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DISSELENETO DE *P*-METOXI FENILA ATENUA O PREJUÍZO COGNITIVO E A INJÚRIA CEREBRAL EM UM MODELO DA DOENÇA DE ALZHEIMER EM ROEDORES

TESE DE DOUTORADO

Simone Pinton

Santa Maria, RS, Brasil
2012

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Simone Pinton

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Orientadora: Profª Drª Cristina Wayne Nogueira
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Co-Orientadora: Profª Drª Cecília M. P. Rodrigues

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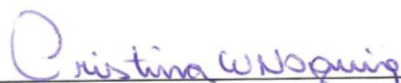

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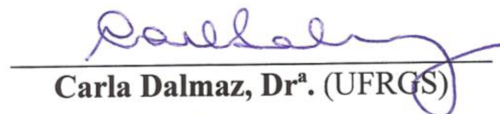

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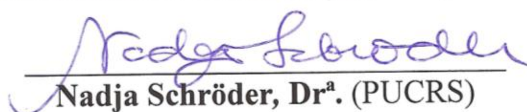

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À minha família.

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"If you can find a path with no obstacles,
it probably doesn't lead anywhere."

Frank A. Clark

RESUMO

Tese de Doutorado
Programa de Pós-Graduação em Ciências Biológicas: Bioquímica Toxicológica
Universidade Federal de Santa Maria

DISSELENETO DE *P*-METOXI FENILA ATENUA O PREJUÍZO COGNITIVO E A INJÚRIA CEREBRAL EM UM MODELO DA DOENÇA DE ALZHEIMER EM ROEDORES

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A doença de Alzheimer (DA) é uma síndrome neurodegenerativa progressiva caracterizada principalmente por uma perda da memória e da capacidade intelectual. Ela é caracterizada pelo depósito de fragmentos β-amilóides; emaranhados neurofibrilares; neuroinflamação; déficit do metabolismo energético; estresse oxidativo e deficiência da neurotransmissão. As múltiplas vias patológicas da DA dificultam sua prevenção e tratamento. Logo, o desenvolvimento de novas terapias para a DA é um desafio. Por esta razão, este trabalho procurou apontar uma nova molécula orgânica contendo selênio, o disseleneto de *p*-metoxi fenila [$(MeOPhSe)_2$], como uma alternativa promissora para o tratamento e prevenção da demência decorrente da DA (DEDA), usando um modelo experimental de demência induzida pela injeção intracerebroventricular (i.c.v.) de estreptozotocina (ETZ) em roedores. Inicialmente, avaliou-se o efeito profilático do $(MeOPhSe)_2$. Para tal, camundongos receberam uma dose oral do organoselênio (25mg/kg, *gavage*) 30 minutos antes da ETZ (2µl de uma solução 2,5mg/ml), esse procedimento foi repetido 48horas depois. Os testes da esquiva passiva, do labirinto em Y e aquático de Morris, que sucederam esse tratamento, revelaram que o $(MeOPhSe)_2$ protegeu os camundongos do prejuízo cognitivo induzido pela ETZ. O $(MeOPhSe)_2$ protegeu o tecido cerebral do aumento das espécies reativas (ER) e da diminuição dos níveis de glutationa (GSH) induzidos pela ETZ, assim como modulou a atividade de enzimas antioxidantes. O $(MeOPhSe)_2$ inibiu a atividade da acetilcolinesterase (AChE), a qual foi estimulada pela ETZ. Posteriormente, investigou-se a efetividade do $(MeOPhSe)_2$ em reverter o prejuízo cognitivo e os danos neuronais induzidos pela ETZ. Para isso, a ETZ foi injetada nos ratos (1µg/8µl, 4µl/ventrículo) em 0 e 48horas. Passados 21dias, iniciou-se uma suplementação dietética com 10ppm de $(MeOPhSe)_2$ durante 30dias. Ao final deste período, observou-se que o $(MeOPhSe)_2$ restaurou as habilidades cognitivas prejudicadas pela ETZ nos ratos, nos testes do labirinto aquático de Morris, esquiva passiva e reconhecimento do objeto. Os resultados referentes aos testes do reconhecimento do objeto e da esquiva passiva apontaram que o $(MeOPhSe)_2$ melhorou *per se* a memória dos ratos. A ETZ aumentou os níveis de ER e de nitração de proteínas no córtex e diminuiu os níveis de GSH no hipocampo dos ratos, o $(MeOPhSe)_2$ reverteu estas alterações. O organoselênio inibiu a atividade da AChE (aumentada pela ETZ) tanto no córtex como no hipocampo dos ratos, mas não modulou o metabolismo da glicose (ETZ diminuiu *ATP-turnover*). O $(MeOPhSe)_2$ evitou a perda neuronal (apoptose) e inibiu os eventos neurodegenerativos (ativação da caspase-3) induzidos pela ETZ. O $(MeOPhSe)_2$ suprimiu a neuroinflamação induzida pela ETZ no hipocampo dos ratos. O organoselênio inibiu a ativação das células gliais e astrócitárias. Baseado nestes resultados, conclui-se que: 1) O $(MeOPhSe)_2$ protegeu e reverteu o declínio das habilidades cognitivas; 2) Os mecanismos envolvidos no efeito neuroprotetor do $(MeOPhSe)_2$ são: antioxidante; inibidor da AChE; supressor da neuroinflamação; 3) O $(MeOPhSe)_2$ não altera o metabolismo energético; e 4) O $(MeOPhSe)_2$ reduziu a morte neuronal. Assim sendo, este trabalho demonstrou que o $(MeOPhSe)_2$ é uma alternativa promissora para o estudo de drogas para o tratamento de desordens cognitivas como a DEDA.

Palavras-chave: Memória; disseleneto de *p*-metoxi fenila; selênio; demênia; doença de Alzheimer; antioxidante, estresse oxidativo, acetilcolinesterase, neuroinflamação.

ABSTRACT

Thesis of Doctor's Degree
Federal University of Santa Maria, RS, Brazil

P,P'-METHOXYL-DIPHENYL DISELENIDE ATTENUATES THE COGNITIVE IMPAIRMENT AND THE BRAIN INJURY IN A SPORADIC DEMENTIA OF ALZHEIMER'S TYPE IN RODENTS

AUTHOR: Simone Pinton
ADVISOR: Cristina Wayne Nogueira
CO-ADVISOR: Gilson Zeni
CO-ADVISOR: Cecília M. P. Rodrigues
PLACE AND DATE OF THE DEFENSE: Santa Maria, September 17, 2012

Alzheimer's disease (AD) is a progressive neurodegenerative disorder, mainly characterized by memory and intellectual capacity loss. AD is characterized by deposition of amyloid-β peptide, neurofibrillary tangles, neuroinflammation, energy metabolism impairment, oxidative stress and synaptic dysfunction and loss. Its multiple pathological pathways contribute to the difficulty of AD treatment and prevention. Thus, the development of new therapies for AD curing or treatment is a challenge. The purpose of this study was to indicate an organoselenium moiety, *p,p'*-dimethoxyl-diphenyl diselenide [$(MeOPhSe)_2$], as a promising alternative for the treatment and prevention of sporadic dementia of Alzheimer-type (SDAT), using an experimental model of dementia induced by intracerebroventricular (i.c.v.) injection of streptozotocin (STZ) in rodents. Initially, it was investigated the prophylactic action of $(MeOPhSe)_2$. For this, mice were treated with $(MeOPhSe)_2$ (25 mg/kg, by gavage) and STZ (2µl of 2,5mg/ml solution; i.c.v.) or vehicles, and 48h after that, the treatment was repeated. The tasks of step-down-type passive-avoidance (SDPA), Y-maze and Morris water-maze (MWM), that followed this treatment, showed that $(MeOPhSe)_2$ protected against the impairment in learning and memory caused by i.c.v. injection of STZ in mice. $(MeOPhSe)_2$ protected against the increase in reactive species (RS) and the reduction of glutathione (GSH) levels, as well as modulated the antioxidant enzymes. $(MeOPhSe)_2$ inhibited the acetylcholinesterase (AChE) activity, which was increased by STZ. Subsequently, it was investigated the effectiveness of $(MeOPhSe)_2$ in reversing the cognitive impairment and neuronal damage induced by STZ. Therefore, rats were injected with STZ (1.0 mg/8µl; 4µl/ventricle) twice, 48h apart. After 21 days of STZ injection, regular diet fed rats were supplemented with 10ppm of $(MeOPhSe)_2$ during 30 days. At the end of this period, it was observed that $(MeOPhSe)_2$ dietary supplementation reversed STZ-induced memory impairment in MWM, SDPA and object recognition tasks. The results on SDPA and object recognition tasks demonstrated that the $(MeOPhSe)_2$ improved memory in rats *per se*. STZ enhanced the RS and protein nitration levels in cortex and decreased GSH levels on hippocampus of rats, $(MeOPhSe)_2$ reversed these alterations. $(MeOPhSe)_2$ normalized AChE activity (which was enhanced by STZ) in both cortex and hippocampus, but did not reverse the deficit in cerebral glucose metabolism (ATP turnover was decrease by STZ). $(MeOPhSe)_2$ was effective in reducing STZ-induced neuronal (apoptosis) loss. Moreover, $(MeOPhSe)_2$ suppressed neuroinflammation induced by STZ in the rats hippocampus. The organoselenium inhibited activation of microglia and astrogliosis. Based on these results, it was concluded that: 1) $(MeOPhSe)_2$ protected and reversed the cognitive abilities decline; 2) the mechanisms involved in the neuroprotective effect of $(MeOPhSe)_2$ are: antioxidant, AChE inhibitor; inflammation suppressor; 3) $(MeOPhSe)_2$ did not alter the energy metabolism; and 4) $(MeOPhSe)_2$ reduced the neuronal death. Therefore, the present study demonstrated that $(MeOPhSe)_2$ is a promising alternative for the drug studies for treatment of cognitive disorders such as SDAT.

Keywords: Memory, *p,p'*-dimethoxyl-diphenyl diselenide, selenium, Alzheimer's disease, antioxidant, oxidative stress, acetylcholinesterase, neuroinflammation.

LISTAS DE FIGURAS

Figura 1: Formação do peptídeo β-amilóide e sua cascata patológica (figura adaptada de Cummings, 2004).. 16

Figura 2: Esquema representativo do estresse oxidativo (figura modificada de Scandalios, 2005). 18

Figura 3: Reação de dismutação do ânion radical superóxido pela enzima superóxido dismutase (SOD).. 21

Figura 4: Reação de eliminação do peróxido de hidrogênio catalisada pela catalase (CAT). 21

Figura 5: Sistema de defesa antioxidante da glutationa. 22

Figura 6: Células gliais normais e ativadas. As imagens A-D mostram o imunoensaio realizado com o Iba1, as imagens A e C são de células em seu estado fisiológico, enquanto a B e D no estado ativado. As imagens de E-G mostram as células astrocitárias, as quais estão ativadas em F e em G com maior detalhe. (Bland et al., 2010; Stozicka et al., 2010). Escala das barras em: A e B 10µm; C e D 25µm, E e F 50µm e G 20µm....................................... 26

Figura 7: Estrutura química da estreptozotocina (ETZ).. 27

Figura 8: Mecanismo de ação proposto para a toxicidade induzida pela ETZ....................... 28

Figura 9: Estrutura química do disseleneto de *p*-metoxi fenila [$(MeOPhSe)_2$]..................... 32

Figura 10: Visão geral dos efeitos do $(MeOPhSe)_2$ em melhorar a cognição de roedores. O organoselênio diminui o EO, a neuroinflamação, a perda e morte neuronal e inibe a atividade da AChE, parâmetros aumentados pela injeção i.c.v. de ETZ. Esta neuroproteção culmina na melhora das habilidades cognitivas dos roedores. O $(MeOPhSe)_2$ não modula o metabolismo da glicose, pois não altera a taxa de *turnover* do ATP a qual foi diminuida pela ETZ. (—) Ação efetiva do $(MeOPhSe)_2$. (---) O $(MeOPhSe)_2$ não interfere. 76

LISTA DE ABREVEATURAS

ACh - Acetilcolina

AChE - Acetilcolinesterase

ALT - Alanina aminotransferase

APP - Proteína precursora do amilóide

AST - Asparato aminotransferase

ATP- Adenosina trifosfato

βA - Fragmento β-amilóide

βA_{1-42} - Fragmento β-amilóide contendo 42 aminoácidos

βA_{1-40} - Fragmento β-amilóide contendo 40 aminoácidos

βA_{15-25} - Fragmento β-amilóide com 11 aminoácidos (derivado do βA_{1-42} e βA_{1-40})

CAT - Catalase

ChAT - Colina acetiltransferase

CNS - Sistema nervoso central

CoA - Coenzima A

CuZnSOD - Superóxido dismutase dependente de cobre e zinco

DA - Doença de Alzheimer

DEDA - Demência esporádica decorrente da doença de Alzheimer

DNA – Ácido desoxirribonucléico

Ebselen - (2-fenil-1,2-benzilselenazol 3(2H)-on)

ENF - Emaranhados neurofibrilares

EO - Estresse oxidativo

ER - Espécies reativas

ERN - Espécies reativas de nitrogênio

ERO - Espécies reativas de oxigênio

ETZ - Estreptozotocina

GFAP - Proteína ácida fibrilar glial (*glial fibrillary acidic protein*)

GPx - Glutationa peroxidase

GR - Glutationa redutase

GSH - Glutationa reduzida

GSSG - Glutationa oxidada

GST - Glutationa S-transferase

i.c.v. - Intracerebroventricular

Iba-1 - Proteína adaptadora ligante de cálcio ionizado (*ionized calcium binding adaptor molecule 1*)

JNK - c-Jun N-terminal quinase

LDDAs - Ligantes difusos derivados da βA,

MAP's - Proteínas associadas aos microtúbulos (*microtubule associated proteins*)

$(MeOPhSe)_2$ - Disseleneto de *p*-metoxi fenila

MnSOD - Superóxido dismutase dependente de manganês

NADPH - Nicotinamida adenina dinucleótido fosfato reduzida

NF- kB - Fator de transcrição nuclear *kappa* B

$(PhSe)_2$ - Disseleneto de difenila

PS - Placas senis

RI - Receptores de insulina

RNA - Ácido ribonucléico

SOD - Superóxido dismutase

SUMÁRIO

1 INTRODUÇÃO 12
1.1 Demência 12
1.2 Doença de Alzheimer (DA) 13
1.3 Mudanças morfológicas na DA 14
1.4 Estresse oxidativo (EO) 17
1.4.1 Espécies reativas (ER) 19
1.4.2 Defesas antioxidantes 20
1.5 Sinalização da insulina e metabolismo energético cerebral 22
1.6 Sistema colinérgico 24
1.7 Neuroinflamação 25
1.8 Modelo animal de DEDA induzida pela estreptozotocina (ETZ) 27
1.9 Selênio (Se) 29
1.10 Compostos orgânicos de selênios 30
2. OBJETIVOS 33
2.1. Objetivo geral 33
2.2. Objetivos específicos 33
3 RESULTADOS 34
3.1 Artigo I 35
2.2 Artigo II 41
3.3 Artigo III 51
3.4 Artigo IV 59
4 DISCUSSÃO 72
5 CONCLUSÕES 77

6 PERSPECTIVAS 78

7 REFERÊNCIAS 79

ANEXOS.. 88

Anexo 1: Outros resultados referentes ao efeito neuroprotetor do disseleneto de p-metoxi fenila 89

Anexo 2: Outros trabalhos publicados 91

1 INTRODUÇÃO

1.1 Demência

A demência é uma síndrome neurológica, geralmente crônica, caracterizada principalmente por uma progressiva e global perda da memória e da capacidade intelectual do individuo, de forma a interferir nas suas atividades sociais ou ocupacionais. Além do prejuízo cognitivo, um comprometimento da linguagem, raciocínio matemático e da capacidade de compreensão e julgamento também são sintomas comuns de pacientes dementes (Janca et al., 2006; Stix, 2010).

Mundialmente existem cerca de 39 milhões de pessoas diagnosticadas com algum tipo de demência e a doença de Alzheimer (DA) é a principal enfermidade associada a este transtorno. Atualmente, estima-se que 60% dos pacientes acima de 65 anos que sofrem de alguma forma de demência no mundo são doentes de Alzheimer e 26% dos pacientes possuem demência vascular, sendo bastante comum a associação de ambas (Janca et al., 2006; Kalaria et al., 2008).

O envelhecimento é o principal fator de risco para a demência, estima-se que apenas 6% dos casos de demência acometem pessoas com idade inferior a 65 anos. Além disso, mulheres são ligeiramente mais suscetíveis ao desenvolvimento da demência. Outros fatores como o estilo de vida, dieta, transtornos que afetam o sistema vascular, como hipertensão, diabetes tipo 2 e obesidade, aumentam o risco de demência (Kalaria et al., 2008).

Mesmo que a idade seja o principal fator de risco para o desenvolvimento de uma demência, o impacto da idade e do sexo é inconclusivo no prognóstico do tempo restante de vida dos pacientes. Variáveis como o tipo de demência e a gravidade da doença são fatores que influenciam diretamente a expectativa média de vida dos pacientes com tal síndrome. Em geral, os pacientes com alguma forma de demência evoluem para a morte em torno de três a dez anos após o diagnóstico (Brodaty et al.,

2012). Infelizmente, ainda não há cura conhecida ou medidas preventivas para a maioria dos tipos de demência.

1.2 Doença de Alzheimer (DA)

Atualmente, cerca de 5% dos homens e 6% das mulheres acima de 60 anos são afetadas pela DA. Dados estatísticos revelam que esta é a doença neurológica com o maior prospectivo de crescimento mundial. Além disso, estudos revelam que esta é a enfermidade que debilita os pacientes por tempo mais prolongado que o câncer ou doenças cardiovasculares, por exemplo. Assim, pacientes com DA requerem cuidados médicos e hospitalares por muito mais tempo, logo, a DA constitui um sério problema de saúde pública, gerando consideráveis custos à economia (Ferri et al., 2005; Stix, 2010).

Estimativas da Organização Mundial da Saúde revelam que o Brasil será o sexto país com maior contingente de idosos em 2025, tendo cerca de 32 milhões de pessoas com mais de 60 anos, esta projeção resulta em um aumento de casos de DA no Brasil. A prevalência e a incidência dessa doença aumenta exponencialmente nos países em desenvolvimento, uma vez que a expectativa média de vida vem aumentando gradativamente nestes países e a prevalência da DA dobra aproximadamente a cada cinco anos após os 65 anos de idade (Scazufca M et al., 2002; Ferri et al., 2005; Kalaria et al., 2008).

A DA foi descoberta pelo Dr. Alois Alzheimer em 1907, e é descrita como uma doença neurológica progressiva e neurodegenerativa. Diferentemente do declínio das habilidades físicas e mentais decorrentes do envelhecimento, na DA o progressivo declínio intelectual é muito mais acentuado (Hardy e Selkoe, 2002; Stix, 2010).

Em um estágio inicial, a DA é mais comumente caracterizada por uma perda insidiosa de memória para fatos recentes, neste estágio, os pacientes também podem apresentar sinais de depressão, ansiedade e mudança de personalidade. Sinais que indicam disfunções cerebrais, tais como a dificuldade de linguagem, a deficiência sensorial, a dificuldade na função motora e a incapacidade de realizar tarefas, são habitualmente observados após alguns anos, e podem afetar a habilidade do paciente na realização de tarefas diárias. Sintomas psicóticos e anormalidades comportamentais

como agressão verbal e motora, ou reclusão, podem se desenvolver com a progressão da doença. Convulsões e mioclonias podem ocorrer nos estágios terminais da doença, e a morte ocorre comumente entre 5 a 10 anos após o diagnóstico (Dooley e Lamb, 2000; Stix, 2010).

Morfologicamente a DA é caracterizada por mudanças neuropatológicas específicas, tais como: o depósito de fragmentos β-amilóide (βA) difusos e/ou organizados em placas senis (PS); a presença de emaranhados neurofibrilares (ENF) decorrentes da hiperfosforilação da proteína Tau; e o surgimento concomitante de sinais claros de inflamação crônica (Hardy e Selkoe, 2002; Moore e O'Banion, 2002; Gotz e Ittner, 2008; Parihar e Brewer, 2010; Stix, 2010). O prejuízo cognitivo em pacientes com a DA é fortemente acompanhado da perda sináptica no neocórtex e no sistema límbico. Os déficits cognitivos relacionados à DA estão principalmente associados à degeneração de neurônios colinérgicos, resultando em déficits da neurotransmissão colinérgica (Ubhi e Masliah, 2012).

Muitas hipóteses etiológicas/patogenéticas vêm sendo estudadas para a DA, entre elas incluem-se a hipótese de defeitos genéticos, causando alterações na clivagem de proteínas precursoras das βAs; deficiência de fatores neurotróficos; defeitos mitocondriais; neurotoxicidade induzida por elementos traço; déficit do metabolismo energético; e neurodegeneração induzida por radicais livres (Markesbery, 1997; Hardy e Selkoe, 2002; Pratico, 2008; Stix, 2010).

Aproximadamente 5% dos casos de DA são familiares, herdados por um modo autossômico dominante de transmissão (mutações nos genes codificadores da proteína precursora da do amilóide (APP), apolipoproteína E, presenilina 1 e 2) (Fridman et al., 2004; Tillement et al., 2011). A maioria dos casos registrados da DA são referidos como uma forma esporádica da doença (demência esporádica decorrente da doença de Alzheimer - DEDA), significando que sua origem é desconhecida. Vários fatores de risco têm sido associados com a DEDA, incluindo a idade, sexo, história familiar, educação, depressão, hipertensão, diabetes, colesterol alto, atividade física e cognitiva baixa e medicamentos. No entanto, o mecanismo pelo qual estes fatores de risco contribuem para a patogênese da DEDA ainda é incerto (Tillement et al., 2011).

1.3 Mudanças morfológicas na DA

As PS e os ENF são os principais biomarcadores da DA. O peptídeo amiloidogênico βA, o qual agrega-se formando oligômeros, é o responsável pela formação das PS. O βA é gerado a partir da clivagem proteolítica da PPA, uma proteína integral de membrana com um grande domínio extracelular (Gotz e Ittner, 2008).

A proteólise da PPA se dá pela ação sequencial de um grupo de enzimas chamadas secretases, estas enzimas podem agir por duas via distintas. A primeira delas é a via não amiloidogênica, na qual a α- e γ-secretase clivam sequencialmente a PPA formando fragmentos solúveis que não tem participação na patofisiologia da DA. No entanto, a PPA pode sofrer ação da β- e γ-secretase e gerar os fragmentos amiloidogênicos βA (mais comumente o $βA_{1-40}$ e o $βA_{1-42}$), e estes, por sua vez, desencadeiam várias cascatas patológicas que levam a disfunções sinápticas e dendríticas, além de ativarem as microglias e os astrócitos e induzirem a morte celular (Figura 1) (Moore e O'Banion, 2002; Cummings, 2004; Selkoe, 2005; Gotz e Ittner, 2008).

Por muito tempo a forma agregada e insolúvel do βA (PS) era considerada a única forma patogênica deste peptídeo, entretanto estudos recentes demonstram que as fibrilas amilóides não são as únicas espécies tóxicas provenientes de monômeros βA, há indícios que formas oligoméricas e solúveis (ligantes difusos derivados da βA, LDDAs) também são capazes de induzir toxicidade e perturbar a transdução de sinal nas sinapses de forma mais rápida e com semelhante grau de toxicidade das PS (Krafft e Klein, 2010). Os LDDAs reduzem a plasticidade sináptica, potencializam a perda de sinapse, perturbam o metabolismo da glicose, contribuem para os danos oxidativos e levam a hiperfosforilação da Tau (Kroner, 2009).

Logo, tanto os LDDAs como o progressivo acúmulo da βA em PS podem estar envolvidos no mecanismo que induz a formação dos ENF (Perez et al., 2008; Pham et al., 2010). Diferentemente das PS, estes ENF são observados dentro do neurônio e são resultados da hiperfosforilação da proteína Tau, uma proteína cuja principal função bioquímica é a estabilização dos microtúbulos (a Tau pertence a família das *MAP's - microtubule associated proteins*).

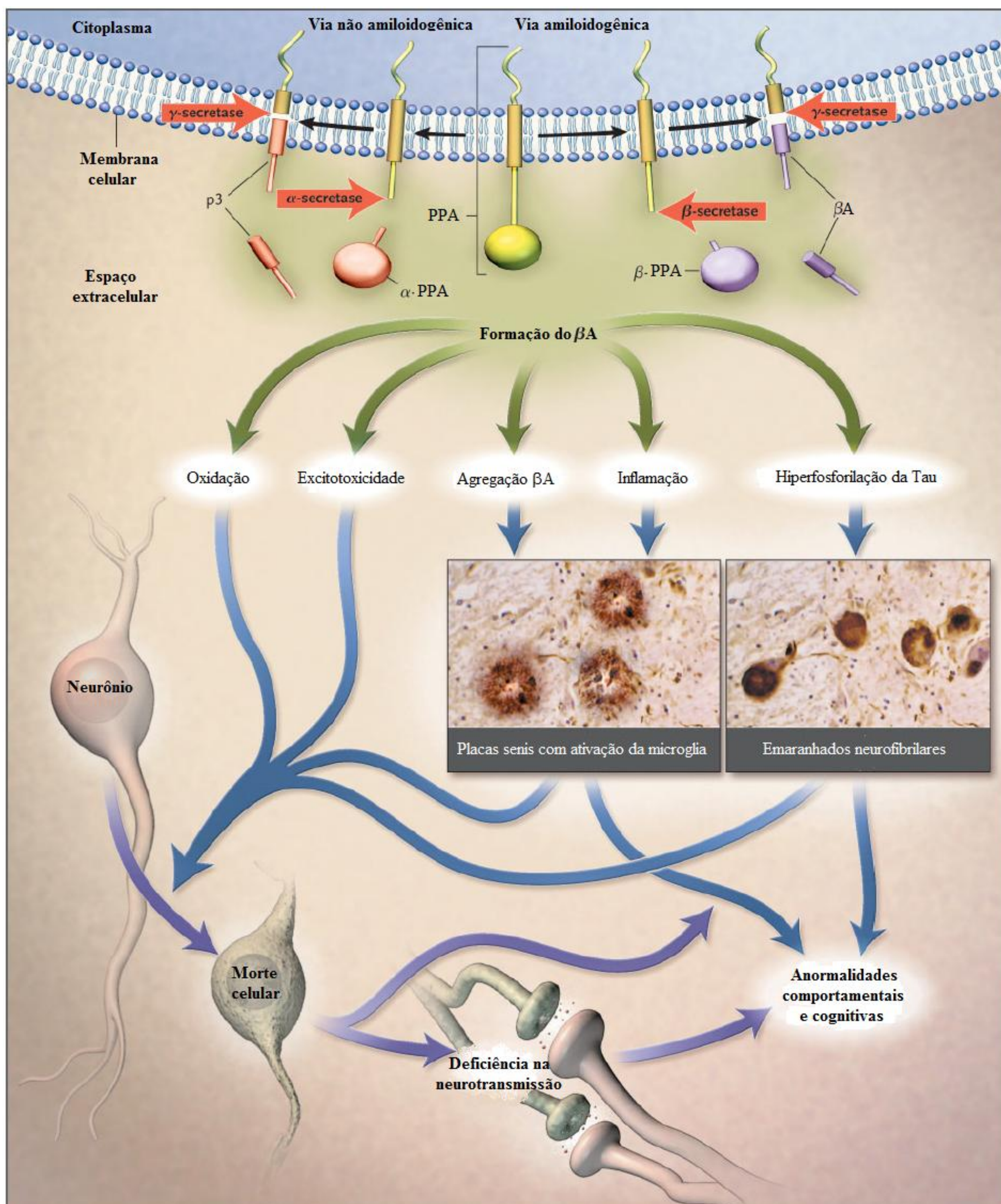

Figura 1: Formação do peptídeo β-amilóide e sua cascata patológica (figura adaptada de Cummings, 2004).

A Tau age ligando-se às unidades de tubulina, possibilitando a organização em hélice das mesmas e o alogamento dos microtúbulos. Sua atividade é regulada por mecanismos de fosforilação e desfosforilação. A interação da Tau com diversas outras proteínas estruturais e funcionais sugere que a Tau pode desempenhar papéis cruciais

não só na arquitetura normal, mas também em transdução de sinal dos neurônios e na regulação da viabilidade celular (Li et al., 2007). O desmonte dos microtúbulos, com a conseqüente desorganização do transporte axonal, aliado a deposição intracelular de proteína Tau hiperfosforilada, acarretam em alterações bioquímicas e morfológicas dos neurônios, culminando em um processo de perda de função e morte neuronal (Hernandez e Avila, 2007).

Assim, embora a patofisiologia da DA não esteja completamente elucidada, assume-se que ambas as alterações morfológicas induzidas pelo Aβ, PS e ENF, iniciam uma cascata patológica que resulta na disfunção e perda sináptica, neuroinflamação, danos oxidativos, alterações na homeostase íons metálico, alterações no metabolismo energético, e, em última análise, a morte neuronal.

1.4 Estresse oxidativo (EO)

Durante o metabolismo celular basal existe uma produção constante de espécies reativas de oxigênio e nitrogênio (ERO/ERN), pró-oxidativas, geradas principalmente durante a respiração celular mitocondrial. Esta geração de espécies reativas (ER) é acompanhada da sua contínua inativação pela ação de antioxidantes, de forma a manter a integridade estrutural e funcional das biomoléculas. O EO é a condição onde há um desequilíbrio entre a formação das ER e seus agentes antioxidantes, em favor das espécies oxidantes, com potencial para ocasionar dano celular. O EO pode ocorrer por um aumento na produção de ER ou por uma diminuição das defesas, ou ambos (veja a Figura 2) (Scandalios, 2005; Halliwell, 2011).

O sistema nervoso central (CNS) é especialmente vulnerável aos danos oxidativos, como um resultado da elevada taxa de consumo do oxigênio pelo cérebro, seu abundante conteúdo lipídico, os elevados níveis de ferro e cobre, e a relativa escassez de enzimas antioxidantes em comparação com outros tecidos (Coyle e Puttfarcken, 1993; Halliwell, 2006). Um grande número de evidências sugere que o EO é uma característica proeminente e precoce na patogênese de doenças neurodegenerativas como a DA (Markesbery, 1997).

O interessante da hipótese do envolvimento do EO nas doenças degenerativas é que o dano oxidativo é consequencia de insultos patológicos que causam, em geral, um

acentuado desequilíbrio entre as ER e antioxidantes. No entanto, o dano oxidativo decorrente do metabolismo também ocorre, de forma muito mais lenta, mas é cumulativo ao longo do tempo e pode ser responsável pelo aparecimento tardio, lento e progressivo destas desordens (Coyle e Puttfarcken, 1993; Markesbery, 1997).

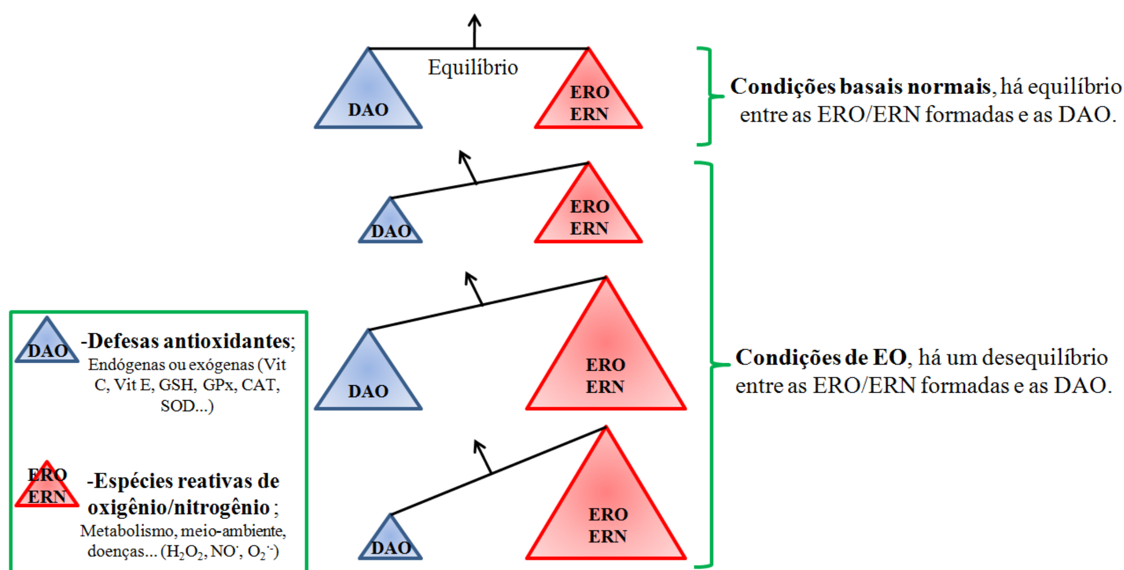

Figura 2: Esquema representativo do estresse oxidativo (figura modificada de Scandalios, 2005).

Há cada vez mais evidências que correlacionam o EO e a disfunção mitocondrial como um fator central na etiologia da AD (Coskun et al., 2012). A toxicidade mitocondrial induzida pelo βA não está completamente elucidada, mas ela pode ocorrer por vários mecanismos, tais como o aumento da permeabilidade das membranas mitocondriais, a perturbação da homeostase do cálcio, a alteração da fosforilação oxidativa com uma consequente produção excessiva de ER (Cummings, 2004; Coskun et al., 2012; Verri et al., 2012). Além disso, a neuroinflamação observada nos pacientes com a DA, (bastante co-localizada com as PS), são ricos em microglia activada. Uma vez estimulada, a microglia libera uma grande variedade de mediadores pró-inflamatórios incluindo citocinas e radicais livres (Moore e O'Banion, 2002; Selkoe, 2011; Verri et al., 2012).

1.4.1 Espécies reativas (ER)

As ER possuem importantes papéis fisiológicos, estas moléculas auxiliam o sistema imunológico atacando microorganismos infecciosos, ativando linfócitos T. Tais espécies também podem agir como sinalizadores e mediadores inflamatórios, por exemplo (Halliwell, 2006; 2011). As ER são intermediários reativos com grande poder de oxidação provenientes do metabolismo fisiológico do oxigênio e nitrogênio ou produzidas por alguma disfunção biológica. As ER correspondem aos radicais livres, espécies químicas que apresentam elétrons não pareados na última camada orbital e são altamente instáveis e reativas, e à espécies não radicalares, as quais são menos reativas mas capazes de gerar radicais. As ER são nocivas ao organismo pois atacam os lipídios, as proteínas e o DNA, oxidando-os e assim induzindo a perda da sua função e/ou estrutura (Halliwell, 2006; 2011).

A respiração celular é a principal fonte endógena geradora de ERO, quando o oxigênio não é completamente reduzido pelos complexos mitocondriais, há a geração de intermediários de oxigênio, como o peróxido de hidrogênio (H_2O_2) e os radicais hidroxila ($HO^•$), ânion superóxido ($O_2^{•-}$). Sendo que o $HO^•$ é a espécie mais deletéria das ERO. O oxigênio singleto ($^1O_2$, o qual possui elétrons não degenerados), o ácido hipocloroso (HClO), a peroxila ($ROO^•$) e alcoxila ($RO^•$) também são exemplos de ERO (Halliwell, 2006).

Dentre as ERN incluem-se o óxido nítrico ($NO^•$), óxido nitroso (N_2O_3), ácido nitroso (HNO_2), nitritos (NO_2^-), nitratos (NO_3^-) e peroxinitritos ($ONOO^-$). O radical $NO^•$ é sintetizado nos organismos pela ação da enzima óxido nítrico sintase (NOS), que converte o aminoácido *L*-arginina em *L*-citrulina liberando o radical $NO^•$. Este é um radical abundante que age em uma variedade de processos biológicos, incluindo relaxação muscular, neurotransmissão, regulação da pressão arterial e controle imune. Diferentemente do $NO^•$, o $ONOO^-$ é um potente agente oxidante, gerado a partir da conjugação do $NO^•$ com o $O_2^{•-}$. O $ONOO^-$ é capaz de nitrar aminoácidos aromáticos (como por exemplo a tirosina, gerando nitrotirosina) e as bases do DNA, em particular a guanina, na qual o produto principal é a 8-nitroguanina (Eiserich et al., 1996).

1.4.2 Defesas antioxidantes

As espécies oxidantes geradas no organismo podem ser detoxificadas por antioxidantes endógenos ou provenientes da dieta (Figura 2). De acordo com Halliwell "*Antioxidante é qualquer substância que, quando presente em baixa concentração comparada à do substrato oxidável, regenera o substrato ou previne significativamente a oxidação do mesmo*". As linhas de defesa do organismo podem ser enzimáticas ou não enzimáticas (Halliwell, 2000).

Dentre as principais enzimas responsáveis pela defesa antioxidante do organismo destacam-se a superóxido dismutase (SOD), a catalase (CAT) e a glutationa peroxidase (GPx). Além destas enzimas, a glutationa (GSH), os peptídeos de histidina, as proteínas ligadas ao ferro (ferritina, tranferrina) e o ácido didrolipóico são exemplos não enzimáticos de defesas produzidas pelo organismo. Defesas exógenas, provenientes da dieta, como o α-tocoferol (vitamina E), o ácido ascórbico (vitamina C), o β-caroteno (pró-vitamina A) e compostos fenólicos como os flavonóides, também possuem um papel importante na desativação das espécies oxidantes (Halliwell, 2000; 2006).

Surperoxido dismutase (SOD)

A atuação do radical ânion superóxido ($O_2^{\bullet-}$) como oxidante direto é irrelevante. Entretanto, o radical ânion superóxido $O_2^{\bullet-}$ presente no organismo é eliminado pela enzima SOD, que catalisa a dismutação de duas moléculas de $O_2^{\bullet-}$ em oxigênio e peróxido de hidrogênio (Figura 3). Este último, quando não eliminado do organismo pelas enzimas peroxidases e catalase, pode reagir com o ferro presente nos tecidos e gerar $HO^{\bullet}$, por essa razão, a atuação da SOD é considerada a primeira linha de defesa endógena de neutralização das espécies reativas (Halliwell, 2006).

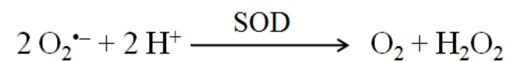

Figura 3: Reação de dismutação do ânion radical superóxido pela enzima superóxido dismutase (SOD).

Os animais possuem isoformas diferentes da SOD, a enzima que contem manganês (MnSOD) no sítio ativo está presente na matriz mitocondrial, enquanto a SOD com cobre e zinco (CuZnSOD) na no espaço mitocondrial intermembranar e no citosol da célula.

Catalase (CAT)

CAT e as peroxidases são responsáveis pela remoção do H_2O_2, e como já foi mencionado, trabalham em conjunto com a SOD. A CAT está presente no SNC e é capaz de eliminar o H_2O_2 em água e oxigênio (veja a equação na Figura 4), integrando assim o sistema de defesa antioxidante do cérebro. Entretanto, a CAT não é muito importante para o cérebro, uma vez que elas não estão presentes nas mitocôndrias (local onde grandes níveis de H_2O_2 são gerados) e, mesmo no citosol, ela encontra-se em baixos níveis (Turrens, 2003; Halliwell, 2006).

Figura 4: Reação de eliminação do peróxido de hidrogênio catalisada pela catalase (CAT).

Sistema da Glutationa (GSH)

As enzimas cerebrais mais importantes na eliminação do H_2O_2 são as GPxs, uma família de enzimas contendo selênio. Este sistema é capaz de eliminar o H_2O_2 acoplando a sua redução à oxidação da GSH, um tripeptídeo tiol contendo glutamato-cisteína-glicina. A GPx catalisa a reação de detoxificação do H_2O_2 através da doação dos equivalentes redutores da GSH. Desta forma, a GSH em seu estado reduzido passa para o seu estado oxidado (GSSG), quando pode sofrer ação da glutationa redutase (GR) e regenerar a GSH, com os equivalentes redutores doados pela nicotinamida adenina dinucleótido fosfato reduzida (NADPH) (Halliwell, 2006).

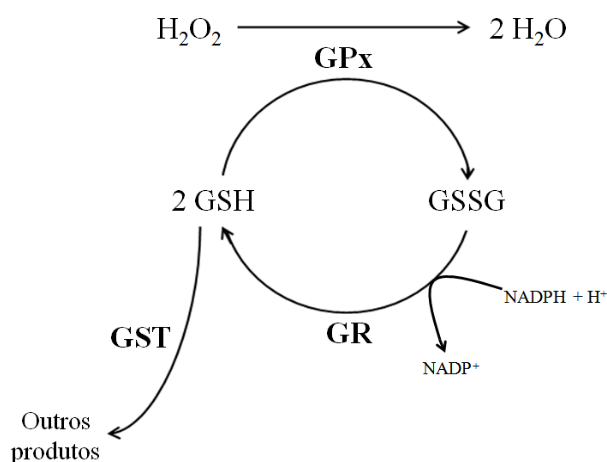

Figura 5: Sistema de defesa antioxidante da glutationa.

A glutationa S-tranferase (GST) participa do sistema de defesa antioxidante dos tecidos, uma vez que ela é uma enzima amplamente distribuída em diversos tecidos que promove a conjugação da GSH com uma variedade de compostos eletrofílicos, resultando na formação substâncias que são facilmente excretadas (Cervello et al., 1992; Luchese e Nogueira, 2010).

1.5 Sinalização da insulina e metabolismo energético cerebral

A proposta de que a sinalização da insulina contribuiria para a patogênese da DA é uma hipótese relativamente recente e desde então, crescentes evidências suportam o

conceito de que AD representa fundamentalmente uma doença metabólica em que a utilização da glicose pelo cérebro e produção de energia são prejudicadas (Henneberg e Hoyer, 1995; Frolich et al., 1998; Bingham et al., 2002; Hoyer, 2002; Marks et al., 2009; Cholerton et al., 2011; de la Monte, 2012). Logo, alterações graves no metabolismo da glicose foram encontradas em todas as formas da DA. A utilização da glicose e os níveis de compostos fosfatados ricos em energia são reduzidos no tecido cerebral de pacientes com DA.

As anormalidades metabólicas cerebrais têm sido associadas à resistência a insulina e ao fator de crescimento semelhante à insulina com interrupção das vias de sinalização que regulam a sobrevivência neuronal, produção de energia, a expressão do gene, e plasticidade sináptica (Frolich et al., 1998; Plum et al., 2005; Kroner, 2009). Estudos demonstraram que os LDDAs causam uma resistência celular a insulina. (Klein, 2002; Kroner, 2009). Corroborando com estes resultados, estudos clínicos e epidemiológicos tem relatado que a diabetes do tipo 2 é um fator de risco para a DA e que a insulina possui efeitos sobre a memória e a cognição (Ronnemaa et al., 2008; Kroner, 2009).

Embora a função da insulina no cérebro não esteja totalmente entendida; o transporte de glicose para as células neuronais seja independente da insulina; e a pouca correlação entre a localização dos receptores de insulina (RI) e a utilização da energia pelos neurônios sugerem um diferente papel fisiológico da insulina no SNC, dados indicam que a cascata de sinalização desencadeada pela insulina/RI está associada a homeostase do metabolismo energético e ao aparecimento e progressão de desordens neurodegenerativas (Hallschmid et al., 2004; Plum et al., 2005; Halmos e Suba, 2011).

No cérebro, a insulina atua como neuromoduladora, controlando processos de liberação de neurotransmissores nas sinapses e ativando vias de sinalização associadas com a aprendizagem e a memória de longo prazo (Bruning et al., 2000; Plum et al., 2005; Benedict et al., 2011). A inibição da sinalização desencadeada pela insulina/RI contribui para a neurodegeneração decorrente da DA por aumentar: i) a atividade de quinases que fosforilam anormalmente a Tau; ii) a expressão da PPA e o acúmulo do peptídeo βA; iii) a disfunção mitocondrial; iv) o ER, pelo aumento da produção de ERO e ERN levando aos danos nos proteínas, RNA, DNA e lipídios; v) a ativação de fatores pró-inflamatórios e pró-apoptóticos; e finalmente vi) a regulação do sistema colinégico, mediando a plasticidade neuronal, memória e cognição (Frolich et al., 1998; Benedict et al., 2011; Cholerton et al., 2011; Halmos e Suba, 2011; de la Monte, 2012).

1.6 Sistema colinérgico

A acetilcolina (ACh) é amplamente distribuída no sistema nervoso onde participa de várias funções vitais. A ativação dos receptores colinérgicos no sistema nervoso periférico tem ações que incluem a redução da frequência e a força da contração cardíaca, o relaxamento de vasos sanguíneos periféricos e a constrição das vias respiratórias. No sistema nervoso central, estão envolvidos no controle da função extrapiramidal, vestibular, em funções cognitivas como memória, aprendizado e atenção, em respostas emocionais, na modulação do estresse, no sono e na vigília (Schliebs e Arendt, 2006).

O neurotransmissor ACh é sintetizado a partir da colina e da coenzima A (CoA) pela ação da enzima colina acetiltransferase (ChAT) principalmente nos neurônios colinérgicos dos núcleos da base de Meynert que se projetam para o córtex e hipocampo (Schliebs e Arendt, 2011). Após sua formação a ACh é liberada na fenda sináptica, onde poderá ser acoplada a dois tipos de receptores, os muscarínicos e nicotínicos. Depois de liberada na fenda sináptica, a ACh é degradada pela enzima acetilcolinesterase (AChE) em colina e acetato (Schliebs e Arendt, 2011).

Com base em evidências experimentais e clínicas, a ACh é considerada um dos mais importantes neurotransmissores envolvidos na regulação das funções cognitivas (Berger-Sweeney, 2003; Schliebs e Arendt, 2006; Agrawal et al., 2009; Ishrat et al., 2009). Durante o envelhecimento, os neurônios colinérgicos sofrem uma moderada degeneração, resultando em uma hipofunção colinérgica, e esta tem sido relaciona com os declínios cognitivos progressivos com o envelhecimento (Schliebs e Arendt, 2011).

Alterações no sistema colinérgico em pacientes com a AD foram documentadas através da avaliação dos principais componentes funcionais e da sinalização dos neurônios colinérgicos. Na DA, evidencias demonstram uma correlação positiva entre a intensidade dos sintomas clinícos de demência com a redução dos marcadores corticais de atividade colinérgica, como os níveis de ChAT, dos receptores muscarínicos e da ACh (Nordberg, 1992; Bierer et al., 1995; Bartus, 2000; Gsell et al., 2004). Estes dados conduziram à formulação da hipótese colinérgica na disfunção da memória na senescência e na AD.

Com base nessa relação, diversos estudos estão desenvolvendo estratégias compensatórias, no intuito de retardar os efeitos da doença, buscando promover aumento dos níveis sinápticos de ACh através, por exemplo, da inibição da AChE. Na clínica, os anticolinesterásicos (inibidores da AChE como a fisostigmina e a donepezila) são usados para o minimizar os sintomas da DA (Bentley et al., 2011).

1.7 Neuroinflamação

A inflamação é uma reação de defesa do organismo contra diversos estímulos nocivos. A resposta inflamatória induz efeitos benéficos para no SNC, limitando a sobrevivência e proliferação de patógenos invasores, além de promover a conservação e o reparo do tecido nervoso (fagocitose de detritos e células apoptóticas, por exemplo). Entretanto, um descontrole ou altos níveis de estímulos inflamatórios resultam na produção de fatores neurotóxicos que agravam a patologia neurodegenerativa (Khandelwal et al., 2011).

A microglia e os astrócitos são as principais células neuronais responsáveis pela inicialização e exacerbação da inflamação no SNC. Os astrócitos são as células mais abundantes do SNC e desempenham muitas funções, como a manutenção da estrutura e conservação da barreira hemato-encefálica e a nutrição e a sustentação do tecido nervoso (Moore e O'Banion, 2002). A microglia é formada por macrófagos especializados, as principais células que compõem o sistema imune do SNC (Ransohoff e Perry, 2009). Em condições fisiológicas, os macrófagos apresentam-se ramificados com grande mobilidade para monitorar o tecido e reconhecer focos de danos ou riscos ao SNC (Nimmerjahn et al., 2005). Nesta condição, a microglia está em um estado basal (inativo) e promove a imunidade do tecido produzindo fatores antiinflamatórios e neurotróficos (Streit, 2002).

Sob condições de estresse, seja ele por invasão de um patógeno, injúria ou anormal acúmulo de proteínas, as células gliais, tanto as microglias como os astrócitos, fornecem uma resposta imune adaptativa, mudando sua morfologia para um fenótipo caracterizado pela presença de um corpo celular mais esférico, pela hipertrofia do núcleo, pelo alongamento/extensão da célula e pela expressão/liberação de fatores e proteínas (Figura 6). Este fenótipo celular mais ativo das células gliais, chamado de

forma amebóide, promove uma resposta inflamatória que estimula o sistema imune a erradicar os estímulos de estresse, contudo, as citocinas e fatores liberados por elas podem agravar e proliferar o estado patológico do tecido (Akiyama et al., 2000; Moore e O'Banion, 2002; Khandelwal et al., 2011).

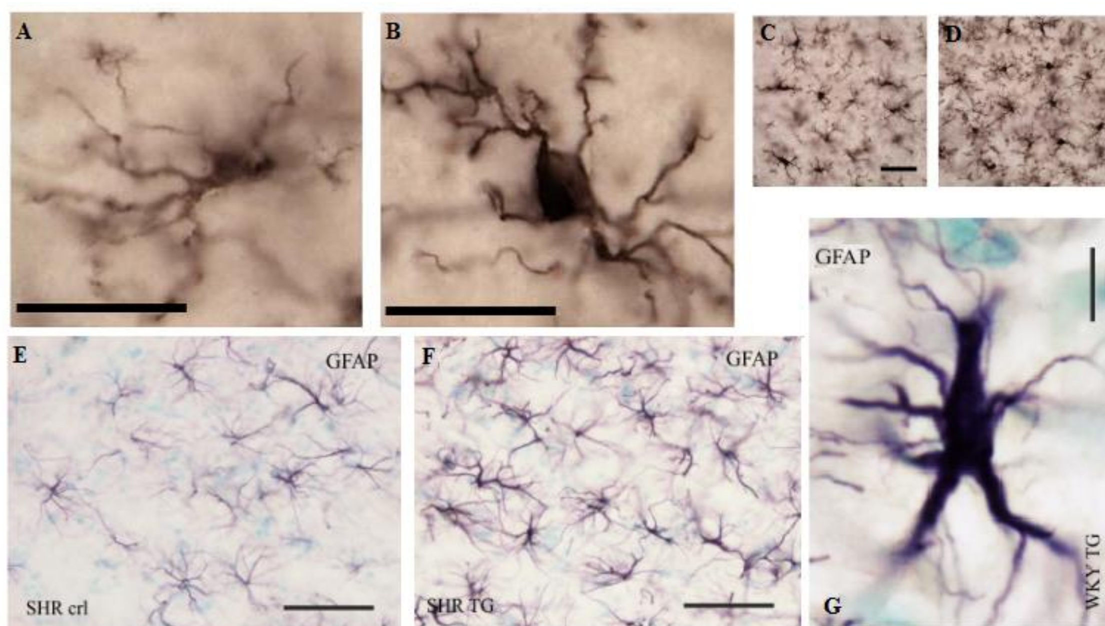

Figura 6: Células gliais normais e ativadas. As imagens A-D mostram o imunoensaio realizado com o Iba1, as imagens A e C são de células em seu estado fisiológico, enquanto a B e D no estado ativado. As imagens de E-G mostram as células astrocitárias, as quais estão ativadas em F e em G com maior detalhe. (Bland et al., 2010; Stozicka et al., 2010). Escala das barras em: A e B 10µm; C e D 25µm, E e F 50µm e G 20µm.

Embora mecanismos diferentes pareçam estar envolvidos na patogênese da DA, muitas evidencias demonstram que a neuroinflamação contribui para a sua progressão e que o peptídeo βA deve induzir um processo inflamatório, uma vez que estudos demonstram que a as células gliais encontram-se ativadas nas regioes que circundam as PS (Akiyama et al., 2000; Glass et al., 2010; Khandelwal et al., 2011).

A ativação das células gliais induzida pelo acúmulo de βA induz mais danos aos neurônios através da produção de mediadores inflamatórios, tais como NO, interleucina 1β, e 6 e fator de transcrição fator nuclear kB (NF- kB) (Moore e O'Banion, 2002; Khandelwal et al., 2011). A GFAP (*glial fibrillary acidic protein*) e o Iba-1 (*ionized calcium binding adaptor molecule 1*) são marcadores de ativação astrocitária e

microglial (Figura 6), respectivamente, e suas produções excessivas são altamente associadas com a geração de um quadro neuroinflamatório e neurodegenerativo (Herman et al., 2012).

1.8 Modelo animal de DEDA induzida pela estreptozotocina (ETZ)

Modelos experimentais que mimetizam e transpõem os sintomas de pacientes com a DA para animais, são ferramentas bastante importantes na busca de novas alternativas para o tratamento desta doença. A injeção intracerebroventricular (i.c.v.) de ETZ, uma glicosamina derivada (Figura 7), em uma dose subdiabetogênica em roedores, tem sido descrita como um modelo apropriado de DEDA, o qual é caracterizado por um progressivo déficit da memória acoplado a diversos efeitos citotóxicos como distúrbios na utilização da glicose e conseqüentemente do metabolismo energético, alterações no sistema colinérgico, EO e neurodegeneração (Lannert e Hoyer, 1998; Lester-Coll et al., 2006; Hoyer e Lannert, 2008; Plaschke et al., 2010; Salkovic-Petrisic et al., 2011; Sharma et al., 2012).

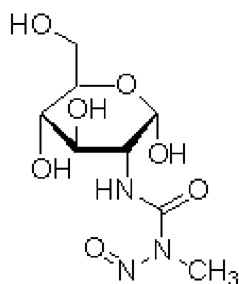

Figura 7: Estrutura química da estreptozotocina (ETZ).

O provável mecanismo pelo qual a ETZ induz citotoxicidade e déficit cognitivo está esquematizado na Figura 8. A injeção i.c.v. de ETZ possivelmente dessensibiliza os receptores neurais de insulina e reduz a atividade de enzimas glicolíticas (Plaschke e Hoyer, 1993; Plaschke et al., 2010). Diversos autores demonstraram que a injeção i.c.v.

de ETZ também diminui a expressão e a densidade dos RI e a síntese cerebral de insulina (Grunblatt et al., 2007; Agrawal et al., 2009; 2011).

A redução do metabolismo energético neural, causado pela ETZ, origina disfunções cognitivas por reduzir a síntese de adenosina trifosfato (ATP) e acetil-CoA, os quais resultam em uma disfunção colinérgica (Henneberg e Hoyer, 1994; Lannert e Hoyer, 1998). Neste modelo de DEDA induzida pela ETZ, a deficiência colinérgica é caracterizada por uma inibição da atividade da ChAT e por um aumento da atividade da AChE (Weinstock e Shoham, 2004; Lester-Coll et al., 2006; Sharma et al., 2008; Ishrat et al., 2009). Além disso, a injeção i.c.v. de ETZ induz um aumento na expressão do gene codificador da AChE (Lester-Coll et al., 2006).

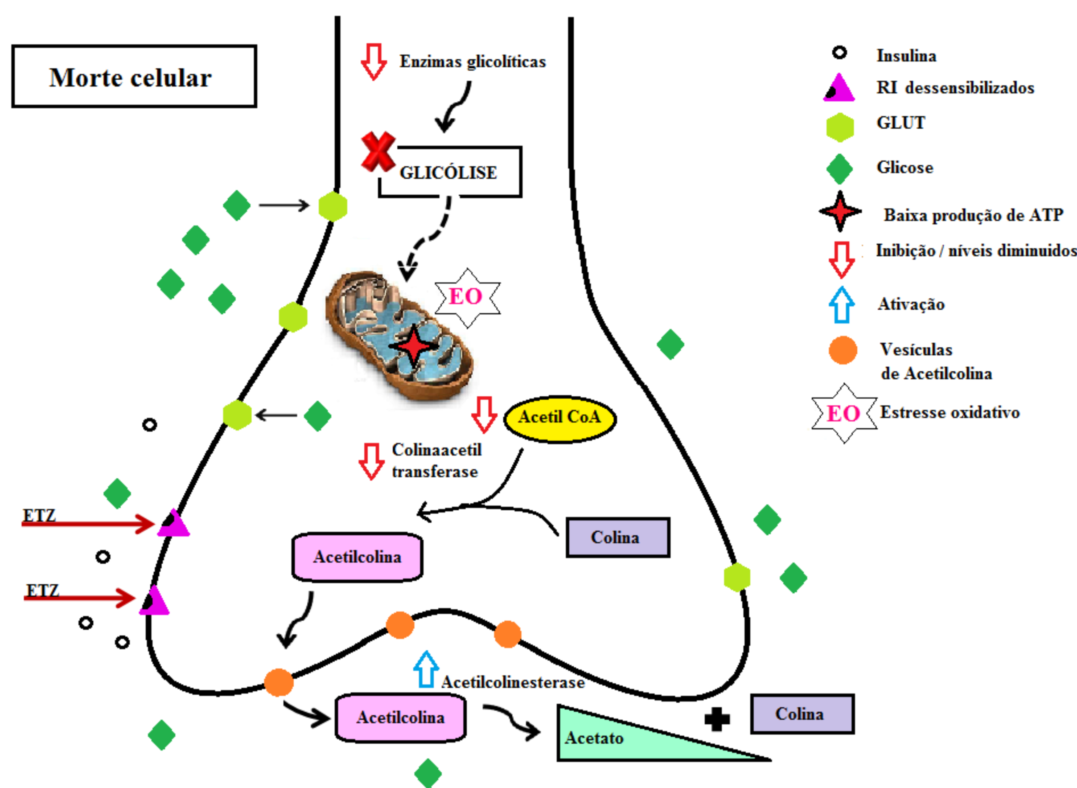

Figura 8: Mecanismo de ação proposto para a toxicidade induzida pela ETZ.

Similar a DA, o EO está envolvido na patogênese do dano neuronal induzido pela ETZ em roedores, desta forma, pode-se admitir que o surgimento de EO está intimamente atrelado aos efeitos deletérios induzidos pela ETZ. Diversos autores já

relataram que há uma maior geração de ER, peroxidação lipídica, carbonilação de proteínas, depleção de defesas antioxidantes não enzimáticas, bem como alterações na atividade de enzimas de detoxificação de ER, como a GPx e a GR (Deshmukh et al., 2009; Ishrat et al., 2009; Prakash e Kumar, 2009; Agrawal et al., 2010; Kumar et al., 2010). Estes relatos confirmam que o surgimento do EO poderia contribuir para a patogênese no modelo de DEDA induzido pela ETZ, reforçando esta hipótese, de que a dessensibilização dos RIs poderia estar diretamente acoplada ao EO. Lester-Coll e colaboradores (2006) demonstraram, usando este modelo de DEDA, que a depleção dos mecanismos de sinalização da insulina e do fator de crescimento semelhante à insulina combinado com o EO são suficientes para causar a neurodegeneração similar à DA.

Por fim, estudos demonstram que além de induzir a morte celular (Lester-Coll et al., 2006; Javed et al., 2011), a injeção i.c.v. de ETZ induz um aumento da hiperfosforilação da Tau (Grunblatt et al., 2007; Plaschke et al., 2010) e da expressão dos genes codificadores da Tau, da APP e dos peptídeos amiloidogênicos (Chu e Qian, 2005; Lester-Coll et al., 2006). As células glias também são ativadas pela ETZ, indicando a presença de neuroinflamação neste modelo (Prickaerts et al., 1999; Weinstock e Shoham, 2004; Rodrigues et al., 2010).

1.9 Selênio (Se)

O elemento Se foi descoberto em 1817 pelo químico sueco Berzelius, por muito tempo o Se foi considerado como sendo apenas um veneno, sem função biológica alguma, até Schwarz e Foltz identificarem-o como um micronutriente em bactérias, mamíferos e pássaros (Schwarz e Foltz, 1957). Recentemente, há um crescente interesse pelo papel do Se no metabolismo, uma vez que ele é um elemento traço essencial de fundamental importância para a saúde humana, com múltiplos papéis no crescimento e funcionamento celular (Papp et al., 2007).

O Se desempenha uma interessante atividade biológica nos organismos ao participar do mecanismo celular de defesa antioxidante, esta influência benéfica pode ser atribuída a presença do Se no sítio ativo de enzimas que possuem atividade antioxidante, como a GPx e a tiorredoxina redutase (Papp et al., 2007). Além de ser conhecido por promover a proteção celular contra o dano induzido pelas ER, no aspecto

terapêutico, o Se é conhecido pelas suas propriedades quimiopreventiva, anti-inflamatória e antiviral (Rayman, 2000; Allmang et al., 2009; Loef et al., 2011).

Estudos têm reportado que o Se é efetivo em prevenir inúmeras condições degenerativas, incluindo a inflamatória e as desordens neurodegenerativas como a DA (Xiong et al., 2007; Loef et al., 2011). Existem dados mostrando que o Se poderia estar envolvido em vias moleculares envolvidas na progressão da DA, embora existam dados controversos associando a suplementação do Se com o prejuízo cognitivo e a patologia da DA, alguns autores demonstraram que a suplementação com Se traria favoráveis efeitos aos pacientes com essa doença (Corrigan et al., 1991; Cornelli, 2010; Loef et al., 2011).

Interessantemente, as concentrações de Se no plasma diminuem com a idade e são ainda menores em pacientes com a DA quando comparado com pacientes saudáveis de mesma faixa etária, indicando que de alguma forma, a diminuição dos níveis de Se poderia contribuir para o declínio das funções neuropsicológicas (Savarino et al., 2001; Akbaraly et al., 2007; Vural et al., 2010). Níveis insuficientes de Se no cérebro tem efeitos potencialmente negativos sobre o seu funcionamento, podendo agravar a perda neuronal e disfunções subseqüentes aos estímulos endógenos ou exógenos, trauma e outras condições neurodegenerativas (Schweizer et al., 2004) além de alterar a taxa de *turnover* de neurotransmissores (Castano et al., 1997). Assim, estes dados sugerem que alterações nas concentrações de Se e/ou das enzimas dependentes de Se poderiam desempenhar um importante papel na etiopatogenese da DA.

Estudos realizados em roedores, nos quais utilizou-se modelos experimentais da DA, o Se foi efetivo em prevenir o dano oxidativo e modular o sistema colinérgico (Ishrat et al., 2009; Lovell et al., 2009; Corcoran et al., 2010; Souza et al., 2010). Finalmente, o Se mostrou-se capaz de reduzir a produção do peptídeo βA e a injúria neuronal induzida por ele em cultura de células (Gwon et al., 2010) e em camundongos transgênicos (Corcoran et al., 2010).

1.10 Compostos orgânicos de selênios

O interesse pela química e bioquímica de compostos orgânicos contendo Se emergiu nas últimas décadas, uma vez que estes demonstraram ter atividades biológicas

e promissoras propriedades farmacológicas. Além disso, evidências sugerem que organoselênios possuem menor toxicidade, maior biodisponibilidade e atividade biológica que compostos inorgânicos de Se (Mahan, 2000). De fato, os organoselênios possuem atividade antitumoral, antimicrobianas, anti-hipertensiva, imunomoduladora, anti-inflamatória, neuroprotetoras entre outras (Mugesh et al., 2001; Nogueira et al., 2004).

Embora poucos estudos tenham focado no efeito neuroprotetror dos organoselênios, evidências demonstraram que a selênio-metionina protege os neurônios da morte celular induzida pelo ferro e pelo βA (Xiong et al., 2007) e que a suplementação dietética com Sel-Plex (formas orgânicas de Se incorporadas por leveduras) atenua a produção e o dano ao DNA induzido pelo βA em camundongos transgênico (APP/PS1) (Lovell et al., 2009).

O Ebselen (2-fenil-1,2-benzilselenazol 3(2H)-on) foi a primeira molécula orgânica de Se a ter sua propriedade neuroprotetora reportada contra a isquemia (Dawson et al., 1995; Namura et al., 2001). O Ebselen possui efeitos neuroprotetores em diferentes modelos experimentais, efeitos estes, atribuídos a sua ação antioxidante (Moussaoui et al., 2000; Rossato et al., 2002; Farina et al., 2003). Além disso, ele reduz a citotoxicidade induzida pelo glutamato em cultura primária de neurônios, o qual deve ser mediado pela inibição da ativação dos receptores glutamatérgicos (Porciuncula et al., 2001). O Ebselen também é efetivo em inibir a fosforilação da Tau induzida pelo ferro em cultura de células devido a inibição do influxo do metal (Xie et al., 2012).

Similares efeitos neuroprotetores tem sido evidenciados pelo disseleneto de difenila [$(PhSe)_2$] e seus análogos substituídos (Nogueira e Rocha, 2010). O $(PhSe)_2$ protege contra o dano oxidativo cerebral induzido pela isquemia/reperfusão (Bruning et al., 2012a) e em um modelo de mania induzido pela ouabaína em ratos (Bruning et al., 2012b). Estes efeitos são atribuídos, pelo menos em parte, a ação antioxidante do $(PhSe)_2$, uma vez que ele possui atividade GPx-*like* e dehidroascorbato-*like* (Luchese e Nogueira, 2010; Nogueira e Rocha, 2010; Bruning et al., 2012a; Bruning et al., 2012b). O $(PhSe)_2$ previne o prejuízo na memória induzido pela escopolamina (antagonista dos receptores muscarínicos) em camundongos (Souza et al., 2010) e reverte o déficit cognitivo induzido pela ovariectomia (da Rocha et al., 2012) e pelo hipotireoidismo (Dias et al., 2012) em ratas. Além disso, o tratamento subcrônico com o $(PhSe)_2$ melhora o desempenho cognitivo de ratos no labirinto aquático de Morris (Stangherlin

et al., 2008) e uma única administração do composto é capaz de melhorar a memória de longa duração no teste do reconhecimento de objeto (Rosa et al., 2003).

Os análogos dissubistituídos do $(PhSe)_2$ apresentaram baixa toxicidade em camundongos (Savegnago et al., 2009). Estes organoselênios são efetivos em prevenir o dano cerebral induzido pelo nitroprussiato de sódio em camundongos (Prigol et al., 2009). O análogo do $(PhSe)_2$ dissubstituído com cloro é efetivo em reverter o prejuízo na memória decorrente do envelhecimento em ratos (Bortolatto et al., 2012).

Por fim, o análogo dissubistituído do $(PhSe)_2$, disseleneto de *p*-metoxi fenila [$(MeOPhSe)_2$] (Figura 11), possui efeitos antioxidantes e propriedades farmacológicas, tais como a hepato e nefroprotetora, antinociceptiva e anti-inflamatória (Pinto et al., 2008; Jesse et al., 2009; Prigol et al., 2009; Wilhelm et al., 2009; Wilhelm et al., 2012). Além disso, $(MeOPhSe)_2$ protege contra o dano oxidativo induzido pelo mercúrio em mitocôndrias de cérebro de camundongos (Meinerz et al., 2011).

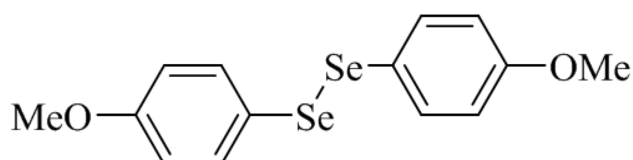

Figura 9: Estrutura química do disseleneto de *p*-metoxi fenila [$(MeOPhSe)_2$].

2. OBJETIVOS

2.1. Objetivo geral

Na tentativa de apontar uma nova e potencial alternativa terapêutica para o tratamento da DEDA e outras formas de demência. O objetivo desta tese foi avaliar o efeito neuroprotetor do $(MeOPhSe)_2$, tanto na prevenção como na terapia da DEDA induzida por ETZ em roedores, através de análises comportamentais, bioquímicas e moleculares.

2.2. Objetivos específicos

- Avaliar a ação profilática do $(MeOPhSe)_2$ em testes de memória e cognição de roedores com declínio cognitivo induzido pela ETZ;
- Verificar se o efeito profilático do $(MeOPhSe)_2$ está relacionado com o sistema colinérgico via modulação da atividade da AChE;
- Inquirir se as propriedades antioxidantes do $(MeOPhSe)_2$ estão envolvidos, preventivamente, no efeito neuroprotetor deste composto;
- Investigar, em diferentes testes comportamentais, o efeito terapêutico do $(MeOPhSe)_2$ contra o prejuízo na memória de roedores induzido por ETZ;
- Avaliar o envolvimento da atividade da AChE, do EO e do metabolismo energético no efeito neuroprotetor do $(MeOPhSe)_2$;
- Determinar se a terapia com $(MeOPhSe)_2$ reverte/impede a neuroinflamação e a neurodegeneração induzida pela ETZ nos roedores.

3 RESULTADOS

Os resultados referentes a esta tese estão apresentados na forma de quatro artigos científicos. Tais resultados foram publicados ou aceitos para a publicação e estão seqüencialmente dispostos abaixo. Os itens Introdução, Materiais e Métodos, Resultados, Discussão e Referências Bibliográficas dos artigos estão organizados de acordo com a recomendação dos periódicos científicos ao quais foram enviados.

3.1 Artigo I

Organoselenium improves memory decline in mice: Involvement of acetylcholinesterase activity

Simone Pinton, Juliana Trevisan da Rocha, Gilson Zeni, Cristina Wayne Nogueira

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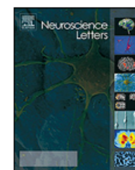

Organoselenium improves memory decline in mice: Involvement of acetylcholinesterase activity

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ABSTRACT

The present study was designed to investigate the possible neuroprotective effect of *p,p'*-methoxyl-diphenyl diselenide [$(MeOPhSe)_2$] in a model of sporadic dementia of Alzheimer's type (SDAT) induced by intracerebroventricular (i.c.v.) injection of streptozotocin (STZ) in mice. Mice were divided into four groups: (I) control, (II) $(MeOPhSe)_2$, (III) STZ, and (IV) $(MeOPhSe)_2$ + STZ. Mice were exposed to $(MeOPhSe)_2$ (25 mg/kg, by gavage) and STZ (2 μl of 2.5 mg/ml solution; i.c.v.) or vehicles. 48 after that the exposure was repeated. Learning and memory were assessed with the step-down-type passive-avoidance (SDPA) and Morris water-maze (MWM) tests at the days 5–6 and 6–9, respectively. At the end of the experimental protocol animals were euthanized and cerebral cortex was removed for acetylcholinesterase (AChE) activity assay. Our results confirmed that i.c.v. STZ caused learning and memory deficits in mice, which were verified using the MWM and SDPA tasks. Furthermore, this study showed that AChE activity was increased in mice that received i.c.v. STZ. The most important findings of the present study are that $(MeOPhSe)_2$ was able to reverse the learning and memory impairments induced by STZ, and to protect against the increase in AChE activity. All these findings support the neuroprotective role of $(MeOPhSe)_2$ in a mice model of SDAT induced by i.c.v. STZ.

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Impaired cerebral glucose utilization and energy metabolism represent very early abnormalities in initial stages of cognitive impairment [12,30]. The reduction in cerebral glucose utilization in cases of dementia has been pointed [13]. Sporadic dementia of Alzheimer's type (SDAT) in particular has been reported as a neurodegenerative disease characterized by progressive memory loss and diminished cognitive ability [11,21,37], reduced levels of glucose utilization and energy rich phosphates [14,18] and acetylcholine synthesis due to lower concentrations of acetyl-coenzyme A [7].

Intracerebroventricular (i.c.v.) injection of streptozotocin (STZ) to rats in a subdiabetogenic dose has been described as an appropriate model for SDAT [8,16] as both are characterized by progressive deterioration of memory, and disturbs on cerebral glucose utilization and energy metabolism [14,18]. I.c.v. STZ possibly desensitizes neuronal insulin receptor and reduced the activities of glycolytic enzyme [24]. It causes reduced cerebral energy metabolism leading to cognitive dysfunction by inhibiting the synthesis of adenosine triphosphate (ATP) and acetyl CoA which results into cholinergic deficiency supported by reduced cholineacetyltransferase (ChAT) activity in hippocampus [14,33] and increased acetylcholinesterase (AChE) activity in rat whole brain [32]. In this context, drugs that improve glucose utilization and metabolism [21] and the enhancement of cholinergic activity by inhibition of AChE enzyme is the mainstay of symptomatic treatment of dementia [34].

Selenium (Se) is an essential biological trace element for mammalian species [6]. The pharmacotherapeutic efficacy of Se has been confirmed in a number of experimental models of brain diseases [11,39]. Evidence has been provided indicating that organoselenium compounds are promising pharmacological agents and possess interesting biological activities. Studies have suggested that they could be considered potential antioxidant and neuroprotective compounds [19].

In fact, acute diphenyl diselenide $(PhSe)_2$ administration enhances cognitive performance of mice in an object recognition paradigm [28]. Accordingly, it has been demonstrated that normal rats sub-chronically exposed to $(PhSe)_2$ presented cognitive improvements in the Morris water-maze task [36].

In this context, the introduction of functional groups in the aromatic ring of diphenyl diselenide can provide alternatives to current therapeutic agents. *p,p'*-methoxyl-diphenyl diselenide [$(MeOPhSe)_2$] was chosen for this study in view of its interesting pharmacological properties already described, such as antioxidant [25] and antinociceptive [23]. However, cognitive studies on this compound still are scarce. In this way, the purpose of the present study was to verify the possible neuroprotective effect of $(MeOPhSe)_2$ in a model of SDAT induced by i.c.v. STZ in mice.

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STZ was purchased from Sigma (St. Louis, MO, USA) and dissolved in artificial cerebral spinal fluid (aCSF). $(MeOPhSe)_2$ was synthesized according to [22]. $(MeOPhSe)_2$ was dissolved in canola oil. All other chemicals were of analytical grade and obtained from standard commercial suppliers.

The experiments were conducted using male Swiss mice (25–35 g), approximately 60 days old, maintained at 22–25 °C with free access to water and food, under a 12:12 h light/dark cycle. All animal experiments were carried out in accordance with the National Institutes of Health Guide for the Care and use of laboratory animals (NIH publication 8023, revised 1996) and with the approval of the local Animal Use Committee.

Mice were divided into four groups: (I) control (n = 10), (II) $(MeOPhSe)_2$ (n = 9), (III) STZ (n = 11), and (IV) $(MeOPhSe)_2$ + STZ (n = 10). Mice were exposed to $(MeOPhSe)_2$ and STZ or vehicles. 48 after that the exposure was repeated. Learning and memory were assessed with the step-down-type passive-avoidance (SDPA) and Morris water-maze (MWM) tests at the days 5–6 and 6–9, respectively. At the end of the experimental protocol animals were euthanized and brain was removed for AChE activity assay.

Mice belonging to groups II and IV received $(MeOPhSe)_2$ (25 mg/kg) by gavage. The dose of $(MeOPhSe)_2$ was chosen based on previous study of our research group which demonstrated that at this dose $(MeOPhSe)_2$ presented neuroprotective effect without causing toxicity [25]. Animals of groups I and III received an application of vehicle by gavage (canola oil, 10 ml/kg of body weight). The same dose of $(MeOPhSe)_2$ or vehicle was repeated 48 h after the first dose.

Thirty minutes after administration of $(MeOPhSe)_2$, groups III and IV received STZ (2 μl of 2.5 mg/ml solution; i.c.v.). Groups I and II received aCSF (2 μl; i.c.v.). I.c.v. injections were given as described by [9] and modified by [15], with the bregma fissure as a reference point [3,35]. The same concentration of STZ was repeated 48 h after the first dose and 30 min after $(MeOPhSe)_2$ administration. The time point of 30 min is enough to the drug reaches to the central nervous system [26,27].

I.c.v. administration was performed under isoflurane anesthesia [3]. Briefly, a 0.4 mm external diameter hypodermic needle attached to a cannula, which was linked to a 25 μl Hamilton syringe. A volume of 2 μl was then administered. The injection site was 1 mm to the right or left from the mid-point on a line drawn through to the anterior base of the ears. To ascertain that the STZ was administered exactly into the cerebral ventricle, the brains were dissected and examined macroscopically after the euthanasia.

Spatial learning and memory were accessed using the MWM task according to the method of [17]. The water-maze consisted of a basin (180 cm × 40 cm) made of black plastic and filled with water (22 ± 2 °C) at a height of 30 cm. Black plastic beads were evenly spread over the water surface in order to camouflage the escape platform (diameter: 10 cm) made of black plastic and covered with a wire mesh grid to ensure a firm grip. The pool was placed in a room with several extra maze visual cues. For the acquisition phase, mice were placed next to and facing the wall successively in north, south, east and west positions. The escape platform was hidden 1 cm below water level in the middle of the northwest quadrant. Behaviors were videotaped via closed circuit TV camera. The experimenter was hidden from the view of the animals, but was able to follow their swimming trajectories on a video monitor, in which the pool was previously separated into four equally spaced quadrants and the platform location was designated. The latency to reach the platform was measured in four trial sessions during 3 days (days 1–3, corresponding to 6–8 days of treatment). The latencies were calculated as mean of total time spent in four trials of each day. The mice remained on the platform for at least 40 s after each trial. Whenever the mice failed to reach the escape platform within the 1 min cut-off period, they were retrieved from the pool and placed on it for 40 s. Twenty-four hours after the acquisition phase (9th day of treatment), a probe trial was conducted by removing the platform and placing the mice next to and facing the north side. The latency to find the place where the platform was and the number of crossings over the former platform position were measured for a single 1-min trial.

Non-spatial long-term memory was investigated using SDPA task according to the method of [29] with modifications in the intensity of electric shock and in the exposure time. During the training session, each mouse was placed on the platform. When it stepped down and placed its four paws on the grid floor, an electric shock (0.5 mA) was delivered for 2 s. The retention test was performed 24 h after training in a similar manner. Each mouse was placed again on the platform and the step-down transfer latency time was recorded.

Spontaneous locomotor activity was measured in the open-field test [38] performed at 8th day of treatment. The floor of the open-field was divided into 9 squares. Each animal was placed individually in the center of the arena and the number of segments crossed (4 paw criterion) and rearings were recorded in a 4-min session.

For AChE activity assay, samples of brain were homogenized in 0.25 M sucrose buffer (1/10, w/v) and centrifuged at 2400 × g at 4 °C for 15 min. Activity of AChE was carried out according to the method of [5], using acetylthiocholine as substrate. The activity of AChE was spectrophotometrically measured at 412 nm. The activity of AChE was expressed as nmol/min/mg protein. Protein concentration was measured according to the method of [4].

At the 9th day of treatment, blood samples were collected from the ventricle of the heart in anesthetized animals, using heparin as the anticoagulant, and plasma was separated by centrifugation (2400 × g) for 15 min. Plasma glucose level was determined by enzymatic colorimetric methods using commercial kits (Labtest Diagnostica, MG, Brazil). Glucose level was expressed as mg/dl.

Results obtained are presented as means ± S.E.M. The statistical significant difference between groups was calculated by means of two-way analysis of variance (ANOVA) followed by Duncan's test when necessary. Probability values less than 0.05 ($p < 0.05$) were considered as statistically significant.

In the MWM, there was no significant difference in the latency to reach platform in the first day of acquisition phase among groups ($F_{1,36} = 0.20$, $p > 0.05$). Two-way ANOVA for second and third days of acquisition phase revealed a significant STZ × $(MeOPhSe)_2$ interaction ($F_{1,36} = 4.32$, $p < 0.05$) and ($F_{1,36} = 3.47$, $p < 0.05$), respectively. Post hoc comparisons demonstrated that STZ injection increased the latency to reach platform in both days (2 and 3). $(MeOPhSe)_2$ treatment protected against the increase of the latency to reach platform caused by STZ in mice (Fig. 1A). In the probe trial, two-way ANOVA of latency to reach the platform showed a significant STZ × $(MeOPhSe)_2$ interaction ($F_{1,36} = 4.28$, $p < 0.05$). Post hoc comparisons demonstrated that STZ injection increased the latency to reach platform. $(MeOPhSe)_2$ prevented memory decline in mice exposed to STZ (Fig. 1B). Two-way ANOVA for the crossing in the platform local revealed a significant STZ × $(MeOPhSe)_2$ interaction ($F_{1,36} = 2.37$, $p < 0.05$). Post hoc comparisons indicated that STZ injection decreased the number of crosses in the platform local. Treatment of mice with $(MeOPhSe)_2$ augmented the number of crosses in the platform local in mice exposed to STZ (Fig. 1C).

During the acquisition phase in the SDPA, there was no difference in the transfer latency time among groups ($F_{1,36} = 0.21$, $p > 0.05$). Two-way ANOVA of the transfer latency time in retention trial showed a significant STZ × $(MeOPhSe)_2$ interaction ($F_{1,36} = 7.15$, $p < 0.05$). Post hoc comparisons yielded that STZ injection decreased the transfer latency time in retention trail. $(MeOPhSe)_2$ prevented the latency time decreased in mice exposed to STZ (Fig. 2).

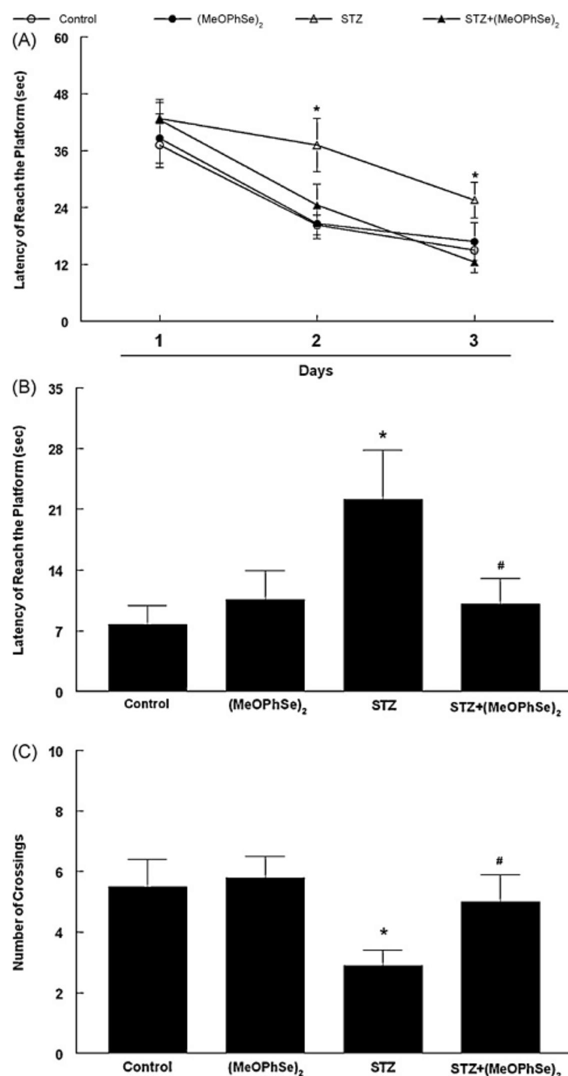

Fig. 1. Effects of $(MeOPhSe)_2$ on STZ induced memory deficit in Morris water-maze test. (A) Latency (s) to reach the platform in the acquisition phase. (B) Latency (s) to reach the platform in the probe test (retention phase). (C) Number of crossings over the former platform position, in the probe test. Data are reported as means ± S.E.M. $^{*}p < 0.05$ as compared to the control group and $^{\#}p < 0.05$ as compared to the STZ group.

The spontaneous locomotor activity measured in the open-field test did not differ significantly among groups. Two-way ANOVA for the number of crossings ($F_{1,36} = 0.43$, $p > 0.05$) and rearings ($F_{1,36} = 0.02$, $p > 0.05$) revealed no significant differences (data not shown).

Two-way ANOVA of AChE activity yielded a significant main effect of STZ. Post hoc comparisons demonstrated that STZ injection increased AChE activity. Treatment of mice with $(MeOPhSe)_2$ restored the increase of AChE activity in mice exposed to STZ ($F_{1,36} = 2.90$, $p < 0.05$) (Fig. 3).

There was no significant difference in blood glucose levels among groups ($F_{1,36} = 0.19$, $p > 0.05$) (data not shown).

In the present study, we investigated the possible neuroprotective effect of $(MeOPhSe)_2$ in a model of SDAT induced by i.c.v. STZ in mice. Our results confirmed that i.c.v. STZ causes cognitive deficits in mice, which were demonstrated using the MWM and SDPA tasks. This study also showed that AChE activity was increased in brains of mice that received i.c.v. STZ. However, the most important finding of the present study is that $(MeOPhSe)_2$ was able to reverse the learning and memory impairments induced by STZ, besides preventing the stimulation in AChE activity.

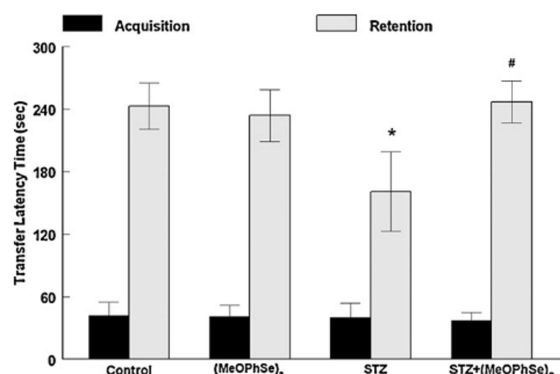

Fig. 2. Effects of $(MeOPhSe)_2$ on STZ induced memory deficit in passive-avoidance test: latency (s) to fall from the platform in the acquisition and retention phase. Data are reported as means ± S.E.M. $^{*}p < 0.05$ as compared to the control group and $^{\#}p < 0.05$ as compared to the STZ group.

Aging is considered as the progressive accumulation of detrimental changes in structure and functions over time accompanied by the end of natural protection against age-related diseases like dementia, cognitive dysfunction and Alzheimer's disease (AD) [2].

Glucose is the brain's main source of energy, and normal glucose metabolism is critical for proper brain functions [11]. Adequate cellular functions are closely dependent on the integrity of cellular energy metabolism [14]. In AD, the age-related reduction in cerebral insulin levels appears to be accompanied by functional disturbances of the insulin receptor, qualifying AD as an Insulin Resistant Brain State [2].

Studies have reported reduced glycolytic enzyme activities such as hexokinase and phosphofructokinase in cerebral cortex and hippocampus after i.c.v. STZ administration in rats [24,12]. In agreement, i.c.v. injection of insulin increases the activities of these enzymes [10]. Data from [20] showed that rats receiving insulin

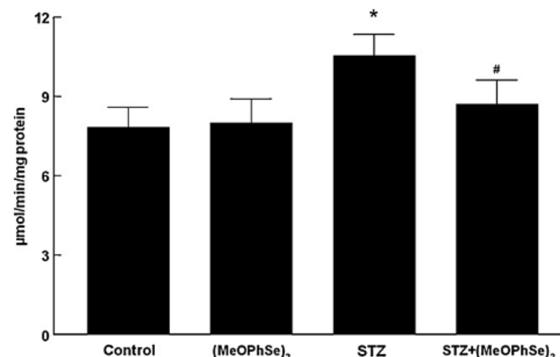

Fig. 3. Effects of $(MeOPhSe)_2$ on STZ induced memory deficit in cerebral cortex acetylcholinesterase activity. Data are reported as means ± S.E.M. $^{*}p < 0.05$ as compared to the control group and $^{\#}p < 0.05$ as compared to the STZ group.

after being shocked in passive-avoidance task had an increased latency to enter the dark compartment, compared to rats that had received saline or heat deactivated insulin after shock. This finding suggests that the role of insulin receptors is essential to learning and memory process.

The cascade of AD neurodegeneration is associated with oxidative stress, mitochondrial dysfunction, impaired energy metabolism, and activation of pro-death signaling pathways [16]. The i.c.v. STZ is known to cause prolonged impairment of brain glucose and energy metabolism by desensitizing neuronal insulin receptors. Corroborating with these data, Lester-coll et al., using a model of i.c.v. STZ in rats, demonstrated that chemical depletion of insulin and insulin-like growth factor signaling mechanisms combined with oxidative injury is sufficient to cause AD-type neurodegeneration. Brain of rats which received i.c.v. STZ exhibited hyperphosphorylated tau protein [8] increased expression of genes encoding acetylcholinesterase, tau, and amyloid precursor protein [16]. Thus, i.c.v. STZ has been approved as an animal model for SDAT [8,12,16].

Consistent with previous studies [8,11,12,16,37], in the present study, i.c.v. STZ in mice caused learning and memory damage, evidenced by the augment in the time required for animals to find the hidden platform in MWM and the decrease in the latency time to stepped down and placed their four paws on the grid floor. Both these tasks are largely employed to evaluate cognitive parameters in animals. MWM evaluates spatial learning and memory [17] while SDPA assesses non-spatial long-term memory [29].

In addition, mice that received $(MeOPhSe)_2$ showed lower time to reach the platform in the MWM, and a raised step-down transfer latency time in passive-avoidance test, both of them similar to the control group. These findings are in agreement with [11] who demonstrated that Se can prevent cognitive deficits in the i.c.v. STZ treated animals. Thus, it is possible suggest that Se may have a therapeutic value for the treatment of SDAT.

Memory impairment induced by i.c.v. STZ in rodents is also associated with defects on cholinergic neurotransmission [1,21,32,33]. Acetylcholine (ACh) is a neurotransmitter required for proper functioning of cholinergic transmission to regulate learning and memory processes [11]. In Alzheimer's disease, brain presents a reduced concentration of acetylcholine in the hippocampus and neocortex, caused by degeneration of cholinergic neurons. Thereat, anticholinesterases are effective in the treatment of Alzheimer's disease, resulting in an improvement in cognitive behavior in patients [31].

Accordingly, the present study showed that $(MeOPhSe)_2$ could be an interesting alternative to treat AD patients, since this compound was able to inhibit the increase in AChE activity in mice that received i.c.v. injections of STZ. Regarding the mechanism by which $(MeOPhSe)_2$ exerts its neuroprotective effect, our data indicate that $(MeOPhSe)_2$ modulates the cholinergic system by inhibiting the activity of AChE in brains of mice. However, we cannot rule out the involvement of $(MeOPhSe)_2$ antioxidant effect [25] in the protection against damage induced by STZ, since this model of dementia causes oxidative stress [1,11].

In summary, we demonstrated that i.c.v. STZ in mice induced a significant impairment on learning and memory, which were evaluated in MWM and SDPA tasks and that this impairment was accompanied by increased cerebral AChE activity. We also showed that $(MeOPhSe)_2$, an organoselenium compound, represents a promising drug to treatment of dementia, since it improved mice performance in cognitive tasks and protected against the increase in cerebral AChE activity. All these findings support the neuroprotective role of $(MeOPhSe)_2$ in a mice model of SDAT induced by i.c.v. STZ.

Acknowledgments

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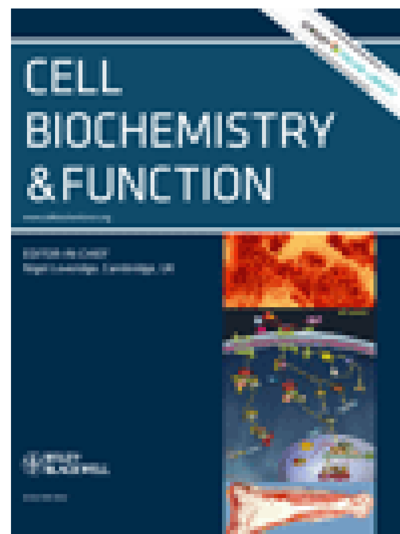
2.2 Artigo II

Neuroprotector effect of p,p'-methoxyl-diphenyl diselenide in a model of sporadic dementia of Alzheimer's type in mice: contribution of antioxidant mechanism

Simone Pinton, Juliana Trevisan da Rocha, Bibiana Mozzaquatro Gai, Marina Prigol, Luiz Vinícius da Rosa and Cristina Wayne Nogueira



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Neuroprotector effect of *p,p'*-methoxyl-diphenyl diselenide in a model of sporadic dementia of Alzheimer's type in mice: contribution of antioxidant mechanism

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The present study investigated whether the antioxidant activity of *p,p'*-methoxyl-diphenyl diselenide [$(MeOPhSe)_2$] is involved in its protective effect against cognitive impairment induced by streptozotocin (STZ) in a model of sporadic dementia of Alzheimer's type (SDAT). Swiss mice were treated with STZ or vehicle [2 μl of 2·5 $mg\,ml^{-1}$ solution; intracerebroventricularly (i.c.v.)] twice, 48 h apart. $(MeOPhSe)_2$ (25 $mg\,kg^{-1}$) or vehicle was orally administered 30 min prior to each STZ treatment. Neuroprotector effect of $(MeOPhSe)_2$ on the behavioral performance of mice on spatial recognition memory consolidation was investigated in the Y-maze test. After that, mouse brains were removed for measuring antioxidant parameters. $(MeOPhSe)_2$ protected against the impairment in learning and memory caused by i.c.v. administration of STZ in mice. $(MeOPhSe)_2$ protected against the increase in reactive species and the reduction of glutathione levels, as well as, the increase in superoxide dismutase and glutathione S-transferase activities caused by STZ in whole brain. These results suggest that antioxidant property is involved, at least in part, in the neuroprotective effect of $(MeOPhSe)_2$ on SDAT induced by STZ in mice. Copyright © 2011 John Wiley & Sons, Ltd.

KEY WORDS — dementia of Alzheimer's type; selenium; streptozotocin; oxidative stress; antioxidant

INTRODUCTION

Oxygen free radicals or, more generally, reactive oxygen species (ROS), are generated continuously during normal oxidative metabolism.[1] Free radical oxidation is responsible for the degradation of fatty acids and their esters, proteins and nucleic acids in biological system.[2] Consequently, this oxidation may play a role in pathologic processes. There has been heightened interest in the role of oxidative stress in neurodegenerative diseases. In this context, there is strong evidence that free radicals play an important role in several neurologic disorders, such as cerebral ischaemia-reperfusion, Parkinson's disease, Huntington's disease, epilepsy, amyotrophic lateral sclerosis, Down's syndrome and Alzheimer's disease.[3]

Selenium (Se) is a nutritionally essential trace element with known antioxidant potential for its role in the expression of several peroxidases and redox enzyme systems, which protect cells from oxidative stress.[4] The interest in organoselenium chemistry and biochemistry has increased mainly due to the fact that these compounds have become attractive synthetic targets because of their chemio-, regio- and stereo-selective reactions.[5]

Studies have suggested that organoselenium compounds could be considered potential antioxidant and neuroprotective compounds.[6–8] Our research group has demonstrated the neuroprotective properties of different organoselenium compounds against some models of neural injury.[9–12] Moreover, diphenyl diselenide [$(PhSe)_2$], a simple organoselenium compound, enhances cognitive performance of mice and rats.[13,14] In this way, the introduction of functional groups in the aromatic ring of $(PhSe)_2$ can provide alternatives to current therapeutic agents, since the introduction of functional groups into this molecule can alter its proprieties.[7,15]

p,p'-Methoxyl-diphenyl diselenide [$(MeOPhSe)_2$] (Figure 1) had already its antioxidant property reported in *in vitro* and *in vivo* studies.[10,15,16] Additionally, we recently demonstrated that $(MeOPhSe)_2$ improves memory in a mouse model of sporadic dementia of Alzheimer's type (SDAT) induced by intracerebroventricular (i.c.v.) injection of streptozotocin (STZ), since it improved mice performance in cognitive tasks.[17] I.c.v. injection of STZ to rodents, in a subdiabetogenic dose, has been proposed as an appropriated model for SDAT, since it results in impairment of brain energy metabolism and oxidative damage, leading to cognitive dysfunction by cholinergic deficiency.[18–21]

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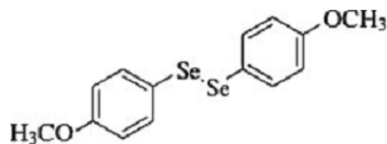

Figure 1. Chemical structure of *p,p*'-methoxyl-diphenyl diselenide

Our previous data show that the mechanism by which $(MeOPhSe)_2$ exerts its neuroprotective effect in the SDAT model is related to the modulation of the cholinergic system by inhibiting acetylcholinesterase (AChE) activity in mouse brain.[17] Thus, the purpose of the present study was to investigate whether the antioxidant activity of $(MeOPhSe)_2$ is involved in its protective effect against cognitive impairment induced by STZ in a model of SDAT.

EXPERIMENTAL PROCEDURE

Chemicals

STZ was purchased from Sigma (St. Louis, MO, USA), which was dissolved in artificial cerebral spinal fluid (aCSF). $(MeOPhSe)_2$ was synthesized according to Paulmier.[22] Analysis of the ^{1}H NMR and ^{13}C NMR spectra showed that $(MeOPhSe)_2$ obtained presented analytical and spectroscopic data in full agreement with its assigned structure. The chemical purity of compound (99·9%) was determined by GC-MS. $(MeOPhSe)_2$ was dissolved in canola oil. All other chemicals were of analytical grade and obtained from standard commercial suppliers.

Animals

Experiments were conducted using male Swiss mice (25–35 g), approximately 60 days old, maintained at 22–25 °C with free access to water and food, under a 12:12 h light/dark cycle (with lights on at 7:00 AM). Mice were acclimatized to the laboratory for at least 1 h before testing. Animals were used according to the guidelines of the Committee on Care and Use of Experimental Animal Resources and with the approval of the Animal Use Committee (# 23081.007005/2010–96), the Federal University of Santa Maria, Brazil.

Experimental protocol

Mice were divided into four groups ($n = 10$ animals per group): (I) control, (II) $(MeOPhSe)_2$, (III) STZ and (IV) $(MeOPhSe)_2 + STZ$. Mice were exposed to $(MeOPhSe)_2$ and STZ or appropriate vehicles. After that (48 h), the exposure was repeated. Nine days after the first dose of STZ and/or $(MeOPhSe)_2$, animals were euthanized and brains were removed.

Administration of $(MeOPhSe)_2$

Mice belonging to groups II and IV received $(MeOPhSe)_2$ ($25\ mg\,kg^{-1}$), per oral route, by gavage. Animals of groups I and III received an application of vehicle by gavage (canola oil, $10\ ml\,kg^{-1}$ of body weight). $(MeOPhSe)_2$ was administered to mice 30 min before i.c.v. STZ injection. The time of pre-treatment and the dose of $(MeOPhSe)_2$ were chosen based on previous study of our research group, which demonstrated that at this dose $(MeOPhSe)_2$ has neuroprotective effect in a mice model of SDAT induced by i.c.v. STZ.[17]

I.c.v. administration of STZ

Thirty minutes after administration of $(MeOPhSe)_2$, groups III and IV received STZ (2 µl of $2{\cdot}5 mg\,ml^{-1}$ solution; i.c.v.). Groups I and II received aCSF (2 µl; i.c.v.). I.c.v. injections were given as described by Haley & McCormick and modified by Laursen & Belknap, with the bregma fissure as a reference point.[23,24] I.c.v. administration was performed under isoflurane anaesthesia. Briefly, a 0·4 mm external diameter hypodermic needle attached to a cannula, which was linked to a 25 µl Hamilton syringe. A volume of 2 µl was then administered. The injection site was 1 mm to the right or left from the mid-point on a line drawn through to the anterior base of the ears. To ascertain that the STZ was administered exactly into the cerebral ventricle, the brains were dissected and examined macroscopically after the euthanasia.

Y-maze apparatus and test

Spatial recognition memory consolidation in the Y-maze was assessed at the eighth day after the first i.c.v. injection of streptozotocin. The Y-maze test was performed as decribed by Zhang and co-workers with some modifications.[25] Y-maze apparatus was made of wood and consisted of three arms ($8 \times 30 \times 15$ cm, width × length × height), with an angle of 120° between each arm. The arms were randomly designated: 'A' arm, in which the mouse began to explore (always open); 'C' arm, which was closed off during the 1st trial, but open in the second trial and 'B' arm (always open). The maze was placed in a quiet, illuminated room and visual cues were placed on the walls of the arms. The maze floor was cleaned with alchool after each trial in order to eliminate olfactory stimulus.

The Y-maze test consists of two trials separated by an inter-trial interval (ITI) to assess spatial recognition memory. The first trial (training) lasted 6 min and allowed the mouse to explore only two arms ('A' and 'B' arms) of the maze, with access to the third arm ('C' arm) blocked off. After 3 h ITI, the second trial (retention) was conducted, during which all three arms were accessible; the mouse was returned to the same starting arm and allowed to explore all three arms for 5 min. The number of entries and time spent in each arm during the retention trial were determined. Spatial recognition memory can be measured by the absolute time spent in the 'C' arm during the 5 min retention period, while the total number of arm visits gives an index of locomotor activity.

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Open field test

Spontaneous locomotor activity was measured in the open-field test performed at eighth day of treatment, immediately after the Y-maze test. The floor of the open-field was divided into nine squares. Each animal was placed individually in the centre of the arena and the number of segments crossed (four paw criterion) and rearings were recorded in a 4-min session.[26]

Tissue preparation

Nine days after the first dose of drugs, the animals were euthanized, blood and brain were removed. Brain was homogenized in 50 mM Tris-Cl, pH 7·4 (1:10, *w/v*). The homogenate was centrifuged at $2400 \times g$ for 15 min at 4 °C and a low-speed supernatant fraction (S_1) was used for assays. Except for protein carbonyl and GSH determinations in which brain homogenates were used. Blood samples were collected directly from the ventricle of the heart in anaesthetized animals, using heparin as the anticoagulant, and plasma was separated by centrifugation ($2400 \times g$) for 15 min.

Reactive species (RS) levels

To estimate the RS production, S_1 was diluted (1:10) in 50 mM Tris-HCl (pH 7·4) and incubated with 10 µl of 2′,7′-dichlorofluorescein diacetate (DCHF-DA; 1 mM), at room temperature for 30 min. The RS levels were determined by a spectrofluorimetric method, using DCHF-DA assay. DCHF-DA is a nonfluorescent compound easily that crosses cell membranes and, in the presence of RS is rapidly oxidized to its highly fluorescent derivative dichlorofluorescein (DCF).[27] 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. The RS levels were expressed as arbitrary unit (AU).

Thiobarbituric acid reactive species (TBARS) levels

TBARS levels were determined as described by Ohkawa *et al*.[28] An aliquot of S_1 was incubated with 0·8% thiobarbituric acid (TBA), acetic acid buffer pH 3·4 and 8·1% sodium dodecil sulphate at 95 °C for 2 h. The colour reaction was measured at 532 nm. TBARS levels were expressed as nmol MDA (malondialdehyde) per mg protein.

Protein carbonyl (PC) determination

Brain homogenates (1:10, *w/v*) were prepared in 50 mM Tris-HCl buffer, pH 7·4. Homogenates were diluted with Tris-HCl buffer, pH 7.4 in a proportion of 1:8 and these aliquots were used. The PC determination was carried out as described by Reznick and Packer.[29] In two tubes, it was added 200 µl of 10 mM 2,4-dinitrophenylhydrazine (DNPH) in 2·0 M HCl. The other tube contains only 200 µl of 2·0 M HCl solution (blank). Tubes were incubated for 60 min at room temperature, in dark and vortexed every 15 min. After that, 0·5 ml of denaturizing buffer (sodium phosphate buffer, pH 6·8, containing 3% SDS), 1·5 ml of ethanol and 1·5 ml of hexane were added. The mixture was vortexed for 40 s and centrifuged for 15 min at $2000 \times g$. The pellet obtained was separated and washed two times with 1 ml of ethanol: ethyl acetate (1:1, *v/v*). The pellet was dissolved in 1 ml of denaturizing buffer solution with mixing. Absorbance was measured at 370 nm. PC levels were expressed as nmol carbonyl per mg protein.

Catalase (CAT) activity

CAT activity in S_1 was assayed spectrophotometrically by the method of Aebi which involves monitoring the disappearance of H_2O_2 in the presence of S_1 at 240 nm.[30] An aliquot of S_1 was added in 50 mM potassium phosphate buffer pH 7·0 and the enzymatic reaction was initiated by adding H_2O_2. One unit of enzyme was defined as the amount of enzyme required for monitoring the disappearance of H_2O_2. The enzymatic activity was expressed as Units (U) per mg protein (1U decomposes 1 µmol H_2O_2 min^{-1} at pH 7 at 25 °C).

Superoxide dismutase (SOD) activity

SOD activity was assayed spectrophotometrically as described by Misra & Fridovich.[31] This method is based on the capacity of SOD in inhibiting autoxidation of epinephrine to adrenochrome. The colour reaction was measured at 480 nm. At the test day, S_1 was diluted 1:10 (*v/v*) for determination of SOD activity. Aliquots of S_1 were added in a 50 mM sodium carbonate (Na_2CO_3) buffer pH 10·3 and the enzymatic reaction was initiated by adding epinephrine. One unit of enzyme was defined as the amount of enzyme required to inhibit the rate of epinephrine autoxidation by 50% at 26 °C. The enzymatic activity was expressed as Units (U) mg^{-1} protein.

Glutathione (GSH) levels

Levels of reduced GSH were determined fluorometrically following Hissin & Hilf using *o*-phthalaldehyde (OPA) as fluorophore.[32] Briefly, the samples were homogenized in 0·1 M perchloric acid ($HClO_4$). Homogenates were centrifuged at $3000 \times g$ for 10 min and the supernatants were separated for measurement of GSH. Supernatant (100 µl) was incubated with 100 µl of OPA (0·1% in methanol) and 1·8 ml of 0·1 M phosphate buffer (pH 8·0) for 15 min at room temperature in dark. Fluorescence was measured with a fluorescence spectrophotometer at excitation wavelength of 350 nm and at emission wavelength of 420 nm. GSH levels were expressed as nmol g^{-1} of tissue.

Glutathione peroxidase (GPx) activity

GPx activity in S_1 was assayed spectrophotometrically by the method of Wendel, through the GSH/NADPH/gluta-

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thione reductase system, by the dismutation of H_2O_2 at 340 nm. S_1 was added in GSH/NADPH/glutathione reductase system and the enzymatic reaction was initiated by adding H_2O_2.[33] In this assay, the enzyme activity was indirectly measured by means of NADPH decay. H_2O_2 is reduced and generates GSSG from GSH. GSSG is regenerated back to GSH by glutathione reductase present in the assay media at the expenses of NADPH. The enzymatic activity was expressed as nmol NADPH $min^{-1} mg^{-1}$ protein.

Glutathione reductase (GR) activity

GR activity in S_1 was determined as described by Carlberg & Mannervik.[34] In this assay, GSSG is reduced by GR at the expense of NADPH consumption, which was followed at 340 nm. GR activity is proportional to NADPH decay. The enzymatic activity was expressed as nmol NADPH $min^{-1} mg^{-1}$ protein.

Glutathione S-transferase (GST) activity

GST activity was assayed spectrophotometrically at 340 nm by the method of Habig *et al.*[35] The reaction mixture contained an aliquot of S_1, 0·1 M potassium phosphate buffer pH 7·4, 100 mM GSH and 100 mM CDNB, which was used as substrate. The enzymatic activity was expressed as nmol CDNB conjugated $min^{-1} mg^{-1}$ protein.

Protein determination

Protein concentration was measured according to the method of Bradford.[36]

Blood glucose estimation

Plasma glucose level was determined by enzymatic colorimetric methods using commercial kit (Labtest Diagnostica, MG, Brazil). Glucose level was expressed as mg dl^{-1}.

Statistical analysis

The results are presented as the means ± S.E.M. The statistical significant difference among groups was calculated by means of two-way analysis of variance (ANOVA) followed by Duncan's test when necessary. Probability values less than 0·05 ($p < 0·05$) were considered statistically significant.

RESULTS

Effects of i.c.v. STZ injection on consolidation of spatial recognition memory in the Y-maze and spontaneous locomotor activity in open field test

Two-way ANOVA of duration spent of mice in the A arm data, the starting arm, demonstrated a significant main effect of STZ ($F_{1,34} = 6·27$; $p = 0·0197$). No changes were found in duration spent in the B arm in all groups tested ($F_{1,34} = 0·07$; $p > 0·05$).

Two-way ANOVA of duration spent in the novel arm data demonstrated a significant STZ × $(MeOPhSe)_2$ interaction ($F_{1,34} = 4·08$; $p = 0·0445$). As shown in Figure 2, the total duration spent by control group in the novel arm (C arm) was increased in comparison to the B arms. Statistical analysis revealed that control mice spent more time (64%) in the novel arm ($p = 0·0174$) when compared to B arm. In

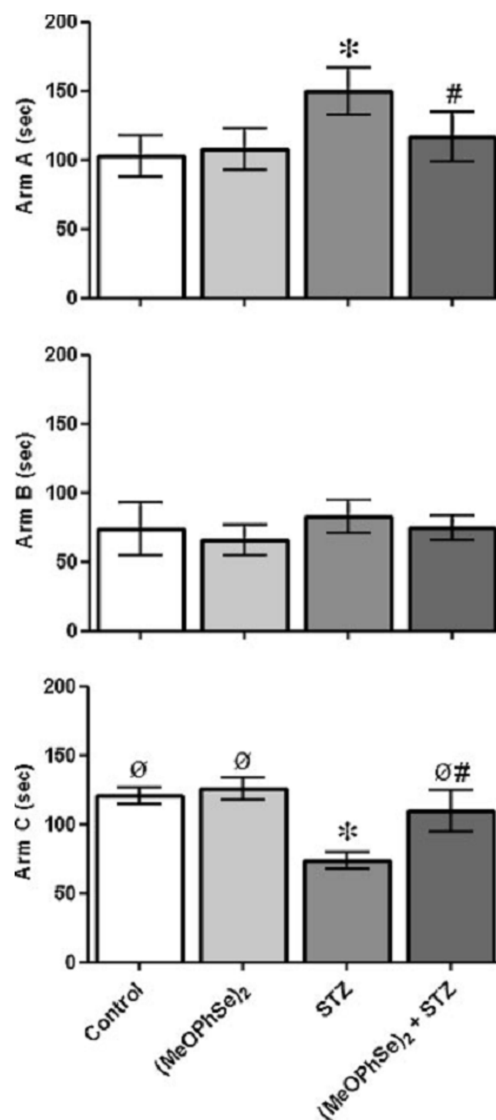

Figure 2. Effects of pre-treatment with $(MeOPhSe)_2$ and of i.c.v. STZ injection on consolidation of spatial recognition memory in the Y-maze: time spent in each arm of Y maze apparatus. Data are reported as means ± S.E.M. *Denotes $p < 0·05$ as compared to the control group; #$p < 0·05$ as compared to the STZ group and Ø$p < 0·05$ as compared to time spent in arm B

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Table 1. Effects of pre-treatment with $(MeOPhSe)_2$ and i.c.v. STZ injection on consolidation of spatial recognition memory in the Y-maze: number of entries in each arm of Y maze

	Control	$(MeOPhSe)_2$	STZ	$(MeOPhSe)_2$ + STZ
Arm A	4·83 ± 0·54	4·50 ± 0·43	4·17 ± 1·02	5·16 ± 0·70
Arm B	4·33 ± 0·43	4·50 ± 0·50	4·40 ± 0·81	5·00 ± 0·77
Arm C	5·17 ± 0·42	5·00 ± 0·81	5·00 ± 0·93	5·83 ± 0·64
Total entries	14·33 ± 0·79	14·00 ± 0·93	12·33 ± 1·65	16·17 ± 2·18

Data are reported as means ± S.E.M. of 10 animals per group.

contrast, the total time spent by STZ-injected group in the C arm was significantly lower than that of control group ($p = 0{\cdot}0243$). $(MeOPhSe)_2$ pre-treatment protected against the impairment of memory.

As shown in Table 1, two-way ANOVA analysis revealed that total number of entries in each arm was not altered among groups, demonstrating no change in the locomotor activity of observed animals in the Y-maze test. In accordance, the spontaneous locomotor activity, which was measured in the open-field test, did not differ significantly among groups. Two-way ANOVA for the number of crossings ($F_{1,34} = 0{\cdot}43$, $p > 0{\cdot}05$) and rearings ($F_{1,34} = 0{\cdot}02$, $p > 0{\cdot}05$) revealed no significant differences (data not shown).

RS levels

Two-way ANOVA of RS levels data demonstrated a significant STZ × $(MeOPhSe)_2$ interaction ($F_{1,34} = 6{\cdot}21$; $p = 0{\cdot}0207$). $(MeOPhSe)_2$ pre-treatment protected against the increase in RS levels caused by STZ in mouse brain (Figure 3).

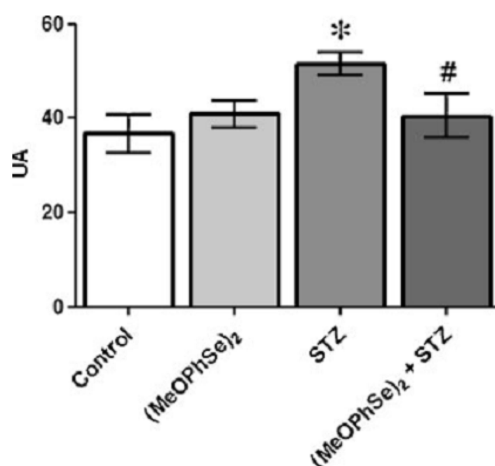

Figure 3. Effects of pre-treatment with $(MeOPhSe)_2$ on RS levels in mouse brain that received i.c.v injection of STZ. Data are reported as means ± S.E.M. *Denotes $p < 0{\cdot}05$ as compared to the control group, and #$p < 0{\cdot}05$ as compared to the STZ group

TBARS levels

Two-way ANOVA of TBARS levels revealed that STZ did not alter this parameter in mouse brain ($F_{1,34} = 1{\cdot}06$; $p > 0{\cdot}05$) (Table 2).

PC levels

Two-way ANOVA for PC levels revealed no significant differences among tested groups ($F_{1,34} = 0{\cdot}00$; $p > 0{\cdot}05$) (Table 2).

CAT activity

Two-way ANOVA for CAT activity ($F_{1,34} = 2{\cdot}47$; $p > 0{\cdot}05$) revealed no significant differences among different groups (Table 2).

SOD activity

Two-way ANOVA of SOD activity data demonstrated a significant STZ × $(MeOPhSe)_2$ interaction ($F_{1,34} = 5{\cdot}73$; $p = 0{\cdot}0271$). Post hoc comparisons showed that $(MeOPhSe)_2$ pre-treatment protected against the increase in SOD activity caused by i.c.v. STZ (Figure 4).

GSH levels

Two-way ANOVA of GSH levels data demonstrated a significant STZ × $(MeOPhSe)_2$ interaction ($F_{1,34} = 4{\cdot}27$; $p = 0{\cdot}0514$). Post hoc comparisons showed that STZ significantly decreased GSH levels (11%) in mouse brain. $(MeOPhSe)_2$ pre-treatment protected against the decrease of the GSH levels caused by STZ (Table 3).

GPx activity

As shown in Table 3, two-way ANOVA of GPx activity yielded a significant main effect of STZ ($F_{1,34} = 12{\cdot}39$, $p = 0{\cdot}0013$). Post hoc comparisons showed that STZ significantly inhibited GPx activity (24%) in mouse brain. $(MeOPhSe)_2$ pre-treatment did not protect against this effect caused by STZ.

GR activity

Two-way ANOVA for GR activity revealed no significant differences among tested groups ($F_{1,34} = 0{\cdot}20$, $p > 0{\cdot}05$) (Table 3).

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Table 2. Effect of pre-treatment with $(MeOPhSe)_2$ on TBARS, PC levels and CAT activity in brains of mice that received i.c.v. injection of STZ

	Control	$(MeOPhSe)_2$	STZ	$(MeOPhSe)_2$ + STZ
TBARS†	11·56 ± 2·62	9·56 ± 3·67	8·98 ± 2·46	10·09 ± 3·63
PC‡	10·67 ± 0·81	9·90 ± 1·02	9·70 ± 0·93	9·01 ± 0·66
CAT§	3·20 ± 0·41	2·93 ± 0·27	2·72 ± 0·46	3·48 ± 0·73

Data are reported as means ± S.E.M. of 10 animals per group.
†Data are expressed as nmol MDA mg^{-1} protein.
‡Data are expressed as nmol carbonyl mg^{-1} protein.
§Data are expressed as U mg^{-1} protein.

GST activity

Two-way ANOVA of GST activity data demonstrated a significant STZ × $(MeOPhSe)_2$ interaction ($F_{1,34} = 4{\cdot}32$; $p = 0{\cdot}0442$). Post hoc comparisons showed that $(MeOPhSe)_2$ pre-treatment protected against the increase of the GST activity caused by i.c.v. STZ in mouse brain (Table 3).

Blood glucose levels

There was no significant difference in blood glucose levels ($mg\,dl^{-1}$) among treated groups ($F_{1,34} = 0{\cdot}22$; $p > 0{\cdot}05$) (data not shown).

DISCUSSION

The i.c.v. injection of STZ has been described as an appropriate animal model for mimic the human SDAT[18] as both are characterized by a progressive impairment in memory and presence of oxidative stress.[20,21,37] Agreeing with these previous literature data, our results confirmed memory loss and oxidative stress caused by i.c.v. injection of STZ in mouse brain. The current study demonstrated that the pre-treatment with $(MeOPhSe)_2$ protected against oxidative

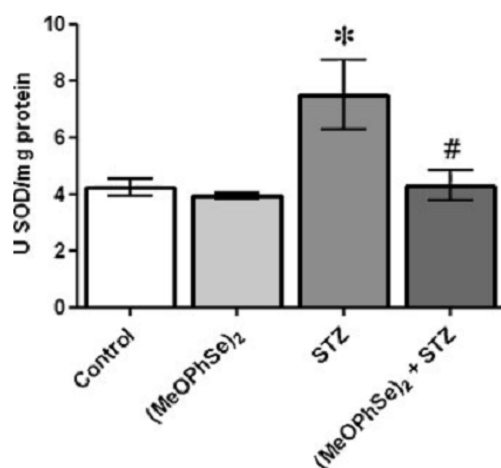

Figure 4. Effects of pre-treatment with $(MeOPhSe)_2$ on SOD activity in mouse brain that received i.c.v injection of STZ. Data are reported as means ± S.E.M. *Denotes $p < 0{\cdot}05$ as compared to the control group, and #$p < 0{\cdot}05$ as compared to the STZ group

stress induced by STZ in mice, characterized by alteration in RS and GSH levels, GPx, GST and SOD activities as well as against impairment of spatial recognition memory consolidation in the Y-maze test. Taken together these results indicate that the antioxidant property of $(MeOPhSe)_2$ is involved in its protective effect against cognitive impairment induced by STZ in a model of SDAT.

I.c.v. injection of STZ possibly desensitizes neuronal insulin receptor (IR) and reduces the activities of glycolytic enzymes.[38] It causes a reduction on cerebral energy metabolism, leading to cognitive dysfunction by inhibiting the synthesis of adenosine triphosphate (ATP) and acetyl CoA, which results into cholinergic deficiency supported by reduced cholineacetyltransferase (ChAT) activity in hippocampus and increased acetylcholinesterase (AChE) activity in rat brain.[19,39] In addition, Agrawal and colleagues suggest that brain IRs may be involved in cognitive functions and may be affected by oxidative stress, they reported in their study that IR is more affected by oxidative stress than by cholinergic changes.[21]

The brain is extremely vulnerable to oxidative stress, in part because it contains a relatively high degree of polyunsaturated fatty acids and is highly enriched with non-haem iron, which is catalytically involved in the production of RS.[40] Some authors have demonstrated that i.c.v. injection of STZ induced an increase in lipid peroxidation and in PC levels in mouse brain.[20,21,37] Different from these data, in this study, TBARS and PC levels were not altered in the STZ group. The most probable explanation for this fact is that the above cited studies were performed by using different doses and regimen of STZ as well as animal species.

Regarding RS determination, DCHF-DA reacts quickly in the presence of reactive species such as $^{\bullet}OH$, H_2O_2 and $O^{\bullet-}$.[27] In this way, there is a clear evidence of an imbalance in neuronal cells, since i.c.v. injection of STZ induced an increase in RS levels, which were kept in the control levels after pre-treatment with $(MeOPhSe)_2$.

Under oxidative stress, protective factors such as CAT, SOD, GPx and GSH are activated in the defence against oxidative injury. Our results showed that i.c.v. injection of STZ increased SOD activity. We hypothesized that this increase in SOD activity was due to increased levels of radicals $O^{\bullet-}$, substrate of this enzyme. SOD is particularly important in protecting the biological cells from oxidative stress because it represents the first line of enzymatic antioxidant defence against $O^{\bullet-}$.[41] $(MeOPhSe)_2$ protected

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Table 3. Effect of pre-treatment with $(MeOPhSe)_2$ on GSH levels, GPx, GR and GST activities in brains of mice that received i.c.v. injection of STZ

	Control	$(MeOPhSe)_2$	STZ	$(MeOPhSe)_2$ + STZ
GSH†	368·83 ± 62·87	360·61 ± 86·93	332·63 ± 77·56*	351·58 ± 112·67**
GPx‡	21·00 ± 1·14	21·19 ± 1·96	16·16 ± 0·65*	17·92 ± 0·73*
GR‡	28·40 ± 4·25	31·70 ± 2·55	24·55 ± 3·21	24·23 ± 4·91
GST§	308·93 ± 34·94	365·98 ± 20·23	408·38 ± 29·74*	339·76 ± 21·40**

Data are reported as means ± S.E.M. of 10 animals per group.
†Data are expressed as nmol GSH mg^{-1} protein.
‡Data are expressed as nmol NADPH $min^{-1} mg^{-1}$ protein.
§Data are expressed as nmol CDNB $min^{-1} mg^{-1}$ protein.
*$p < 0{\cdot}05$ as compared to the control group.
**$p < 0{\cdot}05$ as compared to the STZ group (two-way ANOVA/Duncan).

the cerebral tissue against the increase in RS levels caused by i.c.v. injection of STZ, and as a consequence, it normalized SOD activity.

However, CAT activity was not affected by both i.c.v. injection of STZ and $(MeOPhSe)_2$ in mouse brain. CAT is involved in the detoxification of high concentrations of H_2O_2, whereas GPx is sensitive to lower concentrations. The brain contains less CAT levels and hence GPx has a major role in quenching H_2O_2 and other peroxides which otherwise will lead to the production of hydroxyl and peroxyl radicals in the presence of iron.[40,42]

Reduced GSH is the most abundant non-protein thiol, which buffers free radicals in brain tissue.[43] The redox system of glutathione consists of GSH and an array of functionally related enzymes, of which GR is responsible for the regeneration of GSH, whereas GPx works together with GSH in the decomposition of H_2O_2 and other organic hydroperoxides.[43] Consistent with previous works, in the present study, STZ caused a decrease in GSH levels.[20,37] A reduction in the levels of GSH may impair H_2O_2 clearance and promote formation of $^{\bullet}OH$, the most toxic moiety to the brain, leading to more oxidant load and consequently oxidative damage.[43] Although STZ caused a discrete decrease in GSH levels, the pre-treatment with $(MeOPhSe)_2$ was effective in restoring GSH levels in mouse brain, reinforcing the potential antioxidant activity of this organoselenium compound. Besides, the results demonstrated that i.c.v. injection of STZ caused changes in the activity of GPx and GST, but not in GR. Accordingly, Ishrat *et al*. demonstrated that i.c.v. injection of STZ inhibited GPx activity.[20]

GST, also known as phase II enzymes, are widely distributed catalyzing and binding proteins which promote the conjugation of GSH, with a variety of reactive electrophilic compounds resulting to formation of substances which are easily excreted from the body.[44] Thus, we accept as true that the GST activity was increased in the presence of STZ in an attempt of GST to detoxify xenobiotic. We believed that the stimulation of GST activity observed may be associated with an increase in RS levels. In this way, authors have reported that GST is an antioxidant defence and serves to protect the tissues against oxidative stress.[44] Regarding the antioxidant effect of $(MeOPhSe)_2$ on antioxidant enzymes, the results demonstrated that this organoselenium compound protected against the increase in GST activity, while it was not effective in protecting against GPx inhibition in mouse brain.

Our research group has demonstrated that diphenyl diselenide and its diselenide derivatives, among them $(MeOPhSe)_2$, are good antioxidants. We have also reported that dehydroascorbate reductase-like and GST-like activities are one of the mechanisms behind the antioxidant action of diphenyl diselenide.[45] However, the scavenging activity of radicals seems not to be involved in the antioxidant property of diphenyl diselenide. We believe that $(MeOPhSe)_2$ shares similar like activities with diphenyl diselenide. In addition, $(MeOPhSe)_2$ is a substrate for mammalian thioredoxin reductase, which can explain, at least in part, its *in vivo* antioxidant properties.[46] Based on these data, we suppose that $(MeOPhSe)_2$ acts mainly as an indirect antioxidant (not directly to clear reactive oxygen species) modulating enzymes and antioxidant systems.

In the Y-maze test, there are two contrasting explanations for the behavioural performance of animals of STZ group in the Y-maze test: (i) i.c.v. injection of STZ induced a cognitive impairment in mice.[47] In this test, it is expected that the animals spend more time exploring the new (i.e. arm C), however, injured animals with STZ did not spend more time exploring the new arm and this is may indicate a memory impairment; or (ii) i.c.v. injection of STZ induced an anxious behaviour in mice.[48] By this hypothesis, the mouse has normal or even enhanced recognition of the most familiar arm A, and avoids entering arm C because it is novel and potentially dangerous.

Although we know that Y-maze test should not be unquestionably accepted as a measure of memory alone, the results presented here are in agreement with data previously showed by us, using the same experimental procedure.[17,47] In the previous study, we demonstrated that $(MeOPhSe)_2$ protected against memory impairment induced by i.c.v. STZ, since it improved mouse performance in cognitive tasks (Morris water maze and step-down-type passive-avoidance tests).[17] In agreement, the results obtained in this current study demonstrated that $(MeOPhSe)_2$ was effective in protecting against behaviour alteration induced by i.c.v. STZ. Taken together, these two contrasting behavioural data from the STZ group deserve more detailed studies in which memory and anxiety behaviours could be dissociated.

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CONCLUSION

The present study showed that $(MeOPhSe)_2$ was effective in protecting mouse brain against oxidative stress induced by i.c.v. injection of STZ. Moreover, the neuroprotective property of $(MeOPhSe)_2$ was further reinforced since it improved mice behavioural performance in the Y-maze test. These results suggest that antioxidant property is involved, at least in part, in the neuroprotective effect of $(MeOPhSe)_2$ on SDAT induced by STZ in mice.

CONFLICT OF INTEREST

None known.

ACKNOWLEDGEMENTS

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3.3 Artigo III

Therapeutic effect of organoselenium dietary supplementation in a sporadic dementia of Alzheimer's type model in rats

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Therapeutic effect of organoselenium dietary supplementation in a sporadic dementia of Alzheimer's type model in rats

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Abstract

It is known that selenium (Se) might play different roles in the progression of Alzheimer's disease (AD), but there is a lack of evidence that proves whether supplementation with Se is beneficial or not for the treatment of AD. Thus, the aim of the current study was to investigate the therapeutic effect of *p,p'*-methoxyl-diphenyl diselenide [$(MeOPhSe)_2$], an organoselenium compound, against streptozotocin (STZ)-induced sporadic dementia of Alzheimer's type (SDAT) in rats. Male Wistar rats received STZ twice daily (1.0 mg/8 μl; 4 μl/ventricle) for 21 days. After 21 days of STZ injection, regular-diet-fed rats were supplemented with 10 ppm of $(MeOPhSe)_2$ during 30 days. At the end of this period, the rats were challenged in the Morris water maze and step-down passive avoidance tasks. The activity of acetylcholinesterase (AChE), deficit in cerebral energy metabolism (measurement of adenosine 5-triphosphate and adenosine 5-diphosphate levels), and oxidative and nitrosative stress were determined in the cortex and hippocampus of rats. The results demonstrated that $(MeOPhSe)_2$ dietary supplementation reverted STZ-induced memory impairment of rats in both cognitive tasks. The findings also indicated that $(MeOPhSe)_2$ dietary supplementation reverted oxidative stress in the STZ group (decreased reactive species and tyrosine nitration levels and enhanced nonprotein thiol levels). Moreover, $(MeOPhSe)_2$ dietary supplementation normalized AChE activity, which was enhanced by STZ injection, but did not revert the deficit in cerebral energy metabolism caused by STZ. The results of the present study indicated the therapeutic effect of the $(MeOPhSe)_2$-supplemented diet in a rat model of SDAT.
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Keywords: Alzheimer's disease animal model; Selenium; Nitration; Oxidative stress; Energy metabolism

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1. Introduction

Alzheimer's disease (AD) is estimated to affect approximately 39 million people worldwide, being the neurological disorder with a greater prospect for growth in the world [1–3]. There is currently no accurate description of the etiology of AD; moreover, its pathophysiology is complex and involves multiple pathways of neuronal damage [1]. It is characterized by the accumulation of extracellular amyloid beta (Aβ) into aggregated amyloid plaques and the hyperphosphorylation of tau leading to neurofibrillary tangles. This pathologic process is also associated to neuroinflammation and oxidative stress. In this context, there is strong evidence that free radicals play an important role in AD [1,4].

Recently, there has been heightened interest in the role of the trace element selenium (Se) in health and neurologic disorders. Se is an essential trace mineral nutrient with multiple roles in the growth and functioning of living cells of animals. This trace element is known to provide protection from free-radical-induced cell damage [5,6]. There are data showing that Se is involved in most of the molecular pathways that are important in the progression of AD. Although there are controversial data associating the supplementation of Se with cognitive improvement and AD pathophysiology, some authors demonstrated favorable aspects of Se supplementation in AD patients [6–8]. Vural and colleagues [9] have reported that plasma Se levels are lower in AD patients when compared to healthy patients. Furthermore, Se status decreases with age and may contribute to decline in neuropsychological functions among aging people [10]. These data further suggest that alterations in the Se concentration and its related enzymes may play a role in the etiopathogenesis of AD. Se has been associated to the reduction of Aβ production and of Aβ induction of neuronal death in cells culture [11]. In animal models of AD, Se prevented oxidative damage and modulated the cholinergic system [12,13].

A piece of evidence indicates that Se organic compounds have a higher biodisponibility and biological activity than Se inorganic compounds [14]. For this reason, the interest in organoselenium chemistry and biochemistry has increased in the last decades. In fact, some studies have demonstrated the neuroprotective action, among other properties, of these compounds [15,16]. In this way, diphenyl diselenide [$(PhSe)_2$], a simple organoselenium compound, ameliorates memory impairment induced by scopolamine in mice [13] and enhances cognitive performance in rodents without inducing neurotoxicity [17,18].

Moreover, preadministration of *p,p'*-methoxyl-diphenyl diselenide [$(MeOPhSe)_2$], a substituted analogue of $(PhSe)_2$, improves

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memory of mice in the model of sporadic dementia of Alzheimer's type (SDAT) induced by intracerebroventricular (icv) injection of streptozotocin (STZ) [19,20]. Icv injection of STZ to rodents has been reported as an appropriate SDAT model, characterized by an impairment of memory [21]. Icv injection of STZ reduces the glucose utilization and energy-rich phosphate levels [21]. It also alters the cholinergic system, leading to a reduction in choline acetyltransferase (ChAT) activity [12] and an increase in acetylcholinesterase (AChE) activity [22]. There have also been data indicating the involvement of oxidative stress in STZ-induced SDAT model [12,23]. Based on these positive results [19,20], we planned this study to investigate the therapeutic effect of $(MeOPhSe)_2$ against STZ-induced SDAT model. The involvement of AChE activity, deficit in cerebral energy metabolism, and oxidative and nitrosative stress in the therapeutic effect of $(MeOPhSe)_2$ in the cognitive impairment induced by STZ was examined.

2. Materials and methods

2.1. Drugs

$(MeOPhSe)_2$ was synthesized according to the previously published method [24]. Analysis of the ^1H NMR and ^{13}C NMR spectra showed that the compound obtained presented analytical and spectroscopic data in full agreement with its assigned structure. The chemical purity of $(MeOPhSe)_2$ (99.9%) was determined by gas chromatography–mass spectrometry. 3-Nitrotyrosine (3-NT), tyrosine and STZ were obtained from Sigma Chemical (St. Louis, MO, USA). All other chemicals were obtained in an analytical grade or from standard commercial suppliers.

2.2. Animals

Experiments were conducted using male Wistar rats (350–400 g) about 7 months old. Animals were maintained at 22°C–25°C with free access to water and food under a 12-h:12-h light/dark cycle with lights on at 7:00 a.m. All manipulations were carried out between 8:00 a.m. and 4:00 p.m. Animals were used according to the guidelines of the Committee on Care and Use of Experimental Animal Resources, the Federal University of Santa Maria, Brazil. All efforts were made to minimize animals' suffering and to reduce the number of animals used in the experiments.

2.3. Experimental design

Fig. 1 illustrates the experimental design of this study. The animals were separated into four groups: (a) sham, (b) STZ, (c) $(MeOPhSe)_2$ and (d) STZ+$(MeOPhSe)_2$. The animals used in the step-down passive avoidance task at days 21 and 51 were the same, but they were not the same used in the Morris water maze task. All protocol steps used in this study are described below.

2.4. Surgery

Animals were anesthetized under intraperitoneal Equithesin (1% phenobarbital, 2% magnesium sulfate, 4% chloral hydrate, 42% propylene glycol, 11% ethanol; 3 ml/kg) injection and placed in a stereotaxic frame (Stoelting, Wood Dale, IL, USA). The scalp was shaved and swabbed with iodine, and an incision was made along the midline of the scalp, exposing the bregma. Burr holes were drilled in the skull, and cannulae were implanted on both sides over the lateral ventricles using the following coordinates: 1.0 mm posterior to bregma, 2.0 mm lateral to sagittal suture (both right and left), 4.0 mm beneath the surface of the brain at day 0 [25]. Rats received injections of STZ (1.0 mg/8 μl; 4 μl/ventricle) at days 1 and 3. The sham group received only the icv injection of vehicle (aCSF — 147 mM NaCl, 2.9 mM KCl, 1.6 mM MgCl, 1.7 mM CaCl and 2.2 mM dextrose) (4 μl/site). Injections were carried out at 1 μl/min using a Hamilton 10-μl syringe with a 26-gauge needle.

2.5. Dietary supplementation

Animals were fed daily with 50 g/animal standard diet chow or standard chow supplemented with 10 ppm of $(MeOPhSe)_2$ during 30 days. The supplementation began 21 days after the icv injection of STZ. The concentration of 10 ppm of $(MeOPhSe)_2$ was chosen based on previous study of toxicity in rats (data not shown). The preparation of supplemented standard chow was based on a previous study published by de Bem et al. [26]. The standard diet was pulverized with ethyl alcohol, whereas the supplemented diet was pulverized with $(MeOPhSe)_2$ [1 mg of $(MeOPhSe)_2$/100 g standard chow] dissolved in ethyl alcohol (1 mg/10 ml). The standard and supplemented diets were stored at room temperature for 3 h to evaporate the alcohol and then kept at 4°C for no more than 1 week.

2.6. Behavioral tests

2.6.1. Step-down passive avoidance task

The step-down passive avoidance task has been used to study nonspatial long-term memory [27]. The apparatus consisted of a single box where the floor was made of a metal grid connected to a shock scrambler, and in its lateral, there was a safe platform. During the training session (acquisition trial), each rat was placed on the platform; usually, the rat often stepped down from the platform to explore the box. When it stepped down and placed its four paws on the grid floor, an electric shock (0.5 mA) was delivered for 2 s. Some seconds later, the rat was removed from the step-down passive avoidance apparatus and returned to its home cage. The retention trial was performed 24 h after training. Each rat was placed again on the platform, and the transfer latency time (i.e., time took to step down from the platform) was measured in the same way as in the acquisition trial, but foot shock was not delivered and the transfer latency time was recorded to a maximum of 600 s. The criterion for learning was taken as an increase in the transfer latency time on retention (second) trial as compared to the acquisition (first) trial. So, short transfer latencies indicate poor retention.

2.6.2. Morris water maze task

Spatial learning and memory were assessed using the Morris water maze task according to the method of Morris [28]. The water maze consisted of a basin (diameter: 180 cm, wall height: 40 cm) made of black plastic and filled with water (22°C±2°C) at a height of 30 cm. The pool was placed in a room with several extra-maze visual cues, such as counters, posters, a dangling wire and a pole. For the acquisition phase, the rats were submitted to four trials [starting in the north (N), south (S), east (E) and west (W)] for 4 consecutive days. The escape platform was hidden 1 cm below water level in the middle of the northwest (NW) quadrant. The rats remained on the platform for at least 40 s after each trial. Whenever the rats failed to reach the escape platform within the 1-min cutoff period, they were retrieved from the pool and placed on it for 40 s. The latencies to reach the platform were calculated as the mean of total time spent in four trials of each day. Twenty-four hours after the acquisition phase, a probe trial was conducted by removing the platform and placing the rat next to and facing the S side. The time spent in each quadrant, the number of crossings over the former platform position, and the times spent in the platform quadrant and in the opposite quadrant were measured for a single 1-min trial.

2.6.3. Open field

Spontaneous locomotor activity was measured in the open field test [29]. The floor of the open field was divided into nine squares. Each animal was placed individually in the center of the arena, and the number of segments crossed (four-paw criterion) and rearings were recorded in a 4-min session.

2.7. Ex vivo assays

Fifty-one days after the first injection of STZ, the animals were killed by decapitation, and the cerebral cortex and hippocampus were dissected. Blood samples were collected for glucose determination to further confirm that 1 mg/site of STZ is a subdiabetogenic dose. The cortex and hippocampus were homogenized in 50 mM Tris–

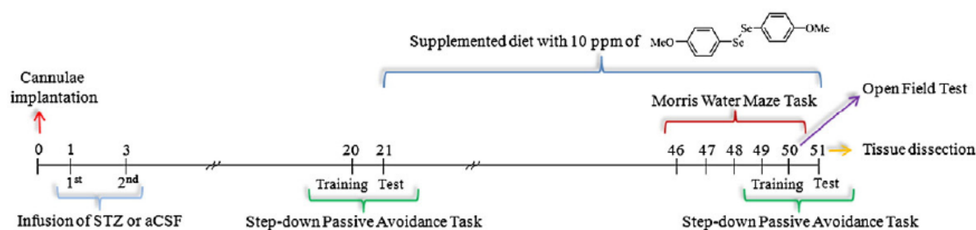

Fig. 1. Experimental procedure.

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HCl, pH 7.4 (1/10, w/v). The homogenate was centrifuged at 2400g for 10 min at 4°C, and a low-speed supernatant fraction (S_1) was used for the following determinations: reactive species (RS) and nonprotein thiol (NPSH) levels.

2.7.1. RS levels

The RS production was determined by diluting S_1 (1:10) in 50 mM Tris–HCl (pH 7.4). S_1 was incubated with 10 µl of 2′,7′-dichlorofluorescein diacetate (DCHF-DA; 1 mM) at room temperature for 30 min. The RS levels were determined by a spectrofluorometric method using the DCHF-DA assay. DCHF-DA is a nonfluorescent compound that easily crosses cell membranes and, in the presence of RS, is rapidly oxidized to its fluorescent derivative dichlorofluorescein (DCF) [30]. 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. The RS levels were expressed as arbitrary unit (AU).

2.7.2. NPSH levels

The NPSH levels were determined by the method of Ellman [31]. S_1 was mixed (1:1) with 10% trichloroacetic acid. 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 5,5′-dithiobis-(2-nitrobenzoic acid). The color reaction was measured at 412 nm. NPSH levels were expressed as µmol NPSH/g tissue.

2.7.3. Tyrosine nitration levels

Determination of 3-NT and tyrosine in rat cortex was performed by high-performance liquid chromatography/ultraviolet (HPLC-UV) detection method based on Erdal and colleagues [32]. Tyrosine nitration was not determined on hippocampus since 3-NT levels were below the method detection limit.

Briefly, cortex samples were homogenized in 50 mM Tris–HCl, pH 7.4 (1/10, w/v), and an aliquot was hydrolyzed in HCl (12 N; 1:1 v/v) at 60°C for 24 h. Digested samples were filtered through a membrane (0.45-µm pore size; Millipore) before injection onto the HPLC instrument. Samples were analyzed on a Shimadzu HPLC apparatus. The analytical column was a 5-µm particle and 100-Å pore size Phenomenex ODS-2 C_{18} reverse-phase column (4.6×250 mm, Allcrom, Brazil). The mobile phase was 50 mM sodium acetate, 50 mM sodium citrate and 8% (v/v) methanol, pH 3.1 (corrected with 12 N HCl). The HPLC analysis was performed under isocratic conditions at a flow rate of 1 ml/min and with the UV detector set at 274 nm. 3-NT levels were expressed as 3-NT (µM)/total tyrosine (µM).

2.7.4. Adenosine 5-triphosphate (ATP) and adenosine 5-diphosphate (ADP)

The energy-rich phosphate (ATP, ADP) in rat cortex and hippocampus was determined by the HPLC-UV detection modified method of Özogul and colleagues [33]. Briefly, the cortex and hippocampus samples were homogenized in 0.6 mM perchloric acid (1/5, w/v) and centrifuged at 2400g at 4°C for 10 min. The supernatant fraction was neutralized to pH 6–6.5 with 1 M potassium hydroxide. The neutralized fractions were kept on ice for 30 min to ensure the total precipitation of potassium crystals. After that, they were filtered through a membrane (0.45-µm pore size; Millipore) before injection onto the HPLC instrument. Samples were analyzed on the same apparatus described for 3-NT and tyrosine determinations. The mobile phase was 0.04 M potassium dihydrogen orthophosphate and 0.06 M dipotassium hydrogen orthophosphate dissolved in purified distilled water and adjusted to pH 7 with 0.1 M potassium hydroxide. The HPLC analysis was performed under isocratic conditions at a flow rate of 1 ml/min and with the UV detector set at 257 nm. The results were expressed as ATP concentration/ADP concentration ratio.

2.7.5. AChE activity

Samples of cortex and hippocampus were homogenized in 0.25-M sucrose buffer (1/10, w/v) and centrifuged at 2400g at 4°C for 15 min. The activity of AChE was carried out according to the method of Ellman and colleagues [34] using acetylthiocholine as substrate. The activity of AChE was spectrophotometrically measured at 412 nm. The activity of AChE was expressed as nmol/min/mg protein. Protein concentration was measured according to the method of Bradford [35].

2.7.6. Plasma glucose levels

Plasma glucose levels were determined by enzymatic colorimetric method using a commercial kit (Labtest Diagnostica, MG, Brazil). Glucose levels were expressed as mg/dl.

2.8. Statistical analysis

The behavioral data were analyzed by the nonparametric test (Kruskal–Wallis analysis of variance) followed by the Dunn's multiple comparison test when necessary or *t* test analysis (GraphPad software, San Diego, CA, USA; for the step-down passive avoidance at 21 days). The behavioral data are given as the median±interquartile range. Data from *ex vivo* assays were calculated by means of two-way analysis of variance followed by the Duncan's test when necessary. Experimental results of *ex vivo* assays are given as the mean±S.E.M. Probability values less than .05 ($P<.05$) were considered to be statistically significant.

3. Results

$(MeOPhSe)_2$ improved memory decline induced by STZ in the rat Morris water maze and step-down passive avoidance tasks. At the 21st day after the STZ infusion, STZ induced an impairment in memory of rats ($P=.0334$) in the step-down passive avoidance task (Fig. 2A), which is in agreement with the literature data.

Thirty days of $(MeOPhSe)_2$ dietary supplementation were effective in improving memory of rats since they took more time to descend from the platform in the step-down passive avoidance task [$H(3)=13.93$; $P<.005$] (Fig. 2B, see the retention phase). Animals of the sham group supplemented with $(MeOPhSe)_2$ diet had a memory of the first shock preserved [$H(3)=12.86$; $P<.005$] (Fig. 2B, in the acquisition phase). These findings support the hypothesis that $(MeOPhSe)_2$ diet recovered and enhanced the memory of rats.

On the Morris water maze task, the analysis of the spatial learning behavior on the memory acquisition phase revealed a significant effect of training days. These results showed that rats of all groups learned to find the platform during the 4 days of training. There was no significant difference in the latency to reach platform in all days of the acquisition phase among groups, except at the fourth day [$H(3)=9.497$, $P<.05$] (Fig. 3). The results showed that STZ induced an impairment on spatial memory of rats because rats from the STZ group spent less time in the platform quadrant [$H(3)=9.034$; $P<.05$] (Fig. 4C) and more time in the opposite platform quadrant when compared to the sham group [$H(3)=14.98$; $P<.005$] (Fig. 4D). $(MeOPhSe)_2$-supplemented diet not only reverted this behavior but also decreased the latency to reach platform in the probe [$H(3)=9.497$, $P<.05$] (Fig. 4A). There was no significant difference in the

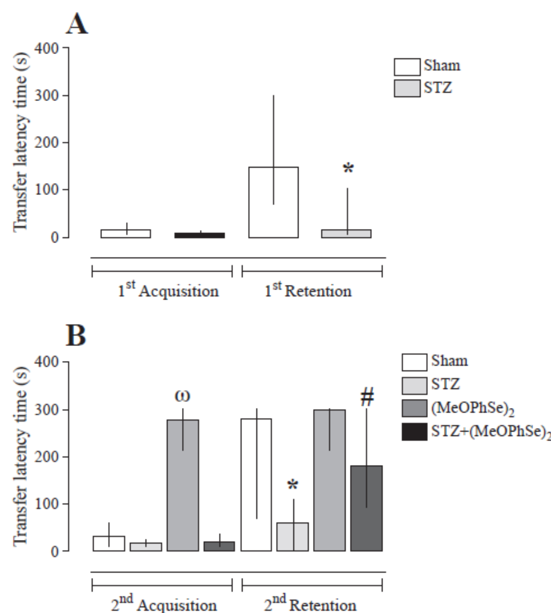

Fig. 2. Effects of $(MeOPhSe)_2$ on STZ-induced memory deficit in passive avoidance test: (A) transfer latency time (s) to fall from the platform in the acquisition and retention phase at 20–21 days after STZ infusion; (B) transfer latency time(s) to fall from the platform in the acquisition and retention phases after $(MeOPhSe)_2$ diet supplementation (50–51 days after STZ infusion). Data are median and interquartile range for $n=12$–13 animals per group (task at 21 day) and $n=6$–7 in each group (task at 51 day). $^{\omega}P<.05$ as compared to the sham group in the acquisition phase; $^{*}P<.05$ as compared to the sham group; $^{\#}P<.05$ as compared to the STZ group in the retention phase.

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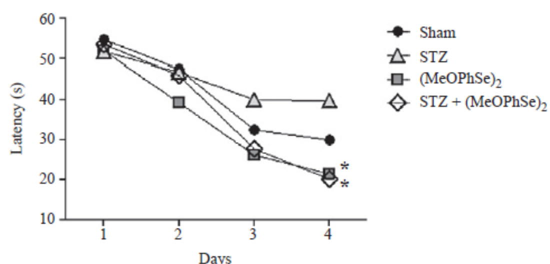

Fig. 3. Effects of $(MeOPhSe)_2$ on STZ-induced memory deficit in the Morris water maze test: latency (ies) to reach the platform in the acquisition phase. Data are median and interquartile range for n=8 in each group. *P<.05 as compared to the STZ group.

number of crossings in the platform local among groups [$H(3)$=5.779; P>.05] (Fig. 4B).

STZ- and/or $(MeOPhSe)_2$-supplemented diet caused alteration neither in the spontaneous locomotion [$H(3)$=2.104; P>.05] nor in exploratory activities [$H(3)$=1.241; P>.05] in the open field test (data not shown).

The dose of STZ did not induce diabetes. The results confirmed that 1 mg/site of STZ is a subdiabetogenic dose since there was no significant difference in plasma glucose levels among groups [STZ×$(MeOPhSe)_2$ interaction ($F_{1,36}$=0.322; P>.05)] (data not shown).

$(MeOPhSe)_2$ supplementation alleviated oxidative and nitrosative stress induced by STZ in rats. As shown in Table 1, STZ induced an increase of RS levels in cortex (by ~110%), and $(MeOPhSe)_2$ was effective against this increase [STZ×$(MeOPhSe)_2$ interaction ($F_{1,25}$=4.341; P<.05)]. There was no significant difference in RS levels in hippocampus of rats from all groups [STZ×$(MeOPhSe)_2$ interaction ($F_{1,25}$=0.000; P>.05)]. STZ induced a decrease of NPSH levels in hippocampus (by ~15%), and the $(MeOPhSe)_2$-supplemented diet reverted this reduction [main effect of STZ ($F_{1,25}$=7.036; P<.05) and $(MeOPhSe)_2$ ($F_{1,25}$=8.999; P<.01)]. In the cortex, the results revealed an effect of $(MeOPhSe)_2$-supplemented diet on NPSH levels [main effect of $(MeOPhSe)_2$ ($F_{1,25}$=7.031; P<.05)]. The icv injection of STZ increased tyrosine nitration in cerebral cortex of rats, and the $(MeOPhSe)_2$-supplemented diet was effective against the increase [STZ×$(MeOPhSe)_2$ interaction ($F_{1,8}$=7.125; P<.05)] (Table 1).

$(MeOPhSe)_2$ inhibited AChE activity but did not change the energy metabolism. No significant difference was found in ATP/ADP ratio in the cerebral cortex of rats [STZ×$(MeOPhSe)_2$ interaction ($F_{1,28}$=0.000; P>.05)] (Table 2). In the hippocampus, the $(MeOPhSe)_2$-supplemented diet was not effective in restoring the decrease in ATP/ADP ratio caused by STZ [main effect of STZ ($F_{1,28}$=7.517; P<.05)]. The $(MeOPhSe)_2$-supplemented diet inhibited AChE activity in both structures: cortex [main effect of STZ ($F_{1,27}$=14.028; P<.05) and $(MeOPhSe)_2$ ($F_{1,27}$=6.348; P<.05)] and hippocampus of rats [STZ×$(MeOPhSe)_2$ interaction ($F_{1,27}$=4.356; P<.05)] (Table 2).

4. Discussion

In the present study, we demonstrated the therapeutic effect of $(MeOPhSe)_2$ dietary supplementation on memory and learning of rats in a model of SDAT induced by STZ. The results indicate that the $(MeOPhSe)_2$-supplemented diet rescued spatial learning and memory and nonspatial long-term memory in STZ-treated rats. $(MeOPhSe)_2$ restored AChE activity and had antioxidant and antinitrosative effects in rats. The therapeutic action of $(MeOPhSe)_2$, the improvement of cognitive function, could be tentatively explained by its antioxidant property. The therapeutic effect of $(MeOPhSe)_2$ dietary supplementation seems not to be related to the energetic metabolism because $(MeOPhSe)_2$ did not alter the levels of

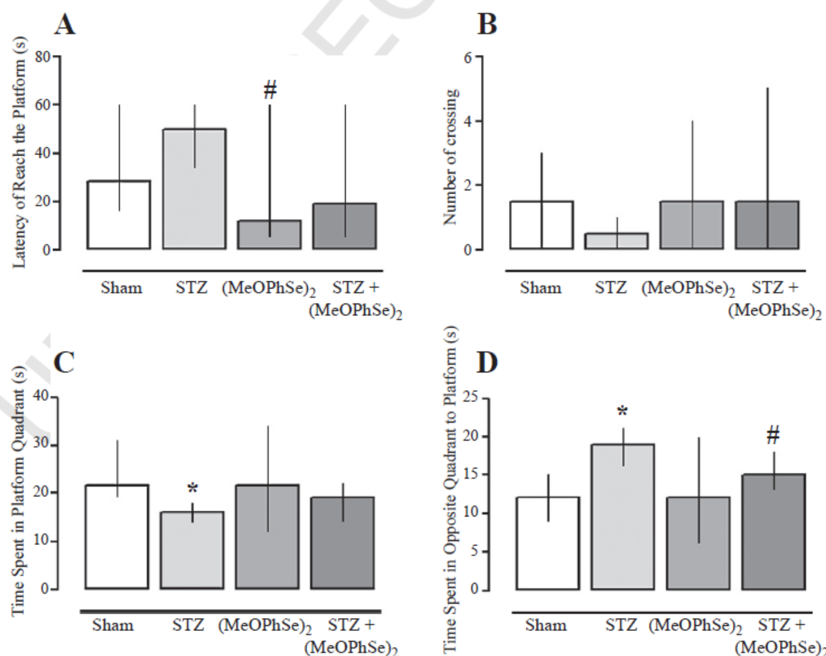

Fig. 4. Effects of $(MeOPhSe)_2$ on STZ-induced memory deficit in the Morris water maze test: (A) latency (ies) to reach the platform in the probe test; (B) number of crossing over the former platform position; (C) time (s) spent in the platform quadrant; (D) time (s) spent in the opposite quadrant to platform. Data are median and interquartile range for n=8 in each group. *P<.05 as compared to the sham group; #P<.05 as compared to the STZ group.

Table 1
Effect of $(MeOPhSe)_2$ diet supplementation on RS, NPSH and 3-NT levels in cerebral cortex and hippocampus of rats that received the icv injection of STZ

	RS [a]		NPSH [b]		3-NT [c]
	Cortex	Hippocampus	Cortex	Hippocampus	Cortex
Sham	104.3±23.7	160.2±21.0	5.59±0.12	5.79±0.16	0.207±0.02
STZ	220.14±29.8 *	187.2±43.3	5.21±0.26	5.02±0.11 *	0.291±0.01 *
$(MeOPhSe)_2$	120.14±40.8	158.1±19.6	5.98±0.20 #	6.37±0.22 #	0.153±0.01 *
STZ+$(MeOPhSe)_2$	118.2±24.7 #	185.0±36.1	5.74±0.09	5.87±0.33 #	0.140±0.03 *#

Data are reported as the mean(s)±S.E.M. for n=7–8 animals per group in RS and NPSH determinations and for n=3 animals per group in 3-NT determination.
[a] Data were expressed as AU.
[b] Data were expressed as μmol NPSH/g tissue.
[c] Data were expressed as 3-NT (μM)/total tyrosine (μM).
* P<.05 as compared to the sham group.
# P<.05 as compared to the STZ group.

ATP and ADP. Moreover, the findings presented here also indicate that the $(MeOPhSe)_2$-supplemented diet improved memory of nontreated rats.

At 21 days after injection of STZ, rats were challenged in the step-down passive avoidance to further confirm the memory impairment. After that, rats were submitted to the $(MeOPhSe)_2$-supplemented diet for 30 days and were retested in step-down passive avoidance. Surprisingly, even after 30 days, in the second acquisition phase, rats supplemented with the $(MeOPhSe)_2$ diet practically did not step down on the grid floor. These findings support the hypothesis that the $(MeOPhSe)_2$-supplemented diet induced cognitive enhancement in rats. In this context, organoselenium compounds have been reported as memory enhancers [17,18].

Moreover, in this study, the therapeutic effect of $(MeOPhSe)_2$ dietary supplementation on STZ-induced SDAT in rats was investigated in Morris water maze and step-down passive avoidance tasks. Although these are different paradigms — Morris water maze evaluates spatial learning and memory, while step-down passive avoidance assesses nonspatial long-term memory — the therapeutic effect of the $(MeOPhSe)_2$-supplemented diet was demonstrated. In fact, the $(MeOPhSe)_2$-supplemented diet was effective in improving spatial learning and memory and nonspatial long-term memory in STZ-treated rats without altering the spontaneous locomotor activity of these animals.

Consistent with previously published data [22,23,36], in the present study, the icv injection of STZ in rats caused learning and memory impairment. The learning and memory impairment was demonstrated by a clear trend to increase the latency to find a platform, a reduction in the time spent by the animals in the quadrant where the platform was formerly located, an augmentation in the time spent in the opposite quadrant in Morris water maze and a decrease in the latency time to step down and place the four paws on the grid floor in step-down passive avoidance. The $(MeOPhSe)_2$-supplemented diet ameliorated the performance of rats in the Morris water maze and passive avoidance tasks. These findings are in agreement with those previously reported, which demonstrated that

Table 2
Effect of $(MeOPhSe)_2$ diet supplementation on AChE activity and ATP turnover levels in the cerebral cortex and hippocampus of rats that received the icv injection of STZ

	AChE [a]		[ATP]/[ADP] ratio	
	Cortex	Hippocampus	Cortex	Hippocampus
Sham	7.35±0.94	9.23±0.62	0.779±0.023	0.913±0.039
STZ	11.34±1.14 *	11.90±1.06 *	0.695±0.047	0.771±0.042 *
$(MeOPhSe)_2$	6.46±0.45	9.90±0.89	0.787±0.057	0.969±0.082
STZ+$(MeOPhSe)_2$	8.30±0.73 #	9.03±0.71 #	0.731±0.066	0.786±0.010 *

Data are reported as the means±S.E.M. for n=7–8 animals per group.
[a] Data were expressed as nmol/min/mg protein.
* P<.05 as compared to the sham group.
# P<.05 as compared to the STZ group.

inorganic Se [12] and organic Se [13,17,18] are effective in preventing, improving or ameliorating memory of rodents.

AD is associated with progressive death of neurons, particularly in the cortex and hippocampus [1]. This neurodegenerative process is coupled to oxidative stress, mitochondrial dysfunction, impaired energy metabolism and activation of prodeath signaling pathways [1,37]. In fact, there is strong evidence that free radicals play an important role in AD [38]. In this context, the icv injection of STZ has been reported as an appropriate animal model to mimic the human SDAT characterized by the presence of oxidative stress [36].

A reduction in the level of GSH may impair H_2O_2 clearance and promote the formation of OH, the most toxic moiety to the brain, leading to more oxidant load and consequently oxidative damage [39]. In this way, the results found in the current study demonstrated that the icv injection of STZ in rats caused an increase in RS levels in the cortex and a depletion of NPSH levels in the hippocampus, which were kept at normal levels after dietary supplementation with $(MeOPhSe)_2$. These results suggest that the antioxidant property could be involved in the $(MeOPhSe)_2$ neuroprotective effect on SDAT induced by STZ in rats. Accordingly, the neuroprotective effect of the antioxidant $(MeOPhSe)_2$ was reported [19].

Moreover, it has been reported that organoselenium moieties are good antioxidants, increasing NPSH levels and promoting RS detoxification [16,19]. Recently, the property of mimicking the activity of glutathione peroxidase, glutathione-*S*-transferase and dehydroascorbate reductase was related to the antioxidant action of $(PhSe)_2$. The data provided in this study further support the idea that $(PhSe)_2$ is not a radical scavenger [40]. In addition, Freitas and colleagues [41] demonstrated that $(MeOPhSe)_2$ was a substrate for mammalian thioredoxin reductase (TxR), which may explain, at least in part, its antioxidant properties. Based on these data, the antioxidant effect of $(PhSe)_2$ and its substituted analogues, like $(MeOPhSe)_2$, has been attributed to the property of mimicking the activity of antioxidant enzymes and by acting as a substrate for TxR.

The rapid interaction between oxygen (O_2) and nitric oxide (NO) produces peroxynitrite ($ONOO^-$). Peroxynitrite is a potent nitration mediator and strong oxidant implicated in AD pathogenesis [42]. It modifies tyrosine residues in protein and thus generates a stable compound, namely, 3-NT. The concentration of 3-NT is markedly elevated in the brains of AD patients and is positively correlated with decreased cognitive functions in these patients [42]. In support of this latter assertion, Horiguchi and colleagues [43] demonstrated that the tau protein is nitrated and co-localized with neurofibrillary tangles in AD brains. Moreover, Zhang and colleagues [44,45] reported that $ONOO^-$ and NO cause modification, accumulation and hyperphosphorylation of tau protein in rat brain. Thus, the inhibition of inducible nitric oxide synthase (iNOS) activity or nitrosative species scavenger could be an alternative to prevent the neurodegeneration in AD.

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The results demonstrated here indicate that $(MeOPhSe)_2$ protected against protein nitration induced by icv injection of STZ in cortex of rats. These results support the hypothesis that the neuroprotector effect of $(MeOPhSe)_2$ is attributed to its antinitrosative property. In this way, previous data have demonstrated that the neuroprotective action of organoselenium compounds is related to the decrease in cerebral nitrate/nitrite levels [46] and inhibition of iNOS activity [47] in rodents. Thus, if $(MeOPhSe)_2$ is effective in decreasing 3-NT levels induced by STZ in cerebral cortex of rats and if $ONOO^-$ and NO could simultaneously induce tau nitration and hyperphosphorylation, it is plausible to assume that $(MeOPhSe)_2$ minimizes tau hyperphosphorylation induced by STZ [48].

During the early stages of AD, a reduced number of mitochondria in neurons, decreased brain glucose metabolism, and reduced activities of both tricarboxylic acid cycle enzymes and cytochrome *c* oxidase have been reported [37]. Similarly to AD, the icv injection of STZ induces a desensitization of neuronal insulin receptor and a reduction in activities of glycolytic enzymes [49]. It causes a deficit on cerebral energy metabolism, leading to a cognitive dysfunction by inhibiting the synthesis of ATP and acetyl CoA, which results into cholinergic deficiency, supported by reduced ChAT activity and enhanced AChE activity [12,22]. Moreover, the brains of rats which received icv injection of STZ exhibited an increased expression of genes encoding AChE, tau and amyloid precursor protein [48,50].

The results of the present study confirmed an inhibition in ATP synthesis, demonstrating that STZ induced a deficit in energy metabolism in the hippocampus of rats since the ATP/ADP ratio, an indicator of ATP turnover, was reduced. The $(MeOPhSe)_2$-supplemented diet was not effective in restoring ATP levels. Thus, these results rule out a possible interaction of $(MeOPhSe)_2$ with energy metabolism. By contrast, $(MeOPhSe)_2$ was effective in normalizing AChE activity of the cerebral cortex in rats exposed to STZ. This result strengthens the relationship between the $(MeOPhSe)_2$ effect on memory and cholinergic modulation [20]. Thus, the results on AChE activity associated to $(MeOPhSe)_2$ antioxidant properties could explain the positive results on the Morris water maze and step-down passive avoidance tasks.

In summary, the most relevant additional findings of the present study are that therapeutic $(MeOPhSe)_2$ dietary supplementation (a) reverted STZ-induced memory impairment of SDAT in rats; (b) reverted oxidative stress; (c) normalized AChE activity, which was increased by STZ injection; and (d) did not alter the deficit in cerebral energy metabolism induced by STZ. Thus, the use of $(MeOPhSe)_2$-supplemented diet should be encouraged for the treatment of SDAT due to its therapeutic values.

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3.4 Artigo IV

p,p'-Methoxyl-diphenyl diselenide prevents neurodegeneration and glial cell activation induced by streptozotocin in rats

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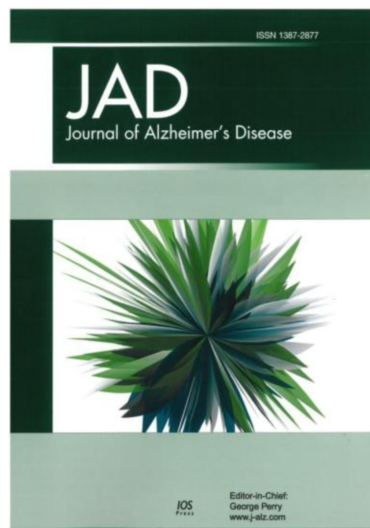

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p,p′-Methoxyl-Diphenyl Diselenide Prevents Neurodegeneration and Glial Cell Activation Induced by Streptozotocin in Rats

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Abstract. The purpose of this study was to investigate possible molecular targets involved in the neuroprotective effect of *p,p′*-methoxyl-diphenyl diselenide [$(MeOPhSe)_2$], using a streptozotocin (STZ)-induced sporadic dementia of Alzheimer's type rat model. Male Wistar rats were injected with STZ (1.0 mg/8 μl; 4 μl/ventricle). After 21 days of STZ injection, regular diet-fed rats were supplemented with 10 ppm of $(MeOPhSe)_2$ during 30 days. At the end of this period, rats performed object recognition and step-down passive avoidance tasks. Apoptosis was assessed by TUNEL staining and active caspase-3. Glial fibrillary acidic protein, ionized calcium binding adaptor molecule 1, and microtubule associated protein 2 were determined by immunofluorescence in rat hippocampus. The results demonstrate that the $(MeOPhSe)_2$ dietary supplementation reversed STZ-induced memory impairment by enhancing memory in sham rats. $(MeOPhSe)_2$ was also effective in reducing STZ-induced apoptosis and preserving dendrites and synapses. Moreover, $(MeOPhSe)_2$ inhibited activation of microglia and astrogliosis induced by STZ in the rat hippocampus. We conclude that the $(MeOPhSe)_2$ neuroprotective action is related to inhibition of apoptosis and suppression of inflammation.

Keywords: Apoptosis, glial cells, memory, neuroinflammation, organoselenium compounds, streptozotocin

INTRODUCTION

Alzheimer's disease (AD) is a progressive neurodegenerative disorder, mainly characterized by memory and intellectual capacity loss, frequently accompanied by non-cognitive neuropsychiatric symptoms. AD affects more than 24 million people worldwide and is described as the neurological disorder with greater prospect for growth in the world, constituting a serious public health problem [1]. Nevertheless, there is currently no precise description of the etiology of AD and its pathophysiology is complex, involving multiple pathways of neuronal damage [2]. Histopathological studies of AD have revealed the presence of senile plaques and neurofibrillary tangles, which result from deposition of amyloid-β peptide (Aβ) and hyperphosphorylated tau protein, respectively [3]. It has been assumed that these morphological changes initiate a pathological cascade that results in synaptic

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dysfunction and loss, inflammation, oxidative damage, altered metallic ion homeostasis and, ultimately, neuronal death [3, 4].

Selenium (Se) is an essential trace element in mammalian species, with multiple roles in cell growth and functioning. It has been established that Se is effective in preventing a number of degenerative conditions, including inflammatory and neurological diseases, such as AD [5, 6]. Interestingly, Se concentration decreases with age [7] and is lower in AD patients when compared to healthy patients [8]. Furthermore, Se supplementation is associated with memory improvement in AD models [6, 9]. Some studies have reported that Se attenuates Aβ production and Aβ-induced neuronal toxicity [10, 11] and lowers tau phosphorylation [12]. In addition, Se prevents oxidative damage and modulates the cholinergic system in animal models of AD [9, 13].

We and others have reported that organoselenium compounds ameliorate memory function in rodents, without inducing neurotoxicity [13–15]. In addition, we recently demonstrated that *p,p′*-methoxyl-diphenyl diselenide [$(MeOPhSe)_2$], an organoselenium compound, improves memory in mice and rats [16–18] in the model of sporadic dementia of Alzheimer's type (SDAT) induced by streptozotocin (STZ) [19]. Collectively, these studies have demonstrated the protective therapeutic effects of $(MeOPhSe)_2$, which are attributed to antioxidant and antinitrosative properties as well as to acetylcholinesterase inhibitory action [16–18]. Since little is known about the specific mode of action of $(MeOPhSe)_2$, the purpose of this study was to investigate possible molecular targets of its neuroprotective role in a SDAT model. Behavior tasks were performed and apoptosis, neural integrity and inflammation were evaluated in rat hippocampus.

MATERIALS AND METHODS

Animals

Experiments were conducted using male Wistar rats (350–400 g) approximately seven months old. Animals were maintained at 22–25°C with free access to water and food, under a 12 : 12 h light/dark cycle, with lights on at 7 : 00 a.m. All manipulations were carried out between 8 : 00 a.m. and 4 : 00 p.m. Animals were used according to the guidelines of the Committee on Care and Use of Experimental Animal Resources and with the approval of the Animal Use Committee (23081.007005/2010-96), Federal University of Santa Maria, Brazil. All efforts were made to minimize animal suffering and to reduce the number of animals used in the experiments.

Experimental design

Rats were divided into four different groups ($n = 7$–8), including: I) Sham; II) STZ; III) $(MeOPhSe)_2$; and IV) STZ + $(MeOPhSe)_2$. The experimental procedure was performed as previously reported [18]. Briefly, animals were submitted to cannulae implantation at day 0 and STZ was infused at days 1 and 3. The supplementation with $(MeOPhSe)_2$ diet began at day 21 after cannulae implantation and remained until day 51, when the animals were killed by decapitation.

Surgery

Animals were anesthetized under intraperitoneal (i.p.) injection of equitesin (1% phenobarbital, 2% magnesium sulfate, 4% chloral hydrate, 42% propylene glycol, 11% ethanol; 3 ml/kg) and placed in a stereotaxic frame (Stoelting, Wood Dale, IL, USA). The scalp was shaved and swabbed with iodine, and an incision was made along the midline of the scalp exposing bregma. Burr holes were drilled in the skull and cannulae were implanted on both sides over the lateral ventricles using the following coordinates: 1.0 mm posterior to bregma; 2.0 mm lateral to sagittal suture (both right and left); 4.0 mm beneath the surface of brain at day 0 [20]. Rats received injections of STZ (Sigma-Aldrich, St. Louis, USA) (1.0 mg/8 μl; 4 μl/ventricle) at days 1 and 3. The sham group received intracerebroventricular (i.c.v.) injection of vehicle (artificial cerebrospinal fluid containing 147 mM NaCl; 2.9 mM KCl; 1.6 mM MgCl, 1.7 mM CaCl and 2.2 mM dextrose) (4 μl/site). Injections were carried out at 1 μl/min, using a Hamilton 10 μl syringe with a 26-gauge needle.

Dietary supplementation

$(MeOPhSe)_2$ was synthesized as previously reported [21]. Analysis of the ^{1}H NMR and ^{13}C NMR spectra showed analytical and spectroscopic data in full agreement with its assigned structure. The chemical purity of 99.9% was determined by gas chromatography-mass spectrometry. Rats in groups III and IV were submitted to a dietary supplementation with $(MeOPhSe)_2$. For this, the standard chow was pulverized with $(MeOPhSe)_2$ (1 mg/100 g standard chow) dissolved in ethyl alcohol (1 mg/10 ml), whereas the normal diet of groups I and

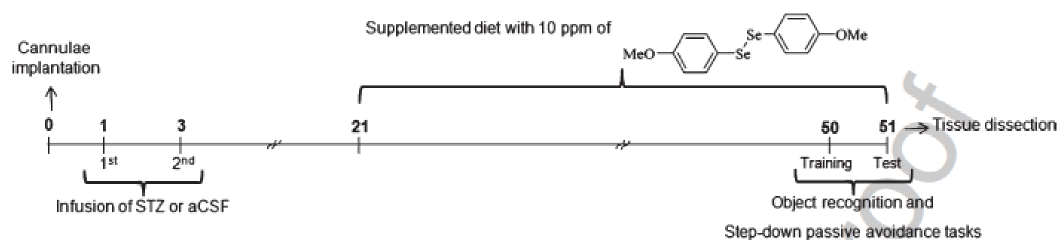

Fig. 1. Experimental protocol.

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II was pulverized with ethyl alcohol only (see Fig.1). Standard and supplemented diets were stored at room temperature for 3 h to evaporate the alcohol and then kept at 4°C for no more than 1 week. Animals were fed daily with 50 g/animal standard diet chow or standard chow supplemented with (MeOPhSe)$_2$ for 30 days. The supplementation began at 21 days after the cannulae implantation. A concentration of 10 ppm of (MeOPhSe)$_2$ was chosen on the basis of our previous study of toxicity in rats (data not shown). The preparation of supplemented standard chow was based on a previous study [22].

Behavioral tests

Object recognition task

The object recognition task took place in a $40 \times 50\,\text{cm}^2$ open field surrounded by 50 cm high walls, made of brown plywood with a frontal glass wall. All animals were given a habituation session, where they were left to freely explore the open field arena for 5 min. No objects were placed in the box during the habituation trial; thus, only the locomotor activity was recorded. Twenty-four hours after habituation, training was conducted by placing individual rats for 5 min into the field, in which two identical objects (objects 1 and 2) were positioned in two adjacent corners, 10 cm from the walls. In a short-term memory (STM) test, given 1.5 h after training, rats explored the open field for 5 min in the presence of one familiar (object 1) and one novel object (object 3). All objects presented similar textures, colors, and sizes, but distinctive shapes. The percentage of the total exploration time that the animal spent investigating each object was the measure of recognition memory. Between trials, the objects were washed with 10% ethanol solution. In a long-term memory (LTM) test, given 24 h after training, the same rats explored the field for 5 min in the presence of familiar object 1 and a novel object (object 4). Recognition memory was evaluated as for the STM test. Exploration was defined as sniffing or touching the object with nose and/or forepaws.

Step-down passive avoidance task

The step-down passive avoidance task has been used to study non-spatial long-term memory [23]. The apparatus consisted of a single box where the floor was made of a metal grid connected to a shock scrambler and in its lateral, there was a safe platform. During the training session (acquisition trial), each rat was placed on the platform, and usually the rat stepped down off the platform to explore the box. When it stepped down and placed its four paws on the grid floor, an electric shock (0.5 mA) was delivered for 2 s. Some seconds later, the rat was removed from the step-down passive avoidance apparatus and returned to its home cage. The retention trial was performed 24 h after training; each rat was again placed on the platform and the transfer latency time (i.e., time it took the rat to step down off the platform) was measured in the same way as in the acquisition trial, but foot shock was not delivered and the transfer latency time was recorded to a maximum of 600 s. The criterion for learning was taken as an increase in the transfer latency time on the retention (2nd) trial as compared to the acquisition (1st) trial. Thus, short transfer latencies indicate poor retention.

Plasma glucose levels

Plasma glucose levels were determined by an enzymatic colorimetric method using a commercial kit (Labtest Diagnostica, MG, Brazil). Glucose levels were expressed as mg/dl.

Ex vivo assays

Tissue preparation for immunohistochemistry and apoptosis

After behavioral tests, animals were anesthetized with an injection of equitesin (i.p., 3 ml/kg). Perfusion

and fixation of brains were performed through transcardiac perfusion with 4% paraformaldehyde. Dehydrated brains were embedded in paraffin and serial 5 µm-thick coronal brain sections were cut on a microtome and mounted on SuperFrost-Plus glass slides (Thermo Scientific, Rockford, IL, USA).

Apoptosis measurement

DNA fragmentation in brain sections was detected using an ApopTag peroxidase *in situ* apoptosis detection kit (Serologicals Corp., Norcross, GA, USA) for transferase mediated dUTP-digoxigenin nick-end labeling (TUNEL) staining, according to the manufacturer's instructions. In brief, tissue sections were deparaffined, rehydrated, and treated with 3% hydrogen peroxide to quench endogenous peroxidase activity. After adding the equilibration buffer, sections were treated with terminal deoxynucleotidyl transferase and digoxigenin-dNTPs for 60 min at 37°C. Specimens were then treated with antidigoxigenin-peroxidase for 30 min at 37°C, colorized with 3,3′-diaminobenzidine (Sigma-Aldrich) substrate, and counterstained with 0.5 % methyl green. Finally, slides were rinsed, dehydrated, and mounted.

Immunohistochemistry

Paraffin-embedded brain sections were deparaffined, rehydrated, and boiled 3 times in 10 mM citrate buffer, pH 6. Sections were then incubated for 60 min in blocking buffer, containing 10% (v/v) normal donkey serum (Jackson ImmunoResearch Laboratories Inc., West Grove, PA, USA) in sodium phosphate buffer with 0.1% (v/v) Triton X-100 (Sigma-Aldrich), and subsequently in diluted primary antibodies overnight at 4°C. After rinsing, the primary antibody was developed by incubating with DyLight 488 (Jackson ImmunoResearch) or Alexa Fluor 594 (Invitrogen, Grand Island, NY, USA) conjugated secondary antibodies against the corresponding species, for 2 h at room temperature. The following primary antibodies were used: astrocytes were immunostained with a mouse monoclonal glial fibrillary acidic protein (GFAP) antibody (GA5; Millipore Corporation, Temecula, CA, USA; 1 : 400); microglia was immunostained with a rabbit polyclonal ionized calcium binding adaptor molecule 1 (Iba-1) antibody (Wako Pure Chemicals, Richmond, VA, USA; 1 : 100). For neurons, neuronal cell bodies and dendrites were labeled with a rabbit polyclonal microtubule associated protein 2 (MAP2) antibody (Millipore; 1 : 100). In order to confirm the apoptotic phenotype, we used a rabbit polyclonal active caspase-3 antibody (R&D Systems, Lille, France; 1 : 100). No staining was observed in control sections, where primary antibody was replaced by blocking buffer.

Image analysis and semiquantification of immunofluorescence

Images were acquired with an Axioskop fluorescence microscope (Carl Zeiss GmbH, Hamburg, Germany). Semiquantitative analysis of mean fluorescence intensities (MFIs) of GFAP and Iba-1 were performed using NIH Image J software. Eight images of slides were obtained per hippocampal region. Images were converted into an 8-bit format, and the background subtracted. An intensity threshold was set and kept constant for all images analyzed. MFI per square millimeter area was calculated by dividing the MFI units by the area of outlined regions and was presented as a bar graph. The length of neuron was calculated by measuring the axon length on MAP2 assay using Image J software. Eight images of slides were obtained per hippocampal region and the length of ten random neurons was measured. Finally, apoptotic cells were examined and counted using the Image J software and results expressed as the number of TUNEL positive cells/high-power field (×400) in at least five high-power fields.

Statistical analysis

The normality of data was analyzed using a D'Agostino and Pearson omnibus normality test. Behavioral data of step-down passive avoidance were analyzed using a Scheirer-Ray-Hare test (an extension of Kruskal-Wallis test) followed by Dunns post-hoc test; these behavioral results are given as the median ± interquartile range. Other data (immunohistochemistry, TUNEL, plasma glucose levels, and object recognition task) were calculated by means of two-way analysis of variance (ANOVA) followed by the Newman-Keuls's test when necessary. These experimental results are given as the mean (s) ± S.E.M. Probability values less than 0.05 ($p < 0.05$) were considered to be statistically significant.

RESULTS

$(MeOPhSe)_2$ ameliorates memory decline induced by STZ in rats on object recognition and step-down passive avoidance tasks

In order to confirm the protective effect of $(MeOPhSe)_2$ on rat memory, we performed the object

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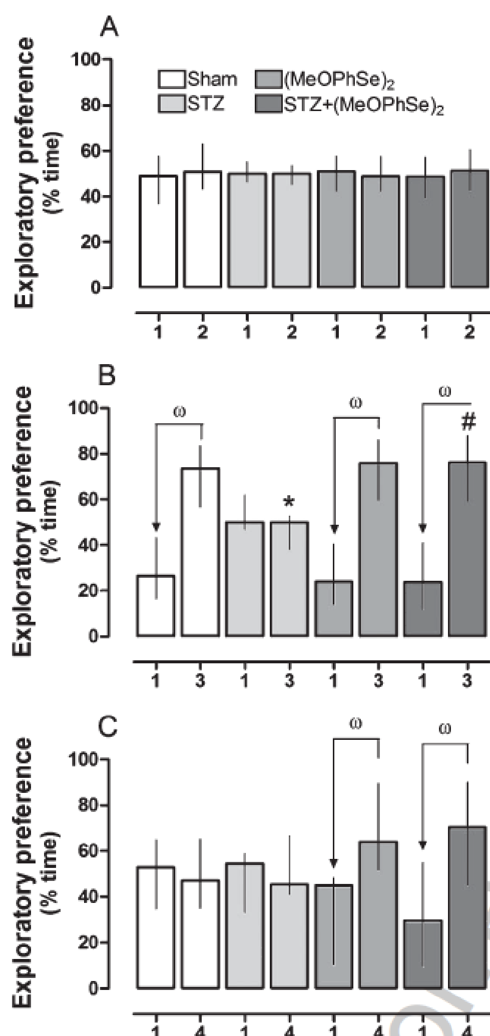

Fig. 2. Effect of $(MeOPhSe)_2$ on a novel object recognition task in rats with memory impairment induced by STZ. A) Exploratory preference during training (two identical objects, 1 and 2). B) Exploratory preference in the STM test carried out 1.5 h after training (when 3 is a novel object). C) Exploratory preference in the LTM test carried out 24 h after training (when 4 is a novel object). Data are expressed as mean ± S.E.M of percentage of time exploring the objects using 7-8 animals per group. $^{\omega}p < 0.05$ as compared to the old object into the same group; $^{*}p < 0.05$ as compared to the sham group and $^{\#}p < 0.05$ as compared to the STZ group in the retention phase.

recognition task. Initially, we observed that STZ injection and/or $(MeOPhSe)_2$ supplemented diet neither caused alteration in the spontaneous locomotion nor in exploratory activities during the habituation phase (data not shown). In addition, there was no significant

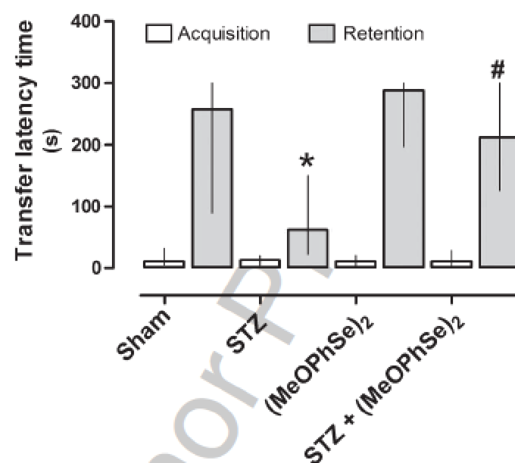

Fig. 3. Effect of $(MeOPhSe)_2$ on a step-down passive avoidance task in rats with memory impairment induced by STZ. Data are expressed as median and interquartile range of percentage of time exploring the objects using 7-8 animals per group. $^{*}p < 0.05$ as compared to the sham group and $^{\#}p < 0.05$ as compared to the STZ group.

difference among groups in the time exploring both objects (1 and 2) during the training session (Fig. 2A). In contrast, our data revealed that all animals, except from the STZ group, showed a significant preference toward the novel object (Fig. 2B) [STZ × $(MeOPhSe)_2$ interaction ($F_{1,26} = 12.10$; $p < 0.05$)]. Thus, these results provided evidence that STZ induces memory deficits in STM in rats, which were rescued by $(MeOPhSe)_2$.

As shown in Fig. 2C, no significant difference was observed between sham and STZ groups in the LTM test. Sham rats explored equally both objects, which may be explained by the advanced age of these animals. More importantly, similarly to the STM test, animals on the $(MeOPhSe)_2$ dietary supplementation, groups III and IV, had a preference toward the novel object in LTM tests [main effect of $(MeOPhSe)_2$ ($F_{1,26} = 21.98$; $p < 0.005$)]. These findings support the hypothesis that $(MeOPhSe)_2$ diet recovered and enhanced memory of rats.

Accordingly, the $(MeOPhSe)_2$ dietary supplementation was effective in improving memory of rats in the step-down passive avoidance task (Fig. 3). During the acquisition trial, there was no difference in the latency to get off from the platform among groups. By contrast, STZ injection decreased the transfer latency time of rats in the retention trial and $(MeOPhSe)_2$ dietary supplementation reverted this effect. Thus, these data are in agreement with our previous results indicating

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that $(MeOPhSe)_2$ rescues rodents memory on the step-down passive avoidance task [16, 18].

To provide extra data about the model characterization, plasma glucose levels were measured in rats; as expected, the STZ did not induced diabetes in rats [STZ × $(MeOPhSe)_2$ interaction ($F_{1,26} = 0.64$; $p > 0.05$)]. The results confirmed that 1 mg/site of STZ is a subdiabetogenic dose, since there was no significant difference in plasma glucose levels among groups (data not shown).

$(MeOPhSe)_2$ reduces STZ-induced neural loss and apoptosis via suppression of neuroinflammation

DNA fragmentation was determined by the TUNEL assay to evaluate the neuroprotective effect of $(MeOPhSe)_2$ on STZ-induced apoptosis. Importantly, $(MeOPhSe)_2$ supplemented diet protected rats from STZ-induced apoptosis (Fig. 4A and B). In fact, the number of TUNEL-positive cells in the ventricular region was markedly decreased by $(MeOPhSe)_2$ [STZ × $(MeOPhSe)_2$ interaction ($F_{1,10} = 13.91$; $p < 0.005$)]. In selected specimens, the modulation of apoptosis was further confirmed by evaluation of active caspase-3 immunoreactivity. The $(MeOPhSe)_2$ supplemented diet prevented against caspase-3 activation induced by STZ (Fig. 4A).

We next evaluated neuronal cell body and dendrite marker in rat hippocampus, MAP2. The results revealed a significant shortening of dendrite extension caused by STZ, which was prevented by $(MeOPhSe)_2$ [main effect of STZ ($F_{1,10} = 10.25$; $p < 0.05$)] (Fig. 5A, B). These data provide direct evidence that $(MeOPhSe)_2$ reduced STZ-induced apoptosis and preserved dendrites and synapses.

Other studies have suggested that neuroinflammation is involved in the complex pathological cascade that leads to neuronal dysfunction and senility in AD [3, 24, 25]. In fact, infiltration of activated astrocytes and microglia is a common feature in AD brains and transgenic mouse models. Thus, the levels of glial activation in the STZ model and the possible effect of $(MeOPhSe)_2$ were investigated. The results revealed that STZ increased GFAP activation by 312%, which was prevented by $(MeOPhSe)_2$ supplemented diet [STZ × $(MeOPhSe)_2$ interaction ($F_{1,10} = 12.96$; $p < 0.01$)] (Fig. 6A, B). Consistently, $(MeOPhSe)_2$ reduced STZ-induced Iba-1 activation [STZ × $(MeOPhSe)_2$ interaction ($F_{1,10} = 37.70$; $p < 0.005$)] (Fig. 6A, C). Therefore, these results show that $(MeOPhSe)_2$ inhibited the activation of microglia and astrocytes induced by STZ in the rat hippocampus, indicating that the $(MeOPhSe)_2$ neuroprotective action is related to its effect in suppressing inflammation.

DISCUSSION

Organoselenium compounds are effective in ameliorating memory in several animal models of cognitive deficits [13, 16–18, 26]. However, their mechanism of action is still unclear. Thus, the purpose of this study was to investigate possible targets involved in the neuroprotective effect of $(MeOPhSe)_2$ in a model of STZ-induced neurodegeneration and memory impairment. Our results clearly demonstrated that the $(MeOPhSe)_2$ dietary supplementation reduces STZ-induced apoptosis and neuronal loss. In addition, $(MeOPhSe)_2$ was capable of preventing the activation of microglia and astrocytes induced by STZ in the rat hippocampus, further protecting neurons. Accordingly, the behavior data provided evidence that $(MeOPhSe)_2$ dietary supplementation ameliorates memory of rats in object recognition and step-down passive avoidance tasks.

Consistent with previous results [26–28], STZ caused memory impairment, which was demonstrated in the STM test. Importantly, the $(MeOPhSe)_2$ dietary supplementation ameliorated animal performance in the object recognition task. Moreover, better performance in the LTM test was recorded in animal groups where $(MeOPhSe)_2$ dietary supplementation was included, as compared with those with non-supplemented diet. These results are similar to others [14, 18], in which systemic administration of the organoselenium compound induced facilitation of LTM in a novel object recognition task.

Additionally, non-spatial long-term memory was investigated using the step-down passive avoidance task. $(MeOPhSe)_2$ improved memory decline induced by STZ in the rat step-down passive avoidance task, without altering the spontaneous locomotor activity of these animals. In agreement, we recently published, using the same experimental procedure, that $(MeOPhSe)_2$ dietary supplementation ameliorated memory of STZ injected rats in the Morris water maze and step-down passive avoidance tasks [18].

AD is associated with progressive dysfunction and loss of neurons and synapses, particularly in the cortex and hippocampus [30]. This neurodegenerative process is coupled to oxidative stress, mitochondrial dysfunction, impaired energy metabolism, neuroinflammation, and activation of pro-death signaling pathways [31–33]. It has already been demonstrated

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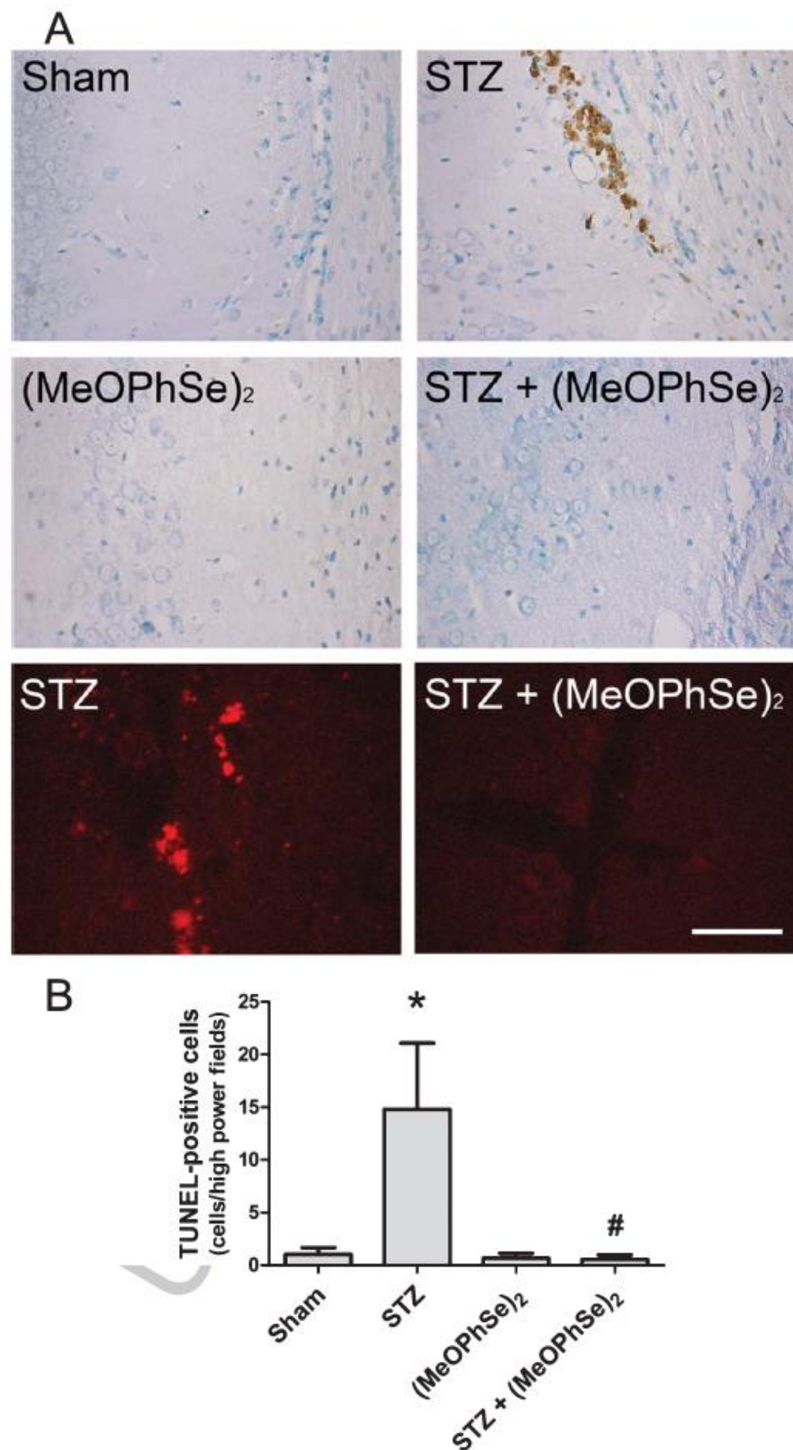

Fig. 4. Effect of $(MeOPhSe)_2$ diet supplementation on DNA fragmentation and caspase-3 activation in brains of rats that received i.c.v. injection of STZ. A) DNA fragmentation assessed by the TUNEL assay (TUNEL-positive cells in brown) and immunofluorescence of active caspase-3 (red). Scale bar: 100 μm. B) Number of TUNEL-positive cells in the ventricular region. The results are expressed as mean ± S.E.M of 4 animals per group. $^{*}p < 0.05$ as compared to the sham group and $^{\#}p < 0.05$ as compared to the STZ group.

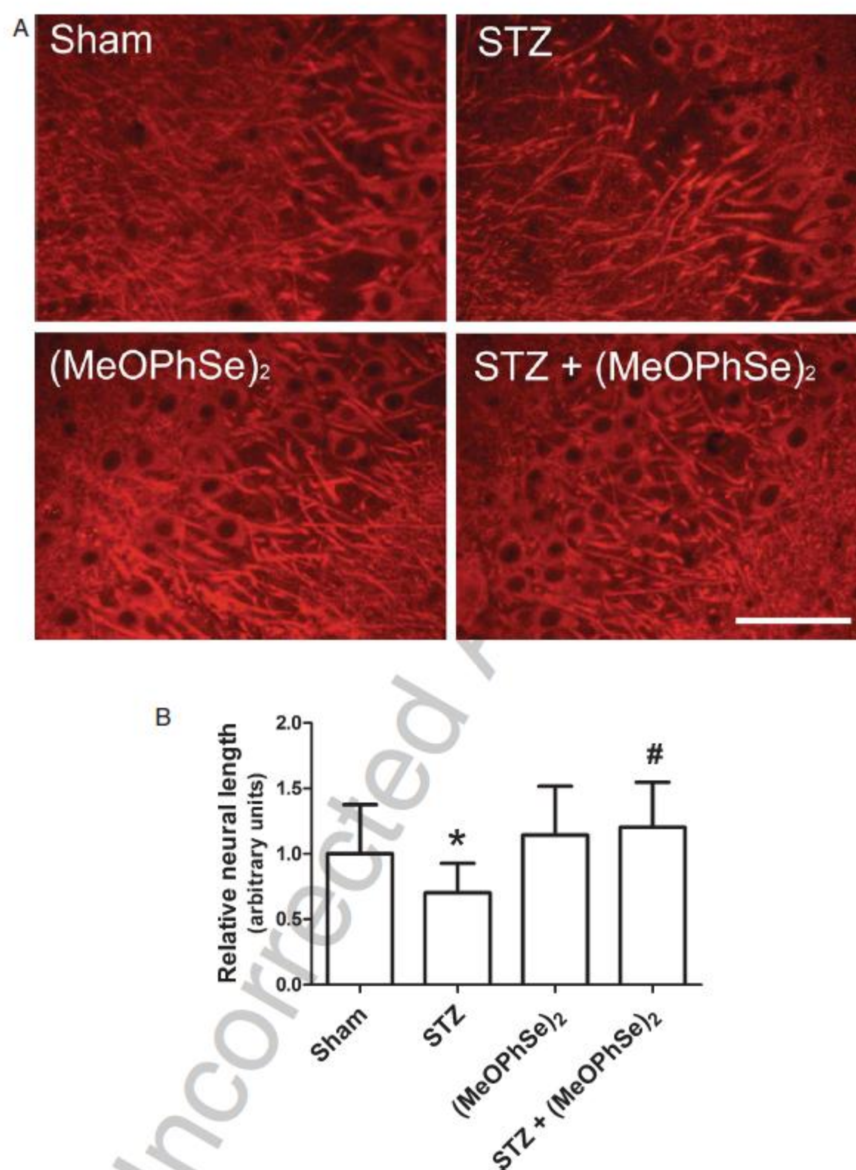

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Fig. 5. Effect of $(MeOPhSe)_2$ diet supplementation on MAP-2 levels in hippocampus of rats that received i.c.v. injection of STZ. A) MAP-2 immunofluorescence. Scale bar: 100 μm. B) Relative neural length. The results are expressed as mean ± S.E.M of 4 animals per group. $^{*}p < 0.05$ as compared to the sham group and $^{\#}p < 0.05$ as compared to the STZ group.

that STZ induces activation of apoptotic cascades, through overexpression of the pro-apoptotic protein Bax [34] and increased caspase-3 activation [35]. Caspase-3 activity is critical for DNA fragmentation and the morphological changes associated with apoptotic cell death. In addition, it has been proposed that caspase-3 activation is an early neurodegenerative event in the progression of AD [36]. Our results confirm that STZ induces DNA fragmentation and caspase-3 activation, which were remarkably attenuated by $(MeOPhSe)_2$. Furthermore, $(MeOPhSe)_2$ efficiently prevented STZ-induced neuronal loss and synaptic dysfunction, as indicated by MAP2 immunostaining. One of the earliest changes in cognitive

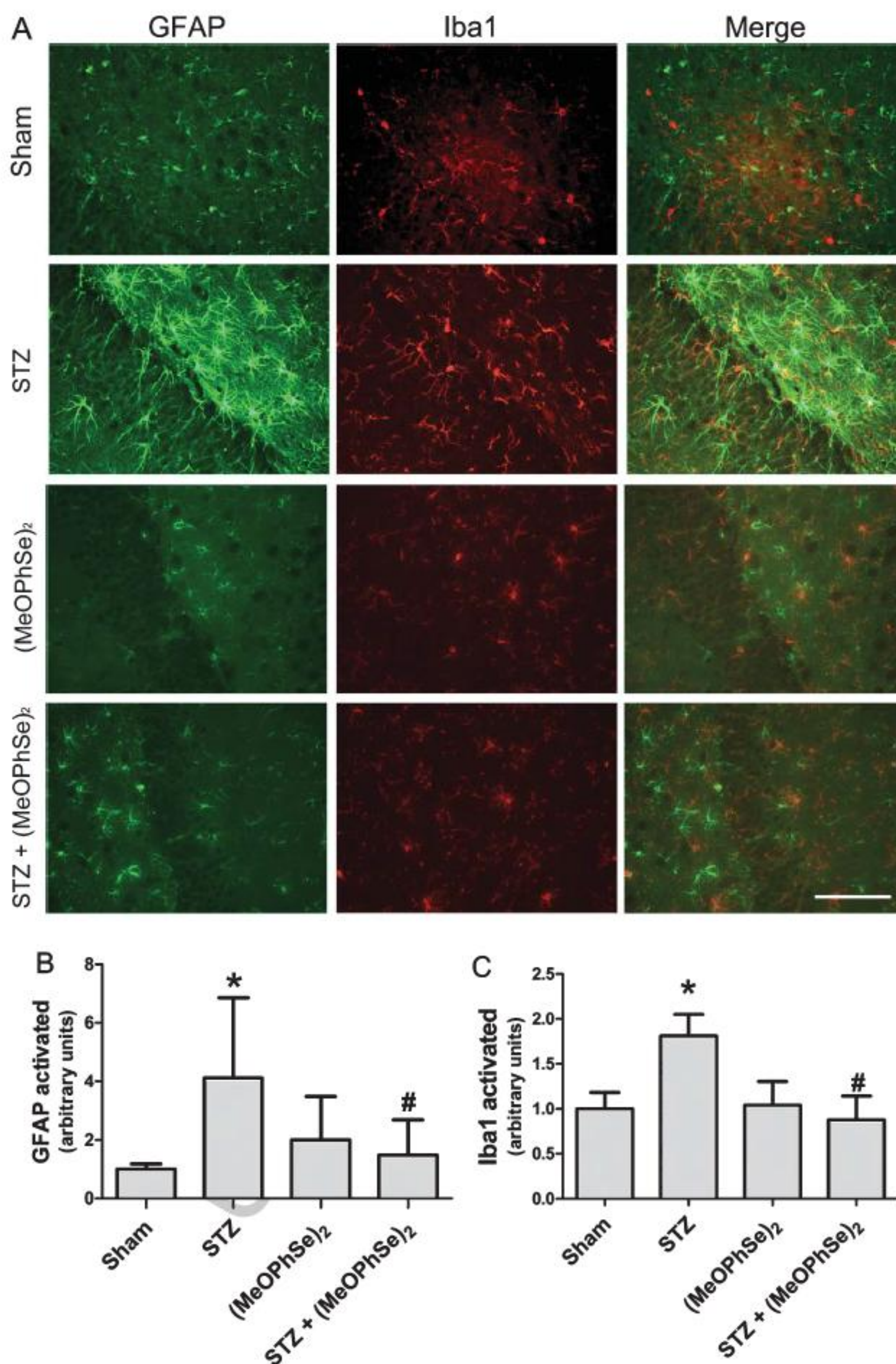

Fig. 6. Effect of (MeOPhSe)$_2$ diet supplementation on GFAP and Iba-1 activation in hippocampus of rats that received i.c.v. injection of STZ. A) Representative images of GFAP (green) and Iba-1 (red) staining. Scale bar: 100 µm. B) GFAP and (C) Iba-1 immunofluorescence. The results are expressed as mean ± SEM of the 4 animals per group. Denotes $^{*}p < 0.05$ as compared to the sham group and $^{\#}p < 0.05$ as compared to the STZ group.

disorders is the loss of dendritic arborization and synapses. Therefore, adult hippocampal neurogenesis and neuronal plasticity are key contributors to the ability of the brain to cope with age-associated cognitive decline [37, 38]. Certainly, this neuronal loss leads to significant deficits in memory and learning in rats. Importantly, by preserving dendrites and synapses, and consequent neuronal loss, (MeOPhSe)$_2$ emerges as an effective agent against STZ-induced neuronal toxicity.

Although different mechanisms appear to be involved in the pathogenesis of AD, it has been demonstrated that neuroinflammation accounts for its progression [32]. Activation of glial cells induced by accumulation of Aβ further damages neurons by producing inflammatory mediators, such as nitric oxide (NO), interleukin 1β, and 6 and transcription factor nuclear factor κB [24, 25]. GFAP and Iba-1 are markers of astrocytes and microglia activation, respectively, and their overproduction is highly associated with the generation of neuroinflammation and neurodegeneration [39]. Previous studies demonstrated that STZ activates glial cells in rat hippocampus [40, 41], although others have reported that GFAP content in rat hippocampus was not changed by the STZ treatment [42]. The present results reinforce the idea that STZ causes an activation of astrocytes and microglia in the hippocampus, when compared to the sham group, favoring the neuroinflammatory response and neuronal loss. Interestingly, GFAP and Iba-1 immunostaining was relatively decreased in the STZ group submitted to the (MeOPhSe)$_2$ dietary supplementation, suggesting that it might be effective in inhibiting astrogliosis and microglial activation, and the subsequent production of inflammatory cytokines. In fact, the reduction of inflammation mediator, such as NO/ONOO$^-$, could be involved in the anti-inflammatory role of (MeOPhSe)$_2$, since this compound is able to reduce cerebral protein nitration levels [18].

The lack of effective drugs in AD treatment and prevention, combining high efficacy and low side effects, has stimulated the search for novel agents that might represent new therapeutic alternatives. Thus, the inhibition of inflammation and the reduction of oxidative stress have been suggested as possible therapeutic strategies to attenuate neurodegenerative events and neuronal cell death in AD [24]. In this context, the antioxidant effect of (MeOPhSe)$_2$ has been previously reported [17]. Although STZ has been widely used as a model of AD [9, 19, 27–29, 40–43], we acknowledge some potential limitations of this study. While the STZ animal model of AD satisfied some criteria, extrapolation of animal studies to humans is difficult because AD is a complex and multifactorial disorder, which involves different etiopathogenic mechanisms.

In conclusion, the present study demonstrates that (MeOPhSe)$_2$ improved rat memory and prevented synapse loss and cell death. Moreover, this organoselenium compound inhibited the activation of microglia and astrocytes and, consequently, inflammatory mediators, which may contribute to attenuate neurodegeneration.

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4 DISCUSSÃO

Entende-se que a DA é uma desordem neurológica bastante abstrusa e suas múltiplas vias patológicas contribuem para a complexidade e dificuldade de tratamento e prevenção desta doença. Uma vez que ainda não há cura para a DA, o desafio maior está no desenvolvimento de novas terapias e/ou alvos terapêuticos para cura ou tratamento sintomático da doença. Desta forma, este trabalho procurou apontar uma nova molécula contendo Se, o $(MeOPhSe)_2$, como uma alternativa promissora para o tratamento da DEDA ou outras formas de demência, usando um modelo experimental de DEDA induzida pela ETZ em roedores.

Os resultados apresentados, em todos os artigos desta tese, demonstraram claramente que a injeção i.c.v. de ETZ induziu um prejuízo na memória e no aprendizado de roedores. Essa conclusão é baseada na piora do desempenho cognitivo desses roedores nos testes do labirinto aquático de Morris (**Artigos 1** e **3**), da esquiva passiva (**Artigos 1**, **3** e **4**), do labirinto em Y (**Artigo 2**) e do reconhecimento do objeto (**Artigo 4**). Mais importantemente, verificou-se que o tratamento prévio ou terapêutico com $(MeOPhSe)_2$ foi efetivo em prevenir e reverter o prejuízo na memória e no aprendizado induzido pela ETZ nestes animais, em todos os testes cognitivos realizados.

Nos **artigos 1** e **2**, os quais possuem o mesmo protocolo experimental, constatou-se que quando os camundongos receberam uma única dose oral do $(MeOPhSe)_2$ antes da infusão de ETZ, eles apresentavam uma performance cognitiva similar aos animais do grupo controle. Os resultados presentes nestes artigos demonstraram que os possíveis mecanismos envolvidos no efeito do $(MeOPhSe)_2$ em melhorar o desempenho cognitivo dos camundongos nos testes do labirinto aquático de Morris (**Artigo 1**) e do labirinto em Y (**Artigo 2**) podem ser atribuídos: i) a uma modulação da neurotransmissão colinérgica (**Artigo 1**); ii) a minimização da injúria tecidual gerada pelo EO no cérebro destes animais (**Artigo 2**).

Certamente, o fato da atividade da AChE cerebral ter aumentado em decorrência da injeção i.c.v de ETZ nos roedores culminou em uma diminuição dos níveis de ACh

na fenda sináptica, prejudicando assim a memória destes animais. O $(MeOPhSe)_2$ inibiu a atividade aumentada desta enzima aos níveis do controle. Por essa razão, nossos resultados sugerem que este organoselênio foi capaz de modular a disfunção na neurotransmissão colinérgica induzida pela ETZ (**Artigo 1**). O efeito antioxidante do $(MeOPhSe)_2$ também esteve envolvido no seu efeito neuroprotetor contra o dano oxidativo induzido pela ETZ, uma vez que o composto preveniu o tecido cerebral do aumento das ER e da diminuição dos níveis de GSH, assim como modulou a atividade das enzimas antioxidantes envolvidas (SOD, GPx, GST) (**Artigo 2**).

Os resultados apresentados nos **artigos 1** e **2** foram promissores e demonstraram bons indícios do papel neuroprotetor do $(MeOPhSe)_2$ na prevenção do desenvolvimento do déficit cognitivo assim como da injúria cerebral induzido pela ETZ. Para mimetizar uma situação onde o prejuízo na memória e o aprendizado, assim como as disfunções bioquímicas induzidas pela ETZ, estivessem bem estabelecidas e assim avaliar o feito terapêutico do $(MeOPhSe)_2$, foram desenvolvidos os **artigos 3** e **4**.

O protocolo experimental usado nos **artigos 3** e **4** visou um tratamento terapêutico com uma suplementação dietética do $(MeOPhSe)_2$. Anterior ao uso da ração suplementada com $(MeOPhSe)_2$ contra o efeito nocivo da ETZ, realizou-se um estudo de toxicidade do composto para a escolha da concentração a ser usada nesta terapia. Durante 30 dias, grupos diferentes de ratos alimentaram-se de ração normal ou suplementada com 3, 10 ou 30 ppm de $(MeOPhSe)_2$. Durante esse período, não observou-se sinais gerais de toxicidade induzida pelo organoselênio, observou-se que não havia diferença significativa para o ganho de peso corporal dos animais, nem quanto a ingestão de água ou ração. Ao final destes 30 dias, também não se notou diferenças entre os grupos nos parâmetros séricos de toxicidade hepática (AST e ALT) ou renal (ureia e creatinina) avaliados (dados não mostrados). Embora nenhuma concentração tenha indicado toxicidade geral nos ratos, a concentração de 10 ppm foi escolhida para o desenvolvimento dos trabalhos futuros a fim de evitar efeitos indesejados pela concentração mais alta (efeitos citotóxicos ou perda da seletividade).

Obviamente, para a realização do tratamento de uma enfermidade, é necessário que a mesma esteja instituída. Logo, para verificar se ETZ induziu um prejuízo na cognição dos ratos e posteriormente tentar reverter este dano com o $(MeOPhSe)_2$, realizou-se o teste da esquiva passiva 21 dias após a infusão da ETZ (**Artigo 3**). Corroborando com dados prévios da literatura, o desempenho cognitivo dos ratos neste testes confirmou que a ETZ causou um déficit na memória destes roedores (Weinstock e

Shoham, 2004; Lester-Coll et al., 2006; Sharma et al., 2008; Ishrat et al., 2009). Após a confirmação da indução do prejuízo na memória, iniciou-se a suplementação dietética destes animais com o $(MeOPhSe)_2$ e após 30 dias, uma nova série de testes cognitivos foram realizados.

Desta forma, observou-se que a suplementação dietética com o $(MeOPhSe)_2$ restaurou as habilidades cognitivas prejudicadas pela ETZ nos ratos, tanto nos testes do labirinto aquático de Morris e da esquiva passiva (**Artigos 3** e **4** – ressaltando que no **Artigo 4** os animais não foram submetidos a tarefa da esquiva passiva 21 dias após a injeção de ETZ) quanto no teste do reconhecimento do objeto (**Artigo 4**). Estes resultados, em conjunto com aqueles apresentados nos **Artigos 1** e **2,** reforçam a ideia de que o $(MeOPhSe)_2$ preserva a memória e é capaz de restaurar as habilidades cognitivas dos roedores.

Surpreendentemente, no **Artigo 3**, quando os animais foram re-convocados para realizar o teste da esquiva passiva, notou-se que os ratos que tiveram suas dietas suplementadas com o organoselênio não desciam da plataforma, ou seja, tinham a memória preservada de um estímulo aversivo adquirido há 30 dias. Logo, a suplementação dietética com o $(MeOPhSe)_2$ não só melhorou a memória dos ratos tratados com ETZ como melhorou a memória dos ratos controle no teste da esquiva passiva (**Artigo 3**). Similar aos achados da esquiva passiva mostrados neste artigo, os resultados referentes ao teste do reconhecimento do objeto reforçam a hipótese de que o $(MeOPhSe)_2$ melhora *per se* a memória dos ratos (**Artigo 4**). Estes resultados corroboram com dados prévios que demonstram que compostos orgânicos de Se são capazes de melhoras a memória de roedores (Rosa et al., 2003; Stangherlin et al., 2008).

As propriedades antioxidantes do $(MeOPhSe)_2$ também foram evidenciadas no **Artigo 3,** onde o composto foi efetivo em reverter todas as alterações oxidativas causadas pela ETZ e testadas neste artigo. Similar ao EO induzidos pela ETZ no **Artigo 2**, no **Artigo 3** a ETZ aumentou os níveis de ER e a nitração de proteínas (nitrotirosina) no córtex e diminuiu os níveis de GSH no hipocampo dos ratos. A suplementação dietética com $(MeOPhSe)_2$ também foi efetiva em inibir a atividade da AChE (aumentada pela ETZ) tanto no córtex como no hipocampo dos ratos (**Artigo 3**).

Os resultados do **Artigo 3** sugerem que a ETZ foi capaz de diminuir a taxa de *turnover* do ATP, uma vez que ela diminuiu a relação ATP/ADP (indicando uma menor síntese de ATP) nas células do hipocampo dos ratos. Estudos realizados com este modelo de DEDA demonstram que é essa redução da taxa de utilização da glicose,

assim como das enzimas glicolíticas que causam uma diminuição da síntese e liberação da ACh e assim o desequilíbrio do sistema colinérgico (Henneberg e Hoyer, 1995). A suplementação dietética com o $(MeOPhSe)_2$ não foi capaz de aumentar a produção de ATP nas células neurais, indicando que o $(MeOPhSe)_2$ não é capaz de modular o metabolismo da glicose (**Artigo 3**). Esse resultado também sugere que a taxa de síntese da acetil-CoA (substrato da ACh) estaria reduzida, desta forma, este resultado é mais um indicativo de que a ação moduladora do sistema colinérgico exercida pelo $(MeOPhSe)_2$ é via modulação direta da enzima AChE (**Artigos 1** e **3**).

A suplementação dietética com o $(MeOPhSe)_2$ evitou a perda sináptica (MAP2) e neuronal (apoptose) e inibiu os eventos neurodegenerativos (ativação da caspase 3) induzidos pela ETZ em ratos (**Artigo 4**). As propriedades antioxidantes do $(MeOPhSe)_2$ são em parte responsáveis pela proteção exercida por este composto contra a neurodegeneração, uma vez que o EO está fortemente associado a morte celular (**Artigo 3**). A atenuação da perda e morte celular $(MeOPhSe)_2$ também deve ser atribuída ao seu efeito anti-inflamatório. A suplementação dietética com o $(MeOPhSe)_2$ suprimiu a neuroinflamação induzida pela ETZ no hipocampo dos ratos. O organoselênio inibiu a ativação das células gliais (Iba1) e dos astrócitos (GFAP) (**Artigo 4**).

Baseado nos resultados apresentados nos **Artigos 1**, **2**, **3** e **4**, é possível admitir que o $(MeOPhSe)_2$ possui propriedades que são favoráveis à conservação e recuperação das habilidades cognitivas em roedores. Pode-se também, atribuir os efeitos neuroprotetores deste composto às suas propriedades antioxidante (**Artigos 2** e **3**), à capacidade de inibir a atividade da AChE (**Artigos 1** e **3**) e suprimir a neuroinflamação e a morte neuronal (**Artigo 4**). A Figura 12 trás um esquema representativo dos alvos da ação neuroprotetora do $(MeOPhSe)_2$.

O uso do modelo de DEDA induzido pela ETZ foi uma importante ferramenta para a descoberta do efeito neuroprotetor do $(MeOPhSe)_2$ e seu modo de ação, contudo, o uso de outros modelos para a indução da DEDA seria interessante para verificar se o efeito deste organoselênio se perpetuaria. Por essa razão, experimentos realizados no nosso grupo de estudo revelaram que o $(MeOPhSe)_2$ reverteu o prejuízo na memória induzido pela injeção i.c.v. do peptídeo βA em camundongos no teste do labirinto aquático de Morris, e esta reversão foi similar ao controle positivo (donepezila) (Anexo 1). Além disso, o $(MeOPhSe)_2$ atenuou a apoptose induzida por esse peptídeo em culturas primárias de neurônios corticais. O $(MeOPhSe)_2$ inibiu a fosforilação da c-Jun N-terminal quinase (JNK) nesses neurônios, o que indica que este é possivelmente um

dos mecanismos moleculares de ação do $(MeOPhSe)_2$ que poderia explicar seus efeitos benéficos sobre a memória e o aprendizado de roedores (Anexo 1). A fosforilação/desfosforilação do JNK está intimamente envolvida na modulação da memória, seu estado desfosforilado favorece a cognição (Ploia et al., 2011; Ramin et al., 2011; Sherrin et al., 2011). Estes resultados não foram apresentados nesta tese pois ainda não foram completamente finalizados, mas reforçam os achados mostrados nos artigos aqui apresentados.

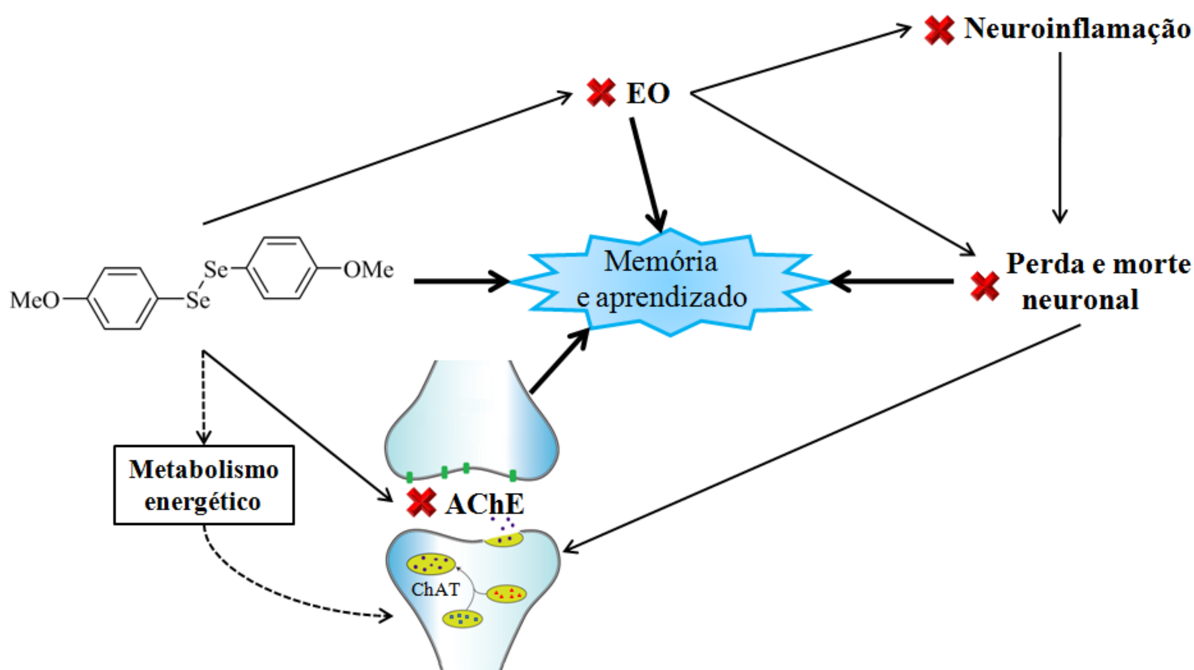

Figura 10: Visão geral dos efeitos do $(MeOPhSe)_2$ em melhorar a cognição de roedores. O organoselênio diminui o EO, a neuroinflamação, a perda e morte neuronal e inibe a atividade da AChE, parâmetros aumentados pela injeção i.c.v. de ETZ. Esta neuroproteção culmina na melhora das habilidades cognitivas dos roedores. O $(MeOPhSe)_2$ não modula o metabolismo da glicose, pois não altera a taxa de *turnover* do ATP a qual foi diminuida pela ETZ. (—) Ação efetiva do $(MeOPhSe)_2$. (---) O $(MeOPhSe)_2$ não interfere.

5 CONCLUSÕES

Baseado nos resultados apresentados nesta tese, pode-se concluir que:

1. O $(MeOPhSe)_2$ foi capaz de proteger e reverter o declínio das habilidades cognitivas induzido pela ETZ em roedores;
2. Os mecanismos envolvidos no efeito neuroprotetor do $(MeOPhSe)_2$ são: i) antioxidante; ii) modulador da atividade da AChE; iii) supressor da neuroinflamação;
3. O déficit no metabolismo energético induzido pela ETZ não é revertido pelo tratamento com o $(MeOPhSe)_2$;
4. O $(MeOPhSe)_2$ mostrou-se capaz de impedir a morte neuronal induzida pela ETZ em ratos;

Assim sendo, este trabalho demonstra que o $(MeOPhSe)_2$, uma molécula orgânica contendo Se, é uma alternativa promissora e potencial para o estudo de drogas para o tratamento de desordens cognitivas como a DEDA.

6 PERSPECTIVAS

Uma vez que o $(MeOPhSe)_2$ mostrou-se promissor no tratamento de desordens cognitivas e neurodegenerativas, as perspectivas para trabalhos posteriores são:

- Avaliar o efeito do $(MeOPhSe)_2$ na atividade da enzima ChAT;
- Verificar se o $(MeOPhSe)_2$ interfere na produção do βA, seja por interação com a APP ou com as enzimas secretases;
- Estudar os efeitos $(MeOPhSe)_2$ no metabolismo de fosforilação e desfosforilação da Tau;
- Investigar outros mecanismos moleculares envolvidos na neuroproteção exercida pelo $(MeOPhSe)_2$;
- Estudar a toxicologia do $(MeOPhSe)_2$, sua absorção, distribuição, metabolização e excreção.

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ANEXOS

Anexo 1

Resultados parciais que indicam que o $(MeOPhSe)_2$ melhora as memória de ratos via inibição da fosforilação da c-Jun N-terminal quinase (JNK) em cultura primária de neurônios corticais de ratos.

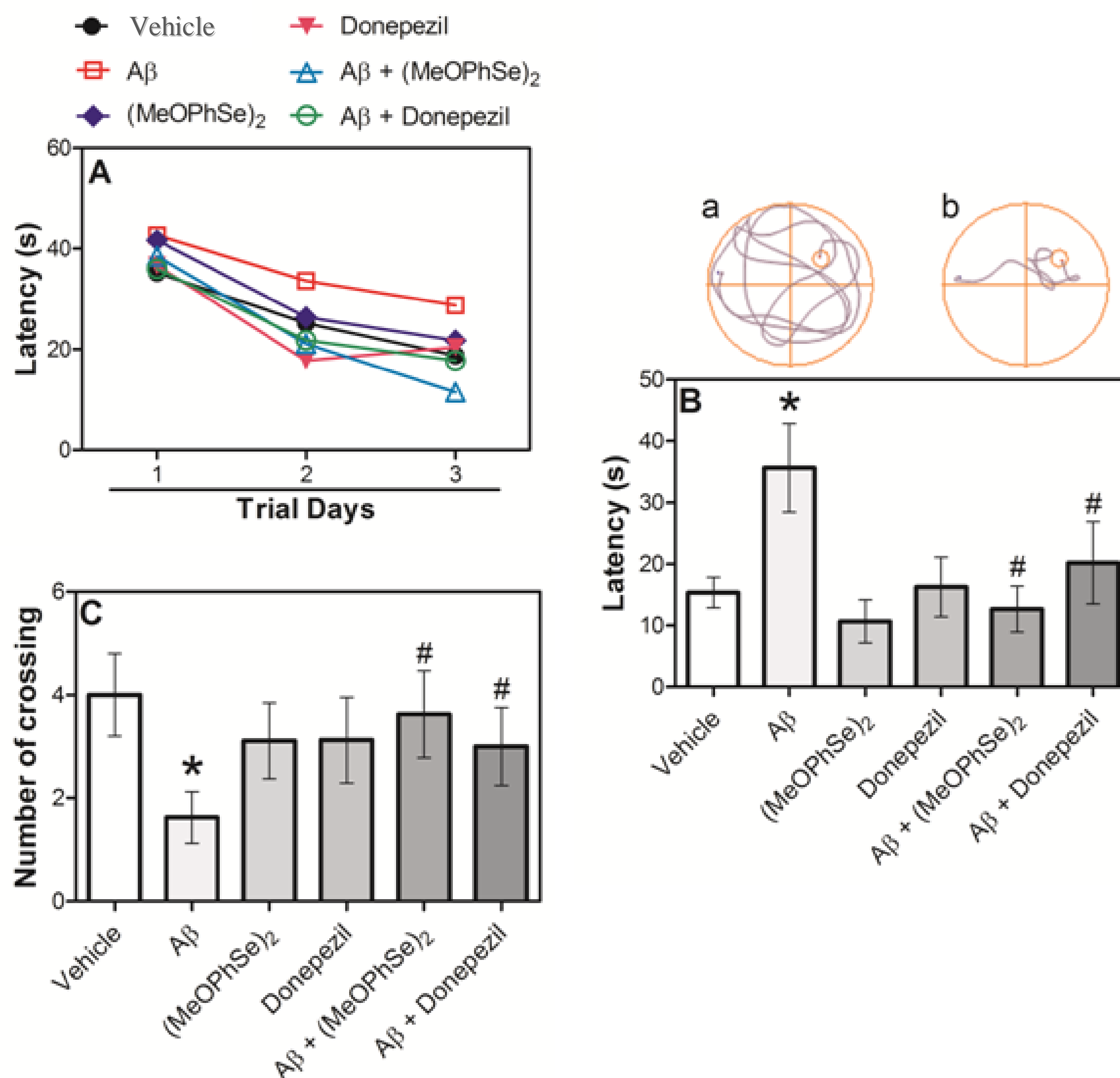

Figura 1. O $(MeOPhSe)_2$ reverteu o prejuízo cognitivo induzido pela infusão i.c.v. de βA_{25-35} em camundongos no teste do labirinto aquático de Morris. (A) Latência (s) para encontrar a plataforma durante as seções de treinamento (fase de aquisição). (B) Latência (s) para chegar ao local onde deveria estar a plataforma submersa no dia do teste (fase de retenção). Em cima, a imagem representativa da performance comportamental dos grupos (a) βA e (b) βA + $(MeOPhSe)_2$. (C) Número de cruzamentos sobre a posição da plataforma no dia do teste. Dados são expressos em media ou média ± desvio padrão de 8-10 animais por grupo. *p < 0.05 quando comparado com o grupo *vehicle* e $^{\#}p < 0.05$ quando comparado com o grupo βA_{25-35}.

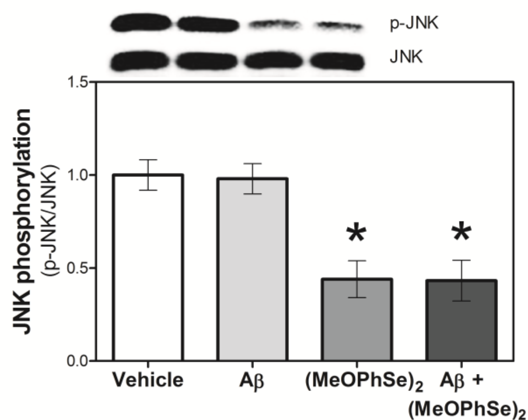

Figure 2: O $(MeOPhSe)_2$ inibiu a fosforilação do JNK em cultura primária de neurônios corticais de ratos. As células foram pré-incubadas com 10 μM de $(MeOPhSe)_2$ ou DMSO (36h antes do ensaio) e posteriormente incubadas com 25 μM do peptídeo βA_{25-35} ou água destilada (24h antes do ensaio). A imagem representativa do *immunoblot* da JNK e JNK fosforilada realizado nas células incubadas com βA_{25-35} ± $(MeOPhSe)_2$ está mostrada acima. Os dados mostrados no gráfico são expressos em média ± desvio padrão de 4 experimentos distintos. *$p < 0.05$ quando comparado com o *vehicle*.

Anexo 2

Participação em outros trabalhos desenvolvidos durante o mestrado e doutorado:

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