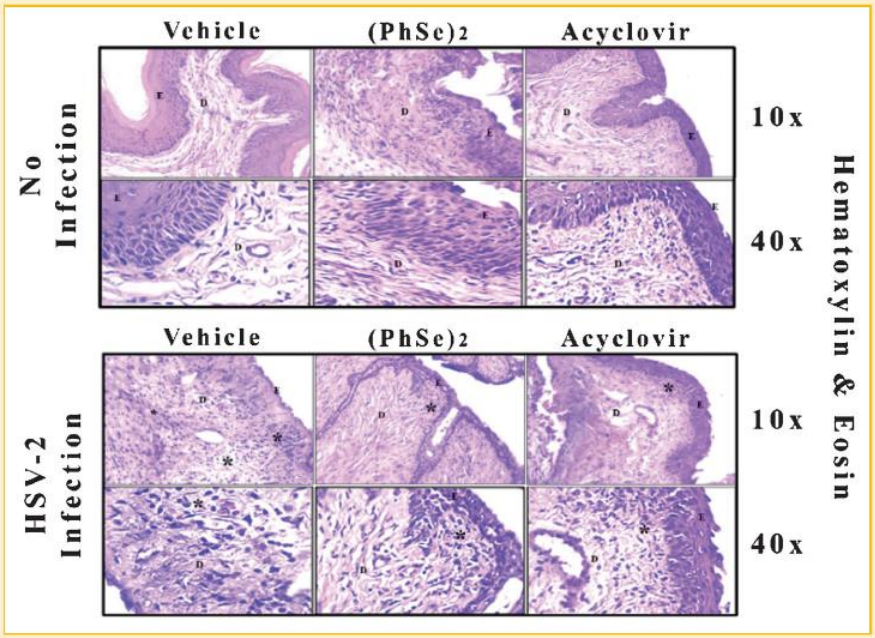


Fig. 3. Histological analyses of vaginal sections of HSV-2-infected and non-infected mice. Cellular infiltration is represented by (*). Epidermis (E); Dermis (D); H.E. 10× and 40×, respectively.

effective against lesions on the fifth day. As expected, the positive control acyclovir protected against the increase in the lesion score means induced by HSV-2 infection during treatment (fifth day: $H(1) = 33.34$; $P < 0.001$), but none of treatments were effective to reverse the lesions. The H (degree of freedom, df) values correspond to the SS value divided by MS_{Total} of the ranked data. In summary, the H value correspond to the F value of two-way ANOVA.

In agreement with the lesion score results, histological analyses of vaginal samples of infected animals treated with $(PhSe)_2$ showed normal tissue appearance with the presence of partial reepithelialization and reduction of cellular infiltration in the dermis and epidermis (Fig. 3). However, vaginal samples obtained from the HSV-2 infected mice showed intense cellular infiltration, containing neutrophils and mononuclear cells in the dermis, with disintegration in all epidermis (Fig. 3, 40×). Mice infected and treated with acyclovir showed complete reepithelialization with intense reduction of cellular infiltration in dermis and epidermis (Fig. 3, 40×). Mice from control group (treated with vehicle) showed epidermis and dermis with normal appearance.

$(PhSe)_2$ TREATMENT REDUCED THE VIRAL LOAD OF HSV-2 INFECTION

One-way analysis indicated that the HSV-2 infected mice showed a high viral load in vaginal tissues (10^5 PFU/ml). Treatment with $(PhSe)_2$ or acyclovir reduced the viral titres (10^4 PFU/ml) ($P < 0.001$) of infected mice (Fig. 4).

$(PhSe)_2$ TREATMENT MODULATED CYTOKINES PRODUCTION AND REDUCED MPO ACTIVITY IN INFECTED ANIMALS

Two-way ANOVA of INF-γ ($F_{(1.8)} = 39.10$; $P < 0.001$) and TNF-α ($F_{(1.8)} = 125.3$; $P < 0.0001$) levels yielded a significant HSV-2 and $(PhSe)_2$ interaction. HSV-2 infection significantly increased INF-γ and TNF-α levels in vaginal tissues of mice when compared to those

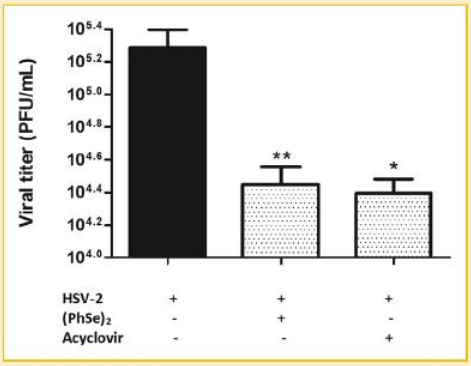


Fig. 4. Effect of treatment with $(PhSe)_2$ at a dose of 5 mg/kg on viral load of vaginal tissues of mice infected with HSV-2. Results (PFU/ml) are expressed as the mean ± S.E.M. ($n = 4$) and were analyzed by ANOVA followed by the Newman–Keuls's multiple comparison test when appropriate; $^*P < 0.01$; $^{**}P < 0.001$ versus HSV-2 group.

of the control group. Treatment with $(PhSe)_2$ potentiated the increase of INF-γ and TNF-α levels in vaginal tissues of mice (Fig. 5A and B).

The mice infected and treated with acyclovir did not have an increase in INF-γ and TNF-α levels when compared with those of the HSV-2 group (Fig. 5A and B).

The Student's *t*-test revealed that acyclovir reduced INF-γ ($P < 0.001$) and TNF-α ($P < 0.0001$) levels in infected mice when compared with those infected and treated with $(PhSe)_2$ (Fig. 5A and B).

Two-way ANOVA of MPO activity showed a significant HSV-2 and $(PhSe)_2$ interaction ($F_{(1.20)} = 9.945$; $P < 0.01$). HSV-2 infection significantly increased MPO activity in vaginal tissues of mice when compared to those of the control group. Treatment with $(PhSe)_2$ was effective in reducing the MPO activity to the control levels. The activity of MPO was reduced in mice infected and treated with acyclovir but these values did not attain the control levels. The Student t-test revealed that the decrease in MPO activity was greater in mice infected and treated with $(PhSe)_2$ than with acyclovir ($P < 0.01$) (Fig. 6).

$(PhSe)_2$ TREATMENT WAS EFFECTIVE TO REDUCE THE OXIDATIVE DAMAGE PRODUCED BY HSV-2 INFECTION

Two-way ANOVA analysis of RS levels revealed a significant main effect of HSV-2 infection. HSV-2 significantly increased RS levels in vaginal tissues of mice when compared to those of the control group. Treatment with $(PhSe)_2$ or acyclovir ($P < 0.05$) attenuated the increase of RS levels in vaginal tissues (Fig. 7A).

Two-way ANOVA of MDA levels indicated a significant HSV-2 and $(PhSe)_2$ interaction ($F_{(1.16)} = 7.05$; $P < 0.05$). HSV-2 infection significantly increased MDA levels when compared to those of the control group. Treatment with $(PhSe)_2$, but not with acyclovir, was effective against the increase in MDA levels in vaginal tissues of mice (Fig. 7B).

Concerning the oxidative damage to proteins, it was not observed alterations in protein carbonyl levels in all groups tested (data not shown).

Two-way ANOVA analysis of NOx levels revealed a significant HSV-2 and $(PhSe)_2$ interaction ($F_{(1.16)} = 4.48$; $P < 0.05$). Posthoc indicated that HSV-2 infection enhanced NOx levels in vaginal tissues of mice. In acyclovir treatment, it was observed the same effect ($F_{(1.16)} = 5.213$; $P < 0.05$). Both treatments reduced NOx levels in vaginal tissues of mice to the levels of control group (Fig. 7C).

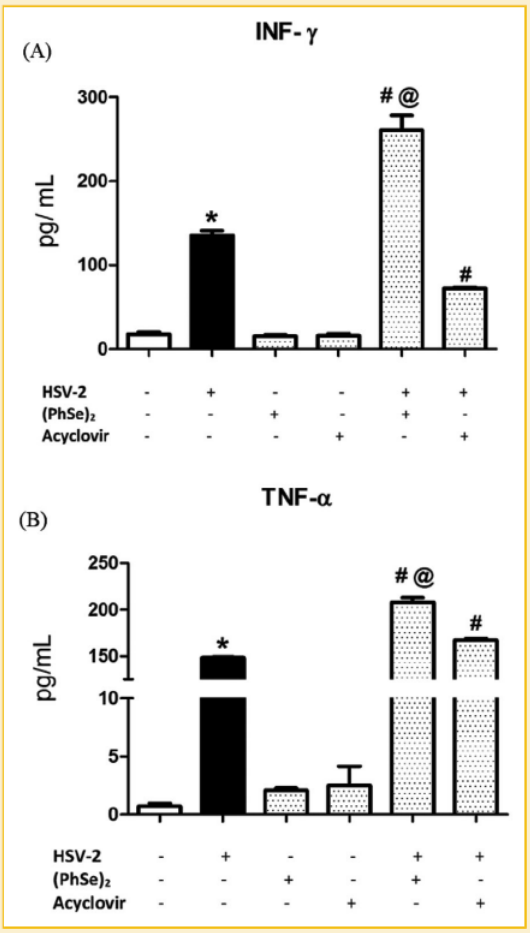


Fig. 5. Flow cytometry analysis of the levels of IFN-γ (A) and TNF-α (B) after HSV-2 infection and treatment with $(PhSe)_2$ at a dose of 5 mg/kg. Data are reported as the mean ± S.E.M. (n = 4) and were analyzed by two-way ANOVA followed by the Duncan's test when appropriate. The Student's *t*-test was used to compare both treatments when appropriate. Results are expressed as pg/ml. *Significant difference when compared to the control group; #Significant difference when compared to the control and to the HSV-2 group; @Significant difference when compared to acyclovir-treated and acyclovir-infected groups ($P < 0.05$).

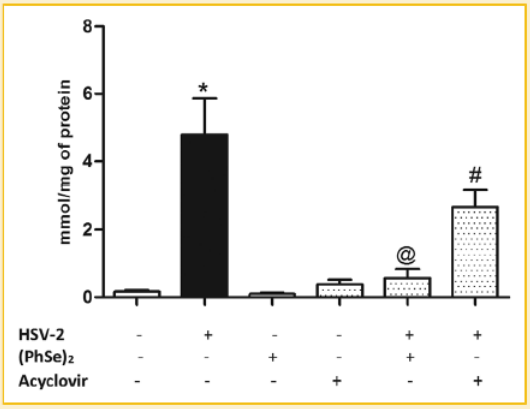


Fig. 6. Effect of treatment with $(PhSe)_2$ at a dose of 5 mg/kg on MPO activity in vaginal tissues of mice infected with HSV-2. Data are reported as the mean ± S.E.M. (n = 5–7) and were analyzed by two-way ANOVA followed by the Duncan's test when appropriate. The Student's *t*-test was used to compare both treatments when appropriate. Results are expressed as mmol MPO/mg of protein. *Significant difference when compared to the control group; #Significant difference when compared to the control and to the HSV-2 group; @Significant difference when compared to acyclovir-treated and acyclovir-infected groups ($P < 0.05$).

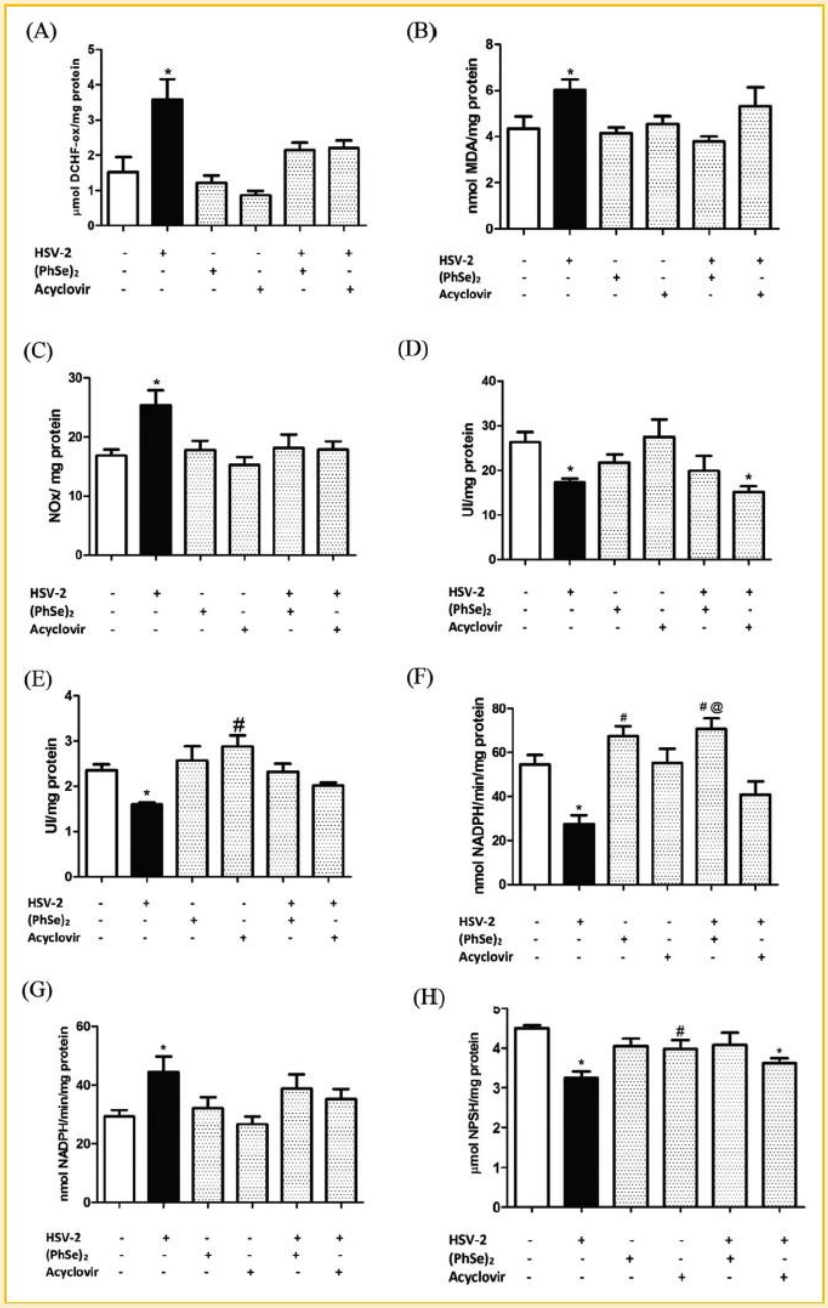


Fig. 7. Effect of treatment with $(PhSe)_2$ at a dose of 5 mg/kg on oxidative stress parameters in vaginal tissues of mice infected with HSV-2. (A) RS levels (results are expressed as µmol/mg of protein); (B) MDA levels (results are expressed as nmol MDA/mg of protein); (C) NOx levels (results are expressed as NOx/mg of protein); (D) SOD activity (results are expressed as U/mg of protein); (E) CAT activity (results are expressed as U/mg of protein); (F) GPx and (G) GR activities (results are expressed as nmol NADPH/min/mg of protein); (H) NPSH levels (results are expressed as µmol NPSH/mg of protein). Acyclovir at a dose of 5 mg/kg was used as positive control. Data are reported as the mean ± S.E.M. ($n = 5$–7) and were analyzed by two-way ANOVA followed by the Duncan's test when appropriate. The Student's *t*-test was used to compare both treatments when appropriate. *Significant difference when compared to the control group; #Ssignificant difference when compared to the control and to the HSV-2 group; @Significant difference when compared to acyclovir-treated and acyclovir-infected groups ($P < 0.05$).

$(PhSe)_2$ TREATMENT REDUCED THE INHIBITION OF ANTIOXIDANT ENZYMES INDUCED BY HSV-2 INFECTION

Two-way ANOVA analysis of SOD activity showed a significant main effect of HSV-2 infection. HSV-2 significantly inhibited SOD activity in vaginal tissues of mice when compared to those of the control group. Treatment with $(PhSe)_2$ ($P = 0.0753$ compared to control and $P = 0.4453$ compared to HSV-2) was partly effective to avoid this inhibition. Similar results could not be observed for the acyclovir treatment (Fig. 7D).

Two-way ANOVA analysis of CAT activity revealed a significant main effect of HSV-2 infection. HSV-2 caused an inhibition of CAT activity in vaginal tissues of mice and both treatments, $(PhSe)_2$ or acyclovir ($P < 0.05$), were effective to avoid this inhibition similar to control levels (Fig. 7E).

$(PhSe)_2$ TREATMENT MODULATED THE GLUTATHIONE SYSTEM IN INFECTED MICE

The two-way ANOVA analysis of GPx activity revealed a significant HSV-2 and $(PhSe)_2$ interaction ($F_{(1.17)} = 11.61$; $P < 0.01$). HSV-2 infection showed a reduction of GPx activity, and $(PhSe)_2$ treatment was effective against this alteration. Treatment with acyclovir did not alter the reduction in GPx activity caused by HSV-2 infection. The Student's *t*-test revealed a significant difference between $(PhSe)_2$ and acyclovir treatments in infected mice ($P < 0.01$) (Fig. 7F).

Regarding the GR activity, it was observed a significant main effect of HSV-2 infection. The HSV-2-infected group had an increase of GR activity, and $(PhSe)_2$ ($P = 0.1475$ compared to control and $P = 0.3596$ compared to HSV-2) or acyclovir ($P = 0.1105$ compared to control and $P = 0.1799$ compared to HSV-2) treatment was partly effective against this effect (Fig. 7G).

The two-way ANOVA of NPSH levels showed a significant HSV-2 and $(PhSe)_2$ interaction ($F_{(1.17)} = 9.98$; $P < 0.01$). HSV-2 infection significantly reduced NPSH levels in vaginal tissues of mice when compared to those of the control group. Treatment with $(PhSe)_2$, but not with acyclovir, was effective against the reduction of NPSH levels (Fig. 7H).

DISCUSSION

The present study demonstrated, for the first time, the antiviral action of $(PhSe)_2$ against HSV-2 in vitro and in vivo. $(PhSe)_2$ showed an antiviral action against infected Vero culture cells and reduced the histological damage, extravaginal lesion scores, and the viral load of vaginal tissue of mice. The antiviral action of $(PhSe)_2$ against HSV-2 infection was related to its immunomodulatory, antioxidant, and anti-inflammatory properties.

$(PhSe)_2$ had antiviral and virucidal actions at a non-cytotoxic concentration. It was observed that $(PhSe)_2$ exhibited antiviral action due to the inhibition of different steps of infection in plaque reduction assay. The infectivity of HSV-2 was reduced by a pre-treatment of the virus with the $(PhSe)_2$ for 1 h prior to the infection of cells, which suggests a direct action of the compound over the viral particle. We assign the antiviral effect at the time of pre-treatment of cells to the major virucidal action of $(PhSe)_2$. It also demonstrated a similar antiviral action after the adsorption period, at the post-treatment (48 h p.i.). However, the inhibitory mechanism of $(PhSe)_2$ remains to be elucidated. Perhaps, it could be due to the binding of compound to viral proteins involved in host cell adsorption or due to damage to the virions possibly their envelopes, injuring their effectiveness to infect host cells.

The mice infected with HSV-2 showed extravaginal signals of lesion, such as swelling, edema, and inflammation, which were similar to those documented in our previous study using this model of infection [Sartori et al., 2012]. The infected group treated with $(PhSe)_2$ demonstrated a slow progression of lesions, with a notable reepithelialization and reduction of leucocyte infiltration in vaginal tissue. We analyzed these herpetic lesions during an acute phase of HSV-2 infection, and it is well characterized in this stage that the main infiltrating cells in the tissue are macrophages and neutrophils [Milligan et al., 2001].

Similar to acyclovir treatment, $(PhSe)_2$ reduced the infiltration of macrophages and neutrophils to the site of infection. However, current data do not allow us to affirm that $(PhSe)_2$ has the same mechanism of action of acyclovir, which directly blocks the viral replication but impairs the propagation of the virus cell in vitro and in vivo. This way, it has been reported that $(PhSe)_2$ suppresses the extent of infiltrating macrophages in an experimental encephalomyelitis model [Chanaday et al., 2011].

In the present study, $(PhSe)_2$ similar to acyclovir reduced HSV-2 titres in vaginal tissue, which is consistent with the delay in the development of vaginal lesions, the reduction in histological damage, and the increase in response to IFN-γ. These results indicate that the most important effect of $(PhSe)_2$ is its antiviral action and that the reduction in oxidative stress and inflammation process seems to be a consequence of lower viral load levels.

Considering that the successful anti-herpes virus drug, acyclovir, has several disadvantages including limited potency and efficacy, particularly against herpes viruses, and that the frequency at which immunocompromised patients develop resistant infections [Weller and Coen, 2012], the demonstration of antiviral action of $(PhSe)_2$ in in vitro and in vivo models is significant to develop new strategies to prevent or treat infections.

The increase in the levels of two cytokines that mediate the inflammatory response, IFN-γ and TNF-α, was an intricate finding of this study. In fact, TNF-α levels of infected mice treated with acyclovir were quite similar to those found in untreated infected mice. This result indicates that even if viral titers and inflammatory cells are reduced, TNF-α continues to be produced at the site of lesion. Similar results were demonstrated in mice infected and treated with $(PhSe)_2$, in which TNF-α levels were higher probably due to the presence of mast cells in the tissue that contribute to this increase. Aoki and collaborators [2013] have reported that under HSV-2 infection, keratinocytes produce IL-33, which lead to the local releasing of TNF-α by mast cells, a phenomenon independent of degranulation, and that TNF-α contributes to the protective antiviral response to HSV-2 in vivo. In agreement with Aoki and collaborators [2013], the histological images of inflamed tissues show a few inflammatory cells in vaginal tissues of mice infected and treated with acyclovir or $(PhSe)_2$.

Moreover, the results on IFN-γ levels of infected and treated mice indicated a direct and positive relationship between this cytokine

and the evolution of HSV-2 infection. The levels of IFN-γ were quite higher in mice infected with HSV-2 and treated with $(PhSe)_2$ if compared to other groups. It is well established that T cells contribute to the clearance of virus and herpetic lesions through the production of IFN-γ and TNF-α and in part by cytolytic mechanisms in genital HSV-2 infection [Milligan et al., 1998, 2004]. It is possible that the high levels of IFN-γ and TNF-α found in mice infected and treated with $(PhSe)_2$ are explained by the findings of Milligan et al. [2004]. In support to this latter possibility, selenium exerts its effects on inflammation and immune responses by the mechanism involving the stimulation of immunological cells, such as T lymphocytes and natural killer cell activity, as well as the virulence of viruses or the inhibitory effect on iNOS [Ferencik and Ebringer, 2003; Hoffmann, 2007; Rayman, 2012].

In agreement with our previous study [Sartori et al., 2012], the inflammatory process caused by herpetic lesions increased MPO activity in the vaginal tissue, and $(PhSe)_2$ was effective against this increase. The reduction in MPO activity in mice infected and treated with $(PhSe)_2$ could reflect the minor production of neutrophils at the site of injury, which in turn corroborates with histological images and lower viral load in the vaginal tissues of mice.

Although the results of this study showed an increase in the levels of IFN-γ and a reduction of MPO activity in mice infected and treated with $(PhSe)_2$, we believe that these are independent mechanisms. These results could indicate the complex action of $(PhSe)_2$ and the interaction between components of the immune system and the target tissue, instead of a direct effect upon a single isolated cell type. Both the innate and the acquired immune systems and the inflammatory response are tightly regulated by the redox state and thus by antioxidant selenoproteins [Chanaday et al., 2011].

RS can contribute to the damage during viral infection by oxidizing lipids and damaging membranes, proteins, and nucleic acids or inactivating critical enzymes [Schwarz, 1996; Hu et al., 2011]. In the present study, $(PhSe)_2$ reduced the levels of RS, nitrate/nitrite, and MDA, markers of oxidative damage, in mice infected not just due to its antioxidant and anti-inflammatory properties [Nogueira et al., 2003; Nogueira and Rocha, 2010] but because this compound reduced viral load in vaginal tissue. Similar to the results found with $(PhSe)_2$, acyclovir reduced the viral load, reducing cells recruitment and consequently the RS and nitrate/nitrite levels.

The overproduction of RS levels caused by virus can disrupt the GSH system and affect the host cell pro-/antioxidant balance by inhibiting the synthesis of antioxidant enzymes, such as SOD and CAT. In vitro cell culture studies revealed that oxidative stress occur in the host cells after the infection with viruses, including HIV [Westendorp et al., 1995], hepatitis C [Abdalla et al., 2005], herpes simplex type 1 [Vogel et al., 2005], and influenza virus [Nencioni et al., 2003]. In the present study, HSV-2 infection reduced CAT, SOD and GPx activities and $(PhSe)_2$ attenuated this effect. These results corroborate with the most reported property of $(PhSe)_2$, the antioxidant [Nogueira and Rocha, 2010].

Moreover, HSV-2 infection increased the activity of GR and reduced the levels of NPSH, a non-enzymatic antioxidant defense that consists in 90% of reduced glutathione, GSH. Probably, the increase in GR activity is a tentative to compensate the reduced GPx activity and NPSH content in HSV-2-infected mice. In agreement with $(PhSe)_2$ antioxidant activity, this compound ameliorated the NPSH content and GR activity in vaginal tissues of mice. It has been reported that an organoselenium compound increased NPSH levels in different tissues of mice [Brandao et al., 2009].

The pharmacological effects of $(PhSe)_2$ demonstrated in this study are as follows: (i) antiviral action in vitro; (ii) antiviral action in vivo through the reduction of viral load in infected mice; (iii) interaction with the immune system; (iv) effectiveness to reduce the inflammatory response caused by viral infection; (v) reduction of virus-induced oxidative injury (See the representative scheme of $(PhSe)_2$ treatment in infected mice in Supplementary Fig. S4). Further analyses are ongoing to establish the possibility to develop it as an innovative therapy to treat HSV infections.

ACKNOWLEDGMENTS

The financial support by FAPERGS, CAPES, and CNPq is gratefully acknowledged.

REFERENCES

Abdalla MY, Ahmad IM, Spitz DR, Schmidt WN, Britigan BE. 2005. Hepatitis C virus-core and non structural proteins lead to different effects on cellular antioxidant defenses. J Med Virol 76:489–497.

Aebi H. 1984. Catalase in vitro. Methods Enzymol 105:121–126.

Ahmed HJ, Mbwana J, Gunnarsson E, Ahlman K, Guerino C, Svensson LA, Mhalu F, Lagergard T. 2003. Etiology of genital ulcer disease and association with human immunodeficiency virus infection in two tanzanian cities. Sex Transm Dis 30:114–119.

Astani A, Reichling J, Schnitzler P. 2010. Comparative study on the antiviral activity of selected monoterpenes derived from essential oils. Phytother Res 24:673–679.

Biswas S, Field HJ. 2008. Herpes simplex virus helicase-primase inhibitors: Recent findings from the study of drug resistance mutations. Antivir Chem Chemother 19:1–6.

Bradford MM. 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal Biochem 72:248–254.

Brandao R, Santos FW, Oliveira R, Roman SS, Nogueira CW. 2009. Involvement of non-enzymatic antioxidant defenses in the protective effect of diphenyl diselenide on testicular damage induced by cadmium in mice. J Trace Elem Med Biol 23:324–333.

Carlberg I, Mannervik B. 1975. Purification and characterization of the flavoenzyme glutathione reductase from rat liver. J Biol Chem 250:5475–5480.

Chanaday NL, de Bem AF, Roth GA. 2011. Effect of diphenyl diselenide on the development of experimental autoimmune encephalomyelitis. Neurochem Int 59:1155–61162.

Cheng HY, Lin CC, Lin TC. 2002. Antiherpes simplex virus type 2 activity of casuarinin from the bark of *Terminalia arjuna* Linn. Antiviral Res 55:447–455.

Ellman GL. 1959. Tissue sulfhydryl groups. Arch Biochem Biophys 82:70–77.

Ferencik M, Ebringer L. 2003. Modulatory effects of selenium and zinc on the immune system. Folia Microbiol (Praha) 48:417–426.

Ge KY, Wang SQ, Bai J, Xue AN, Deng XJ, Su CQ, Wu SQ. 1987. The protective effect of selenium against viral myocarditis in mice. In: Combs GF, Spalholz JE, Levander OA, Oldfield JE, editor. Selenium in biology and medicine, Part B. New York: Avi Book. pp 761–768.

Grisham MB, Hernandez LA, Granger DN. 1986. Xanthine oxidase and neutrophil infiltration in intestinal ischemia. Am J Physiol 251:G567–G574.

Grotto D, Santa Maria LD, Boeira S, Valentini J, Charao MF, Moro AM, Nascimento PC, Pomblum VJ, Garcia SC. 2007. Rapid quantification of malondialdehyde in plasma by high performance liquid chromatography-visible detection. J Pharm Biomed Anal 43:619–624.

Hayashi K, Hayashi T, Miyazawa K, Tomoda A. 2010. Phenoxazine derivatives suppress the infections caused by herpes simplex virus type-1 and herpes simplex virus type-2 intravaginally inoculated into mice. J Pharmacol Sci 114:85–91.

Hoffmann PR. 2007. Mechanisms by which selenium influences immune responses. Arch Immunol Ther Exp (Warsz) 55:289–297.

Hu S, Sheng WS, Schachtele SJ, Lokensgard JR. 2011. Reactive oxygen species drive herpes simplex virus (HSV)-1-induced proinflammatory cytokine production by murine microglia. J Neuroinflammation 8:123.

Ivec M, Botic T, Koren S, Jakobsen M, Weingartl H, Cencic A. 2007. Interactions of macrophages with probiotic bacteria lead to increased antiviral response against vesicular stomatitis virus. Antiviral Res 75:266–274.

Kavouras JH, Prandovszky E, Valyi-Nagy K, Kovacs SK, Tiwari V, Kovacs M, Shukla D, Valyi-Nagy T. 2007. Herpes simplex virus type 1 infection induces oxidative stress and the release of bioactive lipid peroxidation by-products in mouse P19N neural cell cultures. J Neurovirol 13:416–425.

Loetchutinat C, Kothan S, Dechsupa S, Meesungnoen J, Jay-Gerin JP, Mankhetkorn S. 2005. Spectrofluorometric determination of intracellular levels of reactive oxygen species in drug-sensitive and drug-resistant cancer cells using the 2′,7′-dichlorofluorescein diacetate assay. Radiat Phys Chem 72:323–331.

Milligan GN, Bernstein DI, Bourne N. 1998. T lymphocytes are required for protection of the vaginal mucosae and sensory ganglia of immune mice against reinfection with herpes simplex virus type 2. J Immunol 160:6093–6100.

Milligan GN, Bourne N, Dudley KL. 2001. Role of polymorphonuclear leukocytes in resolution of HSV-2 infection of the mouse vagina. J Reprod Immunol 49:49–65.

Milligan GN, Dudley-McClain KL, Young CG, Chu CF. 2004. T-cell-mediated mechanisms involved in resolution of genital herpes simplex virus type 2 (HSV-2) infection of mice. J Reprod Immunol 61:115–127.

Miranda KM, Espey MG, Wink DA. 2001. A rapid, simple spectrophotometric method for simultaneous detection of nitrate and nitrite. Nitric Oxide 5:62–71.

Misra HP, Fridovich I. 1972. The generation of superoxide radical during the autoxidation of hemoglobin. J Biol Chem 247:6960–6962.

Moriarty AT, Darragh TM, Souers R, Fatheree LA, Wilbur DC. 2008. Performance of herpes simplex challenges in proficiency testing: Observations from the College of American Pathologists (CAP) Proficiency Testing (PAP PT) Program. Cancer Cytopathol 114:394–394.

Nencioni L, Iuvara A, Aquilano K, Ciriolo MR, Cozzolino F, Rotilio G, Garaci E, Palamara AT. 2003. Influenza A virus replication is dependent on an antioxidant pathway that involves GSH and Bcl-2. FASEB J 17:758–760.

Nencioni L, Sgarbanti R, Amatore D, Checconi P, Celestino I, Limongi D, Anticoli S, Palamara AT, Garaci E. 2011. Intracellular redox signaling as therapeutic target for novel antiviral strategy. Curr Pharm Des 17:3898–3904.

Nogueira CW, Quinhones EB, Jung EA, Zeni G, Rocha JB. 2003. Anti-inflammatory and antinociceptive activity of diphenyl diselenide. Inflamm Res 52:56–63.

Nogueira CW, Rocha JBT. 2010. Diphenyl diselenide a janus-faced molecule. J Braz Chem Soc 21:2055–2071.

Nolkemper S, Reichling J, Sensch KH, Schnitzler P. 2010. Mechanism of herpes simplex virus type 2 suppression by propolis extracts. Phytomedicine 17:132–138.

Patel R, Alderson S, Geretti A, Nilsen A, Foley E, Lautenschlager S, Green J, van der Meijden W, Gomberg M, Moi H. 2011. European guideline for the management of genital herpes, 2010. Int J STD AIDS 22:1–10.

Paulmier C. 1986. Selenoorganic functional groups. In: Paulmier C, editor. Selenium reagents and intermediates in organic synthesis. 1st ed. Oxford: Pergamon Press. pp 25–51.

Quenelle DC, Collins DJ, Rice TL, Rahman A, Glazer R. 2011. Efficacy of orally administered low dose N-methanocarbathymidine against lethal herpes simplex virus type-2 infections of mice. Antivir Chem Chemother 22:131–137.

Rayman MP. 2012. Selenium and human health. Lancet 379:1256–1268.

Reznick AZ, Packer L. 1994. Oxidative damage to proteins: Spectrophotometric method for carbonyl assay. Methods Enzymol 233:357–363.

Riss TL, Moravec RA. 1992. Comparison of MTT, XTT, and a novel tetrazolium compound for MTS for in vitro proliferation and chemosensitivity assays. Mol Biol Cell 3:184a.

Sartori G, Pesarico AP, Pinton S, Dobrachinski F, Roman SS, Pauletto F, Rodrigues LCJ, Prigol M. 2012. Protective effect of brown Brazilian propolis against acute vaginal lesions caused by herpes simplex virus type 2 in mice: Involvement of antioxidant and anti-inflammatory mechanisms. Cell Biochem Funct 30:1–10.

Schachtele SJ, Hu S, Lokensgard JR. 2012. Modulation of experimental herpes encephalitis-associated neurotoxicity through sulforaphane treatment. PLoS ONE 7:e36216.

Schrauzer GN, Sacher J. 1994. Selenium in the maintenance and therapy of HIV-infected patients. Chem Biol Interact 91:199–205.

Schwarz KB. 1996. Oxidative stress during viral infection: A review. Free Radic Biol Med 21:641–649.

Spear PG, Roizman B. 1972. Proteins specified by herpes simplex virus. V. Purification and structural proteins of the herpesvirion. J Virol 9:143–159.

Vogel JU, Cinatl J, Dauletbaev N, Buxbaum S, Treusch G, Cinatl J, Jr., Gerein V, Doerr HW. 2005. Effects of S-acetylglutathione in cell and animal model of herpes simplex virus type 1 infection. Med Microbiol Immunol 194:55–59.

Weller SK, Coen DM. 2012. Herpes simplex viruses: Mechanisms of DNA replication. Cold Spring Harb Perspect Biol 4:a013011.

Wendel A. 1981. Glutathione peroxidase. Methods Enzymol 77:325–333.

Westendorp MO, Shatrov VA, Schulze-Osthoff K, Frank R, Kraft M, Los M, Krammer PH, Droge W, Lehmann V. 1995. HIV-1 Tat potentiates TNF-induced NF-kappa B activation and cytotoxicity by altering the cellular redox state. EMBO J 14:546–554.

Yu SY, Zhu YJ, Li WG. 1997. Protective role of selenium against hepatitis B virus and primary liver cancer in Qidong. Biol Trace Elem Res 56:117–124.

SUPPORTING INFORMATION

Additional supporting information may be found in the online version of this article at the publisher's web-site.

SUPPLEMENTARY MATERIAL ARTIGO 1

FIGURE LEGENDS

Figure S1. Chemical structure of diphenyl diselenide.

Figure S2. Evaluation of cytotoxic effects of $(PhSe)_2$ on Vero cells. Results are expressed as the mean ± S.E.M. (n=4) and were analyzed by ANOVA, followed by the Newman-Keuls Multiple Comparison test when appropriate. *Denotes $p < 0.01$, $^{***}p < 0.0001$ as compared to the control cells.

Figure S3. Representative scheme of experimental protocol.

Figure S4. Representative scheme of $(PhSe)_2$ treatment in vaginal tissues of female mice infected with HSV-2.

FIGURES

Figure S1.

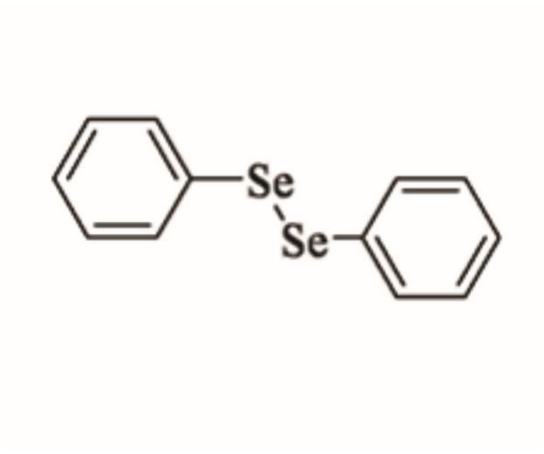


Figure S2.

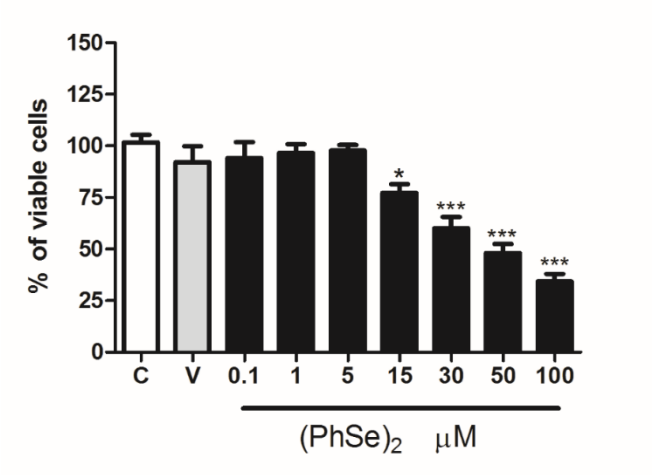


Figure S3.

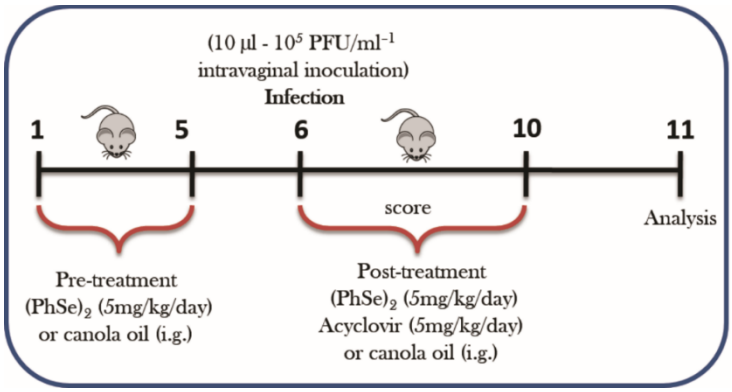
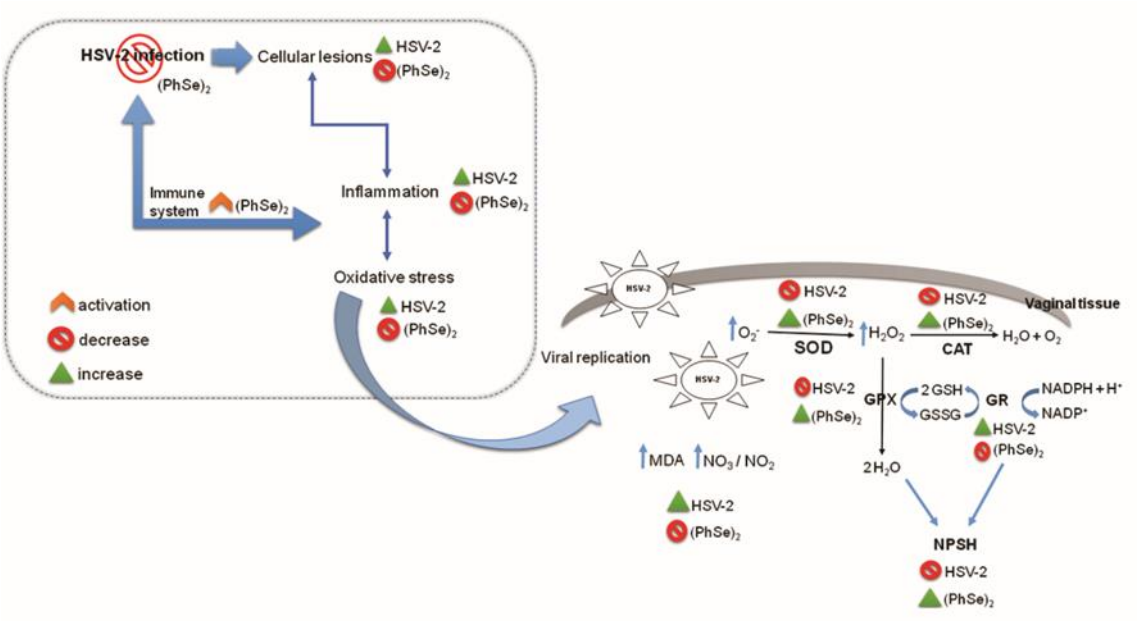


Figure S4.

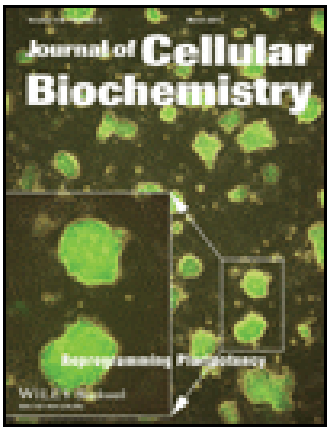


3.2 Artigo 2

Disseleneto de Difenila reduz estresse oxidativo e toxicidade causada pela infecção por HSV-2 em camundongos

DIPHENYL DISELENIDE REDUCES OXIDATIVE STRESS AND TOXICITY CAUSED BY HSV-2 INFECTION IN MICE

Gláubia Sartori, Natália Silva Jardim, Marcel Henrique Marcondes Sari, Eduardo F. Flores, Marina Prigol, Cristina W. Nogueira



Journal of Cellular Biochemistry, 2016 Aug 2, [Epub ahead of print]

DOI: 10.1002/jcb.25667

ARTICLE
Journal of Cellular Biochemistry 9999:1–10 (2016)

Journal of Cellular Biochemistry

Diphenyl Diselenide Reduces Oxidative Stress and Toxicity Caused by HSV-2 Infection in Mice

Gláubia[Q1] Sartori,[1] Natália Silva Jardim,[1] Marcel Henrique Marcondes Sari,[1] Eduardo F. Flores,[2] Marina Prigol,[3] and Cristina W. Nogueira[1]*

[1] *Laboratório de Síntese, Reatividade e Avaliação Farmacológica e Toxicológica de Organocalcogênios, Departamento de Bioquímica e Biologia Molecular, Centro de Ciências Naturais e Exatas, Universidade Federal de Santa Maria, Santa Maria, Rio Grande do Sul CEP 97105-900, Brasil*

[2] *Setor de Virologia, Departamento de Medicina Veterinária Preventiva e Departamento de Microbiologia e Parasitologia, Centro de Ciências Rurais, Universidade Federal de Santa Maria, UFSM, Av. Roraima, no 1000, Santa Maria, Rio Grande do Sul 97105-900, Brasil*

[3] *Departamento de Nutrição, Universidade Federal do Pampa, Campus Itaqui, Rio Grande do Sul CEP 97650-000, Brasil*

ABSTRACT

Herpes simplex viruses can cause uncommon systemic complications as acute liver failure (ALT) or urinary tract dysfunctions. Diphenyl diselenide, $(PhSe)_2$, a classical studied organic selenium compound, has a novel antiviral action against HSV-2 infection and well-known antioxidant and anti-inflammatory properties. This study aimed to investigate if $(PhSe)_2$ reduces oxidative stress and systemic toxicity caused by HSV-2 infection in mice. Adult BALB/c mice were pre-treated with $(PhSe)_2$ (5 mg kg^{-1}/day, intragastric, i.g.) during 5 days; at day 6 mice were infected with HSV-2 (10 µl-10^5 PFU/mL^{-1}) and post-treated with $(PhSe)_2$ for more 5 days. At day 11, they were killed and samples of liver and kidney were obtained to determine: reactive species (RS); malondialdehyde (MDA), and non-protein thiols (NPSH) levels; the activities of antioxidant enzymes, superoxide dismutase (SOD), and catalase (CAT). The activities of adenosine deaminase (ADA), Na^+/K^+-ATPase (liver and kidney); alanine aminotransferase (ALT), aspartate aminotransferase (AST), and the levels of urea (plasma) were determined as markers of hepatic and renal toxicity. The results revealed that $(PhSe)_2$ treatment was effective against the increase of renal and hepatic oxidative stress in infected mice and also normalized hepatic and renal ADA activity. It recovered the activity of Na^+/K^+- and was not effective against the increase in urea levels in infected mice. Different from $(PhSe)_2$, acyclovir (positive control), caused an increase in ADA activity and a decrease in hepatic CAT activity. Considering the interest of alternative therapies to treat HSV-2 infections and secondary complications, $(PhSe)_2$ become a notable candidate. J. Cell. Biochem. 9999: 1–10, 2016.

KEY WORDS: HSV-2; OXIDATIVE DAMAGE; TOXICITY; LIVER; KIDNEY; SELENIUM

Herpes simplex virus 2 (HSV-2), one specie of the Herpesviridae family, is a double-stranded DNA virus enclosed within a protein capsid (diameter of 100–200 nm) covered by a tegument and a glycoprotein-containing envelope. It has the capacity to infect epithelial cells at mucosal surfaces and establish latency in sensory neurons becoming reactivated in a later time. The consequences can result in either symptomatic or asymptomatic virus shedding near the site of original infection [Milligan et al., 2005; Riediger et al., 2009].

Rare complications of recurrent herpetic infections have a considerable medical importance in both immune competent and immune compromised person. The problems include encephalitis, hepatitis, pneumonia, esophagitis, and keratitis. Although, for most patients, the viral factors associated with these severe infections remain unknown [Koelle and Corey, 2008]. Hepatitis is a rare complication of herpes simplex virus (HSV-1 and HSV-2), often leading to acute liver failure (ALF), liver transplantation, and/or death. Usually, the most affected individuals are immunosuppressed

Conflicts of interest: The authors declare that there are no conflicts of interest.
Grant sponsor: FAPERGS; Grant sponsor: CAPES; Grant sponsor: CNPq.
*Correspondence to: Cristina W. Nogueira, Laboratório de Síntese, Reatividade e Avaliação Farmacológica e Toxicológica de Organocalcogênios, Departamento de Bioquímica e Biologia Molecular, Centro de Ciências Naturais e Exatas, Universidade Federal de Santa Maria, Santa Maria, Rio Grande do Sul CEP 97105-900, Brasil.
E-mail: criswn@ufsm.br
Manuscript Received: 2 May 2016; Manuscript Accepted: 1 August 2016
Accepted manuscript online in Wiley Online Library (wileyonlinelibrary.com): 00 Month 2016
DOI 10.1002/jcb.25667 • © 2016 Wiley Periodicals, Inc.

patients and pregnant women, however HSV hepatitis can occur in immunocompetent patients. A high degree of suspicion, even in the absence of skin lesions, combined with early diagnostic modalities (serology, polymerase chain reaction, liver biopsy) may potentially upgrade survival. The appropriate treatment of HSV hepatitis is the systemic therapy with acyclovir. Nevertheless, if a possible resistance occur for some patients, therapy with foscarnet is applied [Riediger et al., 2009].

It has been reported that both susceptibility to infectious disease and the severity if contracted are directly linked to the nutritional status of the host. The absence of one or more nutrients in the diets as selenium element may worsen the consequences of viral and bacterial infections [Nencioni et al., 2011]. The selenium deficiency was associated with more severe lung pathology and altered immune function in mice infected with influenza virus [Beck et al., 2004].

Considering what was mentioned before, we highlight the organoselenium compound, diphenyl diselenide $(PhSe)_2$, that has been reported to have a potential antiviral activity in vitro and in vivo against HSV-2 infection [Sartori et al., 2015]. Moreover, it has been demonstrated that $(PhSe)_2$ has antifungal and fungistatic actions in vitro [Loreto et al., 2011; Denardi et al., 2013; Venturini et al., 2016]. The classical antioxidant and anti-inflammatory properties have been widely studied over the years. $(PhSe)_2$ reproduces endogenous antioxidant enzymes such as glutathione peroxidase (GPx) or is metabolized by thioredoxin reductase to form selenol intermediate, which performs the same function of the antioxidant selenoenzymes [Nogueira and Rocha, 2010].

It is established that the replicative cycle of HSV-2 can trigger multiple intracellular pathways in their hosts, including those induced by oxidative stress [Kavouras et al., 2007; Mathew et al., 2010]. Furthermore, the increase in inflammatory markers and reactive oxygen species (ROS) has been reported during HSV-2 genital infection [Sartori et al., 2012, 2015].

In this view, the present study aimed to investigate if $(PhSe)_2$ diminishes oxidative stress and systemic toxicity caused by HSV-2 infection in mice.

MATERIALS AND METHODS

CHEMICALS

The reagents dichloroflouresceine diacetate (DCFH-DA), *p*-dimethylamino benzaldehyde, epinephrine, dinitrophenyl hydrazine, acyclovir, Ellman's reagent (dithiobis nitro benzoic acid—DTNB) were purchased from Sigma (St. Louis, MO). $(PhSe)_2$ was prepared in our laboratory according to the method described in the literature [Paulmier, 1986]. Analysis of the ^1H nuclear magnetic resonance (NMR) and ^{13}C NMR spectra showed that $(PhSe)_2$ displayed analytical and spectroscopic data in full agreement with its assigned structure. The chemical purity of compound (99.9%) was determined by GC/MS and was stable under storage conditions at room temperature, humidity and light. All other chemicals were obtained of analytical grade or from standard commercial suppliers.

$(PhSe)_2$ was dissolved in canola oil and acyclovir was dissolved in 1% dimethyl sulfoxide (DMSO), then diluted in distilled water.

ANIMALS

The experiments were carried out using female adult BALB/c mice (20–22 g, 8 weeks of age) from our own breeding colony. The animals were kept on a separate animal room, on a 12 h light/dark cycle, at temperature of 22 ± 2°C with free access to food (Guabi, RS, Brazil) and water. The present experimental study was approved by the Committee on Care and Use of Experimental Animal Resources from the Federal University of Santa Maria, Brazil and registered under the number of 006/2013. All efforts were made to minimize animal suffering and to reduce the number of animals used.

VIRUS PRODUCTION AND TITRATION

The HSV-2 strain 333 was amplified and quantitated in Vero cells (African Green monkey kidneycells, ATCC CCL81). Vero cells were grown in Roswell Park Memorial Institute medium (RPMI) containing ampicillin (1.6 mg/L), streptomycin (0.4 mg/L), and amphotericin B (2.25 mg/L), supplemented with 10% fetal bovine serum (FBS) and maintained in incubator with CO_2 at 5% and 37°C. For virus amplification, monolayers of Vero cells were infected with multiplicity of infection of 0.01 (1 plaque formation units per 100 cells) of HSV-2 and incubated at 37°C with CO_2 at 5%. 72 h after, the infected cells were submitted to freeze-thaw and the viral suspension was aliquoted and stored at −80°C. The virus quantitation was performed in duplicate using plates of six wells. For this, the original virus was submitted to limiting dilution (10^{-1} to 10^{-6}) and 400 μL of each dilution were inoculated on Vero cells and incubated at 37°C in 5% CO_2 for 1 h and 30 min. After that, the inoculum was removed and 3 mL of RPMI plus 2% low-melting point agarose were added to each well. After 72 h, viral titers were calculated using PFU/mL method [Spear and Roizman, 1972].

EXPERIMENTAL PROCEDURES

GENITAL INFECTION IN MICE

Mice were anesthetized with ketamine (150 mg/kg) and xylazine (10 mg/kg) injected intraperitoneally (i.p) and inoculated intravaginally by scratching small areas of the skin with the needle of a syringe containing 1×10^5 PFU/mL of HSV-2 in a 10 μL volume. The dose and the method used to induce lesions with HSV-2 333 was based on previous studies [Milligan and Bernstein, 1995; Sartori et al., 2012].

EXPERIMENTAL PROTOCOL

Animals were divided in six groups (n = 7-8): group I—control (vehicle); group II—HSV-2 infected mice; group III—$(PhSe)_2$ (5 mg/kg) treated mice; group IV—acyclovir (5 mg/kg) treated mice; group V—$(PhSe)_2$ treated and HSV-2 infected mice; and group VI—acyclovir treated and HSV-2 infected mice. Five days before infection with HSV-2, groups I and II received canola oil, vehicle, (10 mL/kg), groups III and V received $(PhSe)_2$ at a dose of 5 mg/kg by gavage once a day. At day 5, mice from groups II, V, and VI were infected with HSV-2. Groups I, III, and IV were submitted to the same procedure applied for genital infection but without any viral particle. Animals were treated with vehicle, $(PhSe)_2$, or acyclovir (5 mg/kg) for more 5 days (once a day by gavage) after HSV-2 infection (See the representative scheme of experimental protocol in Figure S1, supplementary material). The doses of $(PhSe)_2$ and acyclovir were chosen based on our previous

study [Sartori et al., 2015]. At the 11th day mice were anesthetized for blood collection (cardiac punction) and the hemolyzed plasma was discarded. Then, the tissues (liver, kidney) were removed for ex vivo experiments (biochemical analysis).

EX VIVO EXPERIMENTS

SAMPLE PREPARATIONS

To determine parameters of stress oxidative and toxicity, livers and kidneys (n = 7–8 per group) were immediately homogenized in cold 50 mM Tris–HCl, pH 7.4 (1/10, w/v). The homogenate was centrifuged for 10 min at 3,000*g* at 4°C to yield a low-speed supernatant (S1). Samples of S1 were used to determine the levels of reactive species (RS), malondialdehyde (MDA) and non-protein thiol (NPSH) and the activities of superoxide dismutase (SOD), catalase (CAT), and adenosine deaminase (ADA).

For Na^+/K^+-ATPase activity analysis, the samples of kidneys were prepared under different conditions detailed on the respective method section.

Samples of plasma were used to determine the levels of urea and the activities of aspartate aminotransferase (AST) and alanine aminotransferase (ALT), markers of toxicity.

PARAMETERS OF OXIDATIVE STRESS

RS LEVELS

To estimate the level of tissue homogenate RS production, an aliquot of S1 (10 μL) was incubated with 10 μL of 2′,7′-dichlorofluorescein diacetate (DCHF-DA; 1 mM). The RS levels were determined by a spectrofluorimetric method. The oxidation of DCHF-DA to fluorescent dichlorofluorescein (DCF) is measured for the detection of intracellular RS. The DCF fluorescence intensity emission was recorded at 520 nm (with 480 nm excitation) 30 min after the addition of DCHF-DA to the medium. RS levels were expressed as nmol DCFH-ox/mg of protein [Loetchutinat et al., 2005].

MDA LEVELS

The determination of MDA levels were performed by high performance liquid chromatography (HPLC)-UV detection method with some modifications [Grotto et al., 2007]. This technique is more specific, reliable and reproducible than the colorimetric method, as MDA is separated from other interfering substances that react with thiobarbituric acid (TBA), such as sugars, amino acids, a variety of aldehydes, and bilirubin. Briefly, an aliquot of 75 μL of S1 or MDA bis (dimethylacetal) solution (as standard, dissolved in distilled water) was added to 25 μL of 3 N NaOH and incubated at 60°C for 30 min. After this, 125 μL of 6% H3PO4 and 125 μL of 0.8% TBA were added and the mixture was heated at 90°C for 2 h. Then the mixture was cooled and kept at −20°C until extraction with n-butanol. For this, samples were added to 50 μL of 10% sodium dodecyl sulfate (SDS) and 300 μL of n-butanol, vortex-mixed for 1 min and centrifuged at 3,000 × *g* for 10 min. The TBA-MDA adduct of butanol layer was analyzed on a Shimadzu HPLC apparatus. The analytical column was 5 μm particles and 100 A pore size, Phenomenex ODS-2 C18 reverse-phase column (4.6 9 150 mm, Allcrom, BR). The mobile phase was a mixture of Milli-Q water and methanol (50:50; v/v). HPLC analysis was performed under isocratic conditions at a flow rate of 0.6 mL/min and UV detector set at 532 nm, with a sample volume injection of 20 μL. The lipid peroxidation was expressed as nmol MDA/mg of protein.

NPSH LEVELS

S1 was mixed (1:1) with 10% trichloroacetic acid and centrifuged at 4000*g* for 10 min. After the centrifugation, the protein pellet was discarded and free -SH groups were determined in the clear supernatant. An aliquot of supernatant was added in 1 M potassium phosphate buffer pH 7.4 and 10 mM DTNB [Ellman, 1959]. The color reaction was measured at 412 nm. NPSH levels were expressed as mmol NPSH/mg of protein.

SOD ACTIVITY

The enzymatic reaction of SOD was initiated by adding an S1 aliquot (150 μL) of the homogenized tissue and the substrate (epinephrine) at a concentration of 4 mM in a medium containing 50 mM bicarbonate buffer, pH 10.3. The color reaction was measured at 480 nm. One unit of enzyme was defined as the amount of enzyme required to inhibit the rate of epinephrine autooxidation by 50% at 26°C [Misra and Fridovich, 1972]. The enzymatic activity was expressed as U/mg of protein.

CAT ACTIVITY

The enzymatic reaction was initiated by adding an aliquot (S1) of 20 μL and the substrate (H_2O_2 0.3 mM) in a medium containing 50 mM phosphate buffer (pH 7.0) and was measured at 240 nm. The enzymatic activity was expressed in Units (one Unit decomposes 1 μmol of H_2O_2 per min at pH 7 at 25°C/mg of protein [Aebi, 1984].

PARAMETERS OF RENAL AND HEPATIC TOXICITY

NA^+/K^+-ATPASE ACTIVITY

Concerning the importance of intracellular K^+ to the viral replication, we determined the Na^+/K^+-ATPase activity analysis in kidneys of mice. The homogenates of samples were prepared in 0.05 M Tris–HCl buffer (pH 7.4) (1/10, w/v). The homogenate was centrifuged and supernatant was used for assay of protein Na^+/K^+-ATPase. The reaction mixture for Na^+/K^+-ATPase activity assay contained 3 mM $MgCl_2$, 125 mM NaCl, 20 mM KCl, and 50 mM Tris–HCl, pH 7.4, in a final volume of 500 μL. The reaction was initiated by the addition of adenosine triphosphate (ATP) to a final concentration of 3 mM. Controls were carried out under the same conditions with the addition of 1 mM ouabain. Na^+/K^+-ATPase activity was calculated by the difference between the two assays. Released inorganic phosphate (Pi) was measured by the method of Fiske and Subbarow [Fiske and Subbarow, 1927] .

ADA ACTIVITY

In order to investigate if the ADA activity is affected by inflammatory and infectious processes, we determined the enzyme activity in liver and kidney of infected animals according to Giusti and Gakis [Giusti and Gakis, 1971] . Briefly, 25 μL of S1 was incubated with 21 nmol/L of adenosine (pH 6.5) at 37°C for 60 min. This method is based on the direct production of ammonia when

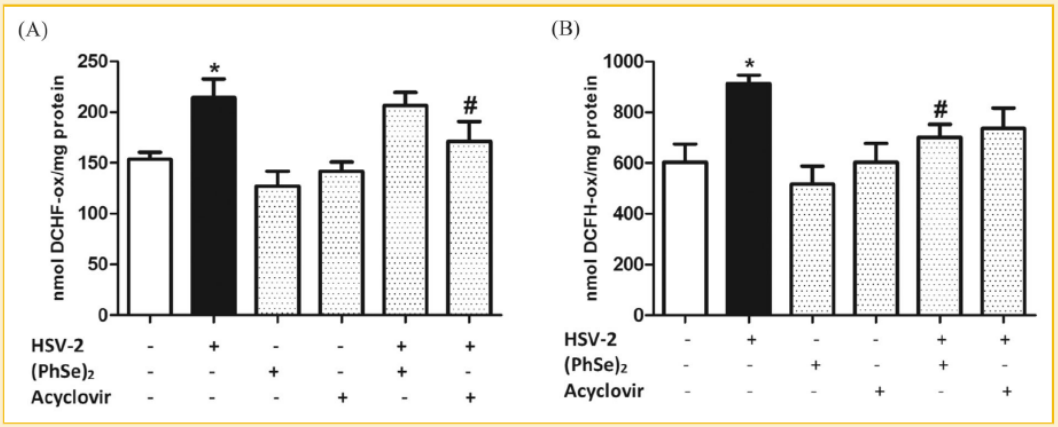


Fig. 1. Effect of treatment with $(PhSe)_2$ at a dose of 5 mg/kg on RS levels in samples of liver (A) and kidney (B) of HSV-2 infected mice. RS levels are expressed as nmol DCHF-ox/mg of protein. Acyclovir at a dose of 5 mg/kg was used as positive control. Data are reported as the mean ± S.E.M. (n = 7–8) and were analyzed by two-way ANOVA followed by the Duncan's test when appropriate. *Denotes significant difference when compared to the control group; #Denotes significant difference when compared to the HSV-2 group ($P < 0.05$).

ADA acts in excess of the adenosine. Results were expressed in units per mg of protein (U/mg protein). One unit (1 U) of ADA is defined as the amount of enzyme required to release 1 mmol ammonia per minute from adenosine at standard assay conditions.

It was performed Pearson's correlation analysis between ADA activity and MDA levels in liver and kidney of HSV-2 infected mice and $(PhSe)_2$ or acyclovir treatment.

TRANSAMINASES ACTIVITY AND UREA LEVELS

The hepatic function was analyzed using plasma ALT and AST activities (LABTEST, MG). Renal function was analyzed through the urea levels (LABTEST, MG).

PROTEIN QUANTIFICATION

Protein concentration was measured by the method of Bradford [Bradford, 1976] using bovine serum albumin as the standard.

STATISTICAL ANALYSIS

Ex vivo data were analyzed using a two-way analysis ($(PhSe)_2 \times$ HSV-2 or acyclovir × HSV-2) of variance (ANOVA), followed by post-hoc comparisons using the Duncan's multiple range test when appropriate. Data were expressed as the mean(s) ± S.E.M. All results were analyzed and approved by the D'Agostino and Pearson omnibus normality test. Correlation analysis of ADA activity and

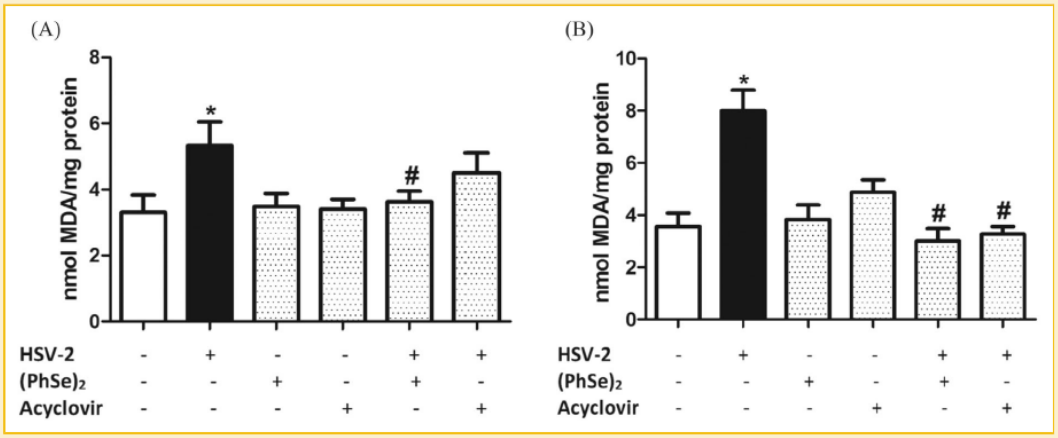


Fig. 2. Effect of treatment with $(PhSe)_2$ at a dose of 5 mg/kg on MDA levels in samples of liver (A) and kidney (B) of HSV-2 infected mice. MDA levels are expressed as nmol MDA/mg of protein. Acyclovir at a dose of 5 mg/kg was used as positive control. Data are reported as the mean ± S.E.M. (n = 7–8) and were analyzed by two-way ANOVA followed by the Duncan's test when appropriate. *Denotes significant difference when compared to the control group; #Denotes significant difference when compared to the HSV-2 group ($P < 0.05$).

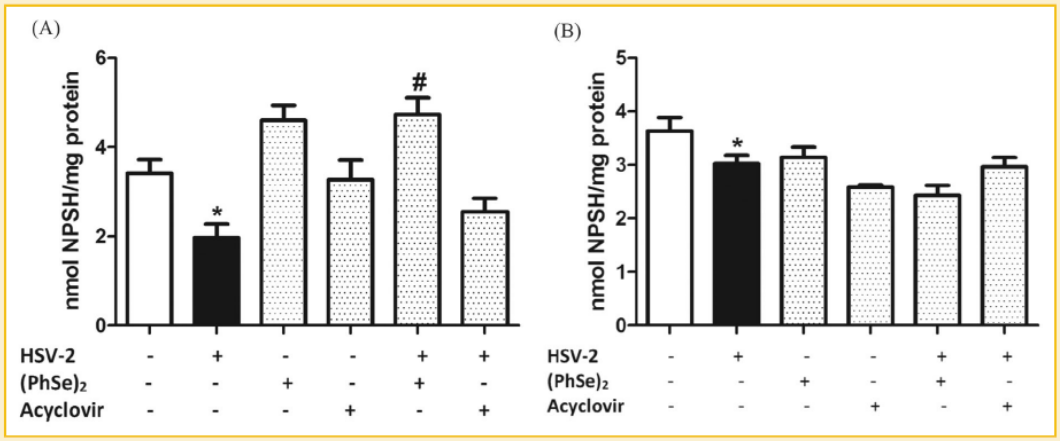


Fig. 3. Effect of treatment with $(PhSe)_2$ at a dose of 5 mg/kg on NPSH levels in samples of liver (A) and kidney (B) of HSV-2 infected mice. NPSH levels are expressed as mmol NPSH/mg of protein. Acyclovir at a dose of 5 mg/kg was used as positive control. Data are reported as the mean ± S.E.M. (n = 7–8) and were analyzed by two-way ANOVA followed by the Duncan's test when appropriate. *Denotes significant difference when compared to the control group; #Denotes significant difference when compared to the HSV-2 group ($P < 0.05$).

MDA levels were performed using Pearson's correlation coefficient (r) followed by the Gaussian distribution (two-tailed *P*-value) to compare the infection versus treatments. Data are reported as a single value for each point. Differences between groups were considered statistically significant when $P < 0.05$.

RESULTS

OXIDATIVE STRESS

Statistical analysis revealed that HSV-2 significantly increased RS levels in liver ($P < 0.01$) and kidney ($P < 0.001$) of mice when compared to those of the control group. $(PhSe)_2$ was effective in reducing RS levels in kidney ($P < 0.05$) (Fig. 1B), but not in liver (Fig. 1A), of infected mice. On the other hand, acyclovir-treated infected group showed a reduction in RS levels in liver (Fig. 1A), but not in kidney (Fig. 1B), of mice ($P < 0.05$).

Analysis of MDA levels showed that HSV-2 infection significantly enhanced lipid peroxidation in both tissues in comparison to control group ($P < 0.01$). Treatment with $(PhSe)_2$ abolished the increase in MDA levels in liver of infected mice ($P < 0.05$), while acyclovir was not effective in reducing MDA levels (Fig. 2A). In kidney, both treatments reduced MDA levels altered by HSV-2 infection ($P < 0.001$) (Fig. 2B).

It was observed that HSV-2 infection significantly reduced NPSH levels in liver of mice in comparison to those of the control group ($P < 0.01$). $(PhSe)_2$ treatment in infected mice avoided this alteration ($P < 0.001$) and increased even more the levels of NPSH. $(PhSe)_2$ treatment in non-infected mice caused an increase of NPSH levels in liver (per se effect). Acyclovir treatment in infected mice did not normalize these levels altered by HSV-2 infection (Fig. 3A). In kidney, both treatments did not protect against the reduction of NPSH levels caused by HSV-2 infection (Fig. 3B). Moreover, acyclovir treatment in non-infected mice caused a decrease in NPSH levels in kidneys of mice (per se effect) (Fig. 3B).

The results demonstrated a reduction in SOD activity in the liver of animals caused by HSV-2 infection in comparison to that of the control group ($P < 0.01$). It was also observed a protection by $(PhSe)_2$ treatment ($P < 0.05$) against this alteration (Fig. 4A). There was no effect of HSV-2 infection and treatments in the SOD activity in kidneys of mice (Fig. 4B).

CAT activity was also reduced by HSV-2 infection when compared to that of the control group ($P < 0.001$) and treatment with $(PhSe)_2$ ($P < 0.05$), but not with acyclovir, was effective to normalize the enzyme activity in the liver of infected mice (Fig. 4C). With respect to kidney, both $(PhSe)_2$ ($P < 0.01$) and acyclovir ($P < 0.001$) treatments protected against the reduction of CAT activity caused by HSV-2 infection (Fig. 4D).

RENAL AND HEPATIC TOXICITY

HSV-2 infection caused an increase in the Na^+/K^+-ATPase activity ($P < 0.001$) in kidneys of mice in comparison to that of the control group and treatment with $(PhSe)_2$ was effective ($P < 0.01$) against this raise. Acyclovir treatment also abolished the increase in the Na^+/K^+-ATPase activity in HSV-2 infected mice ($P < 0.001$) (Fig. 5).

Analysis of ADA showed an increase in this enzyme activity in liver ($P < 0.01$) and kidneys ($P < 0.05$) of HSV-2 infected mice in comparison to that of the control group. Treatment with $(PhSe)_2$ ($P < 0.01$), but not with acyclovir, reduced this activity in both tissues of infected mice (Fig. 6A and B). Acyclovir treatment in non-infected mice increased the hepatic activity of the enzyme (per se effect) (Fig. 6A).

Pearson's correlation analysis of $(PhSe)_2$ treatment versus HSV-2 infection groups revealed a significant positive correlation between hepatic ADA activity and MDA levels ($r = 0.633$, $P < 0.01$) (Fig. 7A). By contrast, acyclovir treatment versus HSV-2 infection revealed a

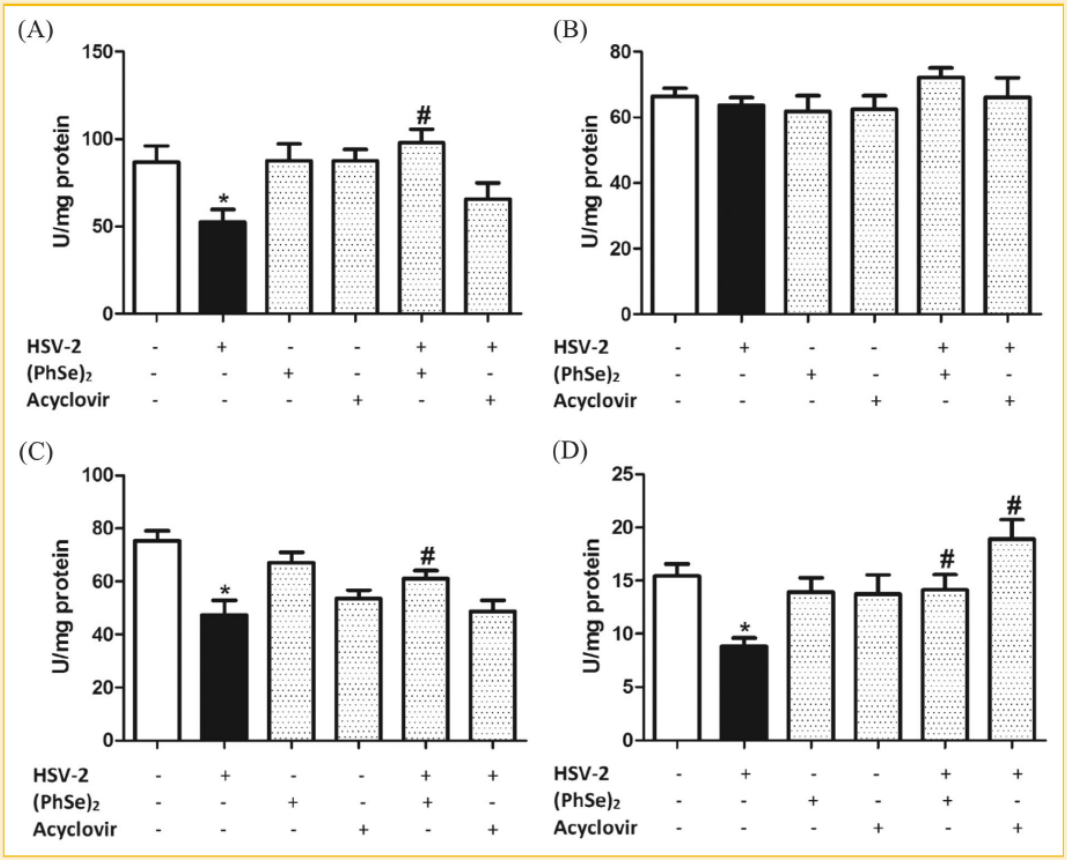


Fig. 4. Effect of treatment with $(PhSe)_2$ at a dose of 5 mg/kg on antioxidant enzyme activities in liver (A and C) and kidney (B and D) of HSV-2 infected mice. SOD (A, B) and CAT (C, D) enzymatic activities were expressed as U/mg of protein. Acyclovir at a dose of 5 mg/kg was used as positive control. Data are reported as the mean ± S.E.M. ($n = 7–8$) and were analyzed by two-way ANOVA followed by the Duncan's test when appropriate. *Denotes significant difference when compared to the control group; #Denotes significant difference when compared to the HSV-2 group ($P < 0.05$).

non-significant correlation between hepatic ADA activity and MDA levels ($r = 0.277$, $P = 0.31$, Fig. 7B). There was not a significant correlation between renal ADA activity and MDA levels for both treatments (data not shown).

Data of transaminases demonstrated an increase in AST ($P < 0.001$) and ALT ($P < 0.01$) activities in plasma of HSV-2 infected animals in comparison to those of the control group. The analysis revealed that both treatments reduced AST and ALT activities ($P < 0.05$) in infected animals (Fig. 8A and B).

Regarding the levels of urea in serum, it was observed an increase of these levels in HSV-2 infected animals when compared to control group ($P < 0.01$) and both treatments were not effective to reduce them (Fig. 8C).

DISCUSSION

The present study demonstrates that treatment with $(PhSe)_2$ minimized hepatic and renal oxidative stress and toxicity caused by HSV-2 infection in mice. In general, the effects of $(PhSe)_2$ on renal and hepatic oxidative stress and toxicity were similar to those obtained with acyclovir, a positive control drug used in this study. But different from $(PhSe)_2$, acyclovir caused an increase in hepatic/renal ADA activity and a decrease in hepatic CAT activity in infected mice.

Infectious diseases are closely related with oxidative stress in several ways as inflammation and organ damage linked to altered metabolism [Nencioni et al., 2011; Tawadrous et al., 2012]. In the experimental model used in this study, oxidative damage is known to play a significant role in the development of tissue injury occurring within a few days from HSV-2 infection. Previous reports have demonstrated that oxidative damage occurs even if the viral load is very low or no detectable [Podlech et al., 1996; Burgos et al., 2005]. In agreement with a previous HSV-2 infection study from our research group [Sartori et al., 2015], HSV-2 caused hepatic and renal oxidative stress reflected by an imbalance of the production of MDA, RS levels, and the antioxidant capacity, through the decrease of

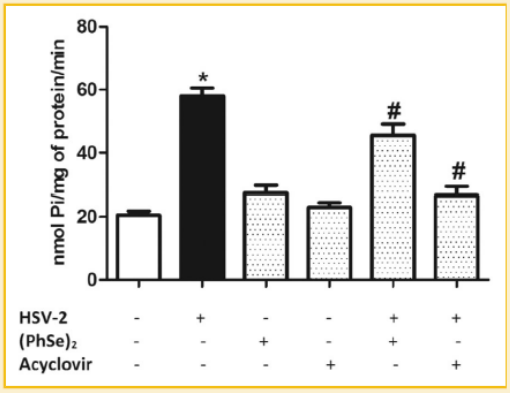


Fig. 5. Effect of treatment with $(PhSe)_2$ at a dose of 5 mg/kg on Na^+/K^+-ATPase activity in kidney of HSV-2 infected mice. The enzymatic activity was expressed as nmol Pi/mg of protein/min. Acyclovir at a dose of 5 mg/kg was used as positive control. Data are reported as the mean ± S.E.M. (n = 7–8) and were analyzed by two-way ANOVA followed by the Duncan's test when appropriate. *Denotes significant difference when compared to the control group; #Denotes significant difference when compared to the HSV-2 group ($P < 0.05$).

NPSH levels and SOD and CAT activities in tissues of mice. Awodele and collaborators reported similar results in humans, in which blood antioxidants, as reduced glutathione, were reduced over the long-term infection in AIDS infected individuals [Awodele et al., 2012].

The classical antioxidant property of $(PhSe)_2$ was evident in liver and kidney of infected mice because $(PhSe)_2$ reduced MDA and RS levels and increased antioxidant defenses. In fact, administration of $(PhSe)_2$ in HSV-2 infected mice restored CAT activity and the NPSH levels in liver and kidneys of these animals. Besides its well reported antioxidant property [Nogueira and Rocha, 2010], it is possible that $(PhSe)_2$ controls oxidative stress in these tissues by reducing the viral load in HSV-2 infected mice. We consider this hypothesis based on our previous study, which demonstrated that $(PhSe)_2$ reduced the viral load, local inflammation and oxidative injury induced by HSV-2 in a vaginal model of infection in mice [Sartori et al., 2015]. Because the HSV-2 viral load was not monitored in this protocol, we assume this as a limitation of the present study.

Regarding oxidative stress, acyclovir treatment was effective in counteracting hepatic MDA and renal RS levels but antioxidant defenses, such as SOD and CAT activities and NPSH levels, seem to be not involved in the antioxidant effect of this drug. This way, GPx activity was carried out in kidney and liver of mice from all experimental groups of this study and this enzyme activity was not altered (data not shown). Therefore, other antioxidant defenses should be responsible for the effectiveness of acyclovir against oxidative stress.

The present findings on infection and overproduction of RS are in agreement with the literature data that report oxidative stress initiating damage to tissues and resulting in unbalanced regulation processes [Peterhans, 1997].

The Na^+/K^+-ATPase enzyme is a plasma membrane cation pump, which is essential for maintenance of intracellular and extracellular Na^+ and K^+ concentrations, cell volume, osmotic balance, and electrochemical gradients. Moreover, Na^+/K^+-ATPase is a central regulator of kidney functions and the infection caused by a number of viruses has been shown to result in an alteration of the cell membrane permeability to ions [Hackstadt and Mallavia, 1982; Mi et al., 2010]. There are not many studies involving HSV infection and Na^+/K^+-ATPase activity, which is reduced by HSV-1 infection in Vero

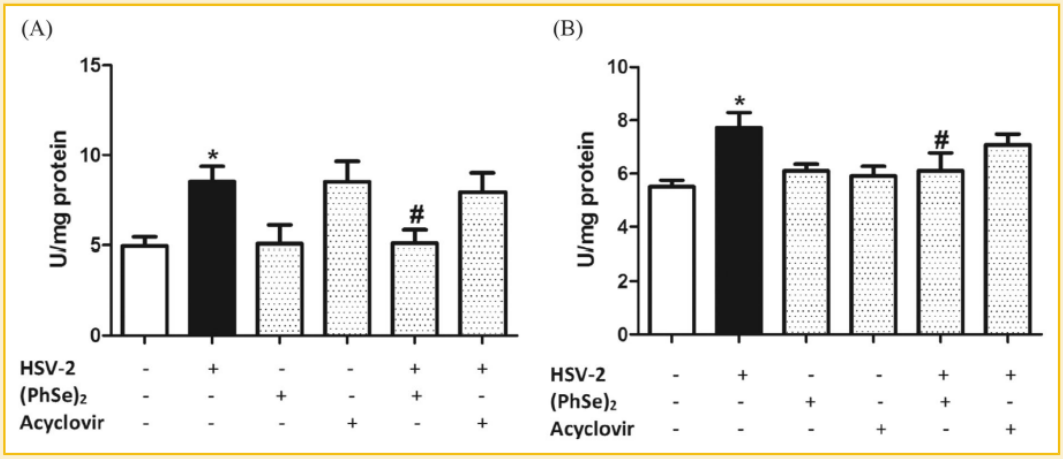


Fig. 6. Effect of treatment with $(PhSe)_2$ at a dose of 5 mg/kg on ADA activity in liver (A) and kidney (B) of HSV-2 infected mice. The enzymatic activity was in units per mg of protein (U/mg protein). Acyclovir at a dose of 5 g/kg was used as positive control. Data are reported as the mean ± S.E.M. (n = 7–8) and were analyzed by two-way ANOVA followed by the Duncan's test when appropriate. *Denotes significant difference when compared to the control group; #Denotes significant difference when compared to the HSV-2 group ($P < 0.05$).

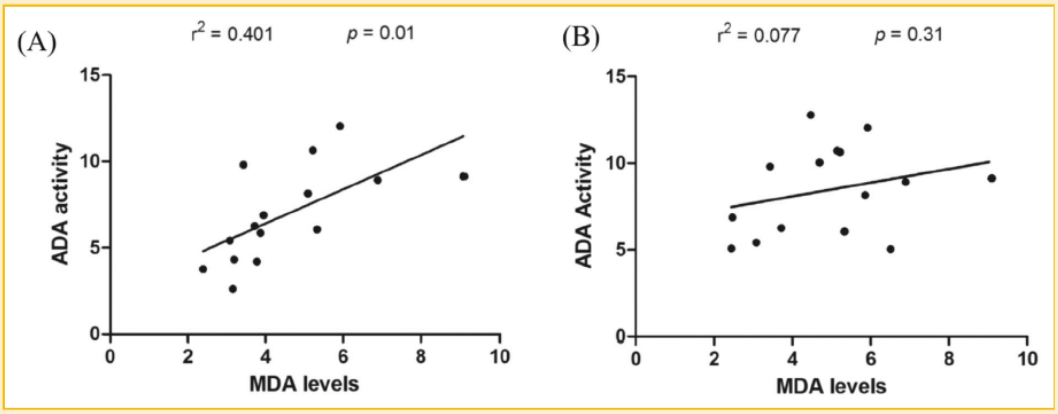


Fig. 7. Correlation analysis between ADA activity and MDA levels in liver of HSV-2 infected mice. (A) shows HSV-2 infected mice and $(PhSe)_2$ treatment. (B) shows HSV-2 infected mice and acyclovir treatment. ADA activity was expressed as units per mg of protein (U/mg protein). MDA levels are expressed as nmol MDA/mg of protein. Data are reported as a single value for each animal (n = 7–8) and were analyzed by Pearson's correlation coefficient (r) followed by the Gaussian distribution (two-tailed *P*-value). Correlation between ADA and MDA analysis were considered statistically significant when $P < 0.05$.

cells and HeLa cell plasma membranes in vitro, though the exact mechanism is still unclear [Schaefer et al., 1982; Mi et al., 2010].

In addition, it has been reported the relevance of intracellular K^+ to the viral replication on in vitro studies [Nagai et al., 1972; Hartley et al., 1993; Hartley et al., 2006]. There is notable evidence indicating modification of cellular membrane structure following HSV infections [Hackstadt and Mallavia, 1982; Palu et al., 1994; Bertol et al., 2011]. The results obtained in our experimental study demonstrated an increase in renal Na^+/K^+ ATPase activity in HSV-2 infected mice, suggesting that HSV-2 interfered in the permeability of cell membranes to ions. Treatment with $(PhSe)_2$ restored Na^+/K^+-ATPase activity, which indicates a cellular electrochemical gradient alteration that could be involved in its mechanism of viral load reduction.

In this scenario, it was demonstrated that HSV-2 infection interfered in renal function, demonstrated in this study by the increase in urea levels. It is possible that the increase in urea levels and Na^+/K^+-ATPase activity caused by infection is an attempt to equilibrate the renal function. It is important to note that although $(PhSe)_2$ or acyclovir treatment reduced Na^+/K^+-ATPase activity they did not counteract the increase in urea levels in infected mice.

HSV infects hepatocytes through different mechanisms: one is exocytosis followed by fusion of viral particles with the cell membrane of an uninfected cell and other direct cell-to-cell spread through intercellular junctions [Holt et al., 2013]. HSV-2 infection increased activities of AST and ALT, markers of hepatic function, in mice. These present data corroborate with those obtained by White and collaborators, who showed an impaired liver function associated

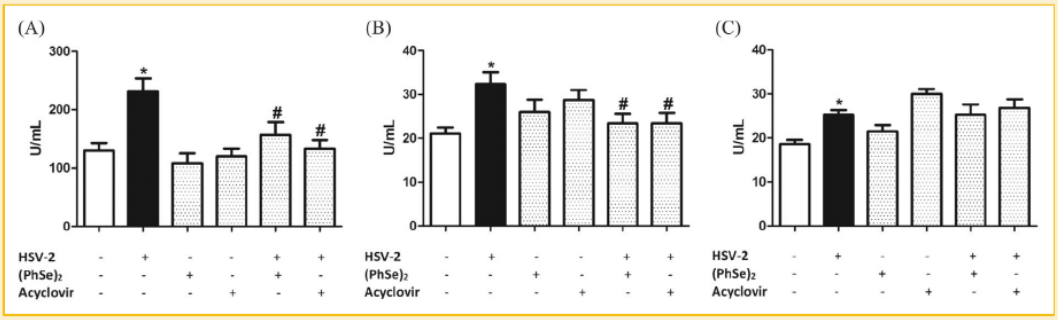


Fig. 8. Effect of treatment with $(PhSe)_2$ at a dose of 5 mg/kg on the activities of AST (A) and ALT (B) and levels of Urea (C) in plasma of HSV-2 infected mice. Results are expressed in units per mililiter (U/mL). Acyclovir at a dose of 5 mg/kg was used as positive control. Data are reported as the mean ± S.E.M. (n = 7–8) and were analyzed by two-way ANOVA followed by the Duncan's test when appropriate. *Denotes significant difference when compared to the control group; #Denotes significant difference when compared to the HSV-2 group ($P < 0.05$).

to the degree of aminotransferase elevation and progression of disease that can lead to death in a human study [White et al., 2011].

Similar to the results obtained in oxidative stress, $(PhSe)_2$ or acyclovir treatment was effective in normalizing AST and ALT activities, decreasing hepatic toxicity caused by infection in mice.

Moreover, in HSV-2 infected mice treated with $(PhSe)_2$ the normalization of ADA activity occurred concomitant to the decrease in oxidative stress. Our findings correlating oxidative stress and the enzyme activity are in accordance with other studies [Bor et al., 1999; Scheller et al., 2006] that suggest a blunt of oxidant processes and a consequent adenosine accumulation which provides protection against inflammatory damage. ADA is widely present in human tissues and is involved with the proliferation and differentiation of immune cells. The increase in ADA activity has been demonstrated in several inflammatory and infectious diseases, such as visceral leishmaniasis, cutaneous antrax, viral and bacterial pneumonia, and HIV infection [Sunnetcioglu et al., 2014]. As far as we know, there are no studies focus on the association between the activity of ADA and HSV-2 infection. With agreement with Williams and Lerner [1975] who demonstrated the antiviral action of inhibitors of ADA activity in vitro, our data showed that ADA activity was increased in HSV-2 infected mice and $(PhSe)_2$ treatment could reduce it [Williams and Lerner, 1975]. This result is very interesting because we already demonstrated an antiviral action of $(PhSe)_2$ on a previous study [Sartori et al., 2015]. It is possible that the decrease of ADA activity caused by $(PhSe)_2$ or acyclovir could contribute to the reduction of hepatic and renal toxicity in HSV-2 infected mice.

We performed a Pearson's correlation analysis in order to test the hypothesis if there is a relationship between ADA activity and MDA levels in liver of infected mice. A significant positive Person's correlation was found between ADA activity and MDA levels in HSV-2 infected mice and treated with $(PhSe)_2$. By contrast, there was no significant Person's correlation between ADA activity and MDA levels in HSV-2 infected mice and treated with acyclovir. Similar to HSV-2 infected mice, an increase in hepatic ADA activity was found in non-infected mice treated with acyclovir.

It is also important to mention that HSV infections can cause constipation, urinary retention and weight loss in animal model of infection [Sanjuan and Zimberlin, 2001] which was also observed in this experimental study. However, our deeper investigations found no alterations in histological (liver and kidney) analysis in this animal model of HSV-2 infection (data not shown). The morphology of hepatic and renal cells of infected mice did not differ from that of control animals showing no modification of cellular structure. Although, with our dataset we suppose that even with hepatic and renal damage, these complications begin in an oxidative molecular level being not so severe in this meantime of analysis. $(PhSe)_2$ treatment could overcome the majority of negative effects caused by infection.

Considering what was mentioned before, we highlight the selenium compound $(PhSe)_2$ as a possible alterative therapy under acyclovir treatment resistance, HSV injury to immunocompromised patients (liver and kidney failure) and HSV hepatitis reports.

ACKNOWLEDGMENTS

The financial support by FAPERGS, CAPES, and CNPq is gratefully acknowledged.

REFERENCES

Aebi H. 1984. Catalase in vitro. Methods Enzymol 105:121–126.

Awodele O, Olayemi SO, Nwite JA, Adeyemo TA. 2012. Investigation of the levels of oxidative stress parameters in HIV and HIV-TB co-infected patients. J Infect Dev Ctries 6:79–85.

Beck MA, Handy J, Levander OA. 2004. Host nutritional status: The neglected virulence factor. Trends Microbiol 12:417–423.

Bertol JW, Rigotto C, de Padua RM, Kreis W, Barardi CR, Braga FC, Simoes CM. 2011. Antiherpes activity of glucoevatromonoside, a cardenolide isolated from a Brazilian cultivar of Digitalis lanata. Antiviral Res 92:73–80.

Bor MV, Durmus O, Bilgihan A, Cevik C, Turkozkan N. 1999. The beneficial effect of 2′-deoxycoformycin in renal ischemia-reperfusion is mediated both by preservation of tissue ATP and inhibition of lipid peroxidation. Int J Clin Lab Res 29:75–79.

Bradford MM. 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal Biochem 72:248–254.

Burgos JS, Ramirez C, Sastre I, Alfaro JM, Valdivieso F. 2005. Herpes simplex virus type 1 infection via the bloodstream with apolipoprotein E dependence in the gonads is influenced by gender. J Virol 79:1605–1612.

Denardi LB, Mario DA, de Loreto ES, Nogueira CW, Santurio JM, Alves SH. 2013. Antifungal activities of diphenyl diselenide alone and in combination with fluconazole or amphotericin B against Candida glabrata. Mycopathologia 176:165–169.

Ellman GL. 1959. Tissue sulfhydryl groups. Arch Biochem Biophys 82:70–77.

Fiske CH, Subbarow Y. 1927. The nature of the "Inorganic Phosphate" in voluntary muscle. Science 65:401–403.

Giusti G, Gakis C. 1971. Temperature conversion factors, activation energy, relative substrate specificity and optimum pH of adenosine deaminase from human serum and tissues. Enzyme 12:417–425.

Grotto D, Santa Maria LD, Boeira S, Valentini J, Charao MF, Moro AM, Nascimento PC, Pomblum VJ, Garcia SC. 2007. Rapid quantification of malondialdehyde in plasma by high performance liquid chromatography-visible detection. J Pharm Biomed Anal 43:619–624.

Hackstadt T, Mallavia LP. 1982. Sodium and potassium transport in herpes simplex virus-infected cells. J Gen Virol 60:199–207.

Hartley C, Hartley M, Pardoe I, Knight A. 2006. Ionic Contra-Viral Therapy (ICVT); a new approach to the treatment of DNA virus infections. Arch Virol 151:2495–2501.

Hartley CE, Buchan A, Randall S, Skinner GR, Osborne M, Tomkins LM. 1993. The effects of lithium and potassium on macromolecular synthesis in herpes simplex virus-infected cells. J Gen Virol 74:1519–1525.

Holt EW, Guy J, Gordon SM, Hofmann JC, Garcia-Kennedy R, Steady SL, Bzowej NH, Frederick RT. 2013. Acute liver failure caused by herpes simplex virus in a pregnant patient: Is there a potential role for therapeutic plasma exchange? J Clin Apher 28:426–429.

Kavouras JH, Prandovszky E, Valyi-Nagy K, Kovacs SK, Tiwari V, Kovacs M, Shukla D, Valyi-Nagy T. 2007. Herpes simplex virus type 1 infection induces oxidative stress and the release of bioactive lipid peroxidation by-products in mouse P19N neural cell cultures. J Neurovirol 13:416–425.

Koelle DM, Corey L. 2008. Herpes simplex: Insights on pathogenesis and possible vaccines. Annu Rev Med 59:381–395.

Loetchutinat C, Kothan S, Dechsupa S, Meesungnoen J, Jay-Gerin JP, Mankhetkorn S. 2005. Spectrofluorometric determination of intracellular levels of reactive oxygen species in drug-sensitive and drug-resistant cancer cells using the 20,70-dichlorofluorescein diacetate assay. Radiat Phys Chem 72:323–331.

Loreto ES, Mario DA, Santurio JM, Alves SH, Nogueira CW, Zeni G. 2011. In vitro antifungal evaluation and structure-activity relationship of diphenyl diselenide and synthetic analogues. Mycoses 54:e572–e576.

Mathew SS, Bryant PW, Burch AD. 2010. Accumulation of oxidized proteins in Herpesvirus infected cells. Free Radic Biol Med 49:383–391.

Mi SF, Li Y, Yan JH, Gao GF. 2010. Na+/K+-ATPase β1 subunit interacts with M2 proteins of influenza A and B viruses and affects the virus replication. Sci China Life Sci 53:1098–1105.

Milligan GN, Bernstein DI. 1995. Generation of humoral immune responses against herpes simplex virus type 2 in the murine female genital tract. Virology 206:234–241.

Milligan GN, Meador MG, Chu CF, Young CG, Martin TL, Bourne N. 2005. Long-term presence of virus-specific plasma cells in sensory ganglia and spinal cord following intravaginal inoculation of herpes simplex virus type 2. J Virol 79:11537–11540.

Misra HP, Fridovich I. 1972. The role of superoxide anion in the autoxidation of epinephrine and a simple assay for superoxide dismutase. J Biol Chem 247:3170–3175.

Nagai Y, Maeno K, Iinuma M, Yoshida T, Matsumoto T. 1972. Inhibition of virus growth by ouabain: Effect of ouabain on the growth of HVJ in chick embryo cells. J Virol 9:234–243.

Nencioni L, Sgarbanti R, Amatore D, Checconi P, Celestino I, Limongi D, Anticoli S, Palamara AT, Garaci E. 2011. Intracellular redox signaling as therapeutic target for novel antiviral strategy. Curr Pharm Des 17:3898–3904.

Nogueira C, Rocha J. 2010. Diphenyl diselenide a janus-faced molecule. J Braz Chem Soc 21:2055–2071.

Palu G, Biasolo MA, Sartor G, Masotti L, Papini E, Floreani M, Palatini P. 1994. Effects of herpes simplex virus type 1 infection on the plasma membrane and related functions of HeLa S3 cells. J Gen Virol 75:3337–3344.

Paulmier C. 1986. Selenoorganic functional groups. In: Oxford PP, editor. Selenium reagents and intermediates in organic Q3synthesis. pp 25–51.

Peterhans E. 1997. Oxidants and antioxidants in viral diseases: Disease mechanisms and metabolic regulation. J Nutr 127:962S–965S.

Podlech J, Hengerer F, Fleck M, Walev I, Falke D. 1996. Replication of herpes simplex virus type 1 and 2 in the medulla of the adrenal gland after vaginal infection of mice. Arch Virol 141:1999–2008.

Riediger C, Sauer P, Matevossian E, Muller MW, Buchler P, Friess H. 2009. Herpes simplex virus sepsis and acute liver failure. Clin Transplant 21:37–41.

Sanjuan NA, Zimberlin MN. 2001. Pathogenesis of herpes simplex virus type 2 experimental genital infection in pregnant mice. FEMS Immunol Med Microbiol 30:197–202.

Sartori G, Jardim NS, Sari MH, Dobrachinski F, Pesarico AP, Rodrigues LC, Jr., Cargnelutti J, Flores EF, Prigol M, Nogueira CW. 2015. Antiviral action of diphenyl diselenide on herpes simplex virus 2 infection in female BALB/c Q4mice. J Cell Biochem.

Sartori G, Pesarico AP, Pinton S, Dobrachinski F, Roman SS, Pauletto F, Rodrigues LC, Jr., Prigol M. 2012. Protective effect of brown Brazilian propolis against acute vaginal lesions caused by herpes simplex virus type 2 in mice: Involvement of antioxidant and anti-inflammatory mechanisms. Cell Biochem Funct 30:1–10.

Schaefer A, Kuhne J, Zibirre R, Koch G. 1982. Poliovirus-induced alterations in HeLa cell membrane functions. J Virol 44:445–449.

Scheller J, Ohnesorge N, Rose-John S. 2006. Interleukin-6 trans-signalling in chronic inflammation and cancer. Scand J Immunol 63:321–329.

Spear PG, Roizman B. 1972. Proteins specified by herpes simplex virus. V. Purification and structural proteins of the herpesvirion. J Virol 9:143–159.

Sunnetcioglu M, Karadas S, Aslan M, Ceylan MR, Demir H, Oncu MR, Karahocagil MK, Sunnetcioglu A, Aypak C. 2014. Serum adenosine deaminase activity in cutaneous anthrax. Med Sci Monit 20:1151–1154.

Tawadrous GA, Aziz AA, Amin DG, Eldemery A, Mostafa MA. 2012. RANTES, TNF-alpha, oxidative stress, and hematological abnormalities in hepatitis C virus infection. J Investig Med 60:878–882.

Venturini TP, Chassot F, Loreto ES, Keller JT, Azevedo MI, Zeni G, Santurio JM, Alves SH. 2016. Antifungal activities of diphenyl diselenide and ebselen alone and in combination with antifungal agents against Fusarium Q5spp. Med Mycol.

White WM, Tran D, Garovic VD, Brost B. 2011. Acute interstitial nephritis proteinuria and herpes simplex virus hepatitis in pregnancy mimic HELLP syndrome (hemolysis, elevated liver enzymes, low platelets). AJP Rep 1:115–118.

Williams BB, Lerner AM. 1975. Antiviral activity of an adenosine deaminase inhibitor: Decreased replication of herpes simplex virus. J Infect Dis 131:673–677.

SUPPORTING INFORMATION

Additional supporting information may be found in the online version of this article at the publisher's web-site.

3.3 Manuscrito 1

Disseleneto de difenila reduz a replicação viral por HSV-1 em culturas de células gliais

Diphenyl Diselenide reduces HSV-1 replication in culture glial cells

Gláubia Sartori[1,2], Simone Agostini[1], Roberta Mancuso[1], Ivana Marventano[1], Marina Saresella[1], Mario Clerici[1,4], Marina Prigol[3],Cristina W. Nogueira[2*]

[1] Laboratory of Molecular Medicine and Biotechnologies, Don Carlo Gnocchi Foundation – ONLUS, Milan, Italy.

[2]Laboratory of Synthesis, Reactivity and Pharmacological Evaluation and Toxicology of Organochalcogens, Department of Biochemistry and Molecular Biology, Federal University of Santa Maria, Santa Maria, CEP 97105-900, RS, Brazil.

[3] Department of Nutrition, Federal University of Pampa, Itaqui campus, Itaqui, CEP 97650-000, RS, Brazil.

[4]Department of Physiopathology and Transplantation, University of Milan, Milan, Italy.

[*]Correspondence should be sent to:

Cristina W. Nogueira

Laboratory of Synthesis, Reactivity and Pharmacological Evaluation and Toxicology of Organochalcogens, Department of Biochemistry and Molecular Biology, Federal University of Santa Maria, Santa Maria, CEP 97105-900, RS, Brazil.

E-mail: criswn@ufsm.br

ABSTRACT

Herpes simplex virus type 1 (HSV-1) infection is prevalent worldwide as this virus is a common pathogen. The best known treatment is acyclovir although an increase in drug resistance has been observed. Because of these considerations it is important to develop new anti-HSV agents; diphenyl diselenide, $(PhSe)_2$, was selected for this study. The organoselenium $(PhSe)_2$ has been reported to have antiviral action against HSV-2 and *in vivo* and *in vitro* antioxidant and anti-inflammatory properties. In the first phase of the study, the *in vitro* MTT assay investigated the cytotoxic effect of $(PhSe)_2$ (0.1 to 100 μM). The *in vitro* antiviral action of $(PhSe)_2$ against HSV-1 (MOI 0.0001 and MOI 0.001) was examined in astrocytomas at 4 h and 24 h post infection using plaque reduction assay (supernatant) and real-time PCR (supernatant and cells). It was also investigated the cytokines expression by PCR (TNF-α and NFK-β) to delineate the effect of selenium compound on inflammation process during infection. In the MTT assay, $(PhSe)_2$ was cytotoxic only at higher concentrations (50 and 100 μM). Results showed a potent antiviral action of $(PhSe)_2$ at 15 μM in both viral concentrations tested; for plaque inhibition, HSV-1 titers were significantly reduced in supernatant and viral DNA copies were also significantly reduced in supernatant and cells. $(PhSe)_2$ inhibited viral replication at different steps of the cycle; reduced the gene expression of viral proteins ICP27, ICP8, ICP0 and viral DNA polymerase. $(PhSe)_2$ modulated the TNF-α expression in HSV-1 infected cells, showing an immunomodulatory effect. We demonstrate that in cultured astrocytomas $(PhSe)_2$ inhibited HSV-1 replication through interference of viral replication steps and modulation of cytokines production.

KEYWORDS: organoselenium; Herpes simplex; cytokines; antiviral; astrocytes.

INTRODUCTION

Herpes simplex virus (HSV) types 1 and 2 are largely prevalent human neurotropic pathogens that cause a variety of diseases, including encephalitis (HSE) with high mortality and neurological morbidity. Members of the *Herpesviridae* family, they establish latency in the sensory ganglia of the peripheral nervous system. In stressful conditions, the virus can reactivate and return to the oral cavity causing lesions (cold sores) and transmission to new hosts (Fatahzadeh and Schwartz, 2007, Weller and Coen, 2012).

The initial attachment of HSV-1 to the cell surface is mediated by glycoproteins B (gB) and C (gC). Viruses are parasitic pathogens that replicate in living cells, exploiting multiple intracellular pathways in their hosts for their own advantage and growth. Consequently, although the severity and outcome of viral infections are indisputably conditioned by host factors like immune system functionality, age, general health, and pharmacological treatments, the metabolic conditions in the cell where the virus replicates can have important repercussions on different steps of the virus life-cycle (Mettenleiter, 2002).

Virus-host interactions are important for the outcome of infections. The final outcome of a viral infection depends on a delicate regulation and timing of these antiviral effector mechanisms in response to the invading virus (Viejo-Borbolla et al., 2012). Indeed, brain inflammation due to infection, aging, and other deleterious processes is associated with activation of the local innate immune system. This could be an important mechanism leading to the neuronal damage observed in different central nervous system (CNS) diseases (Martin et al., 2014).

HSV-1 has been linked to the possible etiology or development of neurodegenerative diseases and virus induced demyelination, such as Alzheimer disease (AD)(Santana et al., 2012, Mancuso et al., 2014, Piacentini et al., 2014). It was reported that HSV-1 reactivates in the CNS and causes a productive infection, perhaps recurrently. It also might periodically reactivate in brain during episodes of stress, immunosuppression or inflammation, causing cumulative damages, including those occurring on DNA (Wozniak et al., 2009, De Chiara et al., 2016).

The most common anti-herpetic compounds in clinical use are nucleoside analogues, such as acyclovir (Jiang et al., 2016). However, an increase in drug resistance mainly in immunocompromised patients can occur after long-term use (Strasfeld and Chou, 2010, Rechenchoski et al., 2016). Thus, the discovery of new non-nucleoside antiviral compounds is a significant alternative to investigate. Supporting this possibility, we have recently reported the *in vitro* and *in vivo* antiviral activity of $(PhSe)_2$ against HSV-2 infection (Sartori et al., 2016a). Our research group demonstrated a reduced viral load in the vaginal tissues of mice related to immunomodulatory, antioxidant and anti-inflammatory properties of (PhSe)2 (Sartori et al., 2016a).

Considering the role of specific and non-specific host defenses against HSV-1, some therapies can work to strengthen and activate the innate and specific immune system components against the virus (Viejo-Borbolla et al., 2012). The aim of this study was to investigate the antiviral action of a selenium compound against HSV-1 replication in human neural culture cells. The relevance of this study is based on the severity of viruses to cause sporadic encephalitis, which even with traditional antiviral therapy, can result in irreversible neurological defects.

MATERIALS AND METHODS

Chemicals

$(PhSe)_2$ was prepared and characterized based on a method carried out by (Paulmier, 1986). Analysis of the ^{1}H nuclear magnetic resonance (NMR) and ^{13}C NMR spectra showed that $(PhSe)_2$ displayed analytical and spectroscopic data in full agreement with its assigned structure. The chemical purity of this organoselenium compound (99.9%) was determined by gas chromatography-mass spectrometry (Shimadzu QP2010PLUS GC/MS combination) and it was stable under storage conditions at room temperature, humidity and light. All other chemicals were obtained of analytical grade or from standard commercial suppliers.

$(PhSe)_2$ was diluted in 0.6% (v/v) dimethyl sulfoxide (DMSO) in the culture medium at different concentrations 0.1 μM; 1 μM; 5 μM; 15 μM; 30 μM; 50 μM and 100 μM.

Cell culture, virus production and titration

The HSV-1(clinical isolate) was amplified and quantitated in Vero cells (*African Green monkey kidney* cells, ATCC CCL81). Vero cells were grown in Dulbecco's modified Eagle medium (DMEM) containing 2 mM L-glutamine and 1 % penicillin (Euroclone, Pero, Milano, Italy), supplemented with 10% fetal bovine serum (FBS) and maintained in incubator with CO_2 at 5% and 37 °C. For virus amplification, monolayers of Vero cells were infected with multiplicity of infection of 0.01 (1 plaque formation units (PFU) per 100 cells) of HSV-1 and incubated at 37 °C with CO_2 at5%. Seventy-two hours after, the infected cells were submitted to freeze-thaw and the viral suspension was aliquoted and stored at -80 ^{0}C. The virus quantitation was performed in duplicate using plates of six wells. For this, the original virus was submitted to limiting dilution (10^{-1} to 10^{-6}) and 400 μL of each dilution were inoculated on Vero cells and incubated at 37 °C

in 5% CO_2 for 1h and 30 min. After that, the inoculum was removed and 3 mL of DMEM plus 2% low-melting point agarose were added to each well. After 72 h, viral titers were calculated using plaque-forming unit/ml (PFU/mL) method (Spear and Roizman, 1972). For the $(PhSe)_2$ antiviral action analysis, human astrocytoma cell line 1321N1 was used and grown in DMEM containing 2 mM L-glutamine and 1 % penicillin (Euroclone), supplemented with 10% FBS and maintained in incubator with CO_2 at 5% and 37 °C.

Cellular toxicity

The viability of astrocytoma cells treated with $(PhSe)_2$ was determined by MTT 3-(4,5- dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay following Mosman protocol (Mosmann, 1983). Briefly, cells were grown in 96-well microplates ($1x10^5$ cells per well) till confluence and the media was replaced by DMEM plus FBS 10% supplemented with $(PhSe)_2$ at different concentrations (0.1 μM;1 μM; 5 μM; 15 μM; 30 μM; 50 μM and 100 μM). Cells were incubated at 37 °C with CO_2 at 5% by 48h. After this time, the compound with old medium was replaced by new DMEM plus 10% FBS and 20 μL (5mg/mL in phosphate-buffered saline, PBS) of ready MTT dye was added in each well and mixed in shaker for 1-2 min at 50-60 rpm. The plate was incubated at 37 °C for 4 h, the medium was carefully aspirated and the cells suspended in 200 μL of dimethyl sulfoxide (DMSO). Then, it was mixed in shaker for 5 min at 50-60 rpm. The optical density of each well was determined at 620 nm. Results were expressed by percentage of viable cells calculated in comparison to control cells as the average of four independent experiments.

Virus infection

Astrocytoma cells were incubated with HSV-1 MOI 0.0001 (82 PFU/mL) and MOI 0.001 (820 PFU/mL) (MOI: multiplicity of infection) for 2 h in serum-free DMEM, washed with medium and incubated in DMEM supplemented with 10% FBS plus $(PhSe)_2$ at 15 μM for 2 h and 22 h of treatment at 37 °C with CO_2 at 5%. Then, after 4 h and 24 h of infection, the supernatants of cells and the cells were trypsinized and collected to perform the analysis. Negative controls (composed by DMEM and vehicle 0.6% DMSO) were included in all experiments.

Plaque reduction assay

The antiviral action of $(PhSe)_2$ against HSV-1 (MOI 0.0001 and MOI 0.001) was examined in supernatants of culture cells 24 h post infection using the plaque reduction assay. The samples were submitted to serial dilutions (10^{-1} – 10^{-6}) and aliquots of infected cells and $(PhSe)_2$ treated and infected cells were inoculated on Vero cells for 1h 30 min at 37 °C with CO_2 at 5%. Then, the cells were washed and overlaid with 3 mL of DMEM containing 2% low-melting point agarose and 10% of FBS for 72 h of incubation period at 37 °C, CO_2 at 5%. After this time, these cells were submitted to fixation using 10% formaldehyde and stained with crystal violet 0.05% for 2 h at room temperature. Finally, the crystal violet was removed and plaque counting was performed (by microscope). The viral titers were calculated using the PFU/mL method (Spear and Roizman, 1972).

DNA isolation and detection of HSV-1 viral load by quantitative polymerase chain reaction (qPCR)

DNA was extracted from cell lysates or supernatants of culture cells. DNA was extracted using the spin-columns technique (Nucleospin RNA Virus, Macherey-Nagel,

Duren, Germany) according to the manufacturer instructions. DNA concentrations were measured using spectrophotomer (OD: 260 nm). qPCR was carried out on real time PCR system (Step One, Life Technologies, Foster City, CA) in 25µL of reaction mix containing TaqMan universal master mix (Life Technologies). The HSV-1 DNA polymerase gene (UL30) was amplified to estimate the amount of viral DNA in each sample; for primers and probe, see Table 1. Standard curves were generated using purified HSV-1 DNA. The quantification results were expressed as viral DNA copy numbers per nanogram of genomic DNA. All experiments were performed in triplicate.

RNA isolation, cDNA retrotranscription

Total mRNA was extracted from infected astrocytomas cells after 2 h and 22 h of $(PhSe)_2$ treatment. Total RNA was extracted using the RNA easy Mini extraction kit (Qiagen, Hilden, Germany) and eluted in RNAse-Free water. Total RNA concentration was determined by spectrophotometer (OD: 260 nm). Purity was determined as the 260 nm/280 nm OD ratio with expected values between 1.8 and 2.0. RNA was treated with TURBO DNA free DNAse (Ambion INC, Austin, TX, USA) and retro transcribed with High-capacity cDNA Reverse Transcription Kit (Applied Biosystems), as specified by manufacturers. cDNA samples were stored at –20 $^{\circ}$C until use.

Quantitative PCR (qPCR)

qPCR amplification was performed on real time PCR system (ABI Prism 7000 instrument, Applied Biosystems) in 25µL of reaction mix containing TaqMan universal master mix (Life Technologies), and specific primers and probe for HSV-1 gene (RL2, UL29, UL30 and UL54) (Table 1), and specific TaqMan gene expression assays (Life

Technologies) for human cytokine genes, TNF-α (ID: Hs01113624_g1) and NFK-β (ID: Hs00765730_m1). As reference gene, YWHAZ (Life Technologies, ID: Hs03044281_g1) for viral genes, whereas HPRT for cytokine genes. For HPRT, qPCR assay was designed using the Universal Probe Library (UPL) system (Roche Diagnostic, Mannheim, Germany). Gene-specific primers and the most appropriate universal probe for HPRT transcripts were defined with the ProbeFinder software (Roche Diagnostic). In particular, the probe was #34, whereas the primers were: Forward primer: 5'- TGA CCT TGA TTT ATT TTG CAT ACC -3'; Reverse primer: 5'- CGA GCA AGA CGT TCA GTG CT -3'. Each cDNA template was tested in triplicate by qPCR, with non-template control for each session. For each sample, relative expression of the target mRNA was calculated in relation with the appropriate endogenous reference (ΔCq_{sample}= Cqtarget – Cq_{ref}). Viral gene expression levels are presented as normalization ratio calculated as: fold: $2^{-[\Delta Cq(\text{HSV-1 infected and (PhSe)2 treated cells}) - \Delta Cq(\text{HSV-1 infected cells})]}$. Cytokine genes expression levels are presented as normalization ratio calculated as: fold: $2^{-[\Delta Cq(\text{control cells}) - \Delta Cq(\text{HSV-1 infected cells or HSV-1 infected and (PhSe)2 treated cells})]}$(Fleige et al., 2006).

Statistical Analysis

The Student's t-test was used to analyze HSV-1 viral load data. One-way analysis of variance (ANOVA) was performed followed by post-hoc comparisons using the Newman–Keuls's multiple comparison test when appropriate for cellular toxicity, cytokines and viral genes expression analyses. All results were analyzed and approved by the D'Agostino and Pearson omnibus normality test. Data are expressed as the mean(s) ± S.E.M and analyzed by Graph Pad Prism (version 6). Differences between groups were considered statistically significant when $P< 0.05$.

RESULTS

$(PhSe)_2$ effects on cellular toxicity

MTT assay showed an effect of $(PhSe)_2$ on astrocytes viability only at higher concentrations (50 and 100 μM) ($p< 0.0001$). Based on these data a concentration of 15 μM was chosen for antiviral assays (Figure 1).

$(PhSe)_2$ effects on plaque formation inhibition and HSV-1 DNA synthesis reduction

The antiviral action of $(PhSe)_2$ against HSV-1 (MOI 0.0001 and MOI 0.001) was examined in astrocytes during 24 h post infection. Thus, for plaque inhibition, HSV-1 titers were significantly reduced in supernatants of cells in both virus concentrations ($p< 0.05$) (Figures 2A and 2B). Notably, viral DNA copies were also significantly reduced in supernatants (MOI 0.0001 and MOI 0.001 $p< 0.05$) (Figures 3A and 3B) and cells (MOI 0.0001 $p<0.05$; MOI 0.001 $p< 0.01$) (Figures 3C and 3D).

$(PhSe)_2$ treatment reduces genes expression of viral replication

Infected cells were collected in two distinct times of analysis to identify in which step the compound was effective to inhibit viral replication. At 4 and 24 h p.i., $(PhSe)_2$ treatment reduced ICP27 gene expression at the lowest MOI 0.0001 ($p< 0.01$); however, at MOI 0.001 the decrease was statistically significant only at 4 h p.i.($p< 0.05$) (Figure 4A). As illustrated in figure 4B, the compound inhibited ICP0 expression at 24 h p.i. at MOI 0.0001 ($p< 0.05$). Indeed, treatment with $(PhSe)_2$ reduced ICP8 early gene expression at two periods of analysis and in both virus concentrations ($p< 0.001$) (Figure 4C). Finally, it was also observed a statistical significant decrease of viral DNA polymerase expression at 24 h p.i. in $(PhSe)_2$-treated infected cells in the lowest viral concentration MOI 0.0001 ($p< 0.001$) (Figure 4D).

$(PhSe)_2$ treatment modulates cytokines expression during HSV-1 infection

It was examined the cytokines expression by PCR (TNF-α and NFK-β) to delineate the effect of selenium compound on inflammation process during HSV-1 replication. By comparing infected and non-infected cells, $(PhSe)_2$ treatment was effective to modulate TNF-α expression at 4 h and 24 h post-infection being significantly different ($p< 0.001$) (Figure 5Ai and 5Aii) at MOI 0.0001. Additionally, at MOI 0.001 it was observed a similar situation 4 h p.i. ($p< 0.01$) (Figure 5Bi). Results also showed that treatment with $(PhSe)_2$ partially reduced TNF-α expression at 24 h p.i. the (Figure 5Bii) being statistically different from infected and non-infected cells ($p< 0.001$). In relation to NFK-β expression, the data demonstrated a significant difference among groups only 4 h p.i. at MOI 0.0001 ($p< 0.01$) while infected and $(PhSe)_2$ treated infected cells had minor cytokine expression (Figure 6Ai). The remaining results did not show alterations (Figures 6Aii, 6Bi and 6Bii).

DISCUSSION

Herpesviruses generally infect their hosts throughout life, after the initial infection of epithelial cells, the virions can spread to CNS and establish latent infections in sensory ganglia (Mitchell et al., 2003). In some cases, the virus causes encephalitis or meningitis, implicating a complex process (Whitley, 2006, Reske et al., 2007, Heldwein and Krummenacher, 2008).

In this study we demonstrated the antiviral action of $(PhSe)_2$ against HSV-1 *in vitro*; by inhibiting specific steps of virus life-cycle and modulating the immune system. In particular, $(PhSe)_2$ inhibited plaque formation when added after infection as well as HSV-1 DNA synthesis in both viral concentrations (24 h p.i.). Thus, this result further

confirm that $(PhSe)_2$ reduced the viral load intra and extracellular, indicating that astrocytoma culture cells are entirely permissive for the HSV-1 replicative cycle. We previously demonstrated that this organoselenium compound has antiviral action in VERO culture cells and by reducing the viral load in infected mice against HSV-2 (Sartori et al., 2016a). Moreover, our recent investigation showed that $(PhSe)_2$ decreases oxidative stress and systemic toxicity caused by HSV-2 infection in mice (Sartori et al., 2016b).

In the early phase of the lytic replication cycle, the IE-gene products, besides being transcription factors for the next wave of viral proteins, intimately regulate cellular functions in favor of viral replication and immune limitation (Koelle and Corey, 2003). We then assessed whether $(PhSe)_2$ could affect genes expression of viral replication such as ICP27, ICP0, ICP8 and viral DNA polymerase. In general, the organoselenium controlled the viral replication but the most significant effect was related to reduction of ICP8 early gene expression at 4 h and 24 h p.i. in both virus concentrations. ICP8, encoded by the UL29 gene identified as the major HSV single-strand DNA-binding protein (SSB), participates in viral DNA synthesis, control of viral gene expression, the formation of pre-replicative sites and replication compartments (Weller and Coen, 2012). The reduction of ICP8, an early gene encoding the DNA binding protein, by $(PhSe)_2$ prevents DNA replication and true late-gene expression. Immediate-early gene products in turn activate the transcription of early genes, which are expressed prior to DNA replication. We found that $(PhSe)_2$ decreased the immediate-early genes expression, ICP27 and ICP0 at 24 h p.i at a lower viral concentration. Interestingly, it was not found ICP0 gene expression at 4 h p.i. in our study in both viral concentrations, this result is probably explained by the time of sample collection and the type of culture cells used in our study model. All together, these data indicate that $(PhSe)_2$ inhibited specific steps of

viral replication, and suggest that late stages of viral replication could be also blocked by a long treatment. Thus, we can hypothesize that $(PhSe)_2$ may inhibit the expression of late proteins, by either preventing their transcription or acting on some cell pathway responsible for protein translation or for post-translational cascades.

Indeed, the expression of all viral proteins was reduced when the compound was added to infected cells for a long time (24 h incubation) at lower viral concentration whereas lower inhibition of cytokine protein synthesis was found when $(PhSe)_2$ was added for short treatment (4 h p.i.) for almost all viral concentrations.

In the early hours of a viral infection, the cytokines produced by cells infected or coming into contact with viral products are vital in conduction of the innate immune response to the infection. Thus, we investigated the expression of TNF-α, INF-γ and NF-κB. It is well established that HSV- 1 induces NF-κB activation in different cell types (Santoro et al., 2003). The scientific literature data indicate that the beginning of the replication cycle and the synthesis of viral proteins are necessary for HSV-1-dependent NF-κB activation (Amici et al., 2001, Mogensen and Paludan, 2001). In special, two IE proteins, ICP4 and ICP27 (encoded by the UL54 gene), are important for NF-κB nuclear translocation (Ren et al., 2011), however, the signaling pathway used by the virus to activate the factor has still not been defined. Other early studies from Hargett et al. demonstrated that ICP27 was important for activation of NF-κB (Hargett et al., 2006) as well as for activation of JNK and P38 (Hargett et al., 2005) in HSV-1infected cells.

NF-κB plays an important function in virus-dependent cytokine expression and pathology (Mikloska et al., 1998, Patel et al., 1998). Interestingly, we did not observe a persistent activation of NF-kB expression by HSV-1 in astrocytomas as well as in $(PhSe)_2$ treated infected cells, however the TNF-α expression was increased in HSV-1 cells. Recently, it was discovered that HSV-1 has developed different mechanisms to attenuate

host antiviral elements and facilitate its infection (Su et al., 2016).

An study with HEK293T and Vero cells reported that HSV-1 infection led to suppression of NF-κB activity (Wang et al., 2014) and consequently diminished TNF-α production, which was also described by other members of the herpesvirus family, including Epstein-Barr virus (EBV) (Sylla et al., 1998), varicella-zoster vírus (VZV) (Jones and Arvin, 2006) and Kaposi's sarcoma-associated herpesvirus (KSHV) (Seo et al., 2004). In our study, we believed that the increased TNF-α expression may occur through another signaling pathway activated during infection, such the MAPKs pathway (mitogen-activated protein kinases) as demonstrated by other authors (McLean and Bachenheimer, 1999, Chen et al., 2015).

Our results corroborate with this teory, it was not observed activation of NF-κB in the cells treated with $(PhSe)_2$ 4 h and 24 h after infection and there was still a reduction of TNF-α expression. These findings indicate that the compound exerted some modulation of the immune system, acting upstream signaling of NF-κB pathways or via inhibition of MAPK. A recent study reported the important activation of NF-κB and MAPKs during HSV infection (Chen et al., 2015). Indeed, these data are confirmed by reduced viral load, less synthesis of viral DNA and lower viral proteins expression in astrocytomas treated with $(PhSe)_2$.

On primary HSV infections, the host cells produce a range of cytokines, that include interleukin-1β (IL-1β), IL-2, IL-6, IL-10, IL-12, tumor necrosis factor alpha (TNF-α), IFN-α/β, IFN-γ, and granulocyte-macrophage colony-stimulating factor (GM-CSF) (Mogensen and Paludan, 2001). Astrocytes are neuroectoderm-derived cells and one of the primary sources of cytokines in the CNS (Jack et al., 2005, Haim and Rowitch, 2017). In addition, cytokines and neurotrophic factors are secreted by astrocytes to regulate the immune response (Wilson et al., 2010). However, whether astrocytes are

linked to HSV-1 infection and how they act as immune cells during infection is unclear.

Furthermore, it was not observed IFN-γ gene expression in infected astrocytoma cells in two times of collection in all viral concentrations. It was previously reported by Wang and cols. that HSV-1 protein kinase US3 was a potent inhibitor of IFN response *in vitro*, serving as one of several strategies used by HSV-1 to interrupt the innate immune system (Wang et al., 2014). Another study revealed that HSV infection in dorsal root ganglia (DRG) neuron cultures results in little to no IFN production (Yordy et al., 2012) and infected neuronal cell lines supported restricted transcriptional upregulation of IFN-β and interferon-stimulated genes (ISGs). Perhaps, the absence of IFN-γ expression in our experimental model could be explained by one of these evidences.

In addition, it was already demonstrated that $(PhSe)_2$ has a notable antioxidant activity against HSV-2 infection in mice (Sartori et al., 2016a, Sartori et al., 2016b). In this way, we suggest that $(PhSe)_2$ may interfere with some pathways sensitive to redox changes important for late stages of virus life-cycle in HSV-1 infected cells (Palamara et al., 2004) and also contribute to the immune system response.

In summary, this study demonstrated that astrocytomas was able to play an antiviral role by innate imune system activation when infected with HSV-1, enhancing NF-kB activation and TNF-α production as reported by other authors (Liu et al., 2013) and $(PhSe)_2$ treatment reduced the mRNA expression of TNF-α, in accordance to reduced viral proteins expression and viral load.

Regarding the effect of $(PhSe)_2$ against HSV-2 infection *in vitro* and *in vivo* and also against HSV-1 in culture neuron cells, it could be useful as a potential antiviral drug to treat not only HSV infections but also associated to HSV-1 as a risk factor for pathogenesis of Alzheimer's disease .

Acknowledgments

The financial support by FAPERGS, CAPES, and CNPq is gratefully acknowledged.

Conflict of Interest statement

The authors declare that there are no conflicts of interest.

REFERENCES

Amici C, Belardo G, Rossi A, Santoro MG (2001) Activation of I kappa b kinase by herpes simplex virus type 1. A novel target for anti-herpetic therapy. The Journal of biological chemistry 276:28759-28766.

Chen D, Su A, Fu Y, Wang X, Lv X, Xu W, Xu S, Wang H, Wu Z (2015) Harmine blocks herpes simplex virus infection through downregulating cellular NF-kappaB and MAPK pathways induced by oxidative stress. Antiviral research 123:27-38.

De Chiara G, Racaniello M, Mollinari C, Marcocci ME, Aversa G, Cardinale A, Giovanetti A, Garaci E, Palamara AT, Merlo D (2016) Herpes Simplex Virus-Type1 (HSV-1) Impairs DNA Repair in Cortical Neurons. Frontiers in aging neuroscience 8:242.

Fatahzadeh M, Schwartz RA (2007) Human herpes simplex virus infections: epidemiology, pathogenesis, symptomatology, diagnosis, and management. Journal of the American Academy of Dermatology 57:737-763; quiz 764-736.

Haim LB, Rowitch DH (2017) Functional diversity of astrocytes in neural circuit regulation. Nature reviews Neuroscience 18:31-41.

Hargett D, McLean T, Bachenheimer SL (2005) Herpes simplex virus ICP27 activation of stress kinases JNK and p38. Journal of virology 79:8348-8360.

Hargett D, Rice S, Bachenheimer SL (2006) Herpes simplex virus type 1 ICP27-dependent activation of NF-kappaB. Journal of virology 80:10565-10578.

Heldwein EE, Krummenacher C (2008) Entry of herpesviruses into mammalian cells. Cellular and molecular life sciences : CMLS 65:1653-1668.

Jack CS, Arbour N, Manusow J, Montgrain V, Blain M, McCrea E, Shapiro A, Antel JP (2005) TLR signaling tailors innate immune responses in human microglia and astrocytes. Journal of immunology 175:4320-4330.

Jiang YC, Feng H, Lin YC, Guo XR (2016) New strategies against drug resistance to herpes simplex virus. International journal of oral science 8:1-6.

Jones JO, Arvin AM (2006) Inhibition of the NF-kappaB pathway by varicella-zoster virus in vitro and in human epidermal cells in vivo. Journal of virology 80:5113-5124.

Koelle DM, Corey L (2003) Recent progress in herpes simplex virus immunobiology and vaccine research. Clinical microbiology reviews 16:96-113.

Liu Z, Guan Y, Sun X, Shi L, Liang R, Lv X, Xin W (2013) HSV-1 activates NF-kappaB in mouse astrocytes and increases TNF-alpha and IL-6 expression via Toll-like receptor 3. Neurological research 35:755-762.

Mancuso R, Baglio F, Agostini S, Cabinio M, Lagana MM, Hernis A, Margaritella N, Guerini FR, Zanzottera M, Nemni R, Clerici M (2014) Relationship between herpes simplex virus-1-specific antibody titers and cortical brain damage in Alzheimer's disease and amnestic mild cognitive impairment. Frontiers in aging neuroscience 6:285.

Martin C, Aguila B, Araya P, Vio K, Valdivia S, Zambrano A, Concha MI, Otth C (2014) Inflammatory and neurodegeneration markers during asymptomatic HSV-1 reactivation. Journal of Alzheimer's disease : JAD 39:849-859.

McLean TI, Bachenheimer SL (1999) Activation of cJUN N-terminal kinase by herpes simplex virus type 1 enhances viral replication. Journal of virology 73:8415-8426.

Mettenleiter TC (2002) Herpesvirus assembly and egress. Journal of virology 76:1537-1547.

Mikloska Z, Danis VA, Adams S, Lloyd AR, Adrian DL, Cunningham AL (1998) In vivo production of cytokines and beta (C-C) chemokines in human recurrent herpes simplex lesions--do herpes simplex virus-infected keratinocytes contribute to their production? The Journal of infectious diseases 177:827-838.

Mitchell BM, Bloom DC, Cohrs RJ, Gilden DH, Kennedy PG (2003) Herpes simplex virus-1 and varicella-zoster virus latency in ganglia. Journal of neurovirology 9:194-204.

Mogensen TH, Paludan SR (2001) Molecular pathways in virus-induced cytokine production. Microbiology and molecular biology reviews : MMBR 65:131-150.

Mosmann T (1983) Rapid colorimetric assay for cellular growth and survival: application to proliferation and cytotoxicity assays. J Immunol Methods 65:55-63.

Palamara AT, Brandi G, Rossi L, Millo E, Benatti U, Nencioni L, Iuvara A, Garaci E, Magnani M (2004) New synthetic glutathione derivatives with increased antiviral activities. Antiviral chemistry & chemotherapy 15:83-91.

Patel A, Hanson J, McLean TI, Olgiate J, Hilton M, Miller WE, Bachenheimer SL (1998) Herpes simplex type 1 induction of persistent NF-kappa B nuclear translocation increases the efficiency of virus replication. Virology 247:212-222.

Paulmier C (1986) Selenoorganic functional groups. In: Selenium reagents and intermediates in organic synthesis, pp 25–51 Oxford: Pergamon Press.

Piacentini R, De Chiara G, Li Puma DD, Ripoli C, Marcocci ME, Garaci E, Palamara AT, Grassi C (2014) HSV-1 and Alzheimer's disease: more than a hypothesis. Frontiers in pharmacology 5:97.

Rechenchoski DZ, Faccin-Galhardi LC, Linhares RE, Nozawa C (2016) Herpesvirus: an underestimated virus. Folia microbiologica.

Ren Z, Li S, Wang QL, Xiang YF, Cui YX, Wang YF, Qi RB, Lu DX, Zhang SM, Zhang PZ (2011) Effect of siRNAs on HSV-1 plaque formation and relative expression levels of RR mRNA. Virologica Sinica 26:40-46.

Reske A, Pollara G, Krummenacher C, Chain BM, Katz DR (2007) Understanding HSV-1 entry glycoproteins. Reviews in medical virology 17:205-215.

Santana S, Recuero M, Bullido MJ, Valdivieso F, Aldudo J (2012) Herpes simplex virus type I induces the accumulation of intracellular beta-amyloid in autophagic compartments and the inhibition of the non-amyloidogenic pathway in human neuroblastoma cells. Neurobiology of aging 33:430 e419-433.

Santoro MG, Rossi A, Amici C (2003) NF-kappaB and virus infection: who controls whom. The EMBO journal 22:2552-2560.

Sartori G, Jardim NS, Marcondes Sari MH, Dobrachinski F, Pesarico AP, Rodrigues LC, Jr., Cargnelutti J, Flores EF, Prigol M, Nogueira CW (2016a) Antiviral Action of Diphenyl Diselenide on Herpes Simplex Virus 2 Infection in Female BALB/c Mice. Journal of cellular biochemistry 117:1638-1648.

Sartori G, Jardim NS, Sari MH, Flores EF, Prigol M, Nogueira CW (2016b) Diphenyl Diselenide Reduces Oxidative Stress and Toxicity Caused by HSV-2 Infection in Mice. Journal of cellular biochemistry.

Seo T, Park J, Lim C, Choe J (2004) Inhibition of nuclear factor kappaB activity by viral interferon regulatory factor 3 of Kaposi's sarcoma-associated herpesvirus. Oncogene 23:6146-6155.

Spear PG, Roizman B (1972) Proteins specified by herpes simplex virus. V. Purification and structural proteins of the herpesvirion. J Virol 9:143-159.

Strasfeld L, Chou S (2010) Antiviral drug resistance: mechanisms and clinical implications. Infectious disease clinics of North America 24:413-437.

Su C, Zhan G, Zheng C (2016) Evasion of host antiviral innate immunity by HSV-1, an update. Virology journal 13:38.

Sylla BS, Hung SC, Davidson DM, Hatzivassiliou E, Malinin NL, Wallach D, Gilmore TD, Kieff E, Mosialos G (1998) Epstein-Barr virus-transforming protein latent infection membrane protein 1 activates transcription factor NF-kappaB through a pathway that includes the NF-kappaB-inducing kinase and the IkappaB kinases IKKalpha and IKKbeta. Proceedings of the National Academy of Sciences of the United States of America 95:10106-10111.

Viejo-Borbolla A, Martinez-Martin N, Nel HJ, Rueda P, Martin R, Blanco S, Arenzana-Seisdedos F, Thelen M, Fallon PG, Alcami A (2012) Enhancement of chemokine function as an immunomodulatory strategy employed by human herpesviruses. PLoS pathogens 8:e1002497.

Wang K, Ni L, Wang S, Zheng C (2014) Herpes simplex virus 1 protein kinase US3 hyperphosphorylates p65/RelA and dampens NF-kappaB activation. Journal of virology 88:7941-7951.

Weller SK, Coen DM (2012) Herpes simplex viruses: mechanisms of DNA replication. Cold Spring Harbor perspectives in biology 4:a013011.

Whitley RJ (2006) Herpes simplex encephalitis: adolescents and adults. Antiviral research 71:141-148.

Wilson EH, Weninger W, Hunter CA (2010) Trafficking of immune cells in the central nervous system. The Journal of clinical investigation 120:1368-1379.

Wozniak MA, Mee AP, Itzhaki RF (2009) Herpes simplex virus type 1 DNA is located within Alzheimer's disease amyloid plaques. The Journal of pathology 217:131-138.

Yordy B, Iijima N, Huttner A, Leib D, Iwasaki A (2012) A neuron-specific role for autophagy in antiviral defense against herpes simplex virus. Cell host & microbe 12:334-345.

Legends of figures

Figure 1. Viability of astrocytoma cells treated with $(PhSe)_2$ at a concentration range of 0.1 - 100 μM in 48 h of incubation. Results are expressed as mean ± S.E.M. (n=4) and were analyzed by ANOVA followed by the Newman-Keuls's Multiple Comparison test when appropriate; *** Denotes $p < 0.001$ vs control cells.

Figure 2. Effect of $(PhSe)_2$ at a concentration of 15 μM on supernatants of HSV-1 infected cells at MOI 0.0001 (A) and MOI 0.001 (B) 24 h post-infection by plaque reduction assay. Results (PFU/mL) are expressed as the mean ± S.E.M. (n=3) and were analyzed by the Student t test when appropriate; * Denotes $p < 0.05$ vs. HSV-1 infected cells.

Figure 3. Antiviral activity of $(PhSe)_2$ at a concentration of 15 μM against HSV-1 infection determined by qPCR. Viral DNA was extracted from supernatants of infected cells at MOI 0.0001 (A); at MOI 0.001 (B) and from infected cells at MOI 0.0001 (C); at MOI 0.001 (D) 24 h post-infection. Results were expressed as viral DNA copy numbers per microgram of genomic DNA (n=3) and were analyzed by ANOVA followed by the Student t test when appropriate; * Denotes $p < 0.05$; ** Denotes $p < 0.01$ vs. HSV-1 infected cells.

Figure 4. mRNA viral genes expression of $(PhSe)_2$-treated cells after 4 h and 24 h of HSV-1 infection at MOI 0.0001 and MOI 0.001 by Real-Time PCR. (A) ICP27 viral protein expression; (B) ICP0 viral protein expression; (C) ICP8 viral protein expression and (D) viral DNA polymerase expression. Gene expression was calculated relative to YWHAZ housekeeping gene. The results are shown as relative expression of each target mRNA (n=3) and were analyzed by ANOVA followed by the Newman-Keuls's Multiple Comparison test when appropriate; * Denotes $p < 0.05$; ** Denotes $p < 0.01$; *** Denotes $p < 0.001$ vs. HSV-1 infected cells.

Figure 5. TNF-α mRNA expression of $(PhSe)_2$-treated cells after HSV-1 infection by Real-Time PCR. TNF-α mRNA expression is demonstrated at 4 h (A_i); 24 h (A_{ii}) at MOI 0.0001 and at 4 h (B_i); 24 h (B_{ii}) at MOI 0.001 post-infection. Gene expression was calculated relative to HPRT housekeeping gene. The results are shown as relative expression of each target mRNA (n=3) and were analyzed by ANOVA followed by the Newman-Keuls's Multiple Comparison test when appropriate; * Denotes $p < 0.05$; *** Denotes $p < 0.001$ vs. non infected cells. # Denotes $p < 0.05$ vs. infected cells.

Figure 6. NF-κB mRNA expression of $(PhSe)_2$-treated cells after HSV-1 infection by Real-Time PCR. NF-κB mRNA expression is demonstrated at 4 h (A_i); 24 h (A_{ii}) at MOI 0.0001 and at 4 h (B_i); 24 h (B_{ii}) at MOI 0.001 post-infection. Gene expression was

calculated relative to HPRT housekeeping gene. The results are shown as relative expression of each target mRNA (n=3) and were analyzed by ANOVA followed by the Newman-Keuls's Multiple Comparison test when appropriate; * Denotes $p < 0.05$; ** Denotes $p < 0.01$ vs. non infected cells.

Figures

Figure 1.

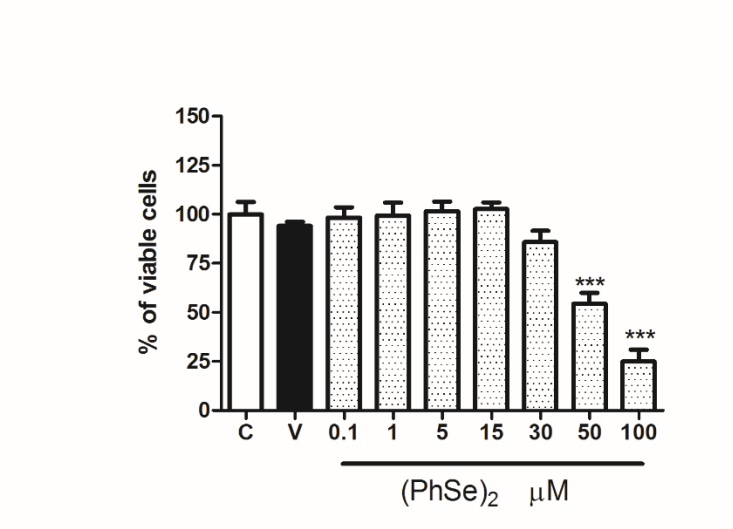


Figure 2.

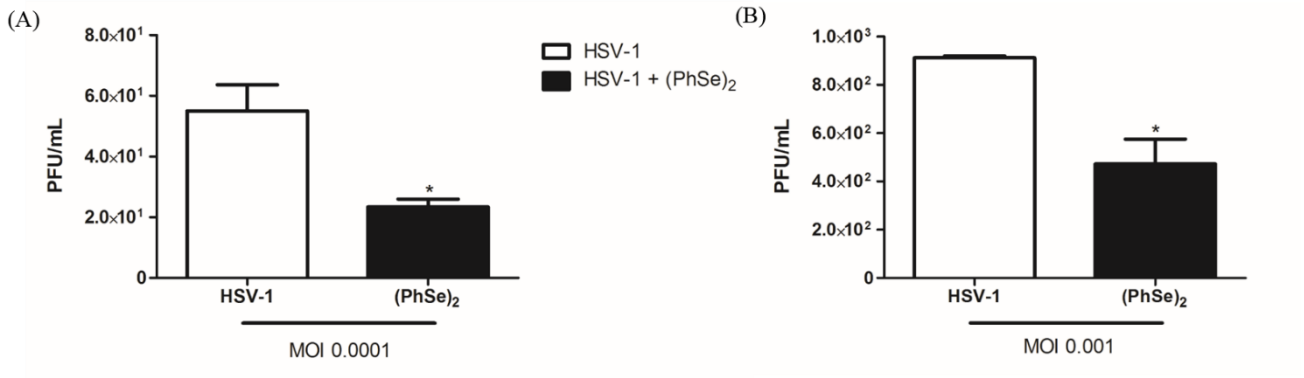


Figure 3.

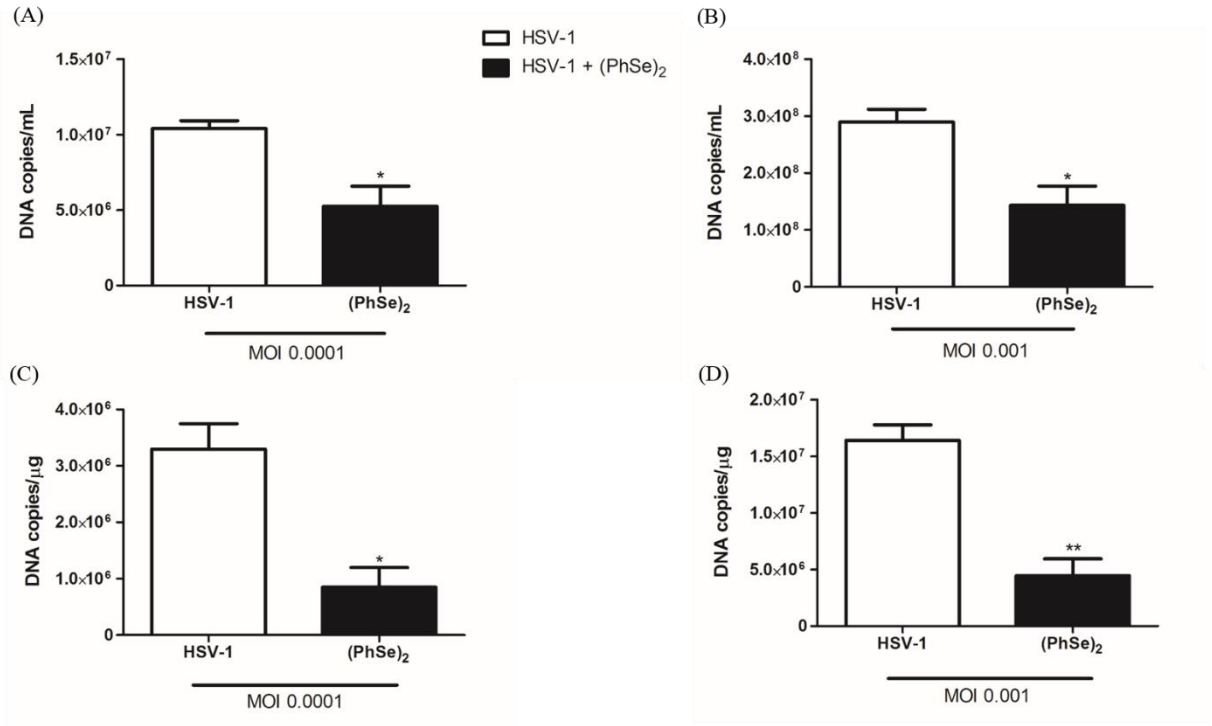


Figure 4.

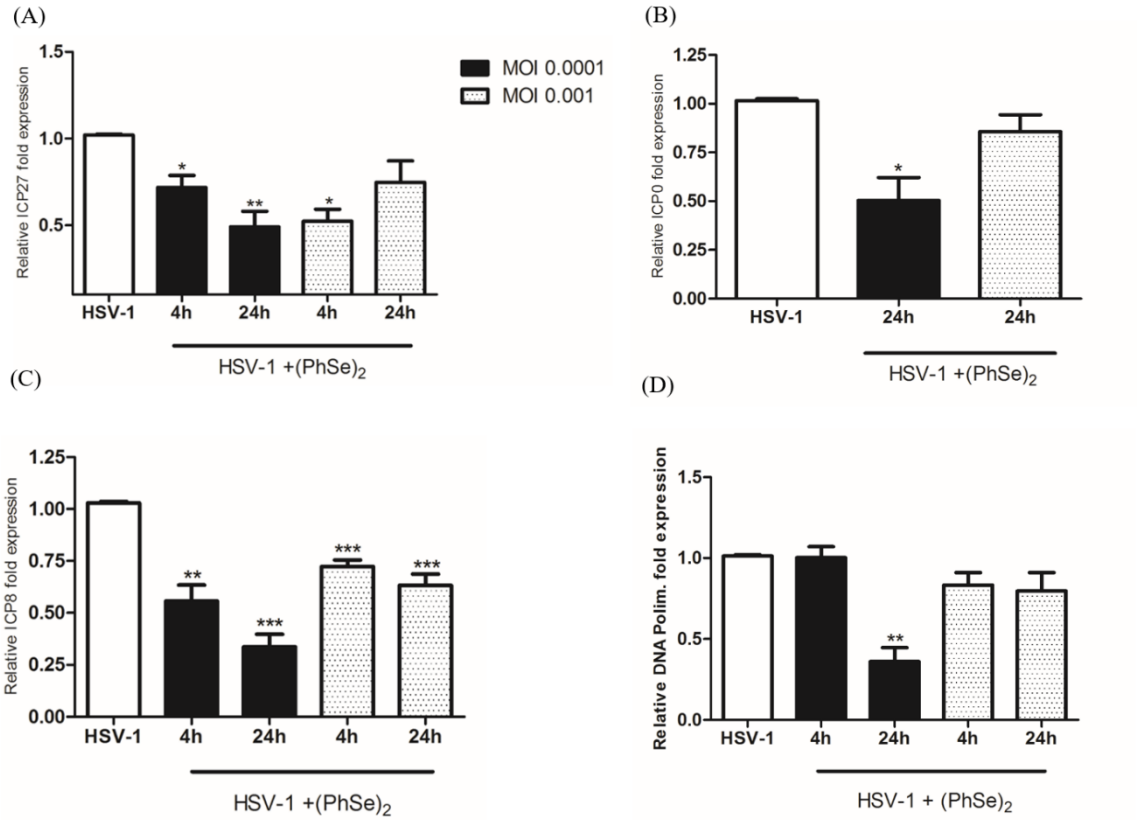


Figure 5.

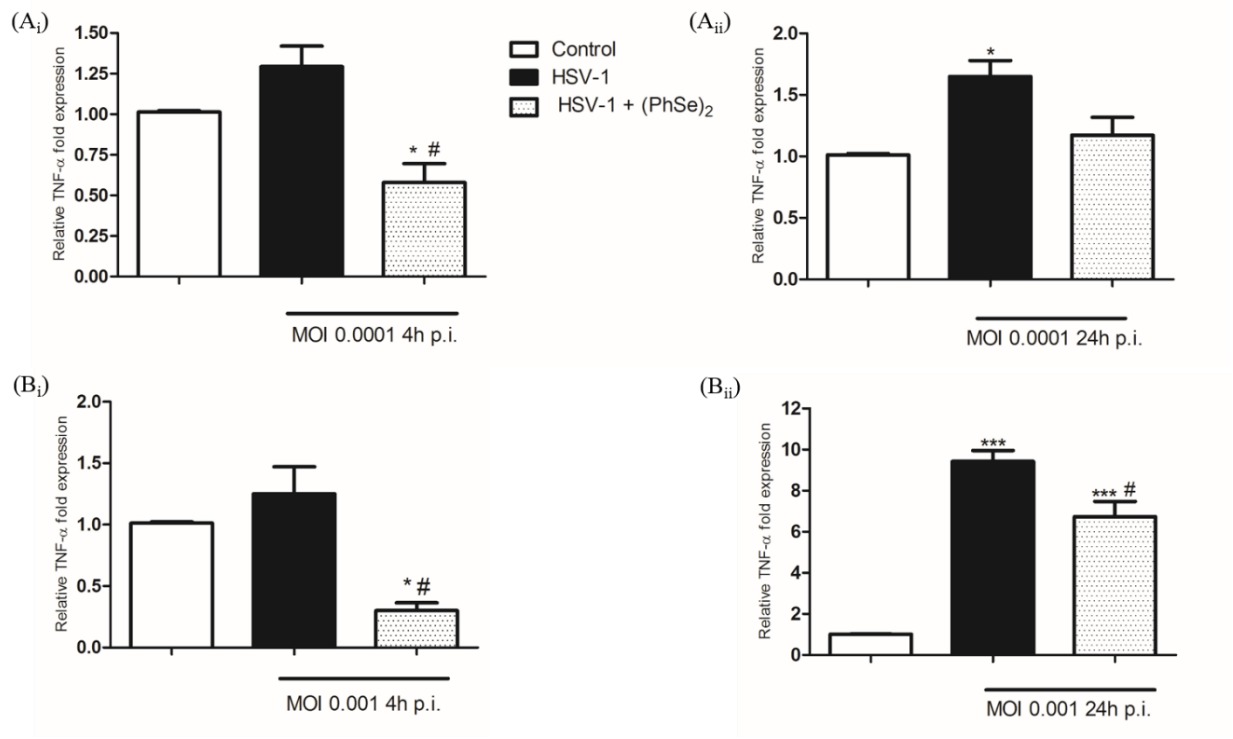
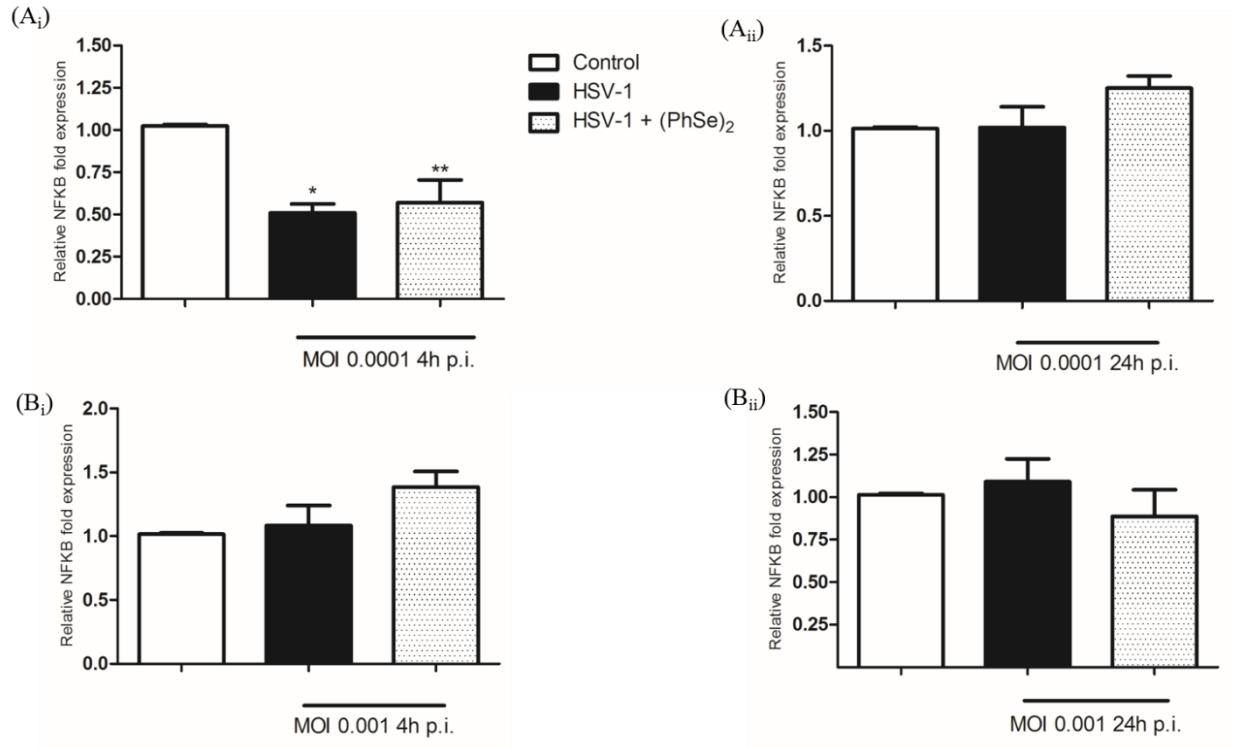


Figure 6.



Table

Table 1. Primers and probes used for quantification of HSV-1 viral genes.

Gene name	Primer and probe name	Sequence
RL2	ICP0- primer F	5'- AACTCGTGGGTGCTGATTGAC -3'
	ICP0- primer R	5'- CAGGTCTCGGTCGCAGGGAAAC -3'
	ICP0- probe	5'FAM- AGCCCGCCCCGGATGTCTGGG –TAMRA 3'
UL29	ICP8- primer F	5'- CACCAGGTTGCGCATCAG -3'
	ICP8- primer R	5'- CTGCATACGGTGGTGAACAAC -3'
	ICP8- probe	5'FAM- ACCTCGCGGTCCACG –TAMRA 3'
UL54	ICP27- primer F	5'- CGCCAAGAAAATTTCATCGAG -3'
	ICP27- primer R	5'- ACATCTTGCACCACGCCAG -3'
	ICP27- probe	5'FAM- CTGGCCTCCGCCGACGAGAC –TAMRA 3'
UL30	UL30- primer F	5'- CATCAGCGACCCGGAGAGGGAC -3'
	UL30- primer R	5'- GGGCCAGGCGCTTGTTGGTGTA -3'
	UL30- probe	5'FAM- CCGCCGAACTGAGCAGACACCCGCGC – TAMRA3'

4. DISCUSSÃO

Infecções por HSV são comuns na população, que, muitas vezes, não tem o conhecimento de que possui a doença. Por ser uma condição comum e não tão perigosa, não causa grande preocupação nos cuidados com sua transmissão (AHMED et al., 2003; MORIARTY et al., 2009). O HSV é uma doença infectocontagiosa crônica e, por vezes, recorrente, tendo como agente etiológico duas cepas diferentes do HSV. Dois quadros distintos podem ser individualizados: o herpes simples extragenital, cujo principal agente é o HSV-1, e as formas perigenitais, correspondente ao HSV-2. Porém, quando não tratada corretamente ou em casos de recorrência deve-se ter mais atenção aos cuidados em relação à evolução da infecção, pois tanto o HSV-1 quanto o HSV-2 pode causar sérios danos ao sistema nervoso central, quando atinge células nervosas (FATAHZADEH et al., 2007).

Como não existe cura para a infecção herpética, há um grande número de pesquisas focadas no desenvolvimento de fármacos antivirais para tratamento das infecções por HSV. Desde modo, buscou-se estudar no **artigo 1** desta tese uma alternativa terapêutica para as lesões causadas pelo HSV-2. Investigou-se, primeiramente, se o $(PhSe)_2$ possui algum efeito antiviral e virucida *in vitro* perante a infecção em células VERO. O efeito antiviral refere-se ao ensaio de redução de placa em dois momentos: um pré-tratamento das células VERO com o composto 1 h antes da inoculação viral; um pós-tratamento destas células após a retirada do vírus por um período de 48 h com o $(PhSe)_2$. Em ambos os tratamentos pode-se observar um efeito inibitório da replicação viral do HSV-2, através da contagem reduzida de placas virais. O composto foi capaz de inativar de forma notável as partículas virais, conforme demonstrado no ensaio virucida. Este resultado sugere um efeito direto sobre a partícula viral. Deste modo, pode-se concluir que o $(PhSe)_2$ exerce seu efeito antiviral *in vitro* de diferentes maneiras, possivelmente através da interferência na ligação de glicoproteínas com seus receptores, interferência nas etapas da replicação viral na célula hospedeira, inativando a partícula viral e prejudicando assim a infectividade na célula hospedeira.

A partir destes resultados, investigou-se no **manuscrito 1** desta tese se o

efeito antiviral do composto seria similar em cultura celular de astrocitomas infectadas por HSV-1 e em quais etapas o mesmo poderia interferir demonstrando seu mecanismo de ação. Assim, observou-se que o $(PhSe)_2$ também inibiu a replicação viral em astrocitomas através da redução da formação de placa viral e síntese de DNA viral 24 h após a infecção por HSV-1. Posteriormente, buscou-se avaliar quais genes virais poderiam estar diminuídos em decorrência do tratamento com $(PhSe)_2$. Constatou-se que o composto conseguiu controlar a replicação viral através da redução da expressão da maioria dos genes estudados como ICP27, ICP0, DNA polimerase viral, exercendo maior efeito sobre o gene ICP8, que bloqueia tanto a síntese do DNA viral quanto a transcrição dos genes tardios, tanto em 4 h como 24 h após a infecção.

A trajetória deste estudo se deve ao fato de que a infecção viral pode atingir células do SNC e causar latência nas regiões dos gânglios nervosos e também no cérebro, o que gera quadros de encefalites ou meningites graves (MITCHELL et al., 2003). Além disso, sabe-se que astrócitos quando estimulados podem expressar uma série de citocinas e quimiocinas inflamatórias, o que culmina na migração de leucócitos que atravessam a barreira hematoencefálica. Desta forma, estas células demonstram um papel importante na estimulação imunológica (DONG et al., 2001) e participam da detecção e da resposta a patógenos invasores como os vírus neurotróficos.

O NF-κB faz a regulação de uma ampla variedade de genes envolvidos em diferentes processos fisiológicos, os quais incluem inflamação, ativação imunológica, sobrevivência celular, diferenciação e proliferação celular, estresse oxidativo, adesão celular e homeostase do sistema imune (HATADA et al., 2000; GILMORE, 2006) . Sua ativação é um processo complexo que pode ser induzido por estímulos como vírus e outros microrganismos, citocinas, dano ao DNA celular, estresse oxidativo e radiação (VALLABHAPURAPU et al., 2009).

Deste modo, analisou-se a expressão do fator de transcrição nuclear NF-κB e da citocina TNF-α, em um período de 4 h e 24 h após a inoculação por HSV-1. Neste modelo de infecção, os astrocitomas desempenharam uma função antiviral através da ativação do sistema imunológico demonstrando um aumento da produção do TNF-α, o que também é mostrado por outros autores (Liu et al., 2013). Porém, não foi observado uma ativação persistente do NF-κB, acredita-

se que este mecanismo pode ser desencadeado pelo proprio vírus a fim de evitar a ativação do sistema imune que possa impedir a replicação viral e indução da apoptose. Evidências mostram que os vírus possuem a capacidade de codificar certas proteinas que podem interferir ou modular a resposta celular do hospedeiro tendo como alvo especifico certos pontos da cascata de sinalização do NF-κB (HISCOTT et al., 2001; SANTORO et al., 2003).

Um estudo de Wang e cols. (2014) corrobora com os achados do **manuscrito 1** desta tese, o qual mostrou uma redução da ativação do NF-κB pelo HSV-1 em células VERO (células de rim de macaco verde africano) e HEK 293T (células embrionárias renais de humanos) (WANG et al., 2014). Inicialmente, 4 h após a infecção, observou-se uma significativa produção do TNF-α que perdurou nas 24 h seguintes de análise nas células infectadas por HSV-1, porém não há ativação significativa do NF-κB. No entanto, é possível que este aumento da expressão do TNF-α possa ocorrer por outra via de sinalização durante a infecção, como a via das MAPKs (proteínas quinases ativadas por mitógenos), conforme demonstrado por outros autores (MCLEAN et al., 1999; CHEN et al., 2015). Isto é reforçado por evidências científicas que demonstram o envolvimento desta via na propagação viral (ZACHOS et al., 1999).

Nas células tratadas com $(PhSe)_2$ 4 h e 24 h após a infecção, não se observou ativação do NF-κB e ainda houve uma redução da expressão do TNF-α. Estes achados indicam que o composto exerceu certa modulação do sistema imunológico, podendo atuar em vias superiores da cascata de ativação do NF-κB ou via inibição da MAPK. Estudos reportam a importante ativação do NF-κB e MAPKs durante a infecção por HSV (CHEN et al., 2015). Estes resultados, por sua vez, demonstram a tentativa do composto em inibir a produção do TNF-α, induzida pelo vírus via sinalização da MAPK, provavelmente. Dados estes que corroboram com os demais resultados de carga viral reduzida, menor expressão das proteinas virais e menor síntese de DNA viral nas culturas de celulas gliais. Pesquisas reportam que variados tipos de vírus como o Epstein-Barr vírus (EBV) (SYLLA et al., 1998), citomegalovírus (MOUTAFTSI et al., 2004), varicella-zoster vírus (VZV) (JONES et al., 2006) e o HSV-1 (AMICI et al., 2006) podem interferir na ativação do NF-κB de diferentes maneiras.

Novas investigações relatam que em alguns casos em que o vírus estabelece latência no SNC, algumas regiões encefálicas podem estar

relacionadas severamente ao desenvolvimento de alterações neurodegenerativas como a DA (SANTANA et al., 2012; MANCUSO et al., 2014b; PIACENTINI et al., 2014). Desta maneira baseado nos resultados obtidos no **manuscrito 1** e nestas evidências, especula-se o possível benefício do $(PhSe)_2$ sobre a patogenia da DA associada ao HSV-1.

Ainda relacionado ao **artigo 1**, além do efeito antiviral *in vitro* e *in vivo* avaliou-se o efeito antioxidante, antiinflamatório e imunomodulador do composto frente as alterações causadas pelo HSV-2 como a produção de espécies reativas, redução da capacidade antioxidante bem como a alteração da atividade de algumas enzimas e resposta imune. O tratamento com $(PhSe)_2$ nos animais infectados mostrou ser efetivo em reduzir a resposta inflamatória através da redução da atividade da mieloperoxidase (MPO) local, interagiu com o sistema imunológico com robusta atividade antiviral devido ao aumento de produção de IFN-γ neste animais e reverteu a maioria dos danos oxidativos induzidos pelo vírus. Além disso, diminuiu as lesões herpéticas avaliadas por escore e por análise histológica bem como a carga viral dosada no tecido vaginal.

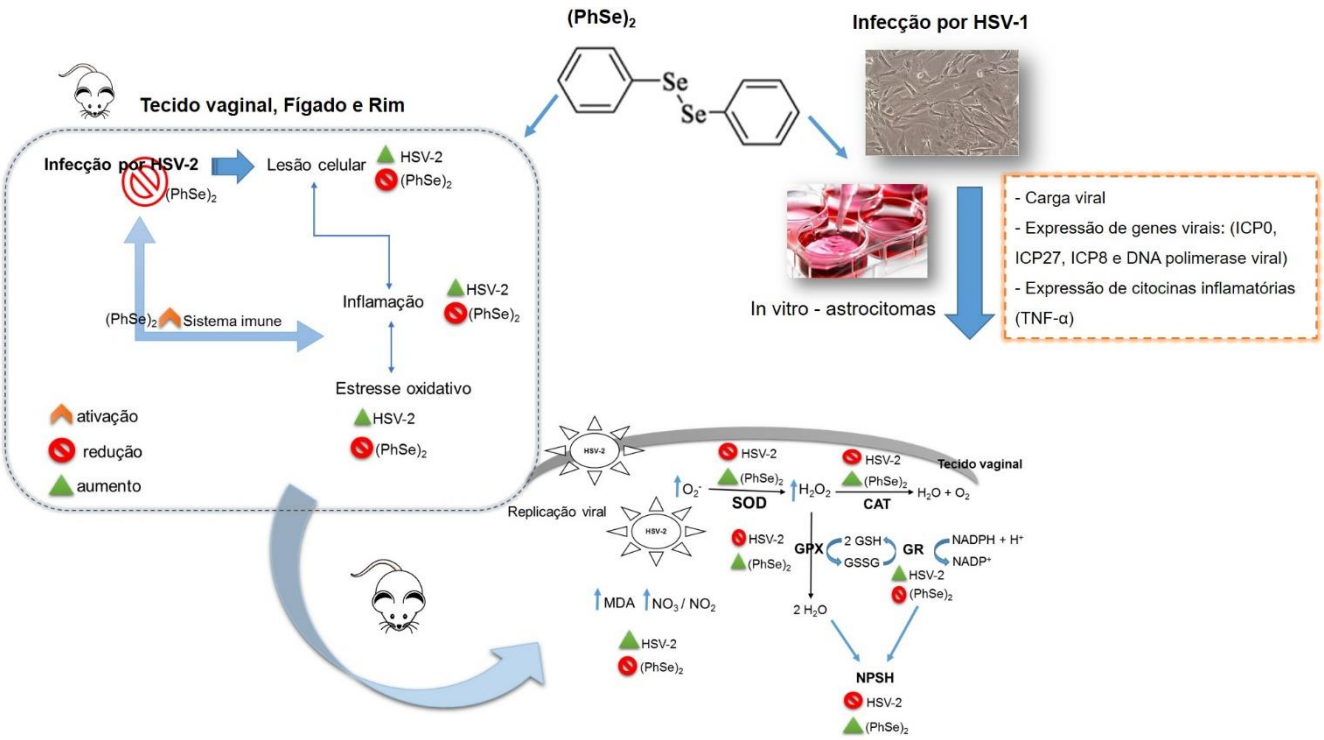
A partir destes resultados promissores obtidos no **artigo 1** e através dos sinais clínicos observados nos animais experimentais como constipação, retenção urinária, coloração alterada dos orgãos internos, perda de peso buscou-se investigar os efeitos tóxicos sistêmicos da infecção por HSV-2 em outros tecidos como rins, fígado e plasma destes animais e se o $(PhSe)_2$ mais uma vez exerceria efeito protetor. Este conjunto de dados constituem **o artigo 2**, no qual foi demonstrado o dano renal e hepático induzido pela infecção através da produção de espécies reativas de oxigênio, peroxidação lipídica, redução da capacidade antioxidante (NPSH) bem como a atividade reduzida das enzimas superóxido dismutase (SOD) e catalase (CAT), semelhante ao que foi mostrado no **artigo 1** no tecido genital.

Já está bem estabelecido que doenças infecciosas estão intimamente relacionadas a geração de estresse oxidativo e inflamação resultando na alteração do funcionamento e metabolismo dos orgãos ou tecidos envolvidos (NENCIONI et al., 2011; TAWADROUS et al., 2012). O tratamento com $(PhSe)_2$ reverteu a maioria dos danos causados pela infecção nos diferentes tecidos, e acredita-se que muitos destes efeitos, de reverter os danos oxidativos e de toxicidade, se devem, pelo menos em parte, ao efeito antiviral constatado no

artigo 1, uma vez que há menor carga viral, consequentemente haverá menor produção de espécies reativas e inflamação e ainda claro, devido ao fato do composto ser um clássico antioxidante (NOGUEIRA et al., 2004).

A partir de todos os dados obtidos, que constituem os **artigos 1** e **2** e o **manuscrito 1** (que estabelece um possível mecanismo de ação para o $(PhSe)_2$, podemos considerar a **ação antiviral** uma nova propriedade deste composto orgânico de selênio, revelando-o como uma alternativa terapêutica para tratar os danos causados pelas infecções herpéticas dos tipos 1 e 2. Um esquema final é demonstrado abaixo com todos os resultados obtidos (Figura 7).

Figura 7 – Esquema representativo dos resultados finais da tese.



5. CONCLUSÃO

Os resultados apresentados nesta tese, que constituem os **artigos 1** e **2** e o **manuscrito 1,** indicam que o $(PhSe)_2$:

I) Apresentou efeito antiviral *in vitro* em células VERO e *in vivo* em modelos de infecção por HSV-2 em camundongos;

II) Exerceu efeito antioxidante através da redução de espécies reativas de oxigênio e de nitrogênio, e da peroxidação lipídica, restaurou níveis de NPSH e as atividades das enzimas antioxidantes em diferentes tecidos dos camundongos infectados por HSV-2;

III) Exerceu efeito imunomodulador através da regulação da produção de citocinas, tais como, o interferon gama (IFN-γ) e o TNF-α em camundongos infectados por HSV-2;

IV) Apresentou efeito anti-inflamatório através da redução da atividade da MPO e ADA em diferentes tecidos dos camundongos infectados por HSV-2;

V) Reduziu a toxicidade hepática e renal causada pela infecção por HSV-2;

VI) Demonstrou efeito antiviral em cultura de células gliais infectadas por HSV-1 através da redução da síntese de DNA viral e expressão dos genes virais de diferentes etapas da replicação;

VII) Demonstrou atividade imunomoduladora através da redução da produção de citocinas (TNF-α) em cultura de células gliais infectadas por HSV-1;

Para dar continuidade ao estudo dos efeitos antivirais do $(PhSe)_2$ pretende-se avaliar com maior complexidade as vias de sinalização envolvidas na infecção por HSV e os efeitos do composto sobre a mesma bem como seus efeitos sobre o processo de neurodegeneração associado a infecção por HSV-1.

6. Referências Bibliográficas

AHMED, H. J., MBWANA, J., GUNNARSSON, E., AHLMAN, K., GUERINO, C., SVENSSON, L. A., MHALU, F. e LAGERGARD, T. (2003). "Etiology of genital ulcer disease and association with human immunodeficiency virus infection in two tanzanian cities." **Sexually transmitted diseases** 30(2): 114-119.

ALBRECHT, M. A., DELUCA, N. A., BYRN, R. A., SCHAFFER, P. A. e HAMMER, S. M. (1989). "The herpes simplex virus immediate-early protein, ICP4, is required to potentiate replication of human immunodeficiency virus in CD4+ lymphocytes." **Journal of virology** 63(5): 1861-1868.

AMICI, C., ROSSI, A., COSTANZO, A., CIAFRE, S., MARINARI, B., BALSAMO, M., LEVRERO, M. e SANTORO, M. G. (2006). "Herpes simplex virus disrupts NF-kappaB regulation by blocking its recruitment on the IkappaBalpha promoter and directing the factor on viral genes." **The Journal of biological chemistry** 281(11): 7110-7117.

ANDRES, A. (2005). "Cancer incidence after immunosuppressive treatment following kidney transplantation." **Critical reviews in oncology/hematology** 56(1): 71-85.

ANZIVINO, E., FIORITI, D., MISCHITELLI, M., BELLIZZI, A., BARUCCA, V., CHIARINI, F. e PIETROPAOLO, V. (2009). "Herpes simplex virus infection in pregnancy and in neonate: status of art of epidemiology, diagnosis, therapy and prevention." **Virology journal** 6: 40.

ARAVALLI, R. N., HU, S. e LOKENSGARD, J. R. (2008). "Inhibition of toll-like receptor signaling in primary murine microglia." **Journal of neuroimmune pharmacology : the official journal of the Society on NeuroImmune Pharmacology** 3(1): 5-11.

ARAVALLI, R. N., HU, S., ROWEN, T. N., GEKKER, G. e LOKENSGARD, J. R. (2006). "Differential apoptotic signaling in primary glial cells infected with herpes simplex virus 1." **Journal of neurovirology** 12(6): 501-510.

ARAVALLI, R. N., HU, S., ROWEN, T. N., PALMQUIST, J. M. e LOKENSGARD, J. R. (2005). "Cutting edge: TLR2-mediated proinflammatory cytokine and chemokine production by microglial cells in response to herpes simplex virus." **Journal of immunology** 175(7): 4189-4193.

ARRIGO, A. P., FIRDAUS, W. J., MELLIER, G., MOULIN, M., PAUL, C., DIAZ-LATOUD, C. e KRETZ-REMY, C. (2005). "Cytotoxic effects induced by oxidative stress in cultured mammalian cells and protection provided by Hsp27 expression." **Methods** 35(2): 126-138.

BEARER, E. L. (2004). "Perspectives on herpes-APP interactions." **Aging cell** 3(2): 81-84.

BELSHE, R. B., HEINEMAN, T. C., BERNSTEIN, D. I., BELLAMY, A. R., EWELL, M., VAN DER MOST, R. e DEAL, C. D. (2014). "Correlate of immune protection against HSV-1 genital disease in vaccinated women." **The Journal of infectious diseases** 209(6): 828-836.

BISWAS, S. e FIELD, H. J. (2008). "Herpes simplex virus helicase-primase inhibitors: recent findings from the study of drug resistance mutations." **Antivir Chem Chemother** 19(1): 1-6.

BOEHMER, P. E. e LEHMAN, I. R. (1997). "Herpes simplex virus DNA replication." **Annual review of biochemistry** 66: 347-384.

BURCH, P. M. e HEINTZ, N. H. (2005). "Redox regulation of cell-cycle re-entry: cyclin D1 as a primary target for the mitogenic effects of reactive oxygen and nitrogen species." **Antioxidants & redox signaling** 7(5-6): 741-751.
CAIRNS, T. M., HUANG, Z. Y., GALLAGHER, J. R., LIN, Y., LOU, H., WHITBECK, J. C., WALD, A., COHEN, G. H. e EISENBERG, R. J. (2015). "Patient-Specific Neutralizing Antibody Responses to Herpes Simplex Virus Are Attributed to Epitopes on gD, gB, or Both and Can Be Type Specific." **Journal of virology** 89(18): 9213-9231.
CARROZZA, M. J. e DELUCA, N. A. (1996). "Interaction of the viral activator protein ICP4 with TFIID through TAF250." **Molecular and cellular biology** 16(6): 3085-3093.
CHAN, T., BARRA, N. G., LEE, A. J. e ASHKAR, A. A. (2011). "Innate and adaptive immunity against herpes simplex virus type 2 in the genital mucosa." **Journal of reproductive immunology** 88(2): 210-218.
CHASSOT, F., POZZEBON VENTURINI, T., BALDISSERA PIASENTIN, F., MORAIS SANTURIO, J., ESTIVALET SVIDZINSKI, T. I. e HARTZ ALVES, S. (2016). "Antifungal activities of diphenyl diselenide and ebselen against echinocandin-susceptible and -resistant strains of Candida parapsilosis." **The new microbiologica** 39(4): 301-303.
CHEN, D., SU, A., FU, Y., WANG, X., LV, X., XU, W., XU, S., WANG, H. e WU, Z. (2015). "Harmine blocks herpes simplex virus infection through downregulating cellular NF-kappaB and MAPK pathways induced by oxidative stress." **Antiviral research** 123: 27-38.
CHEN, K. T., SEGU, M., LUMEY, L. H., KUHN, L., CARTER, R. J., BULTERYS, M., ABRAMS, E. J. e NEW YORK CITY PERINATAL, A. C. T. S. G. (2005). "Genital herpes simplex virus infection and perinatal transmission of human immunodeficiency virus." **Obstetrics and gynecology** 106(6): 1341-1348.
CHENTOUFI, A. A., DERVILLEZ, X., RUBBO, P. A., KUO, T., ZHANG, X., NAGOT, N., TUAILLON, E., VAN DE PERRE, P., NESBURN, A. B. e BENMOHAMED, L. (2012). "Current trends in negative immuno-synergy between two sexually transmitted infectious viruses: HIV-1 and HSV-1/2." **Current trends in immunology** 13: 51-68.
CHUNG, S. M., ADVANI, S. J., BRADLEY, J. D., KATAOKA, Y., VASHISTHA, K., YAN, S. Y., MARKERT, J. M., GILLESPIE, G. Y., WHITLEY, R. J., ROIZMAN, B. e WEICHSELBAUM, R. R. (2002). "The use of a genetically engineered herpes simplex virus (R7020) with ionizing radiation for experimental hepatoma." **Gene therapy** 9(1): 75-80.
Citizendium. Acesso em 01 fev, 2017, Disponível em en.citizendium.org/wiki/Herpes_simplex_virus.
CLINICACISO.NO.COMUNIDADES.NET (2013). "Herpes labial disponivel em: http://clinicaciso.no.comunidades.net/index.php?pagina=1409519295_10."
COWAN, F. M., FRENCH, R. S., MAYAUD, P., GOPAL, R., ROBINSON, N. J., DE OLIVEIRA, S. A., FAILLACE, T., UUSKULA, A., NYGARD-KIBUR, M., RAMALINGAM, S., SRIDHARAN, G., EL AOUAD, R., ALAMI, K., RBAI, M., SUNIL-CHANDRA, N. P. e BROWN, D. W. (2003). "Seroepidemiological study of herpes simplex virus types 1 and 2 in Brazil, Estonia, India, Morocco, and Sri Lanka." **Sexually transmitted infections** 79(4): 286-290.
DESAI, D. V. e KULKARNI, S. S. (2015). "Herpes Simplex Virus: The Interplay Between HSV, Host, and HIV-1." **Viral immunology** 28(10): 546-555.
DOBRACHINSKI, F., DA SILVA, M. H., TASSI, C. L., DE CARVALHO, N. R., DIAS, G. R., GOLOMBIESKI, R. M., DA SILVA LORETO, E. L., DA ROCHA, J. B., FIGHERA, M. R. e SOARES, F. A. (2014). "Neuroprotective effect of diphenyl

diselenide in a experimental stroke model: maintenance of redox system in mitochondria of brain regions." **Neurotoxicity research** 26(4): 317-330.
DONG, Y. e BENVENISTE, E. N. (2001). "Immune function of astrocytes." **Glia** 36(2): 180-190.
DUERST, R. J. e MORRISON, L. A. (2003). "Innate immunity to herpes simplex virus type 2." **Viral immunology** 16(4): 475-490.
FATAHZADEH, M. e SCHWARTZ, R. A. (2007). "Human herpes simplex virus infections: epidemiology, pathogenesis, symptomatology, diagnosis, and management." **Journal of the American Academy of Dermatology** 57(5): 737-763; quiz 764-736.
FERRÁN, M. e PUJOL, R. M. (2006). "Tratamiento del herpes zóster. ." **Actas Dermosifiliogr** 97(3): 25-37.
GHISLENI, G., PORCIUNCULA, L. O., CIMAROSTI, H., BATISTA, T. R. J., SALBEGO, C. G. e SOUZA, D. O. (2003). "Diphenyl diselenide protects rat hippocampal slices submitted to oxygen-glucose deprivation and diminishes inducible nitric oxide synthase immunocontent." **Brain research** 986(1-2): 196-199.
GILMORE, T. D. (2006). "Introduction to NF-kappaB: players, pathways, perspectives." **Oncogene** 25(51): 6680-6684.
GONG, Y., MATTHEWS, B., CHEUNG, D., TAM, T., GADAWSKI, I., LEUNG, D., HOLAN, G., RAFF, J. e SACKS, S. (2002). "Evidence of dual sites of action of dendrimers: SPL-2999 inhibits both virus entry and late stages of herpes simplex virus replication." **Antiviral research** 55(2): 319-329.
GOTTLIEB, S. L. e JOHNSTON, C. (2017). "Future prospects for new vaccines against sexually transmitted infections." **Current opinion in infectious diseases** 30(1): 77-86.
GU, B. e DELUCA, N. (1994). "Requirements for activation of the herpes simplex virus glycoprotein C promoter in vitro by the viral regulatory protein ICP4." **Journal of virology** 68(12): 7953-7965.
HARATAKE, M., YOSHIDA, S., MANDAI, M., FUCHIGAMI, T. e NAKAYAMA, M. (2013). "Elevated amyloid-beta plaque deposition in dietary selenium-deficient Tg2576 transgenic mice." **Metallomics : integrated biometal science** 5(5): 479-483.
HATADA, E. N., KRAPPMANN, D. e SCHEIDEREIT, C. (2000). "NF-kappaB and the innate immune response." **Current opinion in immunology** 12(1): 52-58.
HILL, A., JUGOVIC, P., YORK, I., RUSS, G., BENNINK, J., YEWDELL, J., PLOEGH, H. e JOHNSON, D. (1995). "Herpes simplex virus turns off the TAP to evade host immunity." **Nature** 375(6530): 411-415.
HISCOTT, J., KWON, H. e GENIN, P. (2001). "Hostile takeovers: viral appropriation of the NF-kappaB pathway." **The Journal of clinical investigation** 107(2): 143-151.
HOFFMANN, P. R. e BERRY, M. J. (2008). "The influence of selenium on immune responses." **Molecular nutrition & food research** 52(11): 1273-1280.
Herpesvírus humanos – HSV-1 e HSV-2. Acesso em 02 fev, 2017, Disponível em http://pt-br.aia1317.wikia.com/wiki/Herpesv%C3%ADrus_humanos_%E2%80%93_HSV-1_e_HSV-2.
JACOBS, A., BREAKEFIELD, X. O. e FRAEFEL, C. (1999). "HSV-1-based vectors for gene therapy of neurological diseases and brain tumors: part I. HSV-1 structure, replication and pathogenesis." **Neoplasia** 1(5): 387-401.
JIANG, Y. C., FENG, H., LIN, Y. C. e GUO, X. R. (2016). "New strategies against drug resistance to herpes simplex virus." **International journal of oral science** 8(1): 1-6.
JING, L., LAING, K. J., DONG, L., RUSSELL, R. M., BARLOW, R. S., HAAS, J. G., RAMCHANDANI, M. S., JOHNSTON, C., BUUS, S., REDWOOD, A. J., WHITE, K. D., MALLAL, S. A., PHILLIPS, E. J., POSAVAD, C. M., WALD, A. e KOELLE, D. M.

(2016). "Extensive CD4 and CD8 T Cell Cross-Reactivity between Alphaherpesviruses." **Journal of immunology** 196(5): 2205-2218.
JONES, J. O. e ARVIN, A. M. (2006). "Inhibition of the NF-kappaB pathway by varicella-zoster virus in vitro and in human epidermal cells in vivo." **Journal of virology** 80(11): 5113-5124.
KAVOURAS, J. H., PRANDOVSZKY, E., VALYI-NAGY, K., KOVACS, S. K., TIWARI, V., KOVACS, M., SHUKLA, D. e VALYI-NAGY, T. (2007). "Herpes simplex virus type 1 infection induces oxidative stress and the release of bioactive lipid peroxidation by-products in mouse P19N neural cell cultures." **Journal of neurovirology** 13(5): 416-425.
KHAMENEH, Z. R. e SEPEHRVAND, N. (2008). "Survey of hepatitis B status in hemodialysis patients in a training hospital in Urmia, Iran." **Saudi journal of kidney diseases and transplantation : an official publication of the Saudi Center for Organ Transplantation, Saudi Arabia** 19(3): 466-469.
KOELLE, D. M. e COREY, L. (2003). "Recent progress in herpes simplex virus immunobiology and vaccine research." **Clinical microbiology reviews** 16(1): 96-113.
LAZARINI, P. R., VIANNA, M. F., ALCANTARA, M. P., SCALIA, R. A. e CAIAFFA FILHO, H. H. (2006). "Herpes simplex virus in the saliva of peripheral Bell's palsy patients." **Brazilian journal of otorhinolaryngology** 72(1): 7-11.
LEE, K. M., YEO, M., CHOUE, J. S., JIN, J. H., PARK, S. J., CHEONG, J. Y., LEE, K. J., KIM, J. H. e HAHM, K. B. (2004). "Protective mechanism of epigallocatechin-3-gallate against Helicobacter pylori-induced gastric epithelial cytotoxicity via the blockage of TLR-4 signaling." **Helicobacter** 9(6): 632-642.
LEHMAN, I. R. e BOEHMER, P. E. (1999). "Replication of herpes simplex virus DNA." **The Journal of biological chemistry** 274(40): 28059-28062.
LINDBERG, A. e KREIVI, J. P. (2002). "Splicing inhibition at the level of spliceosome assembly in the presence of herpes simplex virus protein ICP27." **Virology** 294(1): 189-198.
LORETO, E. S., MARIO, D. A., SANTURIO, J. M., ALVES, S. H., NOGUEIRA, C. W. e ZENI, G. (2011). "In vitro antifungal evaluation and structure-activity relationship of diphenyl diselenide and synthetic analogues." **Mycoses** 54(5): e572-576.
LUPI, O. (2011). "Prevalence and risk factors for herpes simplex infection among patients at high risk for HIV infection in Brazil." **International journal of dermatology** 50(6): 709-713.
MANCUSO, R., BAGLIO, F., AGOSTINI, S., CABINIO, M., LAGANA, M. M., HERNIS, A., MARGARITELLA, N., GUERINI, F. R., ZANZOTTERA, M., NEMNI, R. e CLERICI, M. (2014a). "Relationship between herpes simplex virus-1-specific antibody titers and cortical brain damage in Alzheimer's disease and amnestic mild cognitive impairment." **Frontiers in aging neuroscience** 6: 285.
MANCUSO, R., BAGLIO, F., CABINIO, M., CALABRESE, E., HERNIS, A., NEMNI, R. e CLERICI, M. (2014b). "Titers of herpes simplex virus type 1 antibodies positively correlate with grey matter volumes in Alzheimer's disease." **Journal of Alzheimer's disease : JAD** 38(4): 741-745.
MARTIN, C., AGUILA, B., ARAYA, P., VIO, K., VALDIVIA, S., ZAMBRANO, A., CONCHA, M. I. e OTTH, C. (2014). "Inflammatory and neurodegeneration markers during asymptomatic HSV-1 reactivation." **Journal of Alzheimer's disease : JAD** 39(4): 849-859.
MCCORMACK, O., CARBOY, J., NGUYEN, L. e MERCER, J. (2006). "Clinical inquiries. What is the role of herpes virus serology in sexually transmitted disease screening?" **The Journal of family practice** 55(5): 451-452.

MCKENDRICK, M. W., MCGILL, J. I., WHITE, J. E. e WOOD, M. J. (1986). "Oral acyclovir in acute herpes zoster." **Br Med J (Clin Res Ed)** 293(6561): 1529-1532.
MCLEAN, T. I. e BACHENHEIMER, S. L. (1999). "Activation of cJUN N-terminal kinase by herpes simplex virus type 1 enhances viral replication." **Journal of virology** 73(10): 8415-8426.
MITCHELL, B. M., BLOOM, D. C., COHRS, R. J., GILDEN, D. H. e KENNEDY, P. G. (2003). "Herpes simplex virus-1 and varicella-zoster virus latency in ganglia." **Journal of neurovirology** 9(2): 194-204.
MORIARTY, A. T., DARRAGH, T. M., SOUERS, R., FATHEREE, L. A. e WILBUR, D. C. (2009). "Performance of herpes simplex challenges in proficiency testing: observations from the College of American Pathologists proficiency testing program." **Archives of pathology & laboratory medicine** 133(9): 1379-1382.
MOUTAFTSI, M., BRENNAN, P., SPECTOR, S. A. e TABI, Z. (2004). "Impaired lymphoid chemokine-mediated migration due to a block on the chemokine receptor switch in human cytomegalovirus-infected dendritic cells." **Journal of virology** 78(6): 3046-3054.
NENCIONI, L., SGARBANTI, R., AMATORE, D., CHECCONI, P., CELESTINO, I., LIMONGI, D., ANTICOLI, S., PALAMARA, A. T. e GARACI, E. (2011). "Intracellular redox signaling as therapeutic target for novel antiviral strategy." **Current pharmaceutical design** 17(35): 3898-3904.
NOGUEIRA, C. W., QUINHONES, E. B., JUNG, E. A., ZENI, G. e ROCHA, J. B. (2003). "Anti-inflammatory and antinociceptive activity of diphenyl diselenide." **Inflammation research : official journal of the European Histamine Research Society ... [et al.]** 52(2): 56-63.
NOGUEIRA, C. W., ZENI, G. e ROCHA, J. B. (2004). "Organoselenium and organotellurium compounds: toxicology and pharmacology." **Chemical reviews** 104(12): 6255-6285.
NOLKEMPER, S., REICHLING, J., SENSCH, K. H. e SCHNITZLER, P. (2010). "Mechanism of herpes simplex virus type 2 suppression by propolis extracts." **Phytomedicine** 17(2): 132-138.
NOVELLI, F. e CASANOVA, J. L. (2004). "The role of IL-12, IL-23 and IFN-gamma in immunity to viruses." **Cytokine & growth factor reviews** 15(5): 367-377.
PALAMARA, A. T., PERNO, C. F., CIRIOLO, M. R., DINI, L., BALESTRA, E., D'AGOSTINI, C., DI FRANCESCO, P., FAVALLI, C., ROTILIO, G. e GARACI, E. (1995). "Evidence for antiviral activity of glutathione: in vitro inhibition of herpes simplex virus type 1 replication." **Antiviral research** 27(3): 237-253.
PAPP, L. V., HOLMGREN, A. e KHANNA, K. K. (2010). "Selenium and selenoproteins in health and disease." **Antioxidants & redox signaling** 12(7): 793-795.
PAPP, L. V., LU, J., HOLMGREN, A. e KHANNA, K. K. (2007). "From selenium to selenoproteins: synthesis, identity, and their role in human health." **Antioxidants & redox signaling** 9(7): 775-806.
PATEL, R., ALDERSON, S., GERETTI, A., NILSEN, A., FOLEY, E., LAUTENSCHLAGER, S., GREEN, J., VAN DER MEIJDEN, W., GOMBERG, M., MOI, H. e EUROPE, I. W. (2011). "European guideline for the management of genital herpes, 2010." **International journal of STD & AIDS** 22(1): 1-10.
PETRONILHO, F., MICHELS, M., DANIELSKI, L. G., GOLDIM, M. P., FLORENTINO, D., VIEIRA, A., MENDONCA, M. G., TOURNIER, M., PIACENTINI, B., GIUSTINA, A. D., LEFFA, D. D., PEREIRA, G. W., PEREIRA, V. D. e ROCHA, J. B. (2016). "Diphenyl diselenide attenuates oxidative stress and inflammatory parameters

in ulcerative colitis: A comparison with ebselen." **Pathology, research and practice** 212(9): 755-760.
PIACENTINI, R., DE CHIARA, G., LI PUMA, D. D., RIPOLI, C., MARCOCCI, M. E., GARACI, E., PALAMARA, A. T. e GRASSI, C. (2014). "HSV-1 and Alzheimer's disease: more than a hypothesis." **Frontiers in pharmacology** 5: 97.
PINTON, S., BRUNING, C. A., SARTORI OLIVEIRA, C. E., PRIGOL, M. e NOGUEIRA, C. W. (2013). "Therapeutic effect of organoselenium dietary supplementation in a sporadic dementia of Alzheimer's type model in rats." **The Journal of nutritional biochemistry** 24(1): 311-317.
PIRET, J. e BOIVIN, G. (2015). "Innate immune response during herpes simplex virus encephalitis and development of immunomodulatory strategies." **Reviews in medical virology** 25(5): 300-319.
POPIK, W. e PITHA, P. M. (1994). "The presence of tat protein or tumor necrosis factor alpha is critical for herpes simplex virus type 1-induced expression of human immunodeficiency virus type 1." **Journal of virology** 68(3): 1324-1333.
RAYMAN, M. P. (2012). "Selenium and human health." **Lancet** 379(9822): 1256-1268.
RECHENCHOSKI, D. Z., FACCIN-GALHARDI, L. C., LINHARES, R. E. e NOZAWA, C. (2016). "Herpesvirus: an underestimated virus." **Folia microbiologica**.
RIEDIGER, C., SAUER, P., MATEVOSSIAN, E., MULLER, M. W., BUCHLER, P. e FRIESS, H. (2009). "Herpes simplex virus sepsis and acute liver failure." **Clinical transplantation** 23 Suppl 21: 37-41.
RITA CARDOSO, B., APOLINARIO, D., DA SILVA BANDEIRA, V., BUSSE, A. L., MAGALDI, R. M., JACOB-FILHO, W. e COZZOLINO, S. M. (2016). "Effects of Brazil nut consumption on selenium status and cognitive performance in older adults with mild cognitive impairment: a randomized controlled pilot trial." **European journal of nutrition** 55(1): 107-116.
RITA CARDOSO, B., SILVA BANDEIRA, V., JACOB-FILHO, W. e FRANCISCATO COZZOLINO, S. M. (2014). "Selenium status in elderly: relation to cognitive decline." **Journal of trace elements in medicine and biology : organ of the Society for Minerals and Trace Elements** 28(4): 422-426.
ROIZMAN, B. (1996). "The function of herpes simplex virus genes: a primer for genetic engineering of novel vectors." **Proceedings of the National Academy of Sciences of the United States of America** 93(21): 11307-11312.
SANDRI-GOLDIN, R. M. e MENDOZA, G. E. (1992). "A herpesvirus regulatory protein appears to act post-transcriptionally by affecting mRNA processing." **Genes & development** 6(5): 848-863.
SANTANA, S., RECUERO, M., BULLIDO, M. J., VALDIVIESO, F. e ALDUDO, J. (2012). "Herpes simplex virus type I induces the accumulation of intracellular beta-amyloid in autophagic compartments and the inhibition of the non-amyloidogenic pathway in human neuroblastoma cells." **Neurobiology of aging** 33(2): 430 e419-433.
SANTORO, M. G., ROSSI, A. e AMICI, C. (2003). "NF-kappaB and virus infection: who controls whom." **The EMBO journal** 22(11): 2552-2560.
SARTORI, G., JARDIM, N. S., MARCONDES SARI, M. H., DOBRACHINSKI, F., PESARICO, A. P., RODRIGUES, L. C., JR., CARGNELUTTI, J., FLORES, E. F., PRIGOL, M. e NOGUEIRA, C. W. (2016a). "Antiviral Action of Diphenyl Diselenide on Herpes Simplex Virus 2 Infection in Female BALB/c Mice." **Journal of cellular biochemistry** 117(7): 1638-1648.
SARTORI, G., JARDIM, N. S., SARI, M. H., FLORES, E. F., PRIGOL, M. e NOGUEIRA, C. W. (2016b). "Diphenyl Diselenide Reduces Oxidative Stress and Toxicity Caused by HSV-2 Infection in Mice." **Journal of cellular biochemistry**.

SATPUTE-KRISHNAN, P., DEGIORGIS, J. A., CONLEY, M. P., JANG, M. e BEARER, E. L. (2006). "A peptide zipcode sufficient for anterograde transport within amyloid precursor protein." **Proceedings of the National Academy of Sciences of the United States of America** 103(44): 16532-16537.
SCHACHTELE, S. J., HU, S., LITTLE, M. R. e LOKENSGARD, J. R. (2010). "Herpes simplex virus induces neural oxidative damage via microglial cell Toll-like receptor-2." **Journal of neuroinflammation** 7: 35.
SCHRAUZER, G. N. e SACHER, J. (1994). "Selenium in the maintenance and therapy of HIV-infected patients." **Chemico-biological interactions** 91(2-3): 199-205.
SCHWARZ, K. B. (1996). "Oxidative stress during viral infection: a review." **Free radical biology & medicine** 21(5): 641-649.
SHANKAR, S. K., MAHADEVAN, A. e KOVOOR, J. M. (2008). "Neuropathology of viral infections of the central nervous system." **Neuroimaging clinics of North America** 18(1): 19-39; vii.
SINHA, S., ANDERSON, J. P., BARBOUR, R., BASI, G. S., CACCAVELLO, R., DAVIS, D., DOAN, M., DOVEY, H. F., FRIGON, N., HONG, J., JACOBSON-CROAK, K., JEWETT, N., KEIM, P., KNOPS, J., LIEBERBURG, I., POWER, M., TAN, H., TATSUNO, G., TUNG, J., SCHENK, D., SEUBERT, P., SUOMENSAARI, S. M., WANG, S., WALKER, D., ZHAO, J., MCCONLOGUE, L. e JOHN, V. (1999). "Purification and cloning of amyloid precursor protein beta-secretase from human brain." **Nature** 402(6761): 537-540.
SMITH, J. S. e ROBINSON, N. J. (2002). "Age-specific prevalence of infection with herpes simplex virus types 2 and 1: a global review." **The Journal of infectious diseases** 186 Suppl 1: S3-28.
STANFIELD, B. e KOUSOULAS, K. G. (2015). "Herpes Simplex Vaccines: Prospects of Live-attenuated HSV Vaccines to Combat Genital and Ocular infections." **Current clinical microbiology reports** 2(3): 125-136.
SU, C., ZHAN, G. e ZHENG, C. (2016). "Evasion of host antiviral innate immunity by HSV-1, an update." **Virology journal** 13: 38.
SYLLA, B. S., HUNG, S. C., DAVIDSON, D. M., HATZIVASSILIOU, E., MALININ, N. L., WALLACH, D., GILMORE, T. D., KIEFF, E. e MOSIALOS, G. (1998). "Epstein-Barr virus-transforming protein latent infection membrane protein 1 activates transcription factor NF-kappaB through a pathway that includes the NF-kappaB-inducing kinase and the IkappaB kinases IKKalpha and IKKbeta." **Proceedings of the National Academy of Sciences of the United States of America** 95(17): 10106-10111.
TAWADROUS, G. A., AZIZ, A. A., AMIN, D. G., ELDEMERY, A. e MOSTAFA, M. A. (2012). "RANTES, TNF-alpha, oxidative stress, and hematological abnormalities in hepatitis C virus infection." **Journal of investigative medicine : the official publication of the American Federation for Clinical Research** 60(6): 878-882.
VALLABHAPURAPU, S. e KARIN, M. (2009). "Regulation and function of NF-kappaB transcription factors in the immune system." **Annual review of immunology** 27: 693-733.
VASSAR, R. (2001). "The beta-secretase, BACE: a prime drug target for Alzheimer's disease." **Journal of molecular neuroscience : MN** 17(2): 157-170.
VENTURINI, T. P., CHASSOT, F., LORETO, E. S., KELLER, J. T., AZEVEDO, M. I., ZENI, G., SANTURIO, J. M. e ALVES, S. H. (2016). "Antifungal activities of diphenyl diselenide and ebselen alone and in combination with antifungal agents against Fusarium spp." **Medical mycology** 54(5): 550-555.
VOGEL, J. U., CINATL, J., DAULETBAEV, N., BUXBAUM, S., TREUSCH, G., CINATL, J., JR., GEREIN, V. e DOERR, H. W. (2005). "Effects of S-acetylglutathione

in cell and animal model of herpes simplex virus type 1 infection." **Medical microbiology and immunology** 194(1-2): 55-59.
WALD, A., ZEH, J., BARNUM, G., DAVIS, L. G. e COREY, L. (1996). "Suppression of subclinical shedding of herpes simplex virus type 2 with acyclovir." **Annals of internal medicine** 124(1 Pt 1): 8-15.
WANG, K., NI, L., WANG, S. e ZHENG, C. (2014). "Herpes simplex virus 1 protein kinase US3 hyperphosphorylates p65/RelA and dampens NF-kappaB activation." **Journal of virology** 88(14): 7941-7951.
WARD, P. L. e ROIZMAN, B. (1994). "Herpes simplex genes: the blueprint of a successful human pathogen." **Trends in genetics : TIG** 10(8): 267-274.
WEISS, H. (2004). "Epidemiology of herpes simplex virus type 2 infection in the developing world." **Herpes** 11 Suppl 1: 24A-35A.
WHITLEY, R. J. (2002). "Herpes simplex virus infection." **Seminars in pediatric infectious diseases** 13(1): 6-11.
WHITLEY, R. J. (2006). "Herpes simplex encephalitis: adolescents and adults." **Antiviral research** 71(2-3): 141-148.
WHITLEY, R. J. e ROIZMAN, B. (2001). "Herpes simplex virus infections." **Lancet** 357(9267): 1513-1518.
WOJTOWICZ, H., KLOC, K., MALISZEWSKA, I., MLOCHOWSKI, J., PIETKA, M. e PIASECKI, E. (2004). "Azaanalogues of ebselen as antimicrobial and antiviral agents: synthesis and properties." **Farmaco** 59(11): 863-868.
WOZNIAK, M. A., MEE, A. P. e ITZHAKI, R. F. (2009). "Herpes simplex virus type 1 DNA is located within Alzheimer's disease amyloid plaques." **The Journal of pathology** 217(1): 131-138.
YANG, F. L., HUA, K. F., YANG, Y. L., ZOU, W., CHEN, Y. P., LIANG, S. M., HSU, H. Y. e WU, S. H. (2008). "TLR-independent induction of human monocyte IL-1 by phosphoglycolipids from thermophilic bacteria." **Glycoconjugate journal** 25(5): 427-439.
YU, S. Y., ZHU, Y. J. e LI, W. G. (1997). "Protective role of selenium against hepatitis B virus and primary liver cancer in Qidong." **Biological trace element research** 56(1): 117-124.
ZACHOS, G., CLEMENTS, B. e CONNER, J. (1999). "Herpes simplex virus type 1 infection stimulates p38/c-Jun N-terminal mitogen-activated protein kinase pathways and activates transcription factor AP-1." **The Journal of biological chemistry** 274(8): 5097-5103.
ZENG, H. (2009). "Selenium as an essential micronutrient: roles in cell cycle and apoptosis." **Molecules** 14(3): 1263-1278.
ZHU, J., PENG, T., JOHNSTON, C., PHASOUK, K., KASK, A. S., KLOCK, A., JIN, L., DIEM, K., KOELLE, D. M., WALD, A., ROBINS, H. e COREY, L. (2013). "Immune surveillance by CD8alphaalpha+ skin-resident T cells in human herpes virus infection." **Nature** 497(7450): 494-497.

ANEXO A – Carta de Aprovação da Comissão de Ética no Uso de Animais-UFSM:

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

CARTA DE APROVAÇÃO

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

Título do Projeto: ": "Avaliação comparativa dos efeitos antiviral, antioxidante e anti-inflamatório do extrato hidroalcoólico de própolis marrom e do disseleneto de difenila nas lesões vaginais agudas causadas por herpes simplex vírus do tipo 2 em camundongos".".

Número do Parecer: 006/2013

Pesquisador Responsável: Prof. Dra. Cristina Wayne Nogueira

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

OBS: Anualmente deve-se enviar à CEUA relatório parcial ou final deste projeto.

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

DATA DE APROVAÇÃO: 04/10/2013.

Santa Maria, 04 de outubro de 2013.

Alexandre Krause
Coordenador da Comissão de Ética no Uso de Animais- UFSM

Comissão de Ética no Uso de Animais - UFSM - Av. Roraima, 1000 – Prédio da Reitoria - 2º andar - Campus Universitário 97105-900 – Santa Maria – RS - - Tel: 0 xx 55 3220 9362

ANEXO B - Autorização para reprodução do artigo científico:

Antiviral Action of Diphenyl Diselenide on Herpes Simplex Virus 2 Infection in Female BALB/c Mice

JOHN WILEY AND SONS LICENSE TERMS AND CONDITIONS

Feb 03, 2017

This Agreement between GLAUBIA SARTORI ("You") and John Wiley and Sons ("John Wiley and Sons") consists of your license details and the terms and conditions provided by John Wiley and Sons and Copyright Clearance Center.

License Number	4041510758181
License date	Feb 03, 2017
Licensed Content Publisher	John Wiley and Sons
Licensed Content Publication	Journal of Cellular Biochemistry
Licensed Content Title	Antiviral Action of Diphenyl Diselenide on Herpes Simplex Virus 2 Infection in Female BALB/c Mice
Licensed Content Author	Gláubia Sartori,Natália Silva Jardim,Marcel Henrique Marcondes Sari,Fernando Dobrachinski,Ana Paula Pesarico,Luiz Carlos Rodrigues,Juliana Cargnelutti,Eduardo F. Flores,Marina Prigol,Cristina W. Nogueira
Licensed Content Date	Dec 30, 2015
Licensed Content Pages	11
Type of Use	Dissertation/Thesis
Requestor type	Author of this Wiley article
Format	Print
Portion	Full article
Will you be translating?	No
Title of your thesis / dissertation	THERAPEUTIC ACTION OF DIPHENYL DISSELENIDE ON VIRAL INFECTION MODELS CAUSED BY HERPES SIMPLEX VIRUSES TYPES 1 AND 2
Expected completion date	Mar 2017
Expected size (number of pages)	120
Requestor Location	GLAUBIA SARTORI AV RORAIMA n. 1000 predio 18 sala 2424 Santa Maria RS, 97105-900 Brazil Attn: GLAUBIA SARTORI
Publisher Tax ID	EU826007151
Billing Type	Invoice
Billing Address	GLAUBIA SARTORI AV RORAIMA n. 1000 predio 18 sala 2424 Santa Maria RS, Brazil 97105-900 Attn: GLAUBIA SARTORI
Total	**0.00 USD**
Terms and Conditions	

TERMS AND CONDITIONS

This copyrighted material is owned by or exclusively licensed to John Wiley & Sons, Inc. or one of its group companies (each a"Wiley Company") or handled on behalf of a society with which a Wiley Company has exclusive publishing rights in relation to a particular work (collectively "WILEY"). By clicking "accept" in connection with completing this licensing transaction, you agree that the following terms and conditions apply to this transaction (along with the billing and payment terms and conditions established by the Copyright Clearance Center Inc., ("CCC's Billing and Payment terms and conditions"), at the time that you opened your RightsLink account (these are available at any time at http://myaccount.copyright.com).

Terms and Conditions

ANEXO C - Autorização para reprodução do artigo científico:

Diphenyl Diselenide Reduces Oxidative Stress And Toxicity Caused By Hsv-2 Infection In Mice

JOHN WILEY AND SONS LICENSE TERMS AND CONDITIONS

Feb 03, 2017

This Agreement between GLAUBIA SARTORI ("You") and John Wiley and Sons ("John Wiley and Sons") consists of your license details and the terms and conditions provided by John Wiley and Sons and Copyright Clearance Center.

License Number	4041510309858
License date	Feb 03, 2017
Licensed Content Publisher	John Wiley and Sons
Licensed Content Publication	Journal of Cellular Biochemistry
Licensed Content Title	Diphenyl Diselenide Reduces Oxidative Stress and Toxicity Caused by HSV-2 Infection in Mice
Licensed Content Author	Gláubia Sartori,Natália Silva Jardim,Marcel Henrique Marcondes Sari,Eduardo F. Flores,Marina Prigol,Cristina W. Nogueira
Licensed Content Date	Jan 5, 2017
Licensed Content Pages	1
Type of Use	Dissertation/Thesis
Requestor type	Author of this Wiley article
Format	Print
Portion	Full article
Will you be translating?	No
Title of your thesis / dissertation	THERAPEUTIC ACTION OF DIPHENYL DISSELENIDE ON VIRAL INFECTION MODELS CAUSED BY HERPES SIMPLEX VIRUSES TYPES 1 AND 2
Expected completion date	Mar 2017
Expected size (number of pages)	120
Requestor Location	GLAUBIA SARTORI AV RORAIMA n. 1000 predio 18 sala 2424 Santa Maria RS, 97105-900 Brazil Attn: GLAUBIA SARTORI
Publisher Tax ID	EU826007151
Billing Type	Invoice
Billing Address	GLAUBIA SARTORI AV RORAIMA n. 1000 predio 18 sala 2424 Santa Maria RS, Brazil 97105-900 Attn: GLAUBIA SARTORI
Total	**0.00 USD**
Terms and Conditions	

TERMS AND CONDITIONS

This copyrighted material is owned by or exclusively licensed to John Wiley & Sons, Inc. or one of its group companies (each a"Wiley Company") or handled on behalf of a society with which a Wiley Company has exclusive publishing rights in relation to a particular work (collectively "WILEY"). By clicking "accept" in connection with completing this licensing transaction, you agree that the following terms and conditions apply to this transaction (along with the billing and payment terms and conditions established by the Copyright Clearance Center Inc., ("CCC's Billing and Payment terms and conditions"), at the time that you opened your RightsLink account (these are available at any time at http://myaccount.copyright.com).

Terms and Conditions

- The materials you have requested permission to reproduce or reuse (the "Wiley Materials") are protected by copyright.