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EFEITOS FARMACOLÓGICOS DO DISSELENETO DE DIFENILA EM UM MODELO DE MENOPAUSA INDUZIDA POR OVARIECTOMIA EM ROEDORES

TESE DE DOUTORADO

Juliana Trevisan da Rocha

Santa Maria, RS, Brasil
2012

EFEITOS FARMACOLÓGICOS DO DISSELENETO DE DIFENILA EM UM MODELO DE MENOPAUSA INDUZIDA POR OVARIECTOMIA EM ROEDORES

Juliana Trevisan da Rocha

Tese apresentada ao curso de Doutorado do programa de Pós-Graduação em Ciências Biológicas: Bioquímica Toxicológica, Área de Concentração em Bioquímica Toxicológica, da Universidade Federal de Santa Maria (UFSM, RS), como requisito parcial para a obtenção do grau de **Doutor em Bioquímica Toxicológica**.

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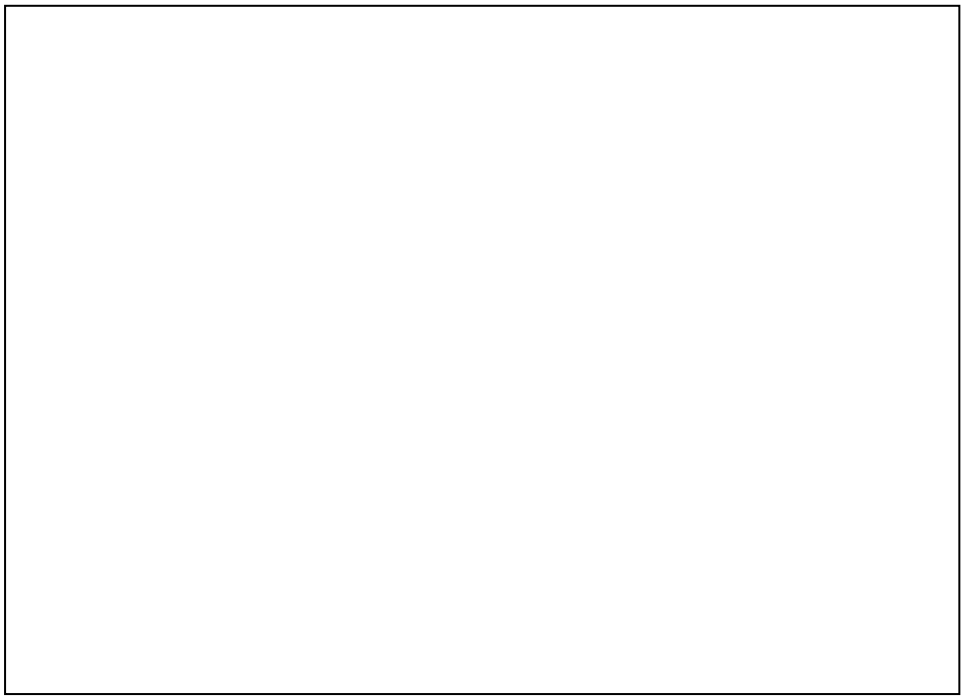

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elaborada por
Juliana Trevisan da Rocha

como requisito parcial para a obtenção do grau de
Doutor em Ciências Biológicas: Bioquímica Toxicológica

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“...nessas horas se percebe cantando as coisas da lida,
que o que se leva da vida é a história que a gente escreve...”

(Jairo Lambari Fernandes)

RESUMO

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

EFEITOS FARMACOLÓGICOS DO DISSELENETO DE DIFENILA EM UM MODELO DE MENOPAUSA INDUZIDA POR OVARIECTOMIA EM ROEDORES

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Data e Local da Defesa: Santa Maria, 18 de setembro de 2012.

A menopausa caracteriza-se pela suspensão irreversível da função ovariana, com declínio da produção de hormônios estrogênicos. A falta desses hormônios culmina no aparecimento de alterações metabólicas, cognitivas e comportamentais. Por essa razão, entender de que maneira a depleção de hormônios ovarianos contribui para o surgimento dos sintomas característicos da menopausa pode ser extremamente útil para o desenvolvimento de tratamentos alternativos à terapia de reposição hormonal. Nesse contexto, os dados aqui apresentados demonstram que ratas Wistar ovariectomizadas que foram tratadas com disseleneto de difenila [$(PhSe)_2$] na dose de 5 mg/kg uma vez ao dia durante um período de 30 dias possuíam reduzidos níveis plasmáticos de triglicerídeos e aumentados níveis de HDL quando comparadas as ratas controle não ovariectomizadas. Além disso, a administração de $(PhSe)_2$ foi capaz de reduzir o ganho de peso e o acúmulo de gordura abdominal nas ratas ovariectomizadas. Também foi observado que o tratamento com $(PhSe)_2$ melhorou parâmetros hepáticos relacionados a estresse oxidativo nas ratas ovariectomizadas (níveis de ácido ascórbico e glutationa reduzida (GSH) e atividade das enzimas glutationa S-transferase e catalase). O tratamento com $(PhSe)_2$ na dose de 5 mg/kg uma vez ao dia durante um período de 30 dias também melhorou o desempenho das ratas Wistar ovariectomizadas no teste do Labirinto Aquático de Morris, possivelmente por impedir o aumento da atividade da enzima acetilcolinesterase cerebral observado nas ratas ovariectomizadas. Dessa forma, os resultados sugerem um possível efeito benéfico do $(PhSe)_2$ para o tratamento do declínio cognitivo associado a menopausa. Ainda com relação aos sintomas apresentados pelas mulheres durante a menopausa, dados da literatura mostram que a transição para a menopausa associa-se a um risco aumentado para o aparecimento de sintomas do tipo depressivos. Com base nisso, foi evidenciado que fêmeas ovariectomizadas de camundongos Swiss submetidas a um protocolo de estresse sub-crônico apresentavam aumento no tempo de imobilidade nos testes de Suspensão da Cauda e do Nado Forçado, ambos preditivos de comportamento do tipo depressivo.Também foi observado que esse prolongamento no tempo de imobilidade apresentado pelas fêmeas ovariectomizadas foi prevenido pelo tratamento com $(PhSe)_2$ na dose de 10 mg/kg administrado 30 minutos antes de cada exposição ao protocolo de estresse. Além disso, evidenciou-se o envolvimento dos receptores de serotonina do tipo 5-$HT_{2A/2C}$ e 5-HT_3 no efeito do tipo antidepressivo apresentado pelo $(PhSe)_2$. Embora o $(PhSe)_2$ tenha inibido a atividade de ambas isoformas da enzima monoamino oxidase (MAO-A e MAO-B) *in vitro*, não foi observada inibição da atividade de tais enzimas quando sua atividade foi determinada *ex vivo*. Com base nesses resultados sugere-se que o tratamento com $(PhSe)_2$ pode influenciar aspectos relacionados ao humor e ao comportamento em mulheres no

período pós-menopausa. No intuito de elucidar os mecanismos envolvidos no efeito hipocolesterolêmico do $(PhSe)_2$ em culturas de células HepG2 observou-se que este composto orgânico de selênio leva a um aumento nos níveis de receptores de LDL e a um aumento no estado de ativação da enzima adenosina monofosfato (AMP) quinase (AMPK), sem inibir diretamente a atividade da HMG-CoA redutase. Esses resultados corroboram com os achados de outros trabalhos, os quais demonstram um efeito hipocolesterolêmico do $(PhSe)_2$ *in vivo*. Além disso, também foi buscado um possível mecanismo para o efeito hipoglicemiante apresentado pelo $(PhSe)_2$ em trabalhos anteriores: resultados obtidos a partir de culturas de células L6 (células de músculo esquelético de ratos), sugerem que o $(PhSe)_2$ aumenta a translocação do transportador de glicose 4 (GLUT4) do citosol para a membrana celular devido a um aumento no estado de ativação da enzima AMPK. Em conclusão, o conjunto de resultados apresentado nesta tese aponta o uso do $(PhSe)_2$ como uma terapia alternativa bastante promissora para o tratamento de algumas das principais conseqüências da menopausa, a saber aumento no ganho de peso, dislipidemia, prejuízos cognitivos e ocorrência de episódios depressivos. Entretanto, faz-se importante mencionar que os efeitos globais do $(PhSe)_2$ ainda precisam ser melhor caracterizados no intuito de verificar a existência de possíveis efeitos adversos.

Palavras-chave: Ovariectomia. Dislipidemia. AMPK. Prejuízo cognitivo. Episódios depressivos. Disseleneto de difenila.

ABSTRACT

Thesis of Doctor's Degree
Programa de Pós-Graduação em Ciências Biológicas: Bioquímica Toxicológica
Federal University of Santa Maria, RS, Brazil

PHARMACOLOGICAL EFFECTS OF DIPHENYL DISELENIDE IN A RODENT MODEL OF MENOPAUSE INDUCED BY OVARIECTOMY

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Data and Place of Defense: Santa Maria, September 18th, 2012.

The physiological state of menopause is characterized by an irreversible loss of ovarian function, with a resulting decrease in estrogen production, leading to the appearance of metabolic, cognitive, and behavioral alterations. For this reason, understanding how the decrease in the estrogen production contributes to the progress of menopausal symptoms can be very useful for the development of alternative therapies than hormone replacement therapy. In this context, the data show that ovariectomized Wistar rats treated with $(PhSe)_2$ at a dose of 5 mg/kg once a day for 30 days presented lower plasma triglyceride levels and increased HDL levels when compared to control ones. Moreover, $(PhSe)_2$ administration reduced the weight gain and fat abdominal accumulation induced by ovariectomy. It was also observed that $(PhSe)_2$ treatment improved hepatic oxidative stress parameters in ovariectomized rats (ascorbic acid and reduced glutathione levels, and glutathione S-transferase and catalase activities). Additionally, $(PhSe)_2$ treatment at a dose of 5 mg/kg once a day for 30 days improved the performance of ovariectomized Wistar rats in the Morris Water Maze test, possibly by inhibiting the increase in brain acetylcholinesterase activity induced by ovariectomy. The present results suggest a promising role of $(PhSe)_2$ against the cognitive decline related to menopause. The transition to menopause is associated with an increased risk of depressed mood. On this bases, the obtained results demonstrate that the prolongation of immobility time in the tail suspension test and forced swimming test in ovariectomized mice submitted to subchronic stress protocol was prevented by $(PhSe)_2$ treatment at a dose of 10 mg/kg. It was also found a possible involvement of the serotonin system in this effect, demonstrated by the modulation of 5-$HT_{2A/2C}$ and 5-HT_3 receptor subtypes. Although $(PhSe)_2$ had inhibited *in vitro* monoamine oxidase A and B activities, treatment of ovariectomized mice with $(PhSe)_2$ did not alter neither MAO-A nor MAO-B *ex vivo* activity. These findings suggest that $(PhSe)_2$ treatment could influence mood and behavior in postmenopausal women. In order to investigate a possible mechanism of action for the hypocholesterolemic effect of $(PhSe)_2$ it was observed that $(PhSe)_2$ treatment increases the LDL receptor levels and augment the adenosine monophosphate (AMP)-activated kinase (AMPK) activation state without inhibiting directly 3-hydroxy-3-methylglutaryl coenzyme A reductase (HMGR) enzyme activity in HepG2 cell cultures. These findings are in accordance with those from *in vivo* studies previously published. In addition, L6 skeletal muscle cells treated with $(PhSe)_2$ presented an augment in glucose transporter 4 (GLUT4) translocation from the cytosol to the cell membrane via an increase in AMPK phosphorylation state, which could be linked to the hypoglycemic properties presented by $(PhSe)_2$ in other studies. In conclusion, the body of

evidence presented in this thesis points to the use of $(PhSe)_2$ as a promising alternative therapy for the management of postmenopausal symptoms. However, it is important to mention that the global net effects of $(PhSe)_2$ still need to be better described in order to identify possible negative side effects.

Key-words: Ovariectomy. Dyslipidemia. AMPK. Cognitive impairment. Depressive state. Diphenyl diselenide.

LISTA DE ILUSTRAÇÕES

LISTA DE TABELAS

SUMÁRIO

1 INTRODUÇÃO

1.1 Menopausa

A menopausa pode ser caracterizada pelo progressivo declínio da função ovariana, resultando na diminuição da produção de estrógenos e no surgimento de alterações metabólicas e hormonais (Tabela 1) (McKinlay e Jeffreys, 1974; Myers et al., 1990). As formas naturais de estrógenos incluem o 17β-estradiol (E_2), a estrona (E_1) e o estriol (E_3) (Bennink, 2004). O E_2, o principal estrógeno, está envolvido em várias funções fisiológicas, dentre as quais, crescimento celular, homeostase energética e funcionamento do sistema reprodutivo (Shi e Clegg, 2009). Entre os efeitos protetores dos estrógenos encontram-se melhora da atividade colinérgica, redução da perda neuronal, redução nos níveis de colesterol e modulção da expressão do gene Apolipoproteína E (Brinton et al., 2000; Chen et al., 2002).

Tabela 1 Sintomas/condições associadas à menopausa.

Sintomas vasomotores	"Ondas de calor" e suor noturno.
Sintomas psicológicos	Depressão, irritabilidade, ansiedade, oscilações de humor, ataques de pânico.
Atrofia genital	Ressecamento vaginal, redução da libido, infecções do trato urinário, dor durante o ato sexual.
Osteoporose/perda de massa óssea	Dor nas costas e nos joelhos, risco aumentado de fraturas.
Prejuízos cognitivos	Esquecimento, desorientação e dificuldade de concentração.
Risco cardiovascular	Palpitações, hipertensão, risco de doença arterial coronariana.
Mudanças metabólicas	Aumento dos níveis de colesterol LDL e colesterol total.
Outros	Insônia/distúrbios do sono, dor de cabeça, fadiga, coceiras na pele, vertigem, visão turva, zumbidos, problemas digestivos e perda de cabelo.

(Adaptado de: Borrelli F, Ernst E. 2010. Alternative and complementary therapies for the menopause. Maturitas 66, 333–343)

1.1.1 Menopausa, Dislipidemias e Risco Cardiovascular

Com o avanço da idade, as mulheres tornam-se mais susceptíveis à ocorrência de aterosclerose e doenças cardiovasculares associadas. A incidência dessas doenças aumenta consideravelmente nas mulheres após os 44 anos quando comparadas a homens de mesma faixa etária (Castelli, 1984). Acredita-se que a diminuição na produção de hormônios sexuais estrogênicos, os quais tem efeito cardioprotetor, está relacionada a uma maior incidência de aterosclerose bem como ao desenvolvimento de dislipidemias (Rossouw, 2002). As mudanças mais freqüentemente observadas no metabolismo lipídico após a menopausa são caracterizadas por uma mudança para um perfil mais aterogênico, isto é, aumento nos níveis de colesterol total, colesterol LDL (low density lipoprotein ou lipoproteínas de baixa densidade) e triglicerídeos, os quais são acompanhados pela diminuição nos níveis de colesterol HDL (high density lipoprotein ou lipoproteínas de alta densidade), o qual é considerado um fator de proteção contra doenças cardiovasculares (Rachoń et al., 2008). Tais alterações devem-se ao fato de que os estrógenos estão envolvidos no metabolismo do colesterol, diminuindo os níveis plasmáticos de LDL e aumentando os de HDL (El-Swefy et al., 2002; Bennink, 2004).

Em decorrência do aumento nos níveis circulantes de colesterol LDL, tais lipoproteínas tornam-se mais susceptíveis à oxidação por radicai livres. Essas LDL oxidadas (oxLDL) acabam por depositar-se na parede dos vasos sangüíneos, alterando tanto a estrutura quanto a função das células endoteliais. Conseqüentemente, as oxLDL podem quimiotaticamente atrair monócitos e macrófagos para o endotélio, os quais fagocitam essas oxLDL originando as "células espumosas" e, por conseguinte, dando início a formação da placa aterosclerótica (Østerud e Bjørklid, 2003).

Além disso, acredita-se que a queda na produção de estrógenos nas mulheres após a menopausa bem como naquelas que foram submetidas à remoção cirúrgica dos ovários leva a um aumento na atividade da enzima lipoproteína lipase (responsável por hidrolisar as moléculas de triglicerídeos contidas no interior das lipoproteínas) e reduzir a atividade dos receptores de LDL (responsáveis por remover, via endocitose, as partículas de LDL da corrente sangüínea para o interior dos tecidos), culminando no acúmulo de LDL na circulação (Wakatsuki and Sagara, 1995).

Dados obtidos de fêmeas de ratos na estropausa (período equivalente a menopausa humana) indicam, além do aumento nos níveis plasmáticos de colesterol, um aumento no estado de ativação da enzima 3-hidroxi-3-metil-glutaril-CoA redutase (HMG-CoA redutase, principal enzima envolvida na biossíntese de colesterol) e uma diminuição na quantidade de

receptores de LDL presentes nas membranas celulares. Tais eventos contribuem, respectivamente, para aumentar a síntese endógena de colesterol e reduzir a captação de LDL pelos tecidos, confirmando a relação existente entre hipercolesterolemia e estropausa (Trapani et al., 2010).

Também tem sido especulado que o aumento no estado de ativação da HMG-CoA redutase observado em fêmeas de ratos durante a estropausa seja dependente de uma menor ativação da enzima AMP quinase (AMPK) (Trapani et al., 2010). A AMPK é uma proteína quinase extremamente importante para a regulação do metabolismo energético. A menos que seja fosforilada por outras quinases, a AMPK encontra-se em sua forma inativa (desfosforilada) (Ingebritsen et al., 1978). Uma vez ativada, a AMPK favorece os processos catabólicos, oxidação de ácidos graxos por exemplo, e suprime processos anabólicos como a síntese de proteínas, glicogênio, ácidos graxos e colesterol (Lu et al., 2010). A AMPK, quando fosforilada inibe a síntese de colesterol e de ácidos graxos por inativar (fosforilar), respectivamente, as enzimas HMG-CoA redutase e acetil-CoA carboxilase (Henin et al., 1995). Em acréscimo aos efeitos rápidos e agudos nos processos anabólicos, a ativação da AMPK também *down regulates* a síntese de ácidos graxos e a gliconeogênese a nível gênico (Hardie 2007). Interessantemente, no tecido muscular, a ativação da AMPK estimula a captação de glicose por aumentar a translocação do GLUT4 do citosol para a membrana celular (Kurth-Kraczek et al. 1999; Hayashi et al., 2000).

Por essa razão, considerando o que foi exposto até aqui, a alta incidência de doença arterial coronariana em mulheres no periodo pós-menopausa tem sido atribuída à diminuição na produção de hormônios esteroidais, pincipalmente estrógenos (Robinson et al., 1959; Rosenberg et al., 1981; Wakatsuki e Sagara, 1995).

1.1.2 Menopausa e Tecido Adiposo

Após o início da menopausa as mulheres em geral tendem a sofrer aumento de peso e passam a acumular gordura na região abdominal (Poehlman et al., 1995; Rachoń et al., 2008). Os estrógenos são reconhecidos como fatores bastante importantes para a regulação e deposição de tecido adiposo tanto em machos quanto em fêmeas, uma vez que o tecido adiposo expressa ambos os receptores α e β para este hormônio em humanos e roedores (Pedersen et al., 1996; Cooke e Naaz, 2004). A ativação dos receptores α é responsável por

modular a lipogênese no tecido adiposo via diminuição da atividade da enzima lípase lipoproteíca (Heine et al., 2000; Cooke e Naaz, 2004). Adicionalmente, os estrógenos podem indiretamente afetar a lipólise por induzir a atividade da enzima lípase hormônio-sensível, contribuindo para reduzir a deposição de tecido adiposo (Cooke e Naaz, 2004).

O tecido adiposo apresenta diferenças gênero-específicas em relação a sua distribuição corporal. Em geral, as mulheres apresentam uma proporção maior de gordura subcutânea que os homens, sendo que a porcentagem média de gordura em indivíduos jovens não obesos é de 15% para homens e 25% para mulheres (Bjorntorp 1996). Nas mulheres, ocorre um acréscimo considerável na deposição de gordura subcutânea a partir da puberdade, indicando que os estrógenos promovem preferencialmente o acúmulo de gordura subcutânea, resultante tanto do aumento do número de adipócitos quanto do aumento do seu tamanho. Contudo, em mulheres no período pós-menopausa verifica-se um maior acúmulo de gordura na região abdominal provavelmente decorrente da queda na produção de estrógenos (Ley et al., 1992; Cooke e Naaz, 2004). Corroborando com tais fatos, foi demonstrado que em fêmeas de roedores submetidas à cirurgia para remoção dos ovários (ovariectomia, OVX), um modelo animal amplamente utilizado para estudar os efeitos decorrentes da menopausa, tanto o acúmulo de gordura abdominal quanto o ganho de peso foram revertidos pela administração de estradiol (Wade et al., 1985; Wegorzewska et al., 2008).

Estudos demonstram a existência de uma relação entre gordura corpórea e riscos de doença cardiovascular. Nas mulheres, geralmente o depósito de gordura ocorre nas regiões do quadril e das coxas, constituindo assim a chamada distribuição ginóide de gordura. Nos homens, o depósito de gordura acontece preferivelmente na região abdominal, a chamada distribuição andróide, a qual relaciona-se diretamente com o desenvolvimento de doenças cardiovasculares e dislipidemias, efeitos não observados na distribuição ginóide (Witteman et al., 1989; Ley et al., 1992; Poehlman et al., 1995; Pedersen et al., 1996).

1.1.3 Menopausa e Estresse Oxidativo

O termo estresse oxidativo corresponde à excessiva formação endógena de radicais livres, os quais podem acarretar alterações nos constituintes celulares, incluindo inativação de enzimas, danos às bases nitrogenadas dos ácidos nucléicos e a proteínas, bem como peroxidação dos lipídios de membrana (Ha et al., 2006). Nesse sentido, o estresse oxidativo

pode ser definido como sendo um desequilíbrio entre a produção de agentes oxidantes e antioxidantes, em favor dos oxidantes, com potencial para ocasionar dano celular (Sies, 1997).

Nesse contexto, sempre que a produção de espécies reativas de oxigênio (EROS) ultrapassa seu índice de decomposição, danos celulares podem ser ocasionados. Por essa razão, com o intuito de manter a homeostase redox, as células desenvolveram sistemas antioxidantes capazes de neutralizar o excesso de EROS (Halliwell e Gutteridge, 1999). Tais sistemas são compostos por enzimas como a superóxido dismutase (SOD), catalase (CAT), glutationa peroxidase (GPx) e glutationa redutase (GR) e por antioxidantes não-enzimáticos com a glutationa (GSH) e as vitaminas C e E (Fridovich, 1999).

Dados da literatura sugerem a existência de uma relação entre níveis de estrógenos e estresse oxidativo (representado pelo aumento na produção de malondialdeído (MDA) e radicais livres) em ratas ovariectomizadas (Ha et al., 2006; Oztekin et al., 2007). Dessa forma, a redução na produção de hormônios pelos ovários resulta no aumento da produção de EROS, podendo resultar em dano ou, até mesmo, morte celular. Sabe-se que os estrógenos apresentam efeito antioxidante por atuarem como *scavengers* de EROS, interrompendo as reações de oxidação de proteínas e lipídios das membranas celulares (Akçay et al., 2000).

Embora estudos demonstrem que os estrógenos são importantes devido a suas propriedades antioxidantes (Huh et al., 1994; Akçay et al., 2000; Thibodeau et al., 2002; Topçuoglu et al. 2009; Borrás et al. 2003, 2010), alguns trabalhos tem sugerido que os estrógenos também podem atuar como agentes pró-oxidantes mesmo em concentrações fisiológicas (Thibodeau et al., 2002; Russo et al. 2003; Okoh et al.2011; Victorino et al., 2012).

1.1.4 Menopausa e Prejuízo Cognitivo

Está bem definida na literatura a associação entre a redução na produção de hormônios sexuais no período pós-menopausa e o surgimento de prejuízos cognitivos (Woods et al., 2000; Wolf e Kirschbaum, 2002; Shumaker et al., 2004; Frick et al., 2010).

Devido ao aumento na expectativa de vida, as mulheres passam uma proporção bastante significativa de suas vidas no período pós-menopausa. Dessa forma, muitas das alterações bioquímicas, estruturais e funcionais que ocorrem no cérebro feminino são

influenciadas pela queda na produção de estrógenos (Morrison et al., 2006). Algumas regiões cerebrais, como o hipocampo e o córtex pré-frontal, são susceptíveis não apenas aos danos decorrentes do envelhecimento, mas também são vulneráveis aos impactos da queda nos níveis de estrógenos circulantes (Keenan et al., 2001; Adams e Morrison, 2003; Daniel e Bohacek, 2010).

Em meio a esse contexto, evidências biológicas sustentam os benefícios dos estrógenos tanto no funcionamento quanto na morfologia cerebral (Amelsvoort et al., 2001). Dentre as funções exercidas pelos estrógenos no cérebro, pode-se citar indução do crescimento neuronal (Ferreira e Caceres, 1991), aumento da expressão da enzima colina acetiltransferase (responsável pela síntese do neurotransmissor acetilcolina) bem como da atividade colinérgica (Luine, 1985; Inestrosa et al., 1998), aumento da densidade de espinhos dendríticos e regulação da sinaptogênese (Gould et al., 1990; Murphy e Segal, 1996), além de proteger os neurônios contra danos oxidativos (Behl, 2002).

Numerosos estudos usando roedores têm confirmado os efeitos benéficos do estradiol para a memória e a função hipocampal (Bimonte-Nelson et al., 2010; Daniel 2006; Dere et al., 2007; Frick, 2009; Frye et al., 2005; Gibbs 2010; Gresack e Frick, 2004, 2006; Harburger et al., 2009; Heikkinen et al., 2002; Vaucher et al., 2002; Walf et al., 2006, 2008). Ainda nesse sentido, existem evidências de que os estrógenos previnem o aparecimento de prejuízos cognitivos associados à menopausa (Heikkinen et al., 2002; Hurn e Brass, 2003; Savonenko e Markowska, 2003; Walf et al., 2006; Brann et al., 2007; Harburger et al., 2009;), reforçando a idéia de que a diminuição da produção de hormônios pelos ovários contribui para o declínio das funções cognitivas em mulheres após a menopausa (Acosta et al., 2009; Bimonte-Nelson et al., 2010).

1.1.5 Menopausa e Sintomas do Tipo Depressivos

Uma série de estudos tem demonstrado que os estrógenos desempenham um importante papel nos transtornos de humor em mulheres (Goodnick et al., 2000; Sloan e Kornstein, 2003; Solomon e Herman, 2009). Soma-se a isso o fato de que considerando que tanto ansiedade quanto depressão manifestam-se através de sintomas psicológicos, fisiológicos e comportamentais, isso pode resultar em impactos negativos sobre a saúde e o bem-estar femininos (Solomon e Herman, 2009). Nesse sentido, uma vez que a prevalência de

desordens depressivas é cerca de duas vezes maior em mulheres que em homens (Seeman, 1997) sugere-se que fatores hormonais podem estar relacionados a isso (Llaneza et al. 2012). Além disso, uma parcela significativa das mulheres apresenta algum tipo de episódio depressivo associado à fase do ciclo menstrual na qual os níveis de estrógenos circulantes encontram-se baixos, durante o período pós-parto e durante a menopausa (Llaneza et al. 2012).

O sentimento de um estado de humor deprimido figura entre os sintomas mais comumente relatados por mulheres durante a menopausa (Freeman et al., 2007). Entretanto, embora o relato de depressão seja comum, é importante mencionar que nem todas as mulheres vivenciam sintomas depressivos, de modo que este não é um sintoma universal (Freeman 2010).

Embora os estrógenos tenham um impacto significativo nas desordens afetivas, outros eventos, incluindo fatores psicossociais a exemplo do estresse, como o provocado pela perda de um parente por exemplo, (Avis e McKinlay, 1991; Kaufert et al., 1992; Hunter, 1992; Solomon e Herman, 2009) e experiências de vida estressantes também estão associados a fisiopatologia da depressão e da ansiedade (Kendler et al., 1995). Nesse contexto, estudos têm demonstrado que a presença de sintomas depressivos em mulheres após a menopausa está freqüentemente associada a fatores como estresse, insônia, problemas de relacionamento, histórico de depressão e mesmo outros sintomas da menopausa, como as “ondas de calor” (McKinlay et al., 1987; Avis e McKinlay, 1991; Kaufert et al., 1992; Dennerstein et al., 1999; Freeman 2010).

Os estrógenos exercem efeitos diretos sobre o sistema nervoso central em áreas relacionadas não apenas à reprodução. O sistema límbico, por exemplo, contém quantidades significativas de receptores de estrógenos, através dos quais os hormônios estrogênicos podem modular os estados de humor (Shughrue et al., 1997). Além disso, estudos *in vitro* têm demonstrado que o estradiol inibe a recaptação de neurotransmissores catecolaminérgicos (Hiemke et al. 1985; Michel et al. 1987), além de interagir com receptores serotonérgicos (Michel et al. 1987; Birzniece et al. 2002; Steiner et al., 2003).

1.2 Modelos Experimentais para o Estudo da Menopausa

Semelhante ao que acontece nas mulheres, as fêmeas de roedores também vivenciam flutuações hormonais à medida que envelhecem, contudo, em roedores esse período denomina-se estropausa (Maffucci e Gore, 2006). Nesse sentido, foram desenvolvidos alguns modelos em roedores no intuito de estudar as modificações metabólicas e cognitivas enfrentadas pelas mulheres durante o período da menopausa.

De maneira geral, pode-se dizer que as fêmeas de roedores são consideradas como fêmeas de meia-idade por volta dos 17 meses de idade e velhas a partir dos 22 meses (Gresack et al., 2007). Por volta de um ano de idade, as fêmeas de roedores entram em um estado de estro persistente, durante o qual a ocorrência de ciclos reprodutivos é interrompida (Frick, 2008). Dessa forma, à medida que o processo de envelhecimento progride, a ovulação cessa e os níveis de estrógenos bem como dos demais hormônios produzidos pelos ovários declinam (Rousseau, 2006). Nesse contexto, o Modelo de Envelhecimento Intacto, o qual utiliza fêmeas de roedores a partir de um ano de idade, permite, além da retenção do tecido ovariano, a presença de um período de transição entre a produção contínua de estrógenos e o seu declínio (Chakraborty e Gore, 2004; Maffucci e Gore, 2006; Rubin, 2000).

O Modelo da Falha Ovariana Acelerada (Mayer et al., 2004; Mayer et al., 2005; Williams, 2005) tem sido empregado como um modelo alternativo para o estudo da menopausa. De acordo com esse modelo injeções de diepóxido de vinilciclohexano são aplicadas em camundongos para acelerar naturalmente a perda dos folículos ovarianos. Durante esse processo, os ovócitos são eliminados por apoptose e a produção de estrógenos pelos ovários é reduzida até cassar mimetizando assim a estropausa (Hoyer e Sipes, 1996; Hu et al., 2001; Mayer et al., 2004; Van Kempen et al., 2011).

O modelo da Ovariectomia, o qual consiste na remoção bilateral dos ovários por intervenção cirúrgica, é uma condição experimental de privação hormonal bem estabelecida na área do envelhecimento (Maffuci e Gore, 2006). A remoção cirúrgica dos ovários é o modelo mais amplamente utilizado por mimetizar de uma maneira geral as características da menopausa (Brinton 2012). Nesse sentido, o emprego de roedores ovariectomizados é reconhecido como o modelo animal mais comumente empregado para caracterizar os efeitos da depleção de hormônios ovarianos que ocorre durante o período pós-menopausa (Savonenko e Markowska, 2003).

1.3 O Papel do Selênio

O elemento químico selênio (Se) é um dos calcogênios que fazem parte do grupo 16 da tabela periódica (Papp et al., 2007; Lu e Holmgren, 2009). Apesar de ser conhecido desde o início do século XIX, foi apenas na década de 1950 que o Se passou a ser tratado como um micronutriente essencial na dieta tanto de seres humanos quanto de animais (Schwarz et al., 1957).

De maneira geral, o Se está presente em várias peroxidases, desempenhando um importante papel na proteção das células contra o estresse oxidativo (Papp et al., 2007). Com relação às funções metabólicas em que o Se atua, dados da literatura evidenciaram que a deficiência de Se leva a um aumento na atividade da enzima HMG-CoA redutase (principal enzima regulatória da biossíntese do colesterol), podendo resultar no aumento das concentrações plasmáticas de colesterol (Nassir et al., 1997). Além disso, Berr et al. (2000) demonstraram que os elementos traço da dieta estão envolvidos em processos metabólicos e reações redox no sistema nervoso central, influenciando as funções cognitivas. De acordo com isso, estudos experimentais (Zhang et al., 2001; Qin et al., 2008) e clínicos (Smorgon et al., 2004; Akbaraly et al., 2007) tem demonstrado um papel positivo do Se na performance cognitiva. Pode-se acrescentar a isso o fato de que alguns estudos também têm sugerido que a deficiência de Se pode afetar aspectos relacionados ao humor e que a suplementação com esse mineral na dieta está associada à melhoras em quadros de depressão (Benton e Cook, 1991; Hawkes e Hornbostel, 1996; Benton, 2001).

1.3.1 Compostos Orgânicos de Selênio

A farmacologia do elemento traço essencial Se vem despertando um crescente interesse, principalmente do ponto de vista da saúde pública (Navarro-Alarcon e Cabrera-Vique, 2008). Por essa razão, o desenvolvimento de compostos orgânicos contendo Se que possuam atividades biológicas e aplicações farmacológicas vem ganhando um destaque crescente nos últimos tempos (Parnham e Graf, 1991; Nogueira et al., 2004, 2010).

1.3.2 Disseleneto de Difenila [$(PhSe)_2$]

1.3.2.1 Farmacologia

Dentre os compostos orgânicos de selênio mais estudados, o $(PhSe)_2$ (**Figura 1**) destaca-se por apresentar diversas propriedades farmacológicas (Nogueira et al., 2004, Nogueira e Rocha, 2010, 2011), podendo-se citar neuroprotetora (Ghisleni et al., 2003), antiinflamatória e antinociceptiva (Nogueira et al., 2003a), antioxidante (Meotti et al., 2004; Santos et al., 2005; Luchese et al., 2009), hepato-protetora (Borges et al., 2005a), hipoglicêmica (Barbosa et al., 2006), antiúlcera (Savegnago et al., 2006), antidepressiva e ansiolítica (Savegnago et al., 2008), hipolipidêmica (De Bem et al., 2009; Da Rocha et al., 2009), além de induzir a melhoras na performance cognitiva de roedores (Rosa et al., 2003; Stangherlin et al., 2008; Souza et al., 2010).

Também já foi demonstrado que o $(PhSe)_2$ inibe a oxidação das LDLs *in vitro* (De Bem et al., 2008), reduz as lesões ateroscleróticas em camundongos hipercolesterolêmicos *knockout* para o receptor de LDL (LDLr -/-) e diminui a infiltração de células inflamatórias na parede vascular (Hort et al., 2011).

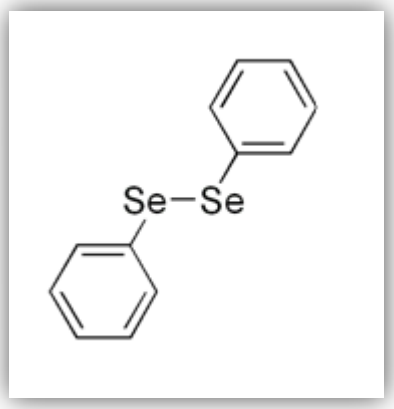

Fig. 1 Estrutura química do $(PhSe)_2$

1.3.2.2 Toxicologia

Embora o $(PhSe)_2$ possua uma baixa toxicidade (Nogueira et al., 2004, Nogueira e Rocha, 2010, 2011), já foi demonstrado que *in vitro* o $(PhSe)_2$ é capaz de interagir com grupamentos tiólicos de diversas proteínas, podendo levar a inibição de importantes enzimas, como δ-aminolevulinato desidratase (δ-ALA-D) (Barbosa et al., 1998; Farina et al. 2002; Nogueira et al., 2003c), Na^+, K^+-ATPase (Borges et al. 2005b; Kade et al. 2008; Santos et al. 2009) e diferentes isoformas da lactato desidrogenase (LDH) (Kade et al. 2009; Lugokenski et al. 2011), além de induzir a disfunções mitocondriais *in vitro* (Puntel et al. 2010). Dados de experimentos *in vivo* e *ex vivo* evidenciaram que a exposição a altas doses de $(PhSe)_2$ leva a depleção de grupos tióis em diferentes tecidos de roedores (Adams et al. 1989; Maciel et al. 2000), bem como à inibição da atividade hepática e cerebral da enzima δ-ALA-D (Maciel et al. 2000; Jacques-Silva et al. 2001; Barbosa et al. 1998; Kade e Rocha 2010). Ainda nesse sentido, também já foi verificado que a administração de $(PhSe)_2$ durante a gestação pode causar toxicidade em fetos de ratas tratadas com este composto (Favero et al., 2005; Weis et al., 2007).

Efeitos neurotóxicos do $(PhSe)_2$ também têm sido relatados (Nogueira et al., 2001; Nogueira et al., 2003b), incluindo a indução de convulsões em roedores (Prigol et al., 2008). Entretanto, o aparecimento de tais efeitos neurotóxicos está relacionado à dose, ao veículo, a rota de administração bem como a idade e a espécie dos animais (Brito et al. 2006; Savegnago et al. 2007; Prigol et al., 2009).

2 OBJETIVOS

2.1 Geral

Tendo em vista que a redução na produção de estrógenos verificada nas mulheres após a menopausa pode levar a prejuízos na capacidade cognitiva, a transtornos de humor e a alterações no perfil lipídico, e, considerando também que o uso de fêmeas de roedores submetidas à remoção bilateral dos ovários mimetiza a condição hormonal pós-menopausa, o presente trabalho visa identificar efeitos farmacológicos do $(PhSe)_2$ em modelos animais de ovariectomia (OVX), bem como elucidar os mecanismos envolvidos nesses efeitos.

2.2 Específicos

I. Investigar os efeitos do $(PhSe)_2$ no ganho de peso corporal, acúmulo de gordura intra-abdominal, perfil lipídico plasmático e estresse oxidativo hepático em fêmeas de ratos Wistar submetidas a OVX;
II. Avaliar a ação do $(PhSe)_2$ na memória espacial de referência em fêmeas de ratos Wistar submetidas a OVX;
III. Demonstrar o efeito do tipo antidepressivo do $(PhSe)_2$ em fêmeas ovariectomizadas de camundongos Swiss submetidas a um protocolo de estresse subcrônico, bem como elucidar o envolvimento do sistema serotonérgico nesse efeito;
IV. Investigar, em culturas de células HepG2 e L6, os mecanismos envolvidos nos efeitos hipolipidêmico e hipoglicêmico do $(PhSe)_2$.

3 ARTIGOS CIENTÍFICOS

Os resultados que fazem parte desta tese estão apresentados sob a forma de artigos e manuscrito científicos. Os itens Materiais e Métodos, Resultados, Discussão dos Resultados e Referências dos artigos encontram-se estruturados de acordo com as normas das respectivas revistas nas quais foram publicados ou submetidos para publicação.

ARTIGO 1

Effects of diphenyl diselenide on lipid profile and hepatic oxidative stress parameters in ovariectomized female rats

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Research Paper

Effects of diphenyl diselenide on lipid profile and hepatic oxidative stress parameters in ovariectomized female rats

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Abstract

Objectives Ovarian hormone decline after menopause is linked to many pathophysiological reactions. Female rats submitted to ovariectomy are employed as a model of postmenopausal condition. This study investigated the effects of diphenyl diselenide $(PhSe)_2$ on body weight gain, intra-abdominal fat deposition, plasma lipid profile and hepatic oxidative stress in ovariectomized rats.

Methods Female adult Wistar rats were ovariectomized (OVX rats) or sham-operated and divided into four groups: (i) sham-operated, (ii) $(PhSe)_2$, (iii) OVX and (iv) OVX + $(PhSe)_2$. $(PhSe)_2$ (5 mg/kg; 5 ml/kg, p.o.) was administered once a day for 30 days to groups (ii) and (iv). After that, rats were anaesthetized for blood sample gathering and submitted to euthanasia.

Key findings $(PhSe)_2$ (5 mg/kg) was effective in preventing the rise in body weight gain and intra-abdominal fat deposition induced in OVX rats. Although $(PhSe)_2$ was not effective in avoiding the increase in plasma total cholesterol and non-HDL levels induced in OVX rats, $(PhSe)_2$ reduced plasma triglycerides and augmented HDL levels in OVX rats. $(PhSe)_2$ also increased hepatic ascorbic acid levels, reduced glutathione content, glutathione S-transferase activity and restored catalase activity in liver of OVX rats.

Conclusions These findings suggest that $(PhSe)_2$ could be a promising alternative to minimize menopause related symptoms.

Keywords cholesterol; menopause; ovariectomy; oxidative stress; selenium

Introduction

Ovarian hormone decline after menopause is linked to many pathophysiological reactions. Dyslipidaemia is often seen in postmenopausal women and is characterized by an overall shift toward a more atherogenic lipid profile: an increase in plasma total cholesterol (TC), low-density lipoprotein cholesterol (LDL) and triglycerides, and a decrease in high-density lipoprotein cholesterol (HDL) levels.[1] The incidence of atherosclerosis in women is lower than in men of the same age group, but its incidence increases after menopause due to decreased oestrogen level, since oestrogens are involved in cholesterol metabolism by lowering LDL and increasing HDL concentrations in plasma.[2] In addition, after menopause, women gain weight and develop abdominal obesity.[1] Oestrogen has long been recognized as a major factor in regulating adipose tissue development and fat deposition in females.[3]

A clear relationship has been reported between oestrogen and malondialdehyde (MDA) levels in ovariectomized rats and that ovariectomy leads to an increase in free radical production.[4] It is well known that oestrogens, acting as free radical scavengers, break the free radical chain formation produced from membrane oxidation processes and hence inhibit lipid and protein oxidation.[5] The ovarian hormone deficiency also increases the generation of reactive oxygen species (ROS), which could result in cell damage or death. In mammalian cells, ROS are normally scavenged by three major types of primary antioxidant enzymes: copper-dependent, zinc-dependent and manganese-dependent superoxide dismutase, catalase (CAT) and glutathione peroxidase (GPx).[4] In this way, it has been shown that increased

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selenium intake induces increased GPx activity and decreased concentrations of lipid parameters, such as TC, LDL and triglycerides in blood of rabbits.[6]

Selenium is an essential trace element for normal growth and development of the mammalian species.[7] The interest in organoselenium pharmacology has increased in the last decades due to a variety of organoselenium compounds that possess biological activity. Accordingly, a number of novel pharmaceutical agents that are selenium-based or that target specific aspects of selenium metabolism are under development.[8] Diphenyl diselenide $(PhSe)_2$, an organoselenium compound, has been reported to be a good candidate for a pharmacological agent[8,9] due to its antioxidant,[10] hypoglycaemic,[11] antinociceptive,[12] anxiolytic,[13] anti-depressant-like[14,15] and hypolipidaemic activity.[16,17] It is important to point out that $(PhSe)_2$ did not display any overt sign of neurotoxicity when administered intraperitoneally, subcutaneously, orally (by gavage) and intracerebroventricularly in adult rats,[9,12,18] even when administered at high doses (500 mg/kg body weight).[19] In summary, the therapeutic potential of $(PhSe)_2$ seems to outweigh its toxic effects.[9]

Thus, considering that the use of female rats submitted to ovariectomy (bilateral removal of the ovaries) is largely employed to simulate a post-menopausal condition characterized by the absence of ovarian hormones, such as oestrogens, the novelty of this study was to investigate the effects of $(PhSe)_2$ on body weight gain, intra-abdominal fat deposition, plasma lipid profile and hepatic oxidative stress parameters in ovariectomized female rats.

Materials and Methods

Drugs

$(PhSe)_2$ was prepared and characterized in our laboratory by the method previously described by Paulmier.[20] Analysis of the ^1H NMR and ^{13}C NMR spectra showed that $(PhSe)_2$ obtained presented analytical and spectroscopic data in full agreement with its assigned structure. Yield: 96%; physical characteristics: yellow solid; ^1H NMR (200 MHz, $CDCL_3$, TMS): $\delta = 7.61–7.57$ (m, 2H), 7.25–7.21 (m, 3H); ^{13}C NMR (100 MHz, $CDCl_3$, TMS): 132.1, 131.2, 129.4, 127.4; IR (KBr), v (cm^{-1}): 3040, 1585, 1475, 790; ^{77}Se NMR ($CDCl_3$, 76.28 MHz) $\delta = 463.1$. The chemical purity of compound (99.9%) was determined by GC/HPLC. $(PhSe)_2$ was dissolved in canola oil. All other chemicals were of analytical grade and obtained from standard commercial suppliers.

Experimental animals

Female adult Wistar rats, 200–250 g, approximately 90 days old, from our own breeding colony were used. The rats were kept on a 12-h light–dark cycle, at room temperature (22 ± 2°C), with free access to food and water. All experiments were approved by and supervised under the Committee on Care and Use of Experimental Animals Resources from the Federal University of Santa Maria, Brazil (Process number 23081.012740/2008-05) and 'The Ethic Committee for Animal and Human Experiment' according to the declaration of Helsinki 1975. All efforts were made to minimize animals' suffering and to reduce the number of animals used in the experiments.

Ovariectomy (OVX)

Rats were ovariectomized by the surgical removal of both ovaries under intraperitoneal ketamine and xylazine anaesthesia (5 : 1; 0.1 ml/100 g). Sham-operated females were only submitted to surgery without removal of the ovaries. The estrous cycle was determined by vaginal swabs during the 10 days prior to OVX, to ensure that rats were cycling normally.[21]

Treatment groups and experimental design

Female rats were assigned to one of the following groups:

Sham-operated (Control) ($n = 9$): rats non-ovariectomized received canola oil (5 ml/kg, p.o. by gavage) once a day for 30 days;

$(PhSe)_2$ ($n = 9$): rats non-ovariectomized received $(PhSe)_2$ (5 mg/kg; 5 ml/kg, p.o.)[9] once a day for 30 days;

OVX ($n = 9$): rats previously ovariectomized received canola oil (5 ml/kg, p.o.) once a day for 30 days;

OVX + $(PhSe)_2$ ($n = 9$): rats previously ovariectomized received $(PhSe)_2$ (5 mg/kg; 5 ml/kg, p.o.) once a day for 30 days.

Rats received these treatments for 30 days, beginning seven days after surgery. After that, female rats were anaesthetized for blood sample gathering and then submitted to euthanasia. The uterine atrophy was verified in all OVX female rats as an indicative of removal of both ovaries.

Body weight measurement

The body weight gain of female rats was monitored weekly during the whole course of the experiment. The body weight gain was calculated according to the following formula: final body weight–initial body weight.

Determination of intra-abdominal fat accumulation

Subsequently to blood sample collection, rats were euthanized by decapitation, and intra-abdominal fat was removed, washed in saline and weighed.

Measurement of plasma lipid values

Blood samples were collected directly from the ventricle of the heart in anaesthetized rats, using heparin as the anticoagulant, and plasma was separated by centrifugation (2400*g*) for 15 min. Plasma TC, HDL and triglycerides were determined by enzymatic colorimetric methods using commercial kits (Labtest Diagnostica, MG, Brazil). Non-HDL-cholesterol values were obtained by the difference between TC – HDL levels. Plasma lipids levels were expressed as mg/dl.

Accomplishment of liver oxidative stress parameters

After euthanasia, the liver was quickly removed and homogenized in 50 mM Tris-HCl, pH 7.4 (1/10, w/v). The homogenate was centrifuged at 2400*g* at 4°C for 15 min and a

low-speed supernatant fraction (S_1) was used for most assays, except to the determination of reduced glutathione (GSH) content.

Thiobarbituric acid reactive species

Thiobarbituric acid reactive species (TBARS), a measure of lipid peroxidation, were determined as described by Ohkawa *et al.*[22] Briefly, a sample of S1 was incubated with 0.8% thiobarbituric acid (TBA), acetic acid buffer pH 3.4 and 8.1% sodium dodecyl sulfate at 95°C for 2 h. The colour reaction was measured at 532 nm. TBARS levels were expressed as nmol MDA (malondialdehyde)/mg protein.

Non-enzymatic antioxidant defences

Ascorbic acid level

Ascorbic acid determination was performed as described by Jacques-Silva *et al.*[23] S1 was precipitated in 10 volumes of a cold 4% trichloroacetic acid solution. A portion of the sample in a final volume of 1 ml of the solution was incubated for 3 h at 38°C then 65% H_2SO_4 (v/v) was added to the medium. The reaction product was determined using colour reagent containing 4.5 mg/ml dinitrophenyl hydrazine and $CuSO_4$ (0.075 mg/ml). The colour reaction was measured spectrophotometrically at 520 nm. Ascorbic acid level was expressed as µg ascorbic acid/g tissue.

Reduced glutathione content

Reduced glutathione (GSH) content was determined fluorometrically following the method of Hissin and Hilf[24] using o-phthalaldehyde (OPA) as fluorophore. Briefly, the samples were homogenized in 0.1 M perchloric acid ($HClO_4$). Homogenates were centrifuged at 3000*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 the dark. Fluorescence was measured with a fluorescence spectrophotometer at an excitation wavelength of 350 nm and an emission wavelength of 420 nm. GSH levels were expressed as nmol/g of tissue.

Antioxidant enzymatic defences

Catalase activity

CAT activity in S1 was assayed spectrophotometrically by the method of Aebi,[25] which involves monitoring the disappearance of H_2O_2 in the presence of S1 at 240 nm. A sample of S1 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)/mg protein (1 U decomposes 1 µmol H_2O_2/min at pH 7 at 25°C).

Glutathione peroxidase activity

GPx activity in S1 was assayed spectrophotometrically by the method of Wendel,[26] through the GSH/NADPH/GR system, by the dismutation of H_2O_2 at 340 nm. S1 was added in GSH/NADPH/GR system and the enzymatic reaction was initiated by adding H_2O_2. In this assay, the enzyme activity is indirectly measured by means of NADPH decay. H_2O_2 is decomposed, generating oxidized glutathione (GSSG) from GSH. GSSG is regenerated back to GSH by glutathione reductase (GR) present in the assay media at the expense of NADPH. The enzymatic activity was expressed as nmol NADPH/min/mg protein.

Glutathione S-transferase activity

Glutathione S-transferase (GST) activity was assayed spectrophotometrically at 340 nm by the method of Habig *et al.*[27] The reaction mixture contained a sample of S1, 0.1 M potassium phosphate buffer pH 7.4, 100 mM GSH and 100 mM 1-chloro-2,4-dinitrobenzene (CDNB), which was used as substrate. The enzymatic activity was expressed as nmol CDNB conjugated/min/mg protein.

Protein

Protein concentration was measured according to the method of Bradford[28] using bovine serum albumin as the standard.

Statistical analysis

The results are presented as the means ± SEM. The statistical significance between groups was calculated by means of two-way analysis of variance followed by Duncan's test when necessary. $P < 0.05$ was considered statistically significant. Main effects are presented only when the second-order interaction was not significant.

Results

Body weight measurement

The analysis of the body weight gain data yielded a significant OVX × $(PhSe)_2$ interaction ($F_{1,32} = 5.16$). Post-hoc comparisons revealed that $(PhSe)_2$ reduced the increase in the body weight induced by OVX (145%) in female rats. $(PhSe)_2$ decreased *per se* the body weight gain (0.1%) in female rats (Figure 1a).

Determination of intra-abdominal fat accumulation

The analysis of the intra-abdominal fat accumulation showed a significant OVX × $(PhSe)_2$ interaction ($F_{1,32} = 3.27$). Post-hoc comparisons revealed that $(PhSe)_2$ decreased the intra-abdominal fat accumulation induced by OVX (68%) in female rats. $(PhSe)_2$ reduced *per se* the intra-abdominal fat accumulation (30%) in female rats (Figure 1b).

Measurement of plasma lipid values

Analysis of the results presented in Table 1 shows that TC and non-HDL-cholesterol levels were significantly affected by OVX. Post-hoc comparisons demonstrated that $(PhSe)_2$ was unable to protect against the rise in plasma TC (27%) and non-HDL-cholesterol (65%) levels induced by OVX in female rats.

As revealed in Table 1, $(PhSe)_2$ exerted its main effect on HDL levels. Post-hoc comparisons showed that $(PhSe)_2$ increased HDL levels (25%) in OVX female rats.

The analysis of triglyceride levels demonstrated a significant OVX × $(PhSe)_2$ interaction. Post-hoc comparisons showed that $(PhSe)_2$ decreased triglyceride levels (29%) in OVX female rats (Table 1).

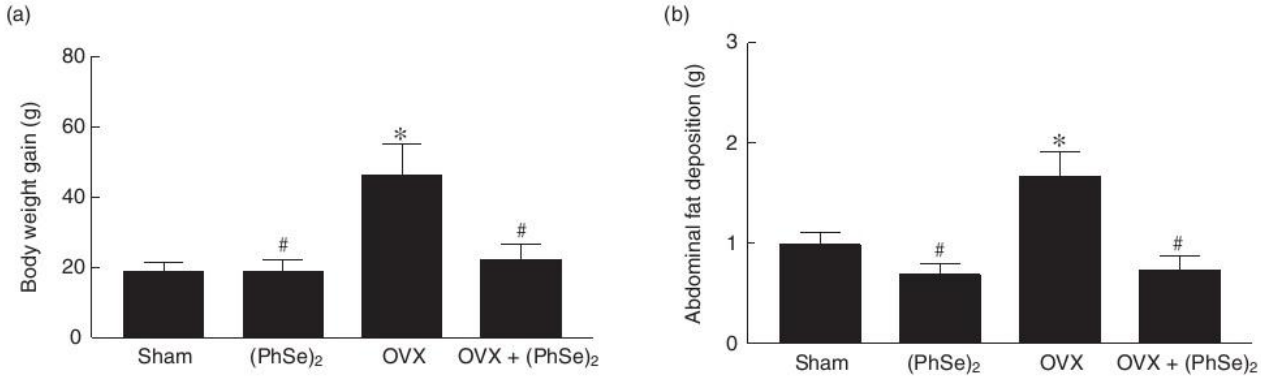

Figure 1 Effect of ovariectomization (OVX) and diphenyl diselenide $(PhSe)_2$ on body weight gain (a) and abdominal fat deposition (b) in female adult rats. Data are reported as means ± SEM for nine rats per group. *$P < 0.05$, compared with Sham-operated group; #$P < 0.05$, compared with OVX group, (two-way analysis of variance/Duncan).

Table 1 Effect of ovariectomization and diphenyl diselenide on plasma lipid values of female adult rats

	Treatment group				*F* value
	Sham	$(PhSe)_2$	OVX	OVX + $(PhSe)_2$	
Total cholesterol	66.00 ± 5.07	68.71 ± 3.03	83.80 ± 6.03*	93.11 ± 5.91*	$F_{1,32} = 17.50$
HDL	44.55 ± 2.82	47.30 ± 3.13	49.09 ± 2.49	55.81 ± 2.37*#	$F_{1,32} = 8.05$
Non-HDL	23.57 ± 3.57	26.50 ± 4.29	39.12 ± 5.21*	42.50 ± 3.94*	$F_{1,32} = 13.53$
Triglycerides	48.55 ± 4.07	48.37 ± 3.14	51.12 ± 5.33	34.50 ± 3.76*#	$F_{1,32} = 4.18$

OVX, ovariectomized; $(PhSe)_2$, diphenyl diselenide. Data are reported as means ± SEM for nine rats per group and expressed as mg/dl. *$P < 0.05$, compared with Sham-operated group; #$P < 0.05$, compared with OVX group (two-way analysis of variance/Duncan).

Table 2 Effect of ovariectomization and diphenyl diselenide on parameters of oxidative stress in liver of female adult rats

	Treatment group				*F* value
	Sham	$(PhSe)_2$	OVX	OVX + $(PhSe)_2$	
TBARS (nmol MDA/mg protein)	52.63 ± 1.02	51.98 ± 3.47	48.98 ± 2.47	54.34 ± 3.46	$F_{1,32} = 2.03$
Ascorbic acid (µg ascorbic acid/g tissue)	264.76 ± 7.64	305.84 ± 9.67*#	253.06 ± 13.16	322.30 ± 12.67*#	$F_{1,32} = 22.32$
GSH (nmol/g tissue)	17.83 ± 0.20	19.03 ± 0.37#	15.84 ± 0.78*	20.07 ± 0.61*#	$F_{1,32} = 7.61$

OVX, ovariectomized; $(PhSe)_2$, diphenyl diselenide. Data are reported as mean ± SEM for 9 rats per group. *$P < 0.05$, compared with Sham-operated group. #$P < 0.05$, compared with OVX group (two-way analysis of variance/Duncan).

Accomplishment of liver oxidative stress parameters

Thiobarbituric acid reactive species

As shown in Table 2, neither OVX nor $(PhSe)_2$ changed TBARS levels in liver of female rats.

Ascorbic acid levels

Ascorbic acid levels were significantly affected by $(PhSe)_2$. Post-hoc comparisons demonstrated that $(PhSe)_2$ increased ascorbic acid levels in liver of $(PhSe)_2$ (15%) and OVX (22%) female rats (Table 2).

Reduced glutathione content

As demonstrated in Table 2, analysis of the GSH content showed a significant OVX × $(PhSe)_2$ interaction. Post-hoc comparisons revealed that $(PhSe)_2$ increased the GSH content (12%) in OVX female rats. $(PhSe)_2$ increased *per se* the GSH content (1%) in female rats.

Catalase activity

Results presented in Table 3 show that CAT activity yielded a significant OVX × $(PhSe)_2$ interaction. Post-hoc comparisons demonstrated that $(PhSe)_2$ restored CAT activity, which was decreased in OVX female rats (18%).

Glutathione peroxidase activity

The analysis of GPx activity revealed that neither OVX nor $(PhSe)_2$ changed this parameter in liver (Table 3).

Glutathione S-transferase activity

As revealed in Table 3, GST activity was significantly affected by $(PhSe)_2$. Post-hoc comparisons showed that $(PhSe)_2$

Table 3 Effect of ovariectomization and diphenyl diselenide on antioxidant enzymatic defences in liver of female adult rats

	Treatment group				*F* value
	Sham	$(PhSe)_2$	OVX	OVX + $(PhSe)_2$	
CAT (U/mg protein)	50.84 ± 1.63	48.03 ± 2.65	41.82 ± 1.27*	46.97 ± 1.20#	$F_{1,32} = 4.98$
GPx (nmol NADPH/min/mg protein)	123.90 ± 11.67	112.87 ± 9.61	135.40 ± 12.10	113.25 ± 12.72	$F_{1,32} = 0.22$
GST (nmol CDNB conjugated/min/mg protein)	78.53 ± 7.53	109.78 ± 15.68*#	64.73 ± 3.80	116.59 ± 11.75*#	$F_{1,32} = 13.23$

OVX, ovariectomized; $(PhSe)_2$, diphenyl diselenide; CAT, catalase; GPx, glutathione peroxidase; GST, glutathione S-transferase. Data are reported as mean ± SEM for 9 rats per group. *$P < 0.05$, compared with Sham-operated group; #$P < 0.05$, compared with OVX group (two-way analysis of variance/Duncan).

increased GST activity (48%) in OVX female rats. $(PhSe)_2$ increased *per se* the GST activity (40%) in female rats.

Discussion

The purpose of this study was to demonstrate the beneficial effects of $(PhSe)_2$ in a mimic model for menopause. Our results showed that OVX rats presented raised plasma TC and non-HDL levels as well as reduced GSH content and CAT activity in liver. $(PhSe)_2$ treatment decreased plasma triglycerides levels and enhanced HDL levels in OVX rats. Moreover $(PhSe)_2$ was able to restrain the increase in body weight gain and abdominal fat accumulation induced by OVX. Although treatment with $(PhSe)_2$ at a dose of 5 mg/kg showed beneficial effects in OVX rats, this dose was not effective in protecting against the increase in plasma TC and non-HDL levels induced by OVX. Another important finding is that $(PhSe)_2$ increased hepatic ascorbic acid levels, GSH content, GST activity and restored CAT activity in liver of OVX rats.

Food intake and body weight regulation are influenced by estradiol in adult females.[29] OVX results in reduction in circulating oestrogen and increases daily food intake and promotes weight gain in rodents.[30] Accordingly, our results demonstrated a greater body weight gain in OVX than in sham-operated rats. $(PhSe)_2$ treatment was effective in avoiding the body weight gain in OVX rats. In agreement with this finding, previous studies have reported that chronic consumption of low doses of selenium can increase the metabolism rate and decrease body weight in men[31] and animals,[32,33] without causing overt signs of toxicity. OVX rats gain fat, specifically visceral fat.[30] Intra-abdominal adipose tissue has adipogenic, pro-atherogenic and pro-thrombotic characteristics.[29] Thus, a very important finding of this study is that $(PhSe)_2$ treatment prevented the abdominal fat deposition induced by OVX in rats.

Wakatsuki and Sagara[34] reported that low levels of endogenous oestrogens enhance plasma lipoprotein lipase activity and may lead to an elevated plasma LDL concentration in postmenopausal and bilaterally oophorectomized women. Considering that non-HDL cholesterol includes LDL and very low density lipoprotein (VLDL), our results corroborate the findings of Van Lenten *et al.*,[35] who demonstrated that OVX female rats exhibit higher TC and non-HDL levels than sham-operated ones. Accordingly, previous data from our research group showed that $(PhSe)_2$ possesses hypolipidaemic activity.[16,17] By contrast, in the present study protocol, treatment with $(PhSe)_2$ at a dose of 5 mg/kg was not enough to lower TC and non-HDL levels, although it was able to decrease triglycerides and augment HDL levels. This finding could suggest that a dose higher than 5 mg/kg might be necessary to restore plasma TC and non-HDL levels increased by OVX.

Oxidative stress, a disparity between the rates of free radical production and elimination, occurs when the antioxidant mechanisms are overwhelmed.[36–41] There is evidence that oxidative imbalance occurs in women after menopause.[42] OVX may induce variations in antioxidant/oxidant status which can be detected in rat liver.[43] Concerning the relationship between OVX and oxidative stress, the present study demonstrated that the menopause-related complications induced by OVX include a decrease in GSH content and CAT activity in liver of OVX rats. However, TBARS and ascorbic acid levels and GPx and GST activity were not altered by OVX in the present protocol.

Ha *et al.*[4] and Topçuoglu *et al.*[44] reported that MDA levels, an indicator of lipid peroxidation, in the liver total homogenate were increased in OVX rats compared with the control group. However, in the present study we did not observe any alteration in MDA levels in liver of OVX rats.

CAT, an enzyme that catalyses the conversion of hydrogen peroxide to water and molecular oxygen, is widely distributed within the living organisms. In the present study CAT activity was found to be decreased in the liver of OVX rats. These data are in accordance with Ha *et al.*,[4] which showed a reduction in CAT activity as a consequence of OVX-induced oxidative stress. In this context $(PhSe)_2$ treatment was able to restore hepatic CAT activity in OVX female rats.

The glutathione system plays a significant role in protecting cells from ROS. GSH constitutes the first line of defence against free radicals. In agreement with Oztekin *et al.*[45] and Topçuoglu *et al.*,[44] our results confirmed that OVX rats showed a decrease in hepatic GSH content, which was restored by $(PhSe)_2$ treatment. It is important to emphasize that $(PhSe)_2$ treatment enhanced *per se* the hepatic GSH content and GST activity. GST, also known as phase II enzymes, are widely distributed in the living organism catalysing and binding proteins which promote the conjugation of GSH with a variety of reactive electrophilic compounds resulting in formation of substances that are easily excreted from the body. So, the increase in GSH content and GST activity are in agreement with Luchese *et al.*,[46] who demonstrated the involvement of the glutathione system in the antioxidant effect of $(PhSe)_2$, suggesting that this organoselenium compound acts as an indirect antioxidant.

The effect of OVX on hepatic GPx activity is contradictory. Kankofer *et al.*[43] demonstrated that GPx activity tends to be high in OVX animals, but Oztekin *et al.*[45] showed that OVX reduces the activity of this enzyme.

It is well-known that ascorbic acid is a component of the first line of antioxidant defence against oxidative processes and participates in free radical scavenging. In this sense, an important finding demonstrated here was that $(PhSe)_2$ treatment enhanced *per se* the hepatic ascorbic acid levels, which could be fundamental for the antioxidant effect of $(PhSe)_2$. In accordance, Luchese *et al.*[46] showed that $(PhSe)_2$ is able to increase ascorbic acid levels, which could indicate an indirect antioxidant mechanism for $(PhSe)_2$. Data from Luchese and Nogueira[47] indicate that $(PhSe)_2$ has dehydroascorbate reductase-like activity which could increase the reduction of dehydroascorbate to ascorbic acid.

Conclusion

The data revealed that $(PhSe)_2$ treatment, at a dose of 5 mg/kg, was effective in preventing the increase in body weight gain and intra-abdominal fat deposition induced by OVX. Although $(PhSe)_2$ treatment was not effective in preventing the increase in plasma TC and non-HDL levels induced by OVX, it was able to decrease plasma triglycerides levels and to increase HDL levels in OVX rats, besides increasing hepatic ascorbic acid levels, GSH content and GST activity and restored CAT activity in liver of OVX rats. Taking together, these findings suggest that $(PhSe)_2$ could be a promising alternative to minimize menopause-related symptoms. However, further studies are warranted to improve the evidence base for clinical practice.

Declarations

Conflict of interest

The Author(s) declare(s) that they have no conflicts of interest to disclose.

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ARTIGO 2

Diphenyl diselenide ameliorates cognitive deficits induced by a model of menopause in rats

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Diphenyl diselenide ameliorates cognitive deficits induced by a model of menopause in rats

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Ovarian hormone loss contributes to cognitive decline in postmenopausal women. Studies have demonstrated a positive role of the level of the element selenium in cognitive performance. The present study investigated the effects of the synthetic organoselenium compound diphenyl diselenide [$(PhSe)_2$] on cognitive functions in ovariectomized rats. Ninety-day-old female Wistar rats were subjected to ovariectomy (OVX) or sham operation. One week after surgery, rats were orally treated with $(PhSe)_2$ (5 mg/kg, per oral route) or vehicle once a day for 30 days. Next, the rats were evaluated in behavioral tests [Morris water maze (MWM) and open-field tests] and biochemical [cerebral acetylcholinesterase (AChE)] analyses were carried out. In MWM probe trial, $(PhSe)_2$ decreased the latency to reach the platform location and increased the number of crossings over the platform location, protecting against cognitive impairment induced by OVX. Furthermore, $(PhSe)_2$ prevented the stimulation of AChE activity caused by OVX. In conclusion, the present study showed a cognition-enhancing effect of $(PhSe)_2$ treatment for 30 days in ovariectomized rats in the MWM test, which could be related to its ability to prevent the stimulation of AChE activity caused by OVX in rats. These findings suggest that $(PhSe)_2$ might have a promising role in preventing the cognitive decline related to menopause. *Behavioural Pharmacology* **00:000–000** © **2011 Wolters Kluwer Health | Lippincott Williams & Wilkins.**

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Introduction

The physiological state of menopause is characterized by a progressive loss of ovarian function, with a resulting decrease in estrogen concentration (McKinlay and Jeffreys, 1974; Myers *et al.* 1990). There are accumulating clinical and basic science data indicating that ovarian hormone loss contributes to cognitive decline in women (Bimonte-Nelson *et al.*, 2010).

Estrogens have numerous neuroprotective effects on the central nervous system (CNS) (Amelsvoort *et al.*, 2001; Bimonte-Nelson *et al.*, 2010; Gibbs, 2010; Walf *et al.*, 2011), including their ability to modulate the function of the basal forebrain cholinergic system affecting learning and memory (Luine, 1985; O'Malley *et al.*, 1987; Savonenko and Markowska, 2003; Gibbs *et al.*, 2004; Gibbs, 2010). There is evidence indicating that beneficial effects of estrogen on brain aging and cognition are related to interactions with cholinergic projections emanating from the basal forebrain. These cholinergic projections play an important role in learning and attentional processes, and their function is known to decline with advanced age and in association with Alzheimer's disease (Gibbs, 2010).

In both rat sexes, gonadectomy, known to impair the hippocampal function, dysregulated the appropriate timing of acetylcholine (ACh) release and attenuated the amount released. In agreement with this concept, female rats with surgically induced ovarian hormone loss exhibited higher hippocampal acetylcholinesterase (AChE) activity relative to ovary-intact animals (Monteiro *et al.*, 2005, 2007; Acosta *et al.*, 2009). However, testosterone in gonadectomized males or estradiol in gonadectomized females restored the timing and maintained the amount of ACh release (Mitsushima, 2010).

Ovariectomy (OVX, bilateral removal of the ovaries) is a widely used model that reflects some, but not all, aspects of menopause, which are characterized by the absence of ovarian hormones such as estrogens (Adam *et al.*, 2008). The OVX model of menopause is well established in the field of aging (Maffuci and Gore, 2006). In recent decades, there have been increased efforts to study the effects of age-related spatial cognitive decline in female rodents (Bimonte-Nelson *et al.*, 2010). The data in the literature indicate that OVX rats present impaired spatial reference memory (Monteiro *et al.*, 2005, 2007, 2008), which is improved by estradiol treatment (Gibbs *et al.*, 2004; Talboom *et al.*, 2008, 2010; Gibbs, 2010).

It has been shown that trace elements are involved in metabolic processes and redox reactions in the CNS and

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may influence cognitive function (Berr *et al.*, 2000). Accordingly, experimental (Zhang *et al.*, 2001; Qin *et al.*, 2008) and clinical (Smorgon *et al.*, 2004; Akbaraly *et al.*, 2007) studies have demonstrated a positive role of the chemical element selenium (Se) level in cognitive performance. Se is a fundamental component of the living cells of a variety of organisms and is found in a limited number of molecules. Se interacts with only a few functional groups of biomolecules (thiol–thiolate groups) or with strong prooxidant peroxides (Araie and Shiraiwa, 2009; Arner, 2009).

The potential role of organoselenium compounds, that is, synthetic organic compounds containing Se in their structures, as therapeutic agents was first suggested in the 1980s, when it was reported that ebselen – a synthetic organic compound containing Se – was a promising antioxidant and mimetic of the antioxidant selenoenzyme glutathione peroxidase (Müller *et al.*, 1984). In this context, our research group initiated investigations of the antioxidant and other pharmacological properties of diphenyl diselenide $(PhSe)_2$, another synthetic organic compound containing Se (Nogueira *et al.* 2004; Nogueira and Rocha, 2010).

Previous data have demonstrated that systemic administration of $(PhSe)_2$ facilitates the formation of long-term object recognition memory in male mice (Rosa *et al.*, 2003) and that subchronic exposure to $(PhSe)_2$ improves the performance of male rats in the Morris water maze (MWM) test (Stangherlin *et al.*, 2008). Recently, it was demonstrated, using the Y-maze and MWM tests in male mice, that $(PhSe)_2$ administration ameliorates the impairment of spatial long-term and short-term memory induced by scopolamine, a muscarinic antagonist (Souza *et al.*, 2010).

Therefore, considering that postmenopausal women have been shown to have lower Se levels (Karita *et al.*, 2008), the present study was designed to determine the effects of $(PhSe)_2$ on the spatial reference memory in OVX female rats and its possible involvement with the cholinergic system, which was evaluated by measuring brain AChE activity.

Methods

Subjects

Female adult Wistar rats (200–250 g, approximately 90 days old) from our own breeding colony were used. The animals were kept on a 12 h light/dark cycle, at a room temperature of 22 ± 2°C, with free access to food and water. All handlings and behavioral tests were carried out between 08:00 and 16:00 h. All experiments were approved and supervised by the Committee on Care and Use of Experimental Animals Resources of the Federal University of Santa Maria, Brazil (Process # 23081.012740/2008–05). All efforts were made to minimize suffering and to reduce the number of animals used in the experiments.

Ovariectomy

Animals were ovariectomized by the surgical removal of both ovaries under intraperitoneal ketamine and xylazine anesthesia (5 : 1; 1 ml/kg). Sham-operated females (non-ovariectomized) were anesthetized and subjected only to surgery without removal of the ovaries. Sham surgery consisted of skin and muscle incision and suture only. The estrous cycle was determined on the basis of the cell-type characteristics of the vaginal epithelium during 10 days before OVX, to ensure that animals were cycling normally (Baker *et al.*, 1979).

Experimental design and drug administration

Female rats were assigned to one of the following groups ($n = 9$/group): sham-operated (control), Sham + $(PhSe)_2$, OVX, and OVX + $(PhSe)_2$.

At day 0, animals were subjected to OVX or sham operation. Animals were treated once a day for 30 days, beginning at the eighth day after surgery, with canola oil [1 ml/kg, by oral gavage (orally)] or $(PhSe)_2$ (5 mg/kg; 1 ml/kg, orally) (Nogueira and Rocha, 2010; Da Rocha *et al.*, 2011). The gavage procedure was carried out as follows: each rat was gently restrained by placing the experimenter's index and middle fingers alongside the rat's head and the experimenter's thumb and ring fingers under the rat's forelegs. The index and the middle fingers were used to secure the rat's head and the remaining fingers to support the chest. Then, the rat was lifted and the rat's head was tilted slightly upward and back to straighten the esophagus. The gavage was positioned onto the right or the left of the mouth and the tube was slowly passed freely into the esophagus, watching for the swallowing reflex. When the desired length of insertion was achieved, the solution (canola oil or $(PhSe)_2$ 1 ml/kg) was injected. The rat was observed after the procedure for signs of distress, such as gasping or frothing of the mouth. The animals were treated daily, always at the same time of the day, for the 30 days before behavioral testing.

Behavioral tests

Open field

Spontaneous locomotor activity was measured in the open-field test (Walsh and Cummins, 1976). The open field was made of plywood and surrounded by walls 30 cm in height. The floor of the open field, 45 cm in length and 45 cm in width, was divided by masking tape markers into 09 squares (3 rows of 3). Each animal was placed individually in the center of the arena and the number of segments crossed and the number of rearings (when the animal stood on its hind legs) were recorded during a 4-min session.

Morris water maze

We used MWM, an apparatus widely used for the study of spatial learning and memory, in which rats learn to escape from water by swimming to a hidden platform located just below the surface of the water. The water maze consisted

of a basin (diameter: 180 cm, wall height: 40 cm) made of black plastic and filled with water (22 ± 2°C) at a height of 30 cm. The height of the water was sufficient to avoid floor walking rather than swimming for the adult rats (Morris, 1984). The pool was placed in a room with several extramaze visual cues, such as counters, posters, a dangling wire, and a pole. The animals were always tested in MWM at the same time of the day. For the acquisition phase, the rats were placed next to and facing the wall successively in north (N), south (S), east (E), and west (W) positions. The escape platform (diameter: 10 cm, made of black plastic) was hidden 1 cm below water level in the middle of the northwest (NW) quadrant. Behavioral data were recorded and analyzed using ANY-maze video tracking software (Stoelting Co., Illinois, USA). 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 time spent to reach the platform and the time spent in the platform quadrant (NW) and the opposite quadrant [southeast (SE)] were measured in four-trial sessions during 4 days (acquisition phase). The latencies were calculated as the mean of the total time spent in four trials of each day. The rats remained on the platform for at least 45 s after each trial. Whenever the rats failed to reach the escape platform within the 1 min cut-off period, they were retrieved from the pool and placed on it for 45 s. After the swim, the rats were kept dry in a plastic holding cage filled with paper towels. 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 N side. The latency to reach the platform position, the number of crossings over the former platform position, the time spent in the platform quadrant (NW), the time spent in the opposite quadrant (SE), the total distance traveled, and the average swim speed were measured for a single 1-min trial.

Biochemical assays

After the behavioral tests, rats were killed by decapitation and the whole brains were quickly removed. Uterine atrophy was verified in all OVX female rats as an indicator of complete removal of both ovaries.

Measurement of acetylcholinesterase activity

Samples of whole brain were homogenized in 0.25 mol/l sucrose buffer (1/10, w/v), centrifuged at 2400*g* at 4°C for 15 min, and stored at −70°C for up 48 h before being assayed. AChE activity was assayed according to the method of Ellman *et al.* (1961), using acetylthiocholine as a substrate and spectrophotometric measurement at 412 nm. AChE activity was expressed as nmol/h/mg protein.

Protein determination

Protein concentration was measured according to the method of Bradford (1976) using bovine serum albumin as the standard.

Drugs

$(PhSe)_2$ was prepared and characterized in our laboratory using the method previously described by Paulmier (1986). Analysis of the ^{1}H NMR and ^{13}C NMR spectra showed that $(PhSe)_2$ obtained presented analytical and spectroscopic data in full agreement with its assigned structure. The chemical purity of the compound (99.9%) was determined by gas chromatography/high-performance liquid chromatography. $(PhSe)_2$ was dissolved in canola oil. Xylazine, acetylthiocholine, bovine serum albumin, and sucrose were purchased from Sigma-Aldrich Co. (St Louis, Missouri, USA). Ketamine was purchased from Aproquímica (Santa Maria, Brazil).

Statistical analyses

Data from the acquisition phase of MWM were analyzed by repeated-measure analysis of variance (ANOVA/MANOVA), with Surgery (Sham or OVX) and Treatment [Vehicle or $(PhSe)_2$] as between-subjects variables and days as the within-subjects variable. Data from MWM probe trial parameters, the open-field test, and AChE activity were analyzed by 2(Sham or OVX) × 2[Vehicle or $(PhSe)_2$] factorial ANOVA. Probe trial data were also analyzed using repeated-measures ANOVA, with Surgery (Sham or OVX) and Treatment [Vehicle or $(PhSe)_2$] as between-subjects variables and maze Quadrant (platform or opposite) as the within-subjects variable. Post-hoc Duncan multiple-range test was run when indicated. Data were expressed as mean ± SEM. Probability values (P-value) less than 0.05 were considered as statistically significant. All analyses were performed using the Statistica for Windows software (StatSoft, Tulsa, Oklahoma, USA), in a PC-compatible computer.

Results

Behavioral tests

Open-field

Neither OVX nor $(PhSe)_2$ altered spontaneous locomotor activity in female rats (Table 1). Two-way ANOVA for the number of crossings [$F(1,32) = 1.84$, NS] and rearings [$F(1,32) = 0.03$] revealed no significant differences.

Morris water maze

In MWM, all animals, regardless of treatment, learned to find the platform as the days progressed, as indicated by a decrease in the distance traveled (main effect of day [$F(3,96) = 99.39$, $P < 0.05$]. There was also a significant Surgery × Treatment interaction [$F(1,32) = 6.30$, $P < 0.05$], because the decrease in the distance traveled was less in the OVX group than that in the other three groups (Table 2).

Similarly, for latency to reach the platform, there was a significant main effect of day [$F(3,96) = 95.49$, $P < 0.05$] and a significant Surgery × Treatment interaction [$F(1,32) = 9.86$, $P < 0.05$]. Post-hoc comparisons demonstrated that there were statistically significant differences

Table 1 Behavioral evaluation of ovariectomy and diphenyl diselenide in the open-field test

	Groups			
	Sham-operated	$(PhSe)_2$	OVX	OVX + $(PhSe)_2$
Crossing	23.80 ± 2.94	24.40 ± 5.46	30.66 ± 3.02	24.16 ± 5.06
Rearing	17.40 ± 3.85	14.66 ± 3.85	20.50 ± 2.02	16.66 ± 2.56

Data are reported as means ± SEM for nine animals per group.
OVX, ovariectomy; $(PhSe)_2$, diphenyl diselenide.

Table 2 Effect of ovariectomy and diphenyl diselenide on distance traveled (m) across training days on the Morris water maze test

	Groups			
	Sham-operated	$(PhSe)_2$	OVX	OVX + $(PhSe)_2$
Day 1	5.36 ± 0.39^{c}	5.18 ± 0.21^{c}	5.22 ± 0.33^{c}	4.85 ± 0.31^{c}
Day 2	3.21 ± 0.39^{d}	$2.70 \pm 0.29^{b,d}$	$4.46 \pm 0.37^{a,c}$	$2.68 \pm 0.30^{b,d}$
Day 3	2.52 ± 0.32^{d}	2.35 ± 0.16^{d}	$3.14 \pm 0.18^{a,d}$	2.56 ± 0.17^{d}
Day 4	1.75 ± 0.21^{e}	$1.71 \pm 0.23^{b,e}$	$2.74 \pm 0.25^{a,d}$	$1.73 \pm 0.24^{b,e}$

Data are reported as means ± SEM for nine animals per group.
OVX, ovariectomy; $(PhSe)_2$, diphenyl diselenide.
[a]Compared with the Sham group on the same day.
[b]Compared with the OVX group on the same day.
[c,d,e]Within the same group, different letters denote a statistically significant difference; $P<0.05$ (two-way analysis of variance/Duncan).

among groups only on days 2 and 4 of the acquisition phase. On these days, rats in the OVX group were less able to find the platform than either sham-treated animals (day 2: $P < 0.005$; day 4: $P < 0.05$) or the OVX + $(PhSe)_2$ group (day 2: $P < 0.001$; day 4: $P < 0.05$) (Fig. 1a).

In the probe trial, two-way ANOVA of the latency to reach the platform position and the number of crossings over the platform position yielded significant Surgery × Treatment interactions [$F(1,32) = 4.74$; $P < 0.05$ and $F(1,32) = 4.66$; $P < 0.05$, respectively]. Post-hoc comparisons indicated that the OVX group showed an increase in the latency to find the platform position (Fig. 1b) and reduced number of crossings over the platform location (Fig. 1c) compared with the Sham group. $(PhSe)_2$ treatment effectively reversed both of these effects. Two-way ANOVA of the time spent in the platform quadrant showed a significant main effect of Surgery [$F(1,32) = 7.94$; $P < 0.05$]. Post-hoc comparisons revealed that the OVX group spent less time in the platform quadrant compared with the Sham group (Fig. 1d). $(PhSe)_2$ treatment tended to reverse this effect but the effect was not significant (Fig. 1d). No significant difference was observed among groups in the time spent in the opposite quadrant [$F(1,32) = 0.46$; NS]. In addition, OVX rats spent roughly equal amounts of time in the platform and the opposite quadrant, whereas all the other groups [Sham, $(PhSe)_2$, and OVX + $(PhSe)_2$] spent most time in the platform quadrant [Surgery × Quadrant interaction: $F(1,32) = 5.85$; $P < 0.05$] (Fig. 1d).

There were no group differences in either the average swim speed [$F(1,32) = 0.00$; NS] or the total distance traveled [$F(1,32) = 1.96$; NS] in the probe trial (data not shown).

Measurement of acetylcholinesterase activity

Two-way ANOVA of AChE activity yielded a significant Surgery × Treatment interaction [$F(1,32) = 4.38$; $P < 0.05$). Post-hoc comparisons showed that OVX increased AChE activity (1.31 times higher than the Sham-operated group) and $(PhSe)_2$ treatment effectively reversed the increase in the AChE activity induced by OVX (Fig. 2).

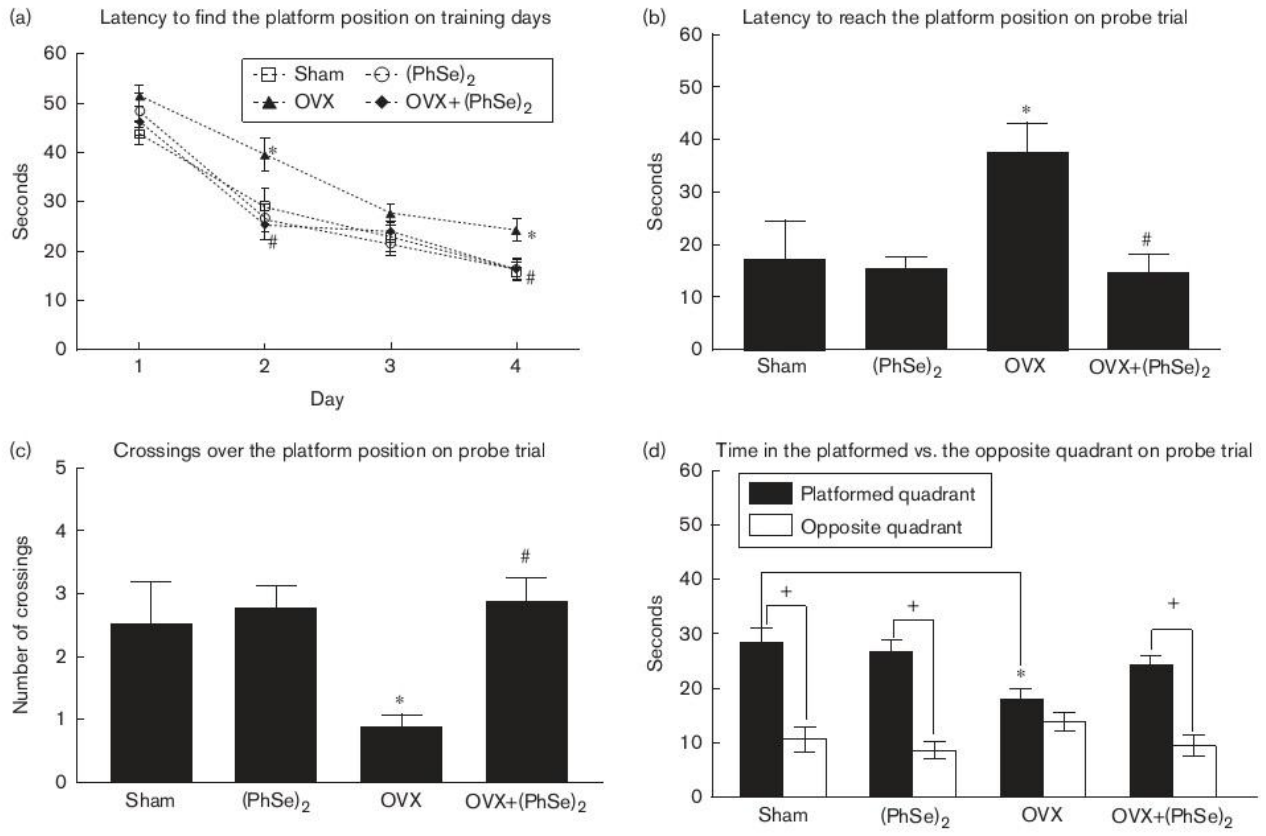
Discussion

The purpose of this study was to demonstrate the beneficial effects of $(PhSe)_2$ in an animal model that reproduces the memory decline related to menopause in women. The study showed a cognition-enhancing effect of $(PhSe)_2$ in OVX rats in MWM, which could be related to its ability to prevent the stimulation of AChE activity caused by OVX in rats. In addition, our results confirmed that OVX causes spatial learning and memory deficits in female rats (Monteiro *et al.*, 2008; Talboom *et al.*, 2008; Acosta *et al.*, 2009; Bimonte-Nelson *et al.*, 2010).

OVX, an experimental condition of hormone deprivation, is the most common animal model of postmenopausal changes in adult female rats (Savonenko and Markowska, 2003). Considering that research in rodents, nonhuman primates, and humans has shown that gonadal hormones are beneficial for the maintenance of cognitive ability (Sandstrom and Williams, 2001; Rapp *et al.*, 2003; Gibbs *et al.*, 2004; Leuner *et al.*, 2004; Talboom *et al.*, 2008, 2010; Acosta *et al.*, 2010; Bimonte-Nelson *et al.*, 2010; Gibbs, 2010), the comparison of memory function in OVX and gonadally intact female rats might provide additional information on the role of ovarian hormones, especially estrogen, in memory function. Accordingly, we observed that $(PhSe)_2$ treatment produced beneficial effects on spatial memory in OVX rats. These effects are similar to those observed in MWM in OVX rats treated with estrogen hormone therapy (Talboom *et al.*, 2008, 2010; Acosta *et al.*, 2009, 2010; Bimonte-Nelson *et al.*, 2010).

OVX female rats showed cognitive improvement after $(PhSe)_2$ treatment, at a dose of 5 mg/kg once a day for 30 days, as demonstrated by their performance in MWM. $(PhSe)_2$ treatment decreased the time required for OVX females to find the platform and increased the number of crossings over the platform location in the probe trial. In accordance with these findings, there is earlier evidence indicating that $(PhSe)_2$ displays cognitive-enhancing properties in the object recognition task in male mice (Rosa *et al.*, 2003) and in the MWM test in male rats (Stangherlin *et al.*, 2008). However, both of these studies used unmanipulated controls (i.e. animals that were not subjected to the stress of a surgical procedure). In the present study, $(PhSe)_2$ had no effect in the sham-

Fig. 1



Effect of ovariectomy (OVX) and [diphenyl diselenide $(PhSe)_2$] on spatial memory parameters in the Morris water maze test. (a) Performance of animals across days in the spatial memory acquisition phase of the Morris water maze test. All animals, regardless of treatment, learned to find the platform as the days progressed. (b) Latency to reach the platform position in the probe trial. (c) Number of crossings over the former platform location in the probe trial. The OVX group showed an increased latency to find the platform position and fewer crossings over the platform location compared with the other groups. (d) Time spent in the platform quadrant vs. the opposite quadrant in the probe trial. OVX rats showed about the same percentage of the time spent in both the platform and the opposite quadrant, whereas the other groups spent most time in the platform quadrant. Data are reported as means ± SEM for nine animals per group. *Compared with the Sham group, #compared with the OVX group, + compared with the same group on the opposite quadrant. $P<0.05$ (two-way analysis of variance/Duncan).

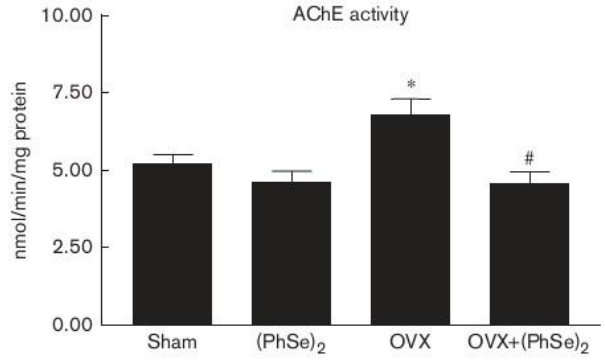
operated group. This divergence could be due to some differences between studies, for example the sex and the species of animals and the doses and routes of $(PhSe)_2$ administration.

Concerning the role of Se in learning and memory processes, it is well established that its content decreases with age. This may contribute to the decline in neuropsychological functioning among the elderly population. In support of this hypothesis, epidemiological studies have reported that low levels of Se were associated with increased cognitive decline (Akbaraly *et al.*, 2007; Gao *et al.*, 2007). Moreover, positive correlations between cognitive ability and Se have been demonstrated in a number of cohort studies (Zhang *et al.*, 2001; Smorgon *et al.*, 2004; Akbaraly *et al.*, 2007; Qin *et al.*, 2008). Corroborating these findings, the present study demonstrated that $(PhSe)_2$ treatment promoted learning and memory enhancement in female rats subjected to OVX.

As neither OVX nor $(PhSe)_2$ altered exploratory activity in female rats evaluated in the open-field test, it is reasonable to suggest that their performance in MWM was not influenced by drug or surgery effects on sensorimotor coordination and could be attributed to the spatial performance of rats.

One of the most convincing and studied neurobiological links between gonadal hormones and memory is the work assessing function and viability of the cholinergic system, with an emphasis on basal forebrain cholinergic neurons (Bimonte-Nelson *et al.*, 2010). Estrogen withdrawal and replacement have been shown to affect the cholinergic system in a variety of brain regions (Simpkins *et al.*, 1997; Gibbs and Aggarwal, 1998; Gibbs, 2010). In female monkeys, OVX decreases the density of cholinergic fibers

Fig. 2



Effect of ovariectomy (OVX) and [diphenyl diselenide $(PhSe)_2$] on whole-brain acetylcholinesterase (AChE) activity. $(PhSe)_2$ treatment effectively reversed the increase in the AChE activity induced by OVX. Data are reported as means + S.M for nine animals per group. *Compared with the Sham group, #compared with the OVX group, $P<0.05$ (two-way analysis of variance/Duncan).

in the prefrontal cortex and estrogen therapy increases synaptic density in this region (Tàng *et al.*, 2004). In addition, it is known that estradiol plays several roles in ovariectomized rats, such as increasing the synthesis of choline acetyltransferase in the medial septal nucleus, nucleus basalis of Meynert, and the frontal cortex (Luine, 1985); increasing high-affinity choline uptake (the rate-limiting step in ACh synthesis) in the cortex and hippocampus (O'Malley *et al.*, 1987); and enhancing the release of ACh in the cortex and hippocampus following potassium stimulation (Gibbs *et al.*, 1997) and during maze learning (Marriott and Korol, 2003).

Accordingly, it was reported that rats subjected to OVX show a significant increase in AChE activity in the hippocampus and the cerebral cortex (Monteiro *et al.*, 2005, 2007). Another recent study showed an augmentation in AChE activity only in the hippocampus of ovariectomized rats (Acosta *et al.*, 2009). The decrease in ACh concentrations caused by stimulation of AChE activity reduces cholinergic activity in the CNS, leading to cognitive deficits. In this context, a direct effect of Se on AChE activity was previously demonstrated (Sen *et al.*, 1995). In the present experimental protocol, we found that after 30 days of treatment, $(PhSe)_2$ protected against the increase in whole-brain AChE activity induced by OVX. As this enzyme is responsible for ACh degradation, the inhibition of AChE activity by $(PhSe)_2$ leads to increased levels of ACh at cholinergic synapses, which could be responsible for the observed improvement in cognitive functions (Lahiri *et al.*, 2003).

In conclusion, the present data revealed that $(PhSe)_2$ treatment for 30 days ameliorated spatial learning and memory deficits in female Wistar rats subjected to OVX, a well accepted model for ovarian hormone deprivation induced by menopause. The improvement in cognitive abilities was evidenced by amelioration in performance in the MWM test. $(PhSe)_2$ treatment was also effective in protecting against the increase in AChE activity induced by OVX, which could be responsible for the observed improvement in cognitive functions. On the basis of these findings, we could suggest that $(PhSe)_2$, an organoselenium compound, might have a promising role against the cognitive decline related to menopause.

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Conflicts of interest

There are no conflicts of interest.

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ARTIGO 3

Effects of diphenyl diselenide on depressive-like behavior in ovariectomized mice submitted to subchronic stress: involvement of the serotonergic system

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ORIGINAL INVESTIGATION

Effects of diphenyl diselenide on depressive-like behavior in ovariectomized mice submitted to subchronic stress: involvement of the serotonergic system

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Abstract
Rationale The transition to menopause is associated with an increased risk of depressed mood.
Objectives This study was conducted to investigate whether diphenyl diselenide [$(PhSe)_2$] treatment could reduce the effects of postmenopausal depression-like behavior in ovariectomized female mice submitted to subchronic stress exposure.
Methods Mice were divided into four groups: sham, $(PhSe)_2$, ovariectomy (OVX), and OVX + $(PhSe)_2$. Animals were ovariectomized/sham-operated and subjected to stress session once a day for 7 days from the fifth to the 11th day after OVX. The behavioral tests (open field, tail suspension (TST), and forced swimming (FST)) were performed on the 14th day after OVX. Mice were treated orally once a day with vehicle (canola oil, 10 ml/kg) or $(PhSe)_2$ (10 mg/kg; 10 ml/kg) 30 min before being exposed to subchronic stress, or from the 11th to the 14th day. Paroxetine (8 mg/kg i.p.) and pargyline (30 mg/kg i.p.) were used as positive controls. The involvement of serotonergic receptor subtypes in the antidepressant-like effect of $(PhSe)_2$ was assessed in the FST using WAY 100635 (0.1 mg/kg s.c.), ritanserin (1 mg/kg i.p.), and ondansetron (1 mg/kg i.p.) as serotonergic antagonists. Monoamine oxidase (MAO) A and B activities were also determined.
Results The prolongation of immobility time in TST and FST in OVX mice submitted to subchronic stress was prevented by $(PhSe)_2$ treatment. Ritanserin and ondansetron blocked the antidepressive-like effect of $(PhSe)_2$, suggesting the involvement of 5-$HT_{2A/2C}$ and 5-HT_3 receptor subtypes. Both paroxetine and pargyline were effective in reducing the immobility time of stressed OVX mice in the FST. No alterations in locomotor activity were observed. Although $(PhSe)_2$ had inhibited in vitro MAO-A and MAO-B activities, none of the groups presented alterations neither in ex vivo MAO-A nor in MAO-B activity.
Conclusions $(PhSe)_2$ treatment could influence mood and behavior, indicating a promising role of this organoselenium compound in the management of postmenopausal depressive symptoms.

Keywords Ovariectomy · Subchronic stress · Depression · Serotonin · Selenium · Diphenyl diselenide

Introduction

Accumulating data indicate that the transition to menopause and its changing hormonal milieu are associated with an increased risk of depressed mood, not only for those with a history of depression but also for some women with no history of depression (Freeman 2010). Depressive symptoms are common during the transition to menopause, and there are suggestive data that estrogen deficiency may increase the susceptibility to mood disorders (Birkhäuser 2002). Estrogen has direct effects on the central nervous system in areas which are not strictly relevant to reproduction. For example, this

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hormone stimulates a significant increase in 5-HT_{2A}-binding sites in areas which are involved in regulating both mood and cognition (Steiner et al. 2003). Moreover, the limbic system contains significant amounts of the estrogen receptors, and through these receptors, estrogen could have numerous effects on mood (Shughrue et al. 1997; Shughrue and Merchenthaler 2001; Rissman 2008; Horst et al. 2009).

There is evidence indicating that ovariectomy (OVX, bilateral removal of the ovaries) significantly prolonged per se the duration of immobility in the forced swimming test (FST) in ICR, C57/BL/6 J, and CD-1 mouse strains, but not in DBA/2 N mice (Bekku and Yoshimura 2005; Bekku et al. 2006). Additionally, Nakagawasai et al. (2009) demonstrated that ovariectomized ddY mice presented no alterations on the duration of immobility time in the FST. Neurochemical studies suggest that brain neural networks might be different among these inbred animals. Furthermore, genetic factors and sex differences among these mouse strains could also contribute to the differences observed in the baseline immobility (Barros and Ferigolo 1998; Urani et al. 2005). Conversely, Nakagawasai et al. (2009) reported that a combination of OVX and subchronic stress caused a significant prolongation of immobility time in the FST in ddY female mice compared to non-ovariectomized ones.

Concerning the etiology of major depression, there is strong support for the role of stress in their development and manifestation (Caspi et al. 2003). Major depression is often described as a stress-related disorder and there is good evidence that episodes of depressive symptoms often occur in the context of some form of stress (Nestler et al. 2002). Accordingly, epidemiological studies also showed that stressful life experiences are associated with the onset of affective disorders like major depression and anxiety disorders (Post 1992; Kendler et al. 1995). In an attempt to mimic excessive human day-to-day stress, several animal models have been developed. Methods using acute stressors, as restraint stress, rely on the ability of an acute stressor to produce behavioral changes (depressive-like effect) that are reversible by antidepressant treatment (Poleszak et al. 2006). In this sense, literature data provided evidence that the association of OVX and subchronic stress exposure in rodents (subchronic restraint–water immersion for example) is an effective method for studying the development and treatment of affective disorders (Nakagawasai et al. 2009).

The involvement of serotonergic (5-HT) system in the neural regulation of mood disorders and in the mechanism of action of antidepressant drugs is well known (Duman et al. 1997; Cryan and Leonard 2000). An enhancement of 5-HT neurotransmission underlies in the therapeutic response to different classes of antidepressant treatment (Ulak et al. 2010). Monoamine oxidase (MAO) is the key enzyme that is associated with the metabolism of monoamines, such as serotonin, regulating its intracellular concentration in the brain. Therefore, the abnormal function of this enzyme is thought to be involved in several psychiatric disorders, such as major depression (Deniker 1983).

Studies have reported that low levels of selenium (Se), an essential trace element, in the diet were associated with higher levels of anxiety, depression, and fatigue and that Se supplementation might be related with amelioration in mood and depression status (Benton and Cook 1991; Benton 2001). Based upon this point of view, diphenyl diselenide $(PhSe)_2$, an organoselenium compound, has been reported as a potential pharmacological agent (Nogueira et al. 2004; Nogueira and Rocha 2010) with antidepressant-like properties (Ghisleni et al. 2008; Savegnago et al. 2007, 2008).

In the present study, we investigated whether $(PhSe)_2$ treatment would be able to reduce the effects of postmenopausal depression-like behavior in ovariectomized female Swiss mice submitted to a protocol of subchronic stress exposure. In view of this fact, previous unpublished data from our research group provided evidence that OVX did not affect the duration of immobility time of female Swiss mice in the FST. Considering that we intended to use ovariectomy as an animal model of menopause, we used the subchronic stress exposure protocol according to Nakagawasai et al. (2009) in order to generate a depressive-like state (prolongation of the immobility time) in OVX Swiss mice. For this reason, in order to justify this type of experimental procedure scientifically and ethically, a time-course analysis of the restraint–water immersion stress-induced depressive-like behavior and the restraint stress-induced depressive-like behavior (without water immersion) was accomplished.

We also examined the participation of the 5-HT receptor subtypes on the effect caused by $(PhSe)_2$. Finally, to ascertain a possible mechanism of action of $(PhSe)_2$, we measured the monoamine oxidase A and B activities in vitro and ex vivo of cerebral cortex and hippocampus from OVX female mice submitted to subchronic stress.

Materials and methods

Drugs

$(PhSe)_2$ was prepared and characterized in our laboratory by the method previously described by Paulmier (1986). Analysis of the ^{1}H NMR and ^{13}C NMR spectra showed that $(PhSe)_2$ showed analytical and spectroscopic data in full agreement with its assigned structure. The chemical purity of the compound (99.9 %) was determined by CG/MS. All other chemicals were of analytical grade and obtained from standard commercial suppliers. For the behavioral tests, all drugs were dissolved in saline except $(PhSe)_2$ that was

dissolved in canola oil. The mice received all drugs in a constant volume of 10 ml/kg of body weight. In order to perform in vitro MAO activity assays, $(PhSe)_2$ was dissolved in dimethyl sulfoxide (DMSO).

Experimental animals

Female adult Swiss mice (25–35 g, approximately 60-day-old) from our own breeding colony were used. The animals were kept on a 12-h light/dark cycle, at a room temperature of 22±2°C, with free access to food and water. All manipulations were carried out between 8:00 a.m. and 4:00 p.m. (Jesse et al. 2010a, b). Investigators who study social behavior are well aware of the effects of the light phase on the nocturnal species and design their studies accordingly, by reversing light/dark cycles so that research personnel can test subjects at an hour that is compatible with normal human activity (which would normally be during the animal's subjective night) (Wersinger and Martin 2009). Since the timing of the animal's activity (including feeding, locomotion, and social behavior) is directly related to the light cycle (Chabot and Menaker 1992; DeCoursey 1986; Hakim et al. 1991; Honma and Honma 1999; Honma et al. 1978; Landau 1975; Shimomura et al. 1998; Sofia and Salama 1970), it is imperative to carefully control this cycle and to test behavior at the appropriate time (Wersinger and Martin 2009). 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 animal suffering and to reduce the number of animals used in the experiments.

Ovariectomy

Animals were ovariectomized by the surgical removal of both ovaries under intraperitoneal (i.p.) Equitesin (1 % phenobarbital, 2 % magnesium sulfate, 4 % chloral hydrate, 42 % propylene glycol, 11 % ethanol; 3 ml/kg, i.p.) anesthesia. Sham-operated females (non-ovariectomized) including all estrous cycle were only submitted to surgery without removal of the ovaries. The success of the operation was confirmed by uterine atrophy verified in all OVX female mice to indicate removal of both ovaries.

Behavioral tests

Open field test

Spontaneous locomotor activity was measured in the open field test (OFT) (Hall 1934; Hall and Ballechey 1932; Walsh and Cummins 1976). The open field was made of plywood and surrounded by walls 30 cm in height. The floor of the open field, 45 cm in length and 45 cm in width, was divided by masking tape markers into 09 squares (three rows of three). Each animal was placed individually in the center of the arena and the number of segments crossed and the number of rearings (when the animal stood on its hind legs) were recorded during a 4-min session.

Tail suspension test

The tail suspension test (TST) is a common screening test for antidepressant drugs (Porsolt et al. 1987). The total duration of immobility induced by tail suspension was measured according to the method described by Steru et al. (1985). Briefly, animals both acoustically and visually isolated were suspended 50 cm above the floor by adhesive tape placed approximately 1 cm from the tip of the tail. Immobility time was recorded during a 6-min period. Mice were considered immobile only when they hung passively and were completely motionless. Mice that pulled themselves up on the tail during the TST or presented tail biting and/or self-mutilation were removed from the experiment. This test is a reliable and rapid screening method for antidepressants including those involving the serotonergic system (Nestler et al. 2002).

Forced swimming test

The FST, as originally described by Porsolt et al. (1977a, b), is the most widely used screening test for antidepressant drugs. In this test, mice were individually forced to swim in an open cylindrical container (diameter 10 cm, height 25 cm), containing 19 cm of water at 25±1°C. The total duration of immobility was recorded during the last 4 min of the 6-min period. Each mouse was judged to be immobile when it ceased struggling and remained floating motionless in the water, making only those movements necessary to keep its head above water. A decrease in the duration of immobility is indicative of an antidepressant-like effect (Porsolt et al. 1977a, b).

Subchronic stress exposure

A time-course analysis of the restraint–water immersion stress-induced depressive-like behavior and the restraint stress-induced depressive-like behavior (without water immersion) was accomplished. For this purpose, mice (ovariectomized and sham-operated) were divided into 12 different groups as described in Table 1.

Animals subjected to restraint–water immersion stress-induced depressive-like behavior were placed in a plastic cylinder (2.5 cm internal diameter, 10 cm length), in which both ends were closed by a wire mesh and immersed to the level of the xiphoid process in a water bath (21°C) for 1 h. The animals were subjected to this stress session once a day

Table 1 Groups for the time-course analysis of the restraint–water immersion stress ($+H_2O$) and restraint stress-induced ($-H_2O$) depressive-like behavior in female Swiss mice

Animal	Water immersion	Days of stress exposure (after ovariectomy)	Day of behavioral tests
Sham	No	1 (5th)	8th
OVX	No	1 (5th)	8th
Sham	Yes	1 (5th)	8th
OVX	Yes	1 (5th)	8th
Sham	No	3 (5th to 7th)	10th
OVX	No	3 (5th to 7th)	10th
Sham	Yes	3 (5th to 7th)	10th
OVX	Yes	3 (5th to 7th)	10th
Sham	No	7 (5th to 11th)	14th
OVX	No	7 (5th to 11th)	14th
Sham	Yes	7 (5th to 11th)	14th
OVX	Yes	7 (5th to 11th)	14th

Sham sham-operated, *OVX* ovariectomized

for 1, 3, or 7 days from the fifth to the 11th day after OVX. The behavioral tests (open field test and FST) were performed on the eighth, tenth, or 14th day after ovariectomy (Table 1).

Animals subjected to restraint stress-induced depressive-like behavior (without water immersion) were placed for 1 h in a plastic cylinder (2.5 cm internal diameter, 10 cm length), in which both ends were closed by a wire mesh. The animals were subjected to this stress session once a day for 1, 3, or 7 days from the fifth to the 11th day after OVX. The behavioral tests (open field test and FST) were performed on the eighth, tenth, or 14th day after ovariectomy (Table 1).

Experimental design

On the basis of the results from the time-course analysis of the restraint–water immersion stress-induced depressive-like behavior, we concluded that the subchronic stress exposure in which animals were immersed to the level of the xiphoid process in a water bath for 1 h once a day for 7 days was the optimal necessary set of parameters to achieve the increase in subsequently measured immobility time in the FST. For this reason, for the next set of experiments, we used the subchronic stress protocol.

Initially, a dose–response curve for $(PhSe)_2$ was performed. For this purpose, ovariectomized mice were treated daily, from the fifth to the 11th day after OVX, with canola oil (10 ml/kg, p.o. by gavage, $n=7$) or different doses of $(PhSe)_2$ (1 to 20 mg/kg; 10 ml/kg, p.o., $n=7$ to each dose) 30 min before being exposed to subchronic stress (Fig. 1a, pretreatment protocol). Paroxetine (8 mg/kg, i.p., a selective serotonin reuptake inhibitor, $n=6$) and pargyline (30 mg/kg, i.p., a MAO-A inhibitor, $n=6$) were used as positive controls. Indeed, in order to evaluate $(PhSe)_2$ effects on the manifestation of depressive-like state after the termination of subchronic stress exposure, mice were treated daily, from the 11th to the 14th day after OVX, with $(PhSe)_2$ (10 mg/kg; 10 ml/kg, p.o., $n=9$) (Fig. 1a, posttreatment protocol). The behavioral tests (OFT and FST) were performed on the 14th day after OVX.

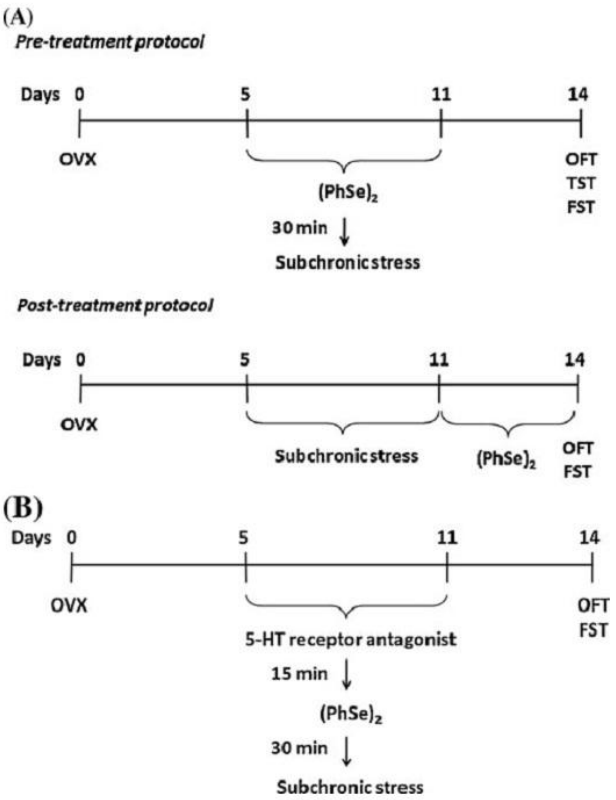

Fig. 1 Experimental design: **a** effects of $(PhSe)_2$ on a combination of OVX and restraint–water immersion stress-induced depressive-like behavior. **b** Evaluation of the role of 5-HT receptor antagonists in the antidepressant-like effect of $(PhSe)_2$. *OVX* ovariectomy, *$(PhSe)_2$* diphenyl diselenide, *OFT* open field test, *TST* tail suspension test, *FST* forced swimming test

After determining $(PhSe)_2$ dose in the dose–response curve, mice were divided into four different groups as follows: sham (n=8), sham + $(PhSe)_2$ (n=8), OVX (n=10), and OVX + $(PhSe)_2$ (n=9). The procedure of subchronic stress exposure (the optimal necessary set of parameters to achieve the increase in subsequently measured immobility time in the FST) was performed as described by Mizoguchi et al. (2000) with some modifications by Nakagawasai et al. (2009) and in accordance to previous results from our research group. Briefly, the animals were placed in a plastic cylinder (2.5 cm internal diameter, 10 cm length), with both ends closed by a wire mesh, and immersed to the level of the xiphoid process in a water bath (21°C) for 1 h. The animals were subjected to this stress session once a day for 7 days from the fifth to the 11th day after OVX. It is very important to state that in this subchronic stress protocol, the animal was not physically compressed and did not experience painful sensations. The behavioral tests (OFT, TST, and FST) were performed on the 14th day after OVX. Mice were treated daily, from the fifth to the 11th day after OVX, with canola oil (10 ml/kg, p.o. by gavage) or $(PhSe)_2$ (10 mg/kg; 10 ml/kg, p.o.) 30 min before being exposed to subchronic stress (Fig. 1a, pretreatment protocol).

To study the possible contribution of serotonergic receptors in reducing the immobility time, we investigated the involvement of 5-HT receptor subtypes in the effect caused by $(PhSe)_2$ in the FST. To this end, we used the following 5-HT antagonists: WAY 100635 (Way, 0.1 mg/kg, s.c., a 5-HT_{1A} receptor antagonist), ritanserin (Rit, 1 mg/kg, i.p., a 5-$HT_{2A/2C}$ receptor antagonist), or ondansetron (Ond, 1 mg/kg, i.p., a 5-HT_3 receptor antagonist). Mice were divided into eight different groups as follows: OVX (n=8), OVX + $(PhSe)_2$ (n=8), OVX + Way (n=6), OVX + Rit (n=6), OVX + Ond (n=6), OVX + $(PhSe)_2$+Way (n=8), OVX + $(PhSe)_2$+Rit (n=8), and OVX + $(PhSe)_2$+Ond (n=8), and they were pretreated daily, from the fifth to the 11th day after OVX. Fifteen minutes after receiving 5-HT receptor antagonists, they received $(PhSe)_2$ (10 mg/kg, p.o.) or vehicle and 30 min after they were subjected to the stress session. The behavioral tests, OFT and FST, were performed on the 14th day after OVX (Fig. 1b). The doses of the antagonists and the administration schedule used in these experiments were chosen on the basis of experiments previously performed (Savegnago et al. 2007; Brüning et al. 2008) and the literature data confirm the selectivity and efficacy of the above-mentioned treatments at the doses used (O'Neill and Conway 2001; Bourin et al. 2009; Machado et al. 2007; Posser et al. 2009).

Monoamine oxidase activity

Preparations of cerebral cortex and hippocampal mitochondria were employed to test, both in vitro and ex vivo, the hypothesis that the antidepressant-like effect of $(PhSe)_2$ could be mediated through an inhibition of MAO-A and/or MAO-B activity.

Mitochondria preparation

Preparations of cerebral cortex and hippocampal mitochondria were used for MAO assay as previously described by Soto-Otero et al. (2001). Cerebral cortices and hippocampus were immediately removed and washed in ice-cold isolation medium (pH 7.4, Na_2PO_4/KH_2PO_4 isotonized with sucrose). Mitochondria from cortex and hippocampus were obtained by differential centrifugation. Briefly, after removing blood vessels and pial membranes, cerebral cortices were manually homogenized with four volumes (w/v) of the isolation medium. Then, the homogenate was centrifuged at 900×g at 4°C for 5 min. The supernatant was centrifuged at 12,500×g for 15 min. The mitochondria pellet was then washed once with isolation medium and recentrifuged under the same conditions. Finally, the mitochondrial pellet was reconstituted in a buffer solution (Na_2PO_4/KH_2PO_4 isotonized with KCl, pH 7.4) and stored in aliquots.

In vitro enzyme assay

MAO activity was determined as described by Krajl (1965) with some modifications of Matsumoto et al. (1984). Aliquots of samples were pre-incubated at 37°C for 5 min in the presence of $(PhSe)_2$ at different concentrations (10–100 μM) or vehicle (DMSO) in a medium containing buffer solution (Na_2PO_4/KH_2PO_4 isotonized with KCl, pH 7.4) and specific inhibitors, selegiline (a MAO-B inhibitor, 250 nM) or clorgiline (a MAO-A inhibitor, 250 nM), at a final volume of 600 ml. Then, kynuramine dihydrobromide (final concentration of 90 mM to MAO-A assay and 60 mM to MAO-B assay) was added to the reaction mixture as substrate. Samples were then incubated at 37°C for 30 min. After incubation, the reaction was terminated by adding 10 % TCA. After cooling and centrifugation at 3,000×g for 15 min, an aliquot of supernatant was added to 1 M NaOH. The fluorescence intensity was detected spectrofluorimetrically with excitation at 315 nm and emission at 380 nm. The concentration of 4-hydroxyquinoline was estimated from a corresponding standard fluorescence curve of 4-hydroxyquinoline. MAO-A and MAO-B activities were expressed as nanomoles of 4-hydroxyquinoline formed per milligram protein per minute.

Ex vivo enzyme assay

The ex vivo assay was conducted in exactly the same way as the in vitro one, except for the fact that samples were not pre-incubated in the presence of $(PhSe)_2$.

Protein determination

The protein concentration was measured by the method of Bradford (1976), using bovine serum albumin (1 mg/ml) as the standard.

Statistical analysis

The time-course analysis of the restraint–water immersion stress (+H_2O) and restraint stress-induced (−H_2O) depressive-like behavior was analyzed using unpaired Student's *t* test. Descriptive statistics data were expressed as the mean(s) ± SEM. Probability values less than 0.05 ($p<0.05$) were considered as statically significant.

Dose–response data for $(PhSe)_2$, paroxetine, and pargyline treatments were analyzed by one-way ANOVA, followed by post hoc Newman–Keuls test. OFT data from animals used in the dose–response data for $(PhSe)_2$, paroxetine, and pargyline pretreatments and $(PhSe)_2$ posttreatments were analyzed by one-way ANOVA, followed by post hoc Newman–Keuls test. Data from TST, FST, OFT (from animals used in FST and TST), and MAO activity ex vivo were analyzed by 2 (sham or OVX)×2 (vehicle or $(PhSe)_2$) factorial ANOVA (analysis of variance); data from the involvement of 5-HT receptor subtypes in the effect caused by $(PhSe)_2$ to OVX mice in the FST and OFT were analyzed by 2 (vehicle or $(PhSe)_2$)×2 (vehicle or 5-HT antagonists) factorial ANOVA (analysis of variance); post hoc Duncan multiple range test was run when indicated. Data from MAO activity in vitro were analyzed by one-way ANOVA; post hoc Newman′Keuls test was run when indicated. Descriptive statistics data were expressed as the mean (s) ± SEM. Probability values less than 0.05 ($p<0.05$) were considered to be statically significant. The main effects of first-order interactions are presented only when interaction was not significant. All analyses were performed using the STATISTICA for Windows software (StatSoft, Tulsa, OK, USA), in a PC-compatible computer.

Results

Subchronic stress exposure

The results from time-course analysis of the restraint–water immersion stress-induced depressive-like behavior and the restraint stress-induced depressive-like behavior (without water immersion) are depicted in Fig. 2. The OVX group had a significantly prolonged immobility time after being exposed to 7 days of restraint–water immersion stress when compared to the corresponding sham-operated group. No locomotor alterations were observed in the open field test (data not shown).

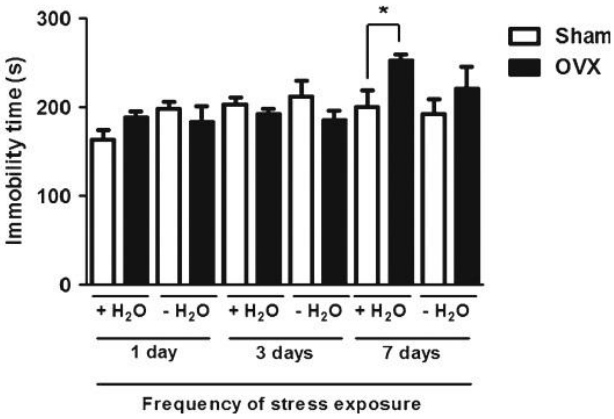

Fig. 2 Time-course analysis of the restraint–water immersion stress (+H_2O) and restraint stress-induced (−H_2O) depressive-like behavior in female Swiss mice in the forced swimming test. Data are reported as means ± SEM. *$p<0.05$, compared with the sham-operated group (Student's *t* test). *Sham* sham-operated, *OVX* ovariectomized

Effects of paroxetine, pargyline, and a dose–response curve for $(PhSe)_2$ in the FST

The dose–response curve of the antidepressant-like effect of $(PhSe)_2$ was accomplished in the FST (Table 2). $(PhSe)_2$ administration decreased the immobility time in the FST at the doses of 10 and 20 mg/kg ($p=0.0001$). Both paroxetine ($p=0.0006$) and pargyline ($p=0.0002$) treatments were able to diminish the immobility time of stressed OVX mice in the FST (Table 2).

The posttreatment protocol yielded a significant OVX× $(PhSe)_2$ interaction ($F_{1,\ 31}=7.03$; $p<0.002$). Post hoc comparisons yielded that mice treated with 10 mg/kg $(PhSe)_2$ (p.o., from the 11th to 14th day after ovariectomy) presented a shorter duration of immobility time in the FST

Table 2 Evaluation of paroxetine, pargyline, and a dose–response curve for $(PhSe)_2$ treatments on a combination of OVX and restraint–water immersion stress-induced depressive-like behavior in the forced swimming test

Group	Immobility time
OVX + oil	231.70±8.91
OVX + $(PhSe)_2$ 1 mg/kg (pretreatment)	212.80±21.28
OVX + $(PhSe)_2$ 10 mg/kg (pretreatment)	138.00±17.64*
OVX + $(PhSe)_2$ 20 mg/kg (pretreatment)	148.20±13.77*
OVX + $(PhSe)_2$ 10 mg/kg (posttreatment)	162.85±16.16*
Paroxetine 8 mg/kg (pretreatment)	192.75±12.06*
Pargyline 30 mg/kg (pretreatment)	141.75±11.82*

Data are reported as means ± SEM for six to eight animals per group

OVX ovariectomized, *$(PhSe)_2$* diphenyl diselenide

*$p<0.05$ (one-way ANOVA/Newman–Keuls test), compared with the OVX + oil group

when compared to ovariectomized mice treated with vehicle (canola oil) (Table 2).

Open field test

The spontaneous locomotor activity did not differ significantly among different groups. Neither OVX nor treatments ($(PhSe)_2$, paroxetine, pargyline, or 5-HT antagonists) changed spontaneous locomotor activity in female mice (data not shown).

Tail suspension test

A significant OVX×$(PhSe)_2$ interaction ($F_{1,\ 31}$=10.34; p< 0.003) was found in the TST. Post hoc comparisons revealed that OVX significantly increased immobility time (135 %) in the TST. $(PhSe)_2$ treatment protected against the increase in the TST caused by OVX (Fig. 3a).

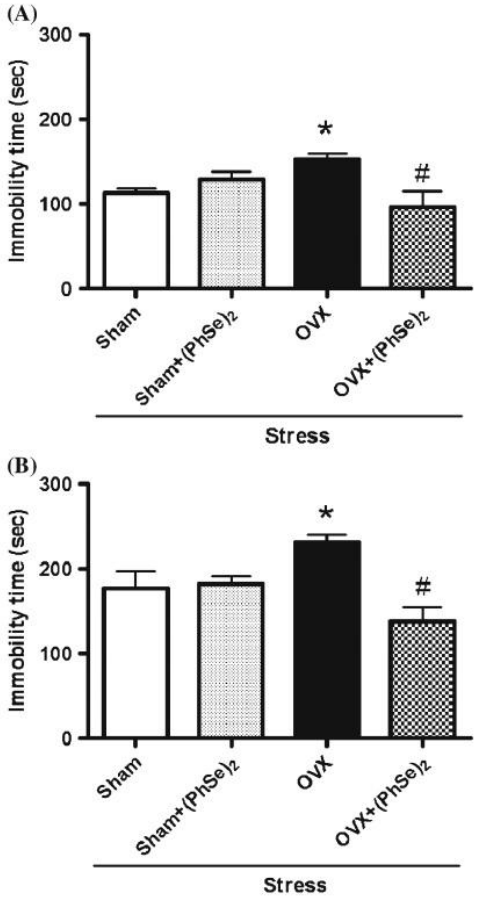

Fig. 3 Effect of $(PhSe)_2$ on a combination of OVX and restraint–water immersion stress-induced depressive-like behavior in **a** tail suspension test, **b** forced swimming test (pretreatment), in female adult mice. Data are reported as means ± SEM for eight to ten animals per group. *Compared with Sham-operated group, #compared with the OVX group, p < 0.05 (two-way ANOVA/Duncan). *OVX* ovariectomized, *$(PhSe)_2$* diphenyl diselenide

Forced swimming test

In the pretreatment protocol, there was a significant OVX× $(PhSe)_2$ interaction ($F_{1,\ 31}$=11.48; p<0.001). Post hoc comparisons provided evidence that $(PhSe)_2$ was able to avoid the increase in immobility time induced in the OVX group (130 %) (Fig. 3b).

Role of 5-HT receptors in the antidepressant-like effect of $(PhSe)_2$

Figure 4 shows that pretreatment of OVX mice with WAY 100635 partially blocked the effect of $(PhSe)_2$ in the FST (main effect of $(PhSe)_2$, $F_{1,\ 26}$=6.43; p<0.017). Pretreatment of mice with ritanserin prevented the antidepressant-like effect caused by $(PhSe)_2$ in the FST (main effect of both ritanserin and $(PhSe)_2$, $F_{1,\ 26}$=27.77; p<0.00001 and $F_{1,\ 26}$=12.88; p<0.001, respectively). Pretreatment with ondansetron completely blocked the effect of $(PhSe)_2$ in the FST ($(PhSe)_2$×ondansetron interaction $F_{1,\ 26}$=9.22; p<0.005).

Effect of OVX and $(PhSe)_2$ on MAO-A and MAO-B activities

As shown in Table 3, $(PhSe)_2$ at the concentration of 100 μM inhibited in vitro both MAO-A and MAO-B activities from the cerebral cortex (p=0.018 and p=0.010, respectively), and MAO-B activity from the hippocampus (p=0.001). Neither OVX nor $(PhSe)_2$ changed the ex vivo MAO-A or MAO-B activity in the cerebral cortex and hippocampus of female mice (data not shown).

Discussion

The purpose of this study was to demonstrate the effects of $(PhSe)_2$ in a model of postmenopausal depressive-like behavior in ovariectomized female mice submitted to a protocol of subchronic stress exposure. Our results demonstrated that the prolongation of immobility time in the TST and FST in OVX mice submitted to subchronic stress protocol was prevented by $(PhSe)_2$ treatment at a dose of 10 mg/kg. We also found a possible involvement of the 5-HT system in this effect, evidenced by the modulation of 5-$HT_{2A/2C}$ and 5-HT_3 receptor subtypes. Although $(PhSe)_2$ had inhibited in vitro MAO-A and MAO-B activities, treatment of OVX mice with $(PhSe)_2$ did not alter neither MAO-A nor MAO-B ex vivo activity.

Evidence from Nakagawasai et al. (2009) indicated that a combination of OVX and subchronic stress exposure (subchronic restraint–water immersion) is an effective method to prolong the duration of the immobility time in OVX mice.

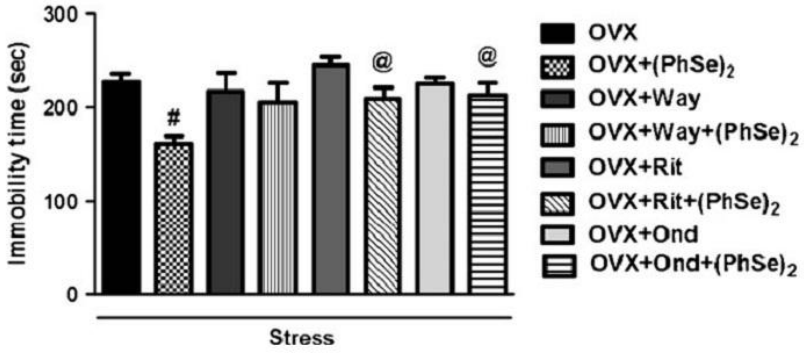

Fig. 4 Effect of $(PhSe)_2$ and pretreatment with WAY 100635 ("Way"; 0.1 mg/kg, s.c.), ritanserin ("Rit"; 1 mg/kg, i.p.), and ondansetron ("Ond"; 1 mg/kg, i.p.) on a combination of OVX and restraint–water immersion stress-induced depressive-like behavior in the mouse forced swimming test. Data are reported as means ± SEM for six to eight animals per group. #$p<0.05$, compared with the OVX group; @$p<0.05$, compared with the OVX + $(PhSe)_2$ group (two-way ANOVA/Duncan). *OVX* ovariectomized, *(PhSe)₂* diphenyl diselenide

Accordingly, the results in the present study demonstrated that OVX mice submitted to subchronic stress exposure exhibited an increase in the immobility time in the TST and FST. In line with this, one of the most important findings of this study is that $(PhSe)_2$ administration to OVX mice before being submitted to subchronic stress prevented the depressive-like behavior assessed in the TST and FST. Thus, the fact that $(PhSe)_2$ treatment was active in both tests, TST and FST, supports the hypothesis that this compound may play a role in the modulation of depressive symptoms associated to the postmenopausal period.

Literature data reported that while chronic stress may affect some aspects of motivation, the motor ability remains functional (Kleen et al. 2006; McLaughlin et al. 2007). Accordingly, in the present study, we did not find any kind of alteration in locomotor coordination of mice submitted to subchronic stress exposure, since there is no difference among groups in the OFT. In this sense, given that neither OVX, $(PhSe)_2$ treatment nor subchronic stress altered the locomotor activity among groups in the OFT, it is reasonable to suggest that their performance in the TST and FST was not influenced by surgery, drug, or stress effects on sensorimotor coordination.

Table 3 Effect of $(PhSe)_2$ on in vitro MAO-A and MAO-B activities (nanomoles of 4-hydroxyquinoline per milligram protein per minute) from the cerebral cortex and hippocampus of female mice

	Cortex		Hippocampus	
	MAO-A	MAO-B	MAO-A	MAO-B
Vehicle	58.73±3.68	89.35±2.39	37.99±2.37	89.70±3.06
$(PhSe)_2$ 10 μM	55.87±4.98	95.34±9.29	35.88±3.25	103.60±5.95
$(PhSe)_2$ 50 μM	46.43±4.48.	76.23±6.32	35.30±3.28	78.61±7.55
$(PhSe)_2$ 100 μM	39.59±3.60*	62.12±6.57*	38.71±5.63	64.05±8.11*

Data are reported as means ± SEM for four to five independent experiments

(PhSe)₂ diphenyl diselenide

*$p<0.05$ (one-way ANOVA/Newman–Keuls test), compared with vehicle

The neurotransmitter serotonin is often involved in mood disorders and many antidepressant drugs act in the 5-HT system (Olausson et al. 2002). Serotonin plays a key role in the coordination of response to stress (Linthorst et al. 2002). In order to investigate a possible contribution of the 5-HT neurotransmission system in the antidepressant-like effect of $(PhSe)_2$ in the FST, OVX mice were treated with pharmacological 5-HT antagonists and $(PhSe)_2$ before being submitted to subchronic stress.

The 5-HT_{1A} receptor subtype is expressed both presynaptically in the raphe nuclei and postsynaptically in limbic and cortical regions (Hensler 2002). Presynaptic 5-HT_{1A} autoreceptors inhibit neuronal firing and thus are particularly important in the regulation of 5-HT neurotransmission, while postsynaptic 5-HT_{1A} receptors also participate in the modulation of the release of other neurotransmitters (Lanfumey and Hamon 2000; Hannon and Hoyer 2008). The results of our study showed that pretreatment of OVX mice with WAY 100635, a selective 5-HT_{1A} receptor antagonist, slightly reversed the immobility-reducing effect of $(PhSe)_2$ in the FST, but this failed to reach a statistically significant level. Contradictorily, Savegnago et al. (2007) demonstrated the involvement of 5-HT_{1A} receptors in the antidepressant-like effect of $(PhSe)_2$ in male rats. So further experiments are needed to clarify the involvement of 5-HT_{1A} receptors in the antidepressant-like effect of $(PhSe)_2$ in OVX mice.

Inhibition of 5-$HT_{2A/2C}$ receptors plays an important role in the antidepressant-like effect of conventional antidepressants in the FST (Redrobe and Bourin 1997; Van Oekelen et al. 2003; Elhwuegi 2004). An evidence of the involvement of 5-$HT_{2A/2C}$ receptors in the antidepressant-like effect of $(PhSe)_2$ in OVX mice submitted to subchronic stress exposure was demonstrated by the fact that the 5-$HT_{2A/2C}$ receptor antagonist ritanserin was effective in preventing the anti-immobility effect of $(PhSe)_2$ in the FST.

The involvement of 5-HT_3 receptors in the mechanism of action of antidepressants was demonstrated by the modulation of 5-HT_3 receptors by different classes of antidepressants (Ishihara and Sasa 2001; Eisensamer et al. 2003). In the present study, we also demonstrated that the antidepressant-like effect of $(PhSe)_2$ on the FST was blocked by pretreatment with ondansetron, a 5-HT_3 receptor antagonist. In agreement with the findings mentioned above, previous data from our research group have already provided evidence of the involvement of 5-HT receptor subtypes in the acute antidepressant-like effect of $(PhSe)_2$ (Savegnago et al. 2007) and other selenium-based compounds (Jesse et al. 2010a; Gay et al. 2010).

MAO is the key enzyme that is associated with the metabolism of central monoamines (serotonin, noradrenaline, and dopamine) regulating their intracellular concentrations in the brain. MAO inhibitors cause an increase in the amount of these amines stored and released from the nerves. An interesting finding of our study was that although $(PhSe)_2$ inhibited in vitro MAO-A and MAO-B activities from the cerebral cortex and MAO-B activity from the hippocampus, neither OVX nor $(PhSe)_2$ administration altered ex vivo MAO-A or MAO-B activities in the cerebral cortex and hippocampus from OVX mice. This discrepancy between our in vitro and ex vivo results could be easily explained by the fact that if the inhibition of MAO by $(PhSe)_2$ is reversible, it could be reverted by the differential centrifugation used to prepare mitochondrial fractions from the cortex and hippocampus. Thus, these findings suggest that $(PhSe)_2$ belongs to a "fourth generation" of MAO inhibitors, which combine reversibility and mixed inhibitory activity toward both MAO-A and MAO-B isoenzymes (Aubin et al. 2004).

Additionally, the effects of selenium would likely be mediated by changes in 5-HT receptor expression or have some effects on MAO-A and MAO-B activities. This could be purely a postsynaptic event but other possibilities can also be considered. Maybe selenium can somehow interfere in the coupling of the estrogen receptor complex to the DNA-binding sites, but this is only a matter of speculation. Considering that $(PhSe)_2$ could act as a prooxidant agent (Nogueira et al. 2004; Nogueira and Rocha 2010), it would be reasonable to assume that $(PhSe)_2$ could disrupt the DNA-binding properties of estrogen receptor by oxidizing its cysteine residues (Hayashi et al. 1997) and, consequently, preventing its genomic effects (Liang et al. 1998; Whittal et al. 2000). Therefore, it is unlikely that the interference of selenium in the coupling of the estrogen receptor complex to the DNA-binding sites is the main mechanism by which $(PhSe)_2$ exerts its anti-depressant-like properties.

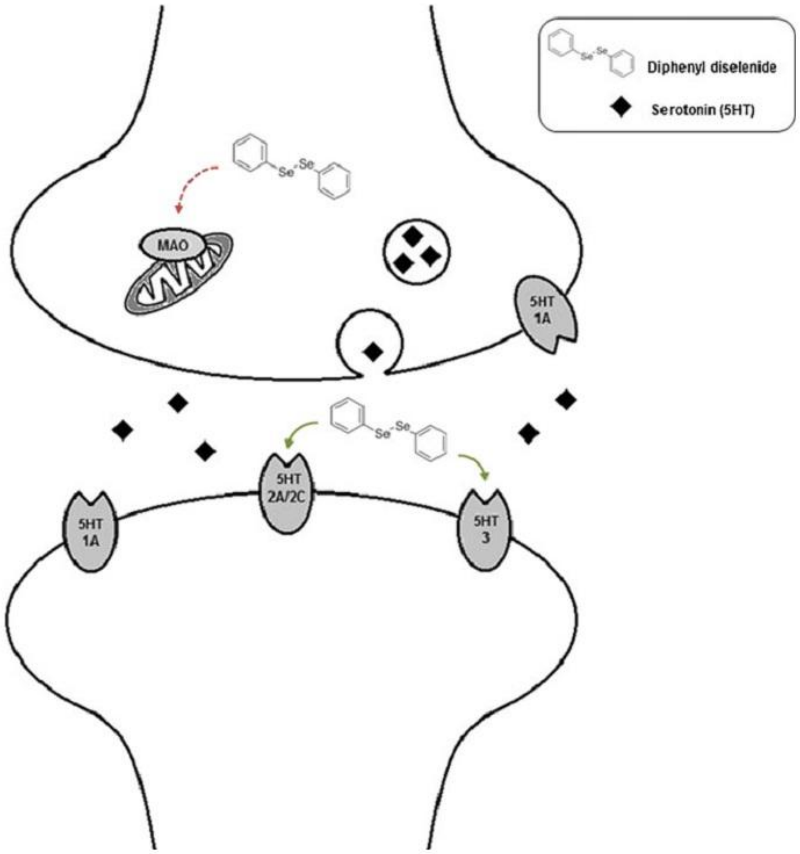

Fig. 5 Effects of $(PhSe)_2$ on serotonergic neurotransmission. $(PhSe)_2$ possibly acts as an agonist in receptors 5-$HT_{2A/2C}$ and 5-HT_3 (*solid line arrows*). Additionally, it seems that $(PhSe)_2$ also inhibits MAO activity (*broken line arrows*)

In conclusion, the prolongation of immobility time in the TST and FST in OVX mice submitted to subchronic stress was prevented by $(PhSe)_2$ treatment. Our findings also suggest a possible involvement of 5-$HT_{2A/2C}$ and 5-HT_3 serotonergic receptor subtypes in the effect caused by $(PhSe)_2$ as well as the participation of MAO-A and MAO-B activities (Fig. 5). Taken together, these findings suggest that $(PhSe)_2$ treatment could influence mood and behavior, indicating a promising role of this organoselenium compound in the management of postmenopausal depressive symptoms.

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Conflict of interest The authors declare they have no conflicts of interest to disclose.

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MANUSCRITO 1

Mechanisms underlying the hypolipidemic and hypoglycemic effects of diphenyl diselenide: a new therapeutic tool for metabolic syndrome?

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Mechanisms underlying the hypolipidemic and hypoglycemic effects of diphenyl diselenide: a new therapeutic tool for metabolic syndrome?

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Running Title: $(PhSe)_2$ effect on glucose and cholesterol regulation.

ABSTRACT

This study was designed to investigate the molecular mechanisms involved in the hypolipidemic activity of diphenyl diselenide ($(PhSe)_2$) on HepG2 cell line in a dose-dependent manner. The protein levels of both total and phosphorylated 3-hydroxy-3-methylglutaryl coenzyme A reductase (HMGR and P-HMGR), low-density lipoprotein receptors (LDLr) and the proteins involved in their regulatory network were analyzed by Western blotting, and the effect of $(PhSe)_2$ on HMGR activity was measured. Since previous data demonstrated a hypoglycemic activity of $(PhSe)_2$, we also evaluated the effects of this compound on glucose transporter type 4 (GLUT4) translocation using fluorescence microscopy in L6 skeletal muscle cell line.

Results demonstrated that $(PhSe)_2$ increased P-HMGR, HMGR, and LDLr protein levels as well as simvastatin treatment, which was used as positive control, without directly affecting HMGR activity. We observed that both long- and short-term HMGR regulation mechanisms are involved in the hypocholesterolemic activity of $(PhSe)_2$, as this compound was able to augment Sterol regulatory element binding proteins (SREBP)-1 and Insulin induced gene (Insig)1 protein levels, and to increase AMP activated kinase (AMPK) activation state. We also found that, in L6 skeletal myotubes, 10 μM $(PhSe)_2$ increases GLUT4 translocation through AMPK activation, which could be linked to the hypoglycemic properties of $(PhSe)_2$.

Taken together, these findings suggest that $(PhSe)_2$ could be a promising alternative for the treatment of hyperglycemia- and hyperlipidemia-related metabolic diseases.

Keywords: AMPK, cholesterol, diphenyl diselenide, glucose, HepG2 cells, HMGR, LDLr.

1. INTRODUCTION

The metabolic syndrome is a common cluster of risk factors for coronary heart disease and type 2 diabetes mellitus that includes obesity, elevated blood pressure, insulin resistance, and dyslipidemia. Metabolic syndrome appears to be an important risk factor for atherogenic cardiovascular disease and diabetes [1]. Type 2 diabetes is a metabolic disorder that is characterized by high blood glucose in the context of insulin resistance and relative insulin deficiency. Hyperlipidemia, and in particular the high cholesterol level in the blood, is one of the major risk factors for atherosclerosis, a progressive disease that leads to an impairment in the vascular functions promoting heart attacks and strokes [2]. Thus the control of glucose and lipid content in the blood is of paramount importance.

Cholesterol is both synthesized by cells and introduced by food intake. The liver represents the principal site for cholesterol homeostasis maintenance [3], carried out by many mechanisms, such as the uptake through low-density lipoprotein (LDL) receptors (LDLr), lipoprotein release in the blood, storage by esterification, conversion into bile acids and the modulation of the key and rate-limiting enzyme of cholesterol biosynthetic pathway, the 3-hydroxy-3-methylglutaryl coenzyme A reductase (HMGR, E.C. 1.1.1.34) [4].

When cells accumulate excess sterols, the activity of HMGR declines by more than 90% and the amount of LDLr decreases. By contrast, upon depletion of intracellular cholesterol, cells increase HMGR transcription and activity and enhance the expression and the membrane exposition of LDLr [4].

HMGR is regulated at multiple levels: the long-term regulation is operated by the control of both the biosynthesis and the degradation of the enzyme, while the short-term regulation is attained by the modulation of phosphorylation or dephosphorylation processes [5-6]. Sterol regulatory element-binding proteins (SREBPs) enhance cholesterol synthesis and uptake by modulating genes encoding cholesterol biosynthetic enzymes, including HMGR

and the LDLr [7]. SREBPs are produced as membrane-bound precursors that require cleavage by a two-step proteolytic process into Golgi apparatus, in order to release into the nucleus their amino-terminal domain (nSREBP) which is responsible for the expression of their target genes involved in cholesterol and fatty acid synthesis [8]. Two proteins, Insig1 and Insig2 (Insulin Induced Gene), are shown to cooperate with sterols to inhibit the migration of SCAP (SREBP cleavage-activating protein)/SREBP complex from the endoplasmic reticulum [9,10]. When cholesterol builds up in the endoplasmic reticulum (ER) membranes, the SREBP/SCAP complex fails to exit the ER, the proteolitic processing of SREBPs is abolished and the transcription of target genes declines. ER retention of SCAP/SREBP is mediated by sterol dependent binding of SCAP/SREBP to Insigs [11]. Intracellular accumulation of sterols also induces HMGR to bind Insigs which promote the ubiquitination and proteasomal degradation of the enzyme [12].

HMGR short-term regulation depends on cellular metabolic state through the AMP-activated kinase (AMPK) that optimizes ATP expenditure during metabolic stress into cells [6,13,14]. Once activated by phosphorylation, AMPK phosphorylates HMGR, inhibiting its activity. On the other hand, subsequent HMGR dephosphorylation by the protein phosphatase 2A (PP2A) fully restores the enzyme activity [15].

The activation of AMPK in some tissues, the liver and the skeletal muscle among others, leads to a number of desirable metabolic responses: increased fatty acid oxidation, glucose uptake and mitochondrial biogenesis in skeletal muscle, and decreased fatty acid and cholesterol synthesis and gluconeogenesis in the liver [16]. Glucose transporters (GLUTs) are known to play pivotal roles in energy metabolism by promoting the glucose uptake into cells. GLUT4 is present in insulin-sensitive tissues, such as brown and white adipose tissues, cardiac and skeletal muscles [17]. Uptake of glucose by skeletal muscles can be activated by the AMPK signaling pathway. Active AMPK (P-AMPK) can increase the translocation rate

of GLUT4 stored in the cytosol to the plasma membranes leading to an increased rate of glucose uptake [18].

To manage metabolic syndrome, changes of lifestyle are often needed, however, if in three to six months the efforts at remedying risk factors are insufficient, pharmacological treatment is required. Although it is generally agreed that first-line clinical intervention for metabolic syndrome is lifestyle change, in many patients this is insufficient to normalize the risk factors, justifying the need of drug therapy. However, at present there are no approved drugs that can reliably reduce all of the metabolic risk factors over the long term, thus the interest in therapeutic strategies that might target multiple risk factors more effectively, minimizing problems with polypharmacy, is growing [19].

The interest in pharmacokinetics and pharmacodynamics of organoselenium compounds has increased in the last decades due to the variety of these molecules that possess biological activity. Diphenyl diselenide ($(PhSe)_2$), an organoselenium compound, has been reported as a potential pharmacological agent due to its hypolipidemic and hypoglycemic properties [20-21]. Moreover, $(PhSe)_2$ was shown to inhibit human LDL oxidation *in vitro* [22], to reduce foam atherosclerotic lesion in hypercholesterolemic LDL receptor knockout (LDLr -/-) mice, to decrease infiltration of inflammatory cells in vessel-wall, and to prevent the upregulation of the proatherogenic monocyte chemoattractant protein-1 [23].

Thus, the aim of the present study was to investigate the mechanisms involved in the hypolipidemic and hypoglycemic activity of $(PhSe)_2$. To this end, two different experimental models were used: HepG2 cell line to study $(PhSe)_2$ effects on the protein network of cholesterol homeostasis and rat myotube (L6) to analyze GLUT4 translocation on cell membrane.

2. MATERIALS AND METHODS

2.1 Materials

All materials used were obtained from commercial sources and were of the highest quality available. All materials with no specified source are obtained from Sigma-Aldrich (Milan, Italy).

2.2 Drugs

$(PhSe)_2$ (Fig. 1) was prepared and characterized in the Laboratório de Síntese, Reatividade e Avaliação Farmacológica e Toxicológica de Organocalcogênios, of the Department of Chemistry, Universidade Federal de Santa Maria, Brazil. Analysis of the ^{1}H NMR and ^{13}C NMR spectra showed that the compound presented analytical and spectroscopic data in full agreement with its assigned structure. The chemical purity of $(PhSe)_2$ (99.9%) was determined by GC/MS.

2.3 Cell culture and experimental procedures

HepG2 cells and rat L6 myoblasts were used as experimental models. HepG2 cells were routinely grown at 5% CO_2 in RPMI-1640 medium, containing 10% (v/v) foetal calf serum, L -glutamine (2 mM), gentamicin (0.1 mg/ml) and penicillin (100 U/ml). Cells were passaged every 3 days and medium changed every 2 days. HepG2 cells were grown to 70% confluence in flasks then stimulated with DMSO (negative control), Simvastatin (1 µM, positive control), or $(PhSe)_2$ (1, 3, 10, or 30 µM) for 24 h.

The concentration of 30 µM $(PhSe)_2$ was chosen as the highest tested concentration since it corresponds to the peak of $(PhSe)_2$ in the plasma of rodents treated with 500 mg/kg $(PhSe)_2$ (which is considered a toxic dose since it induces seizures in these animals) [24].

Undifferentiated L6 myoblasts were purchased from ATCC (Manassas, VA) and were cultured in DMEM containing 10% horse serum, L-glutamine (2 mM), gentamicin (0.1

mg/ml) and penicillin (100 U/ml) at 5% CO_2. Cells were plated in six-well plates or 25 cm^2 dishes at a density of 5,000 cells/cm^2 in DMEM containing 10% horse serum until they grown to ~70% confluence. Then, L6 undifferentiated myoblasts were treated with differentiation medium containing 2% (vol/vol) horse serum; myotubes were obtained 7 days after the reduction of serum concentration. To analyze whether $(PhSe)_2$ was able to increase AMPK phosphorylation in myotubes, cells were stimulated with DMSO (negative control) or $(PhSe)_2$ (3 or 10 µM) for 24 h. In order to analyze the capacity of $(PhSe)_2$ to increase GLUT4 translocation from the cytosol to the cell membrane, myotubes were stimulated with DMSO (negative control) or $(PhSe)_2$ (3 or 10 µM) for 24 h, or 10 nM insulin (positive control) for 15 min.

2.4 Lysate preparation, electrophoresis, and protein level analysis

Twenty-four hours after treatment, cells were lysed in 80 µl lysis buffer (0.25 M Tris pH 6.8, 10% SDS, 0.062 M NaF, and protease inhibitors) by sonication. Protein concentration of each sample was determined by the method of Lowry [25]. Protein profiles were analyzed by Western blotting. Twenty micrograms of protein from cell lysates were resolved by 10% SDS-PAGE at 130 V for 60 min. The proteins were subsequently transferred electrophoretically onto nitrocellulose for 90 min at 100 V. The nitrocellulose membrane was blocked at room temperature with 5% fat-free milk in Tris-buffered saline (138 mM NaCl, 27 mM KCl, 25 mM Tris-HCl, 0.05% Tween-20, pH 6.8), and probed at 4ºC overnight with primary antibodies to HMGR and P-HMGR (Upstate, Lake Placid, NY) ; LDLr (Abcam Cambridge, UK); P-AMPKα and AMPKα (Cell Signaling Technology, Boston, MA , USA); PP2A, Insig-2, (Santa Cruz Biotechnology, Santa Cruz, CA, USA); Insig-1 (Novus Biologicals, Littleton, CO, USA); SREBP-1 and SREBP-2 N-terminal (Abcam, Cambridge, UK). The first incubation was followed by another one for 1 h at room temperature with secondary IgG antibodies coupled to horseradish peroxidase (Bio-Rad Laboratories, Milan,

Italy). The nitrocellulose membrane was then stripped with Restore Western Blot Stripping Buffer (Pierce Chem-ical, Rockford, IL, USA) for 10 min at room temperature and re-probed with anti-vinculin (Novus Biologicals, Littleton, CO, USA) or anti-α-tubulin (MP Biomedicals) antibody. Bound antibodies were visualized using enhanced chemoluminescence detection (GE Healthcare). All images derived from Western blotting were analyzed with ImageJ (NIH, Bethesda, MD, USA) software for Windows. Each value was derived from the ratio between arbitrary units obtained by the protein band and the respective vinculin or α-tubulin band (chosen as housekeeping proteins).

2.5 Immunofluorescence assay

L6 myoblast were grown and differentiated on glass coverslips as previously described and after the treatment, fixed in 4% paraformaldehyde in PBS (phosphate buffer saline) (vol/vol) for 15 min at 4° C and permeabilized with 0.2% Triton X-100 in PBS (vol/vol) for 3 min. Myotubes were incubated at room temperature with 5% BSA in PBS for 1 hour, rinsed three times in PBS for 5 min each wash and then probed at room temperature with primary antibodies to GLUT4 (Santa Cruz Biotechnology, Santa Cruz, CA, USA). Cells were then washed three times with PBS and then the first incubation was followed by another one for 1 h in the dark with Alexa^{546} donkey anti-rabbit immunoglobulin (IgG) obtained from Molucular Probes (Invitrogen, Eugene, OR). After that, the cells were washed three times with PBS for 5 min each wash and counterstained with DAPI for 15 min in the dark. Cells were analyzed by fluorescent microscopy (Olympus BX51).

2.6 HMGR activity assay

The assay was performed by the radioisotopic method, following the production of C^{14}MVA (mevalonate) from 3-[^{14}C]-HMGCoA (specific activity 57.0 mCi/mmol. Amersham-Pharmacia, Little Chalfont, UK). 1 g of rat liver was homogenized in phosphate buffer containing 0.1 M sucrose, 0.05 M KCl, 0.04 M KH_2PO_4, 0.03 M EDTA, pH 7.4. Microsomes

were prepared by centrifugation of the homogenates as already reported [26]. Microsomes were incubated in the presence of co-factors (20 mM glucose-6-phosphate, 20 mM NADP sodium salt, 1 unit of glucose-6-phosphate dehydrogenase, and 5 mM dithiothreitol) and Simvastatin (1 µM) or $(PhSe)_2$ (10 uM). Final volume was 180 µl. The assay was started by the addition of 10 µl (0.088 µ Ci/11.7 nmol) of 3-[^{14}C]-HMG-CoA. The radioactivity of the synthesized [^{14}C]-MVA, isolated by chromatography on AG1-X8 ion exchange resin (BioRad, Italy), was measured and the recovery calculated on the basis of the internal standard (3-[^{3}H]-MVA, specific activity (24.0 Ci/mmol. Amersham-Pharmacia, Little Chalfont, UK) [27].

2.7 Statistical analysis

The obtained data were analyzed by one-way ANOVA (analysis of variance). Post hoc Dunnett's Multiple Comparison Test versus control was run when indicated. Descriptive statistics data were expressed as mean $\pm$ SD of n=3 independent experiments carried out in duplicate. Probability values less than 0.05 ($p<0.05$) were considered as statiscally significant. Statistical analysis was performed using GraphPad Instat3 (GraphPad software, Inc., La Jolla, CA, USA) software for Windows.

3. RESULTS

3.1 $(PhSe)_2$ effects of on LDLr protein levels

To study the mechanism underlying $(PhSe)_2$ hypolipidemic effect, HepG2 cells were treated with 1 µM simvastatin as positive control and $(PhSe)_2$ at doses ranging from 1 to 30 µM for 24 hours. LDLr isoform levels were checked by Western blot analysis: we used an anti-LDLr antibody that reacting with both glycosylated and unglycosylated LDLr forms allows to analyse the expression and the post-translational processing of the receptor. LDLr is highly glycosylated through N- and O-linkages, migrating at 100 kDa (neo-synthesized

receptor), 130 kDa (intermediate form) and 160 kDa (functional and mature form) on SDS-PAGE, and three specific bands can be detected [28].

30 μM $(PhSe)_2$, as expected, exerted toxic effects on HepG2 cells confirming the data previously obtained [24], thus all the following experiments were carried out at doses ranging from 1 to 10 μM The obtained results demonstrate that $(PhSe)_2$ as well as simvastatin was able to increase the expression of LDLr 110 kDa (Fig. 2A), LDLr 130 kDa (Fig. 2B), and LDLr 160 kDa protein levels (Fig 2C).

3.2 $(PhSe)_2$ effects on HMGR protein levels, phosphorylation state, and activity

It is well known that HMGR and LDLr are modulated by intracellular cholesterol content: the expression of both the proteins rises when cholesterol molecule decreases [4]. In order to better investigate whether the observed $(PhSe)_2$-induced LDLr up-regulation was paralleled by a modulation of HMGR as observed after statin treatment [7], the protein levels of the enzymes were analyzed by Western blot analysis after 24 hour treatment of HepG2 cells. As depicted in figure 3(A), 3 and 10 μM $(PhSe)_2$ were able to increase total HMGR protein levels when compared to control cells treated with vehicle. It is interesting to note that $(PhSe)_2$ effect is higher than simvastatin one. Additionally, the analysis of P-HMGR levels (Fig. 3B) showed that the enzyme phosphorylation (inhibition) was generally increased, being statistically significant at 10 μM.

To analyze whether $(PhSe)_2$-induced HMGR and LDLr increase was due to a direct inhibition exerted by the compound, the enzyme activity assay was carried out. The experiment was performed by treating liver microsomes with $(PhSe)_2$ at 10μM (1 hour) and 1 μM simvastatin as positive control. The radioisotopic method revealed that, $(PhSe)_2$ did not affect HMGR activity which was susceptible, as expected, to simvastatin treatment (Fig. 3C).

3.3 $(PhSe)_2$ effects on HMGR long-term regulation

To ascertain how $(PhSe)_2$ treatment could affect HMGR and LDLr long-term modulation, the proteins involved in the regulatory network were investigated. Western blot analyses on total lysates of HepG2 cells treated for 24 hour with $(PhSe)_2$ were performed measuring nSREBP-1, nSREBP-2, Insig1, and Insig2 protein content.

The obtained results revealed that $(PhSe)_2$ treatment was able to increase nSREBP-1 protein levels to a greater extent than simvastatin at all tested concentrations (Fig. 4A). Conversely, nSREBP-2 was not affected by either simvastatin or $(PhSe)_2$ treatment (Fig. 4B).

Additionally, $(PhSe)_2$ induced a higher increase of Insig1 content when compared to the one induced by simvastatin (Fig. 4C), whereas Insig2 protein was barely affected by the treatments being increased only at 1µM $(PhSe)_2$ treatment (Fig. 4D).

3.4 $(PhSe)_2$ effects on HMGR short-term regulation proteins

As shown in figure 3(B), $(PhSe)_2$ treatment seems to increase HMGR phosphorylation state even though this rise is statistically significant only at 10 µM. Thus, the protein levels of AMPK and PP2A, the enzymes responsible of HMGR phosphorylation/dephosphorylation, were analyzed.

The results showed that $(PhSe)_2$ treatment at 3 and 10 µM strongly increased AMPK activation state when compared to control cells. On the contrary, this parameter was not affected by 1 µM simvastatin (Fig 5A). Moreover, 1 and 3 µM $(PhSe)_2$ treatments were both able to induce an increase of PP2A catalytic subunit when compared to control, as well as Simvastatin does (Fig 5B).

3.5 $(PhSe)_2$ effect on GLUT4 translocation and AMPK phosphorylation state in rat skeletal muscle cells (L6)

To investigate the hypoglycemic effect exerted by $(PhSe)_2$ [29], GLUT4 translocation on plasma membrane of rat skeletal muscle cells was studied by using a morphological approach. L6 cells were firstly induced to differentiate and then treated for 24 hours with $(PhSe)_2$ at 3 and 10 µM. Immunofluorescence staining was performed in order to assess GLUT4 membrane translocation. 15 minutes before cells were fixed, a sample was treated with 10 nM insulin, used as positive control for the GLUT4 translocation. As expected, insulin treatment was able to induce an almost complete translocation of GLUT4 protein from the cytoplasm to the membrane compartment as assessed by the loss of fluorescence observed in insulin-treated cells, while the transporter is still present in the cytoplasm of controls. Besides insulin stimulation, a similar effect was also observed in both 3 and 10 µM $(PhSe)_2$ stimulated cells in a dose-dependent manner, strongly suggesting that $(PhSe)_2$ is able to induce the GLUT4 translocation from the cytoplasm to the cellular membrane depending on concentration (Fig. 6). To study the mechanism underlying $(PhSe)_2$–induced GLUT4 translocation, AMPK activation state was measured since it is known to be involved in the rise of glucose uptake [30]. The obtained data showed that the compound induced an increase of AMPK activation that gains statistical significance at 10 µM (Fig. 7).

4. DISCUSSION

The purpose of the present work was to study, on cultured cells, the mechanisms involved in the hypolipidemic and hypoglycemic effects that $(PhSe)_2$ demonstrated to exert "in vivo" [21,29,31,32]. Our results demonstrate that $(PhSe)_2$ exerts hypolipidemic effects through the increase of LDLr and promotes hypoglycemic properties, through the translocation of GLUT4 likely through AMPK activation in skeletal muscle. Indeed, the studied compound is able to activate AMPK, the key sensor of cellular energy status [15], which is deeply involved in the regulation of cellular metabolism.

The strategies widely used to reduce plasma cholesterol levels are based on the inhibition of HMGR activity and on the consequent compensatory increase of LDLr membrane exposure. The newly produced LDLr removes LDL from the blood, and delivers it into the cell where the lipoprotein is digested and released cholesterol becomes available for metabolic needs [4]. In this work, we demonstrated that the LDLr expression was strongly increased by $(PhSe)_2$ treatment, suggesting that this compound could exert its hypocholesterolemic effect through the rise of hepatic LDL clearance. $(PhSe)_2$-induced increase of LDLr is related to the rise of HMGR protein levels: both the results were also observed after simvastatin administration, a drug widely used in therapies against hypercholesterolemia which is able to inhibit the activity of HMGR [33]. These feature is a typical feedback effect, suggesting that $(PhSe)_2$ is somehow able to inhibit intracellular sterol synthesis, as also indicated by the hypolipidemic effects previously observed *in vivo* [21,31,32]. Nevertheless, unlike simvastatin, $(PhSe)_2$ does not directly inhibit HMGR activity. However, we cannot exclude that this compound could exert its hypolipidemic properties through the inhibition of other enzymes downstream of HMGR: this could be very attractive for $(PhSe)_2$ putative clinical implication, since it is desirable to find alternative pharmacological approach to statin treatment able to block cholesterol biosynthetic pathway downstream to HMGR, whose inhibition can affect body health in different tissues, such as the skeletal muscle and the central nervous system [27,34-36]. We did not still try out in which step of cholesterol biosynthetic pathway $(PhSe)_2$ could exerts its effect, and this aspect will be under active investigation in our laboratory.

To ascertain whether the observed results were a consequence of the classical compensatory effect depending on the decrease of cholesterol synthesis, the levels of SREBPs and Insigs were also checked [37].

SREBPs are a family of transcription factors that consist of SREBP-1a, SREBP-1c, and SREBP-2. When cellular sterol content decrease, the trascriptionally active fragment of SREBP (nuclear SREBP), activates the genes of enzymes and proteins responsible for the regulation of cholesterol metabolism, such as HMGR and LDLr [37]. Accordingly, the presented data demonstrated that $(PhSe)_2$-induced HMGR and LDLr increase was accompanied by the rise of SREBP-1 active fraction, thus confirming our hypothesis. We also found that Insig1 protein levels were enhanced in $(PhSe)_2$-treated cell. The increase in Insig1 content is not astounding, since *Insig1* is also a SREBP target gene. Because of the likely insufficient amount of cholesterol, Insig1 protein could accumulate into the cells without affecting both HMGR degradation and SREBP migration into the nuclei, sustaining that cellular cholesterol synthesis and content is reduced by $(PhSe)_2$ treatment [9,10,13].

Additionally, P-HMGR protein levels were found to be higher after $(PhSe)_2$ treatment, thus indicating that this compound was also able to induce a physiological inhibition of the enzyme, which is mediated by the increase in AMPK activation.

AMPK functions as a fuel sensor in the cell and is activated when cellular energy is depleted. AMPK inhibits fatty acid synthesis in rat adipocytes [38], and both fatty acid and cholesterol synthesis in rat hepatocytes by inactivation of acetyl-CoA carboxylase [39] and HMGR [40], respectively. Thus, our results suggest that $(PhSe)_2$-induced AMPK activation might be also involved in other protective events such as fatty acid synthesis, cellular proliferation, atherosclerosis, and cancer: indeed AMPK activation has been described as a logical therapeutic target for several diseases [41].

Together with the increase in AMPK phosphorylation/activation induced by $(PhSe)_2$ treatment, the rise in PP2A catalytic subunit level was also observed. PP2A antagonizes AMPK activity and activates HMGR by dephosphorylation [6]. In this study, we found that both simvastatin and $(PhSe)_2$ treatments increased PP2A catalytic subunit protein levels.

However, we can not speculate about the relevance of this data as the activity of PP2A depends on the presence of several co-activators and is involved in other cellular functions besides HMGR dephosphorylation [42].

In addition to the well characterized hypolipidemic effects, AMPK activation is also known to increase glucose uptake through the enhancement of GLUT4 translocation on myotube plasma membrane [18,30]. The obtained data in the present study showed that $(PhSe)_2$ increased AMPK phosphorylation state and total protein levels together with GLUT4 translocation on myotube plasma membranes, indicating that this mechanism could be responsible for the glycemia decrease observed in the previous work [29]. Nonetheless, these observations do not preclude the existence of other mechanisms involved in the hypoglycemic effect of $(PhSe)_2$.

The growing realization that AMPK could switch metabolism from an anabolic state, defined by the synthesis and the storage of glucose and fatty acids, to a catabolic state, thus favoring the oxidation of these fuel molecules, suggests that AMPK activators might be effective treatments for metabolic diseases such as obesity, type 2 diabetes, and cardiovascular diseases [16,43].

5. CONCLUSION

In conclusion, the mechanisms underlying the hypocholesterolemic effects of $(PhSe)_2$ investigated in HepG2 cells line involve the increase in HMGR phosphorylation/inactivation induced by AMPK activation, and the rise in LDLr protein levels without directly inhibiting HMGR activity. The net consequence of these effects is that plasma cholesterol level is kept low as demonstrated *in vivo*. In addition, we also found that, in L6 skeletal muscle cells, $(PhSe)_2$ augments GLUT4 translocation from cytosol to cell membrane through the increase in AMPK phosphorylation state, which could be linked to the hypoglycemic properties of

$(PhSe)_2$ previously demonstrated *in vivo*. The effects of $(PhSe)_2$ evaluated in this work, crucial for both glycemic and lipidic metabolisms, make the organoselenium compound a promising alternative for the treatment of metabolic syndrome in order to overcome current problems related to polypharmacy.

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Abbreviations

AMPK	AMP activated kinase
ER	endoplasmic reticulum
GLUT4	glucose transporter type 4
HMGR	3-hydroxy-3-methylglutaryl coenzyme A reductase
Insig	Insulin induced gene
LDL	low-density lipoprotein
LDLr	low-density lipoprotein receptor
MVA	mevalonate
PBS	phosphate buffer saline
P-HMGR	phosphorylated HMGR
$(PhSe)_2$	diphenyl diselenide
PP2A	protein phosphatase 2A
SCAP	SREBP cleavage-activating protein
Sim	simvastatin
SREBP	Sterol regulatory element binding protein

Conflict of Interest : Authors declare no conflict of interest

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FIGURE LEGENDS

Figure 1 Chemical structure of Diphenyl Diselenide $(PhSe)_2$. $(PhSe)_2$ is a yellow solid compound, very stable that can be stored in the laboratory, in simple flasks, for a long time.

Figure 2 LDLr protein levels in HepG2 cells treated with 1 µM simvastatin and 1, 3 and 10 µM $(PhSe)_2$ for 24 h. The figure shows the densitometric analysis of LDLr 110 kDa (A), LDLr 130 kDa (B), and LDLr 160 kDa (C) protein levels and a representative Western blot (D). The protein levels were normalized with vinculin content. The data are expressed as arbitrary units obtained analyzing the bands by using the software ImageJ. Data are the mean $\pm$ S.D. of n=3 independent experiments carried out in duplicate. ***P < 0.001 from a Dunnett's Multiple Comparison Test versus control (C). Sim = Simvastatin, $(PhSe)_2$ = Diphenyl diselenide.

Figure 3 HMGR and HMGR phosphorylation state in HepG2 cells treated with 1 µM Simvastatin and 1, 3 and 10 µM $(PhSe)_2$ for 24 h, and HMGR activity from rat hepatic microssomes treated with 10 µM $(PhSe)_2$. The figure illustrates the densitometric analysis of total HMGR (A), P-HMGR (B) protein levels and a representative Western blot (C). The protein levels were normalized with vinculin content. The data are expressed as arbitrary units obtained analyzing the bands by using the software ImageJ. Panel D illustrates HMGR activity performed in HepG2 cells trated with simvastatin or (PhSe)2. The activity of the enzyme is expressed as [14C]-mevalonate production (pmol/min/mg protein) from 3-[14C]-HMG CoA added to the samples. Data are the mean $\pm$ S.D. of n=2 independent experiments carried out in duplicate. *P < 0.05, and ***P < 0.001 from a Dunnett's Multiple Comparison Test versus control (C). Sim = Simvastatin, $(PhSe)_2$ = Diphenyl diselenide.

Figure 4 nSREBP-1, nSREBP-2, Insig1, and Insig2 protein levels in HepG2 cells treated with 1 µM simvastatin and 1, 3 and 10 µM $(PhSe)_2$ for 24 h. Figure illustrates the densitometric analysis of nSREBP-1 (A), nSREBP-2 (B), Insig1(C), and Insig2 (D) protein levels. Panels E and F show representative Western blots of nSREBPs and Insigs, respectively. The protein levels were normalized with vinculin content. The data are expressed as arbitrary units obtained analyzing the bands by using the software ImageJ. Data are the mean $\pm$ S.D. of n=3 independent experiments carried out in duplicate. * P < 0.05, **P < 0.01, and ***P < 0.001 from a Dunnett's Multiple Comparison Test versus control (C). Sim = Simvastatin, $(PhSe)_2$ = Diphenyl diselenide.

Figure 5 AMPK phosphorylation state and PP2A protein level, in HepG2 cells treated with 1 µM simvastatin and 1, 3 and 10 µM $(PhSe)_2$ for 24 h. Figure shows the densitometric analysis of AMPK phosphorylation state calculated as P-AMPK/AMPK/vinculin and PP2A protein levels . Panels C and D show representative Western blots of AMPK and PP2A respectively. The protein levels were normalized with vinculin content. The data are expressed as arbitrary units obtained analyzing the bands by using the software ImageJ. Data are the mean $\pm$ S.D. of n=3 independent experiments carried

out in duplicate. * P < 0.05, **P < 0.01, and ***P < 0.001 from a Dunnett's Multiple Comparison Test with respect to control (C). $(PhSe)_2$ = Diphenyl diselenide, Sim = Simvastatin.

Figure 6 Morphological evaluation of GLUT4 translocation. Evaluation of GLUT4 tranlsocation in L6 skeletal muscle myotubes treated with 3 and 10 µM $(PhSe)_2$ for 24h and 10 nM Insulin for 15 minutes. Panels A, B, C, D show the images of respectively control, Insulin (positive control), 3 and 10 µM $(PhSe)_2$. $(PhSe)_2$ = Diphenyl diselenide.

Figure 7 AMPK phosphorylation state in L6 skeletal muscle cells treated with 1, 3 and 10 µM $(PhSe)_2$ for 24 h. Figure shows the densitometric analysis of AMPK phosphorylation state calculated as P-AMPK/AMPK/vinculin (A) and a representative Western blot (B). The data are expressed as arbitrary units obtained analyzing the bands by using the software ImageJ. Data are the mean $\pm$ S.D. of n=3 independent experiments carried out in duplicate. **P < 0.01 from a Dunnett's Multiple Test with respect to control (C). $(PhSe)_2$ = Diphenyl diselenide

Figures

Figure 1

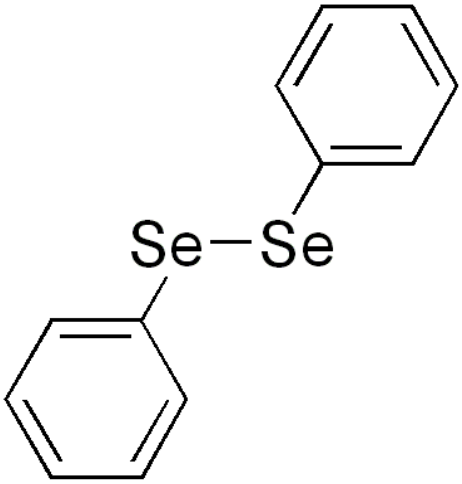


Figure 2

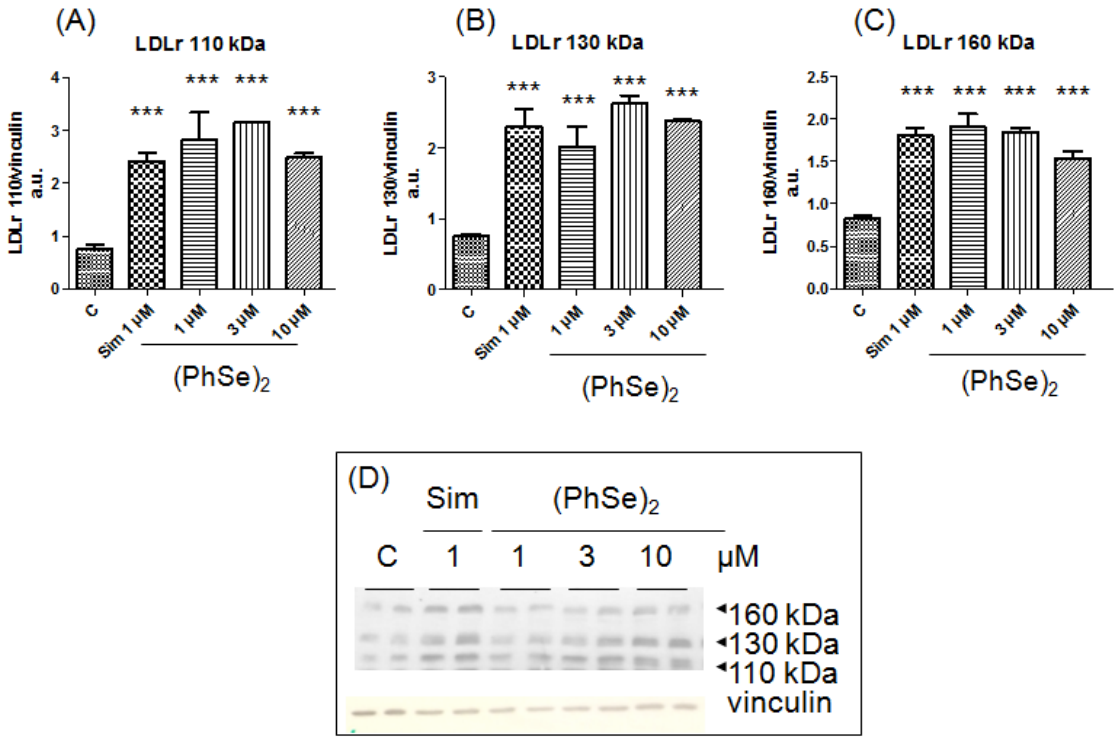

(A)
LDLr 110 kDa
LDLr 110/vinculin a.u.
***
C
Sim 1 µM
1 µM
3 µM
10 µM
(PhSe)2
(B)
LDLr 130 kDa
LDLr 130/vinculin a.u.
(C)
LDLr 160 kDa
LDLr 160/vinculin a.u.
(D)
Sim
(PhSe)2
C
1
1
3
10
µM
160 kDa
130 kDa
110 kDa
vinculin

Figure 3

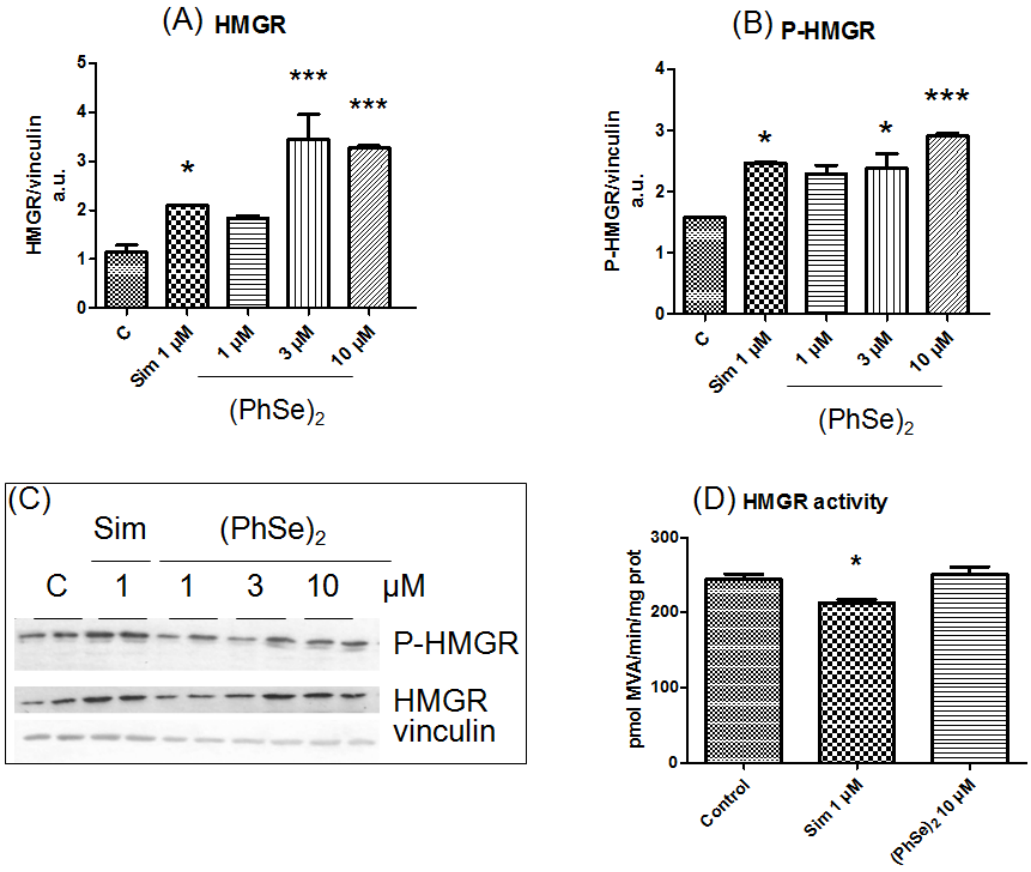

(A) HMGR
HMGR/vinculin a.u.
C
Sim 1 µM
1 µM
3 µM
10 µM
(PhSe)2
(B) P-HMGR
P-HMGR/vinculin a.u.
(C)
Sim
(PhSe)2
C 1 1 3 10 µM
P-HMGR
HMGR
vinculin
(D) HMGR activity
pmol MVA/min/mg prot
Control
Sim 1 µM
(PhSe)2 10 µM

Figure 4

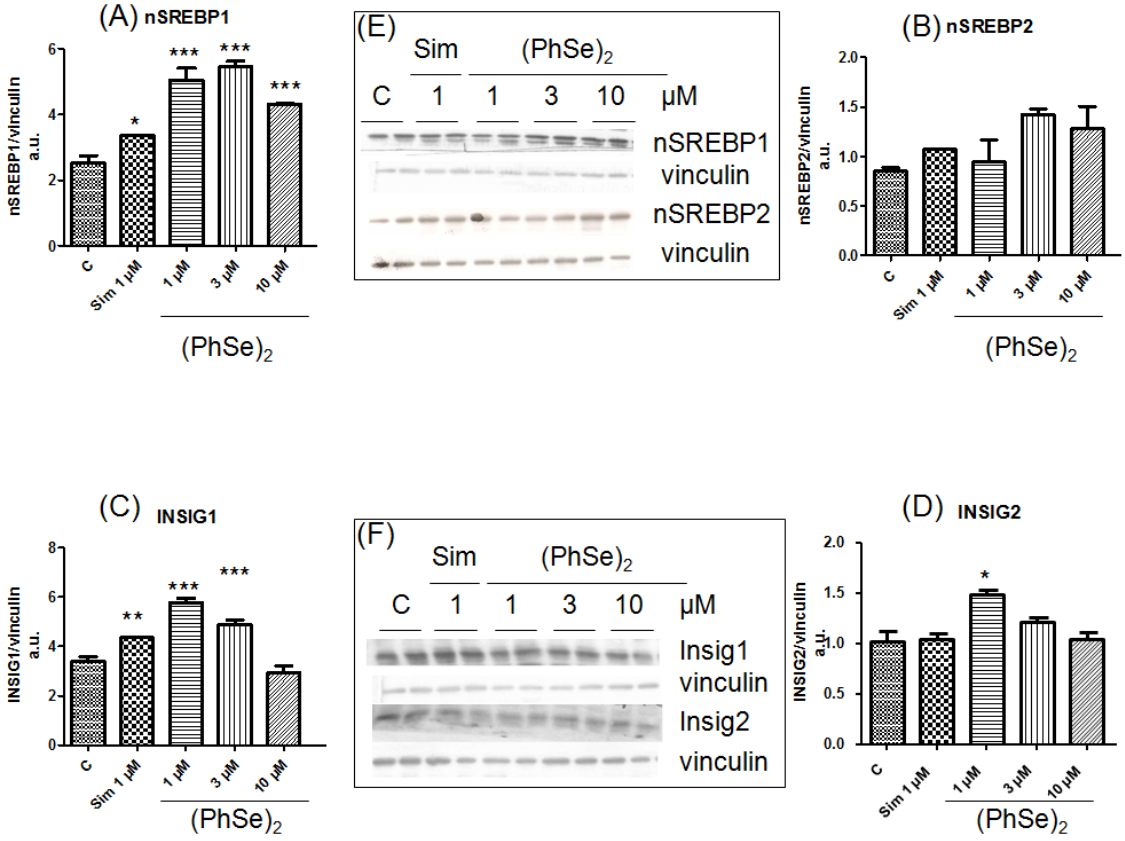

(A) nSREBP1
nSREBP1/vinculin a.u.
C
Sim 1 µM
1 µM
3 µM
10 µM
(PhSe)2
(E)
Sim
(PhSe)2
C 1 1 3 10 µM
nSREBP1
vinculin
nSREBP2
vinculin
(B) nSREBP2
nSREBP2/vinculin a.u.
(C) INSIG1
INSIG1/vinculin a.u.
(F)
Insig1
vinculin
Insig2
vinculin
(D) INSIG2
INSIG2/vinculin a.u.

Figure 5

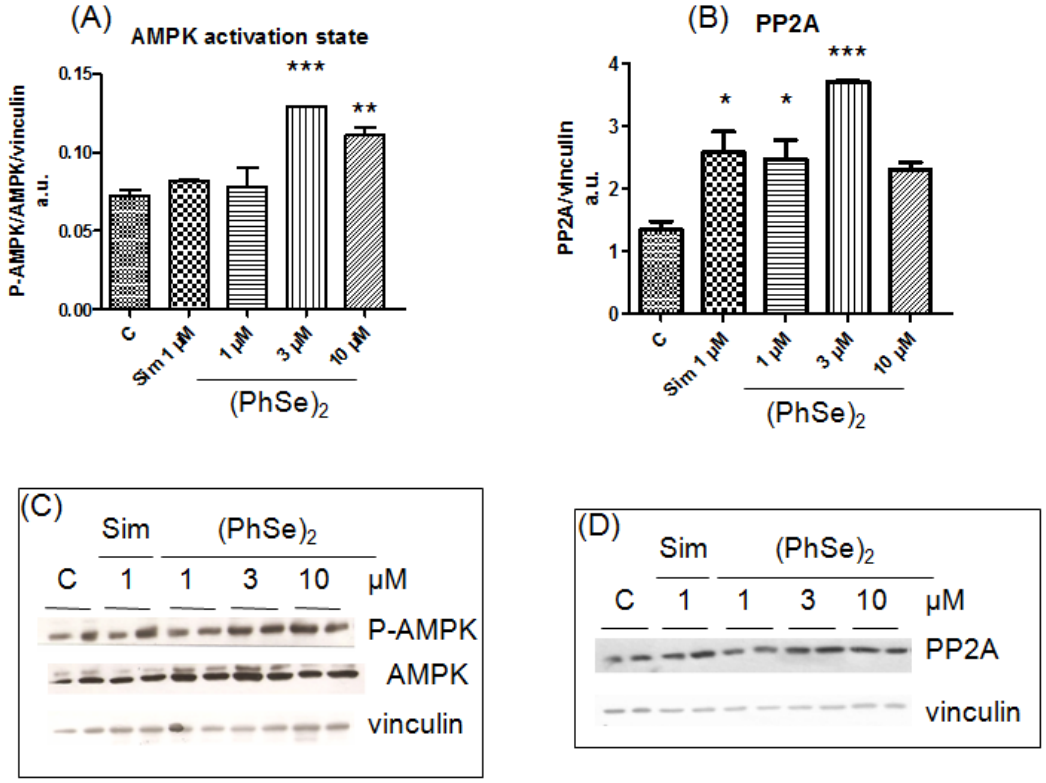

(A)
AMPK activation state
P-AMPK/AMPK/vinculin a.u.
0.15
0.10
0.05
0.00
***
**
C
Sim 1 µM
1 µM
3 µM
10 µM
(PhSe)2
(B)
PP2A
PP2A/vinculin a.u.
4
3
2
1
0
*
*
***
C
Sim 1 µM
1 µM
3 µM
10 µM
(PhSe)2
(C)
Sim
(PhSe)2
C
1
1
3
10
µM
P-AMPK
AMPK
vinculin
(D)
Sim
(PhSe)2
C
1
1
3
10
µM
PP2A
vinculin

Figure 6

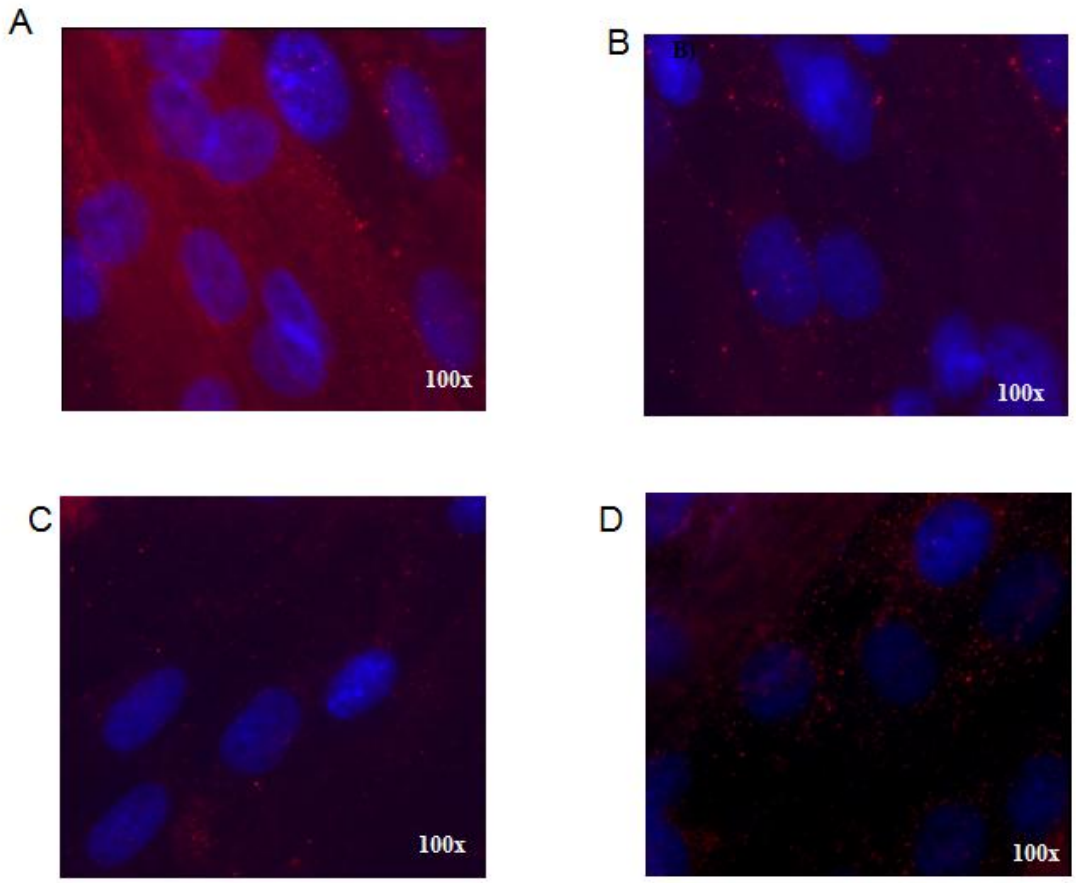
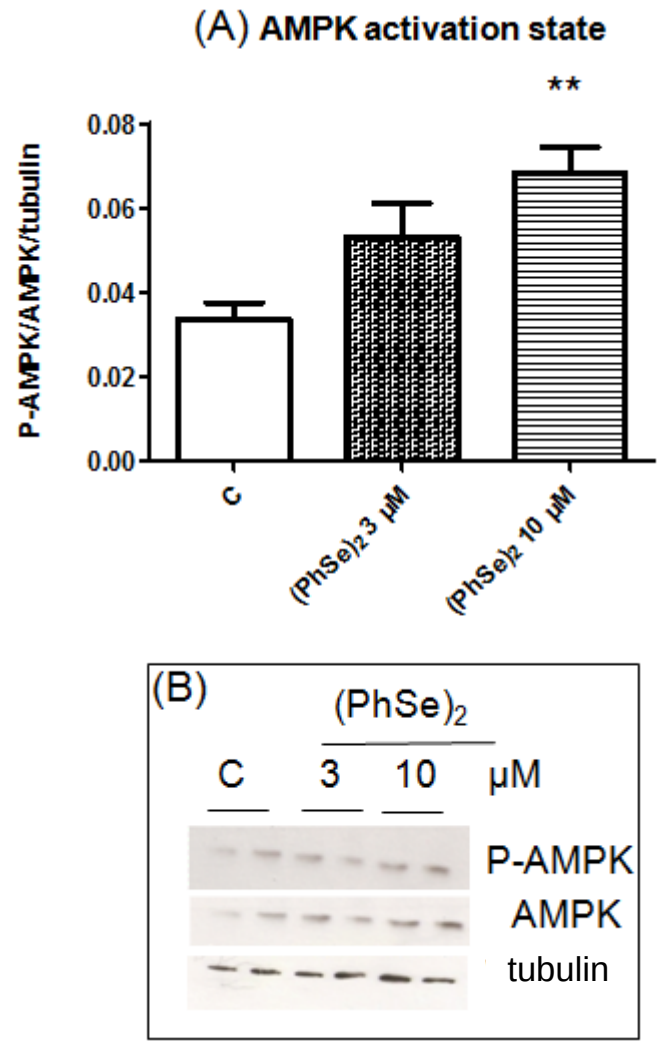


Figure 7


(A) AMPK activation state
**
P-AMPK/AMPK/tubulin
0.08
0.06
0.04
0.02
0.00
C
(PhSe)2 3 µM
(PhSe)2 10 µM
(B)
(PhSe)2
C
3
10
µM
P-AMPK
AMPK
tubulin

4 DISCUSSÃO

A menopausa caracteriza-se pela suspensão irreversível da função ovariana com consequente declínio da produção de hormônios estrogênicos. A falta desses hormônios culmina no aparecimento de alterações metabólicas, cognitivas e comportamentais. Nesse contexto, devido ao aumento na expectativa de vida, as mulheres freqüentemente passam uma porção significativa de suas vidas no período pós-menopausa (Daniel e Bohacek, 2010; Daniel, 2012). Por essa razão, entender de que maneira a depleção de hormônios ovarianos contribui para o surgimento dos sintomas característicos da menopausa pode ser extremamente útil para o desenvolvimento de tratamentos alternativos à terapia de reposição hormonal. Com base nessa premissa, os resultados contidos na presente tese sugerem o emprego do $(PhSe)_2$ como uma promissora opção farmacológica para o tratamento de alguns dos principais sintomas da menopausa.

O aparecimento de alterações pró-aterogênicas no perfil lipídico das mulheres após a menopausa figura entre as principais características da depleção na produção de estrógenos (Rachoń et al., 2008). Dados obtidos de ratas Wistar submetidas a ovariectomia (El-Swefy et al., 2002) ou na estropausa (Trapani et al., 2010; Trapani e Pallottini, 2010) demonstraram que tais alterações pró-aterogênicas incluem aumento nos níveis de colesterol total, LDL e triglicerídeos, acompanhados pela diminuição nos níveis de HDL. Consistente com esses dados da literatura, os resultados contidos no **Artigo 1** desta tese, além de corroborarem com tais informações, ainda mostram um efeito hipolipemiante do $(PhSe)_2$, isto é, o tratamento com $(PhSe)_2$ na dose de 5 mg/kg uma vez ao dia durante um período de 30 dias foi eficaz em diminuir os níveis de triglicerídeos plasmáticos bem como em aumentar os de HDL em fêmeas ovariectomizadas de ratos Wistar.

Ainda nesse sentido, estudos prévios evidenciaram que ratas na estropausa também apresentam um aumento no estado de ativação da enzima HMG-CoA redutase acompanhado pela diminuição nos níveis de receptores de LDL, resultando na diminuição da captação de colesterol e aumento de sua síntese endógena. Acredita-se que essas alterações sejam devidas a modificações na regulação a curto prazo da síntese de colesterol, isto é, ao menor estado de ativação da enzima AMPK, o que levaria a menor fosforilação da HMG-CoA redutase (Trapani e Pallottini, 2010). Portanto, existe uma relação entre a redução na produção de estradiol e a regulação da HMG-CoA redutase. Sugere-se que o efeito protetor desempenhado pelos estrógenos na regulação do metabolismo lipídico seja mediado não apenas pelo aumento

nos níveis de HDL e diminuição nos de LDL, mas também pela regulação do estado de ativação da AMPK, fato que resulta na inibição da atividade da HMG-CoA redutase (Trapani e Pallottini, 2010).

Nesse sentido, os resultados apresentados no **Manuscrito 1**, os quais se propõem a elucidar os mecanismos envolvidos no efeito hipocolesterolêmico do $(PhSe)_2$ investigados em culturas de células HepG2 demonstram que este composto orgânico de Se leva a um aumento nos níveis de receptores de LDL bem como a um aumento no estado de ativação da AMPK, sem inibir diretamente a atividade da HMG-CoA redutase. De uma maneira geral, todas essas modificações levam ao aumento da captação de colesterol e diminuição de sua síntese endógena. Esses resultados corroboram com os achados de outros trabalhos, os quais demonstram um efeito hipocolesterolêmico do $(PhSe)_2$ *in vivo* (De Bem et al., 2009; Da Rocha et al., 2009).

Além disso, o **Manuscrito 1** também sugere um possível mecanismo para o efeito hipoglicemiante do $(PhSe)_2$ já demonstrado em trabalhos anteriores (Barbosa et al., 2006). Os resultados do **Manuscrito 1** sugerem que, em culturas de células L6 (células de músculo esquelético de ratos), o $(PhSe)_2$ aumenta a translocação do GLUT4 do citosol para a membrana celular devido a um aumento no estado de ativação da AMPK, o que provavelmente resulta no aumento da captação de glicose pelo tecido muscular explicando, ao menos em parte, o efeito hipoglicemiante do $(PhSe)_2$ *in vivo* (Barbosa et al., 2006) (**Figura 2**).

Está bem descrito na literatura que a ovariectomia leva tanto ao ganho significativo de peso corporal quanto ao acúmulo de gordura abdominal, efeitos resultantes, em parte, devido ao aumento na ingestão de alimentos e menor gasto energético (Poehlman et al., 1995; Rachoń et al., 2008; Saengsirisuwan et al., 2009; Rogers et al., 2009). Em conformidade a isso, os dados contidos no **Artigo 1** demonstram claramente que as ratas ovariectomizadas apresentaram maior ganho de peso quando comparadas as ratas controle. Interessantemente, o tratamento com $(PhSe)_2$ na dose de 5 mg/kg uma vez ao dia durante um período de 30 dias foi capaz de impedir o ganho de peso nas ratas ovariectomizadas tratadas com esse composto. Além disso, a administração de $(PhSe)_2$ também reduziu consideravelmente o acúmulo de gordura abdominal induzido pela ovariectomia. Quando analisados em conjunto, os dados em questão mostram-se bastante promissores, uma vez que, no presente momento, existem poucas terapias eficazes para o controle da obesidade em mulheres no período pós-menopausa, de modo que o $(PhSe)_2$ constituiria uma alternativa bastante interessante.

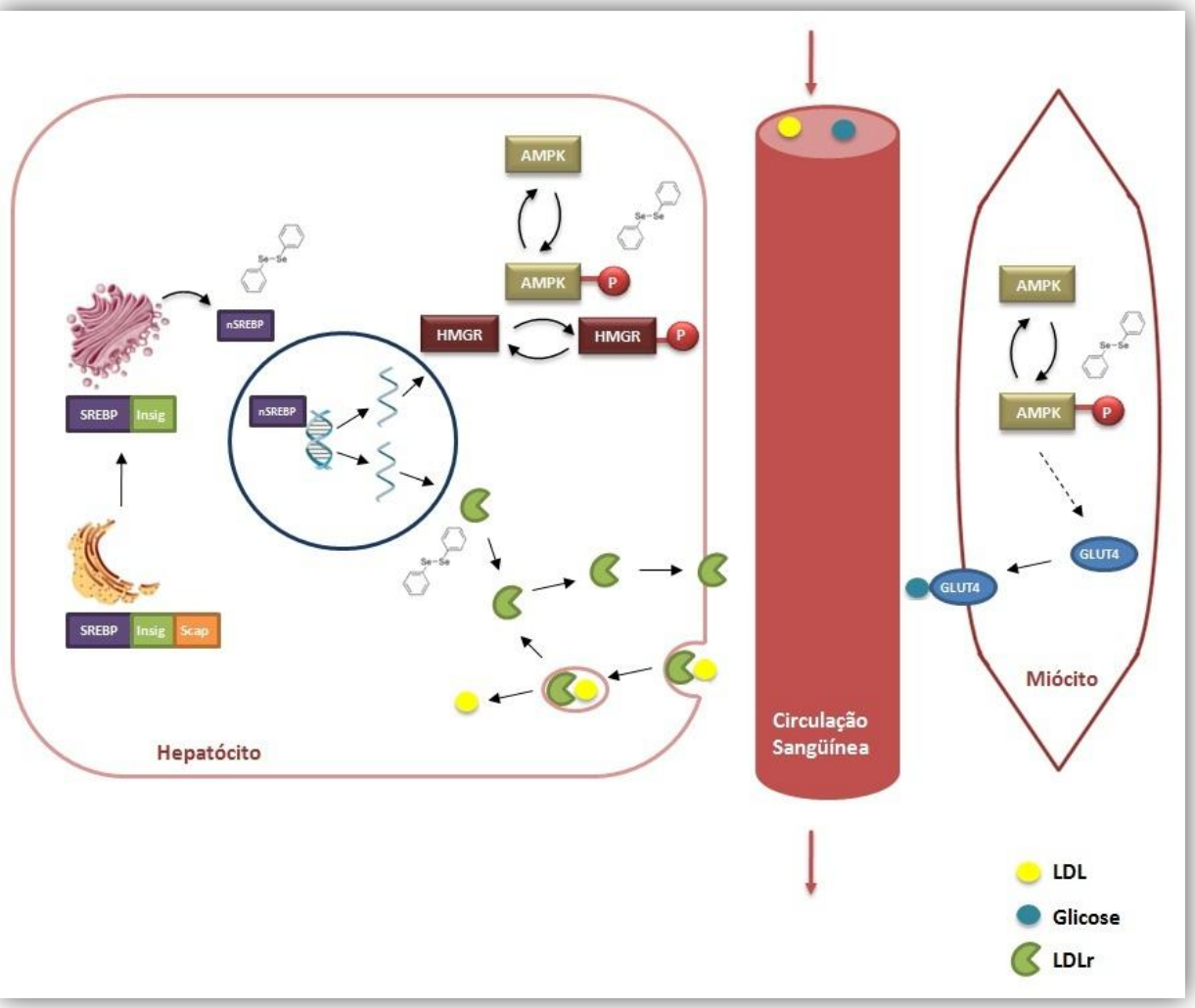


Figura 2 Esquema geral dos efeitos do $(PhSe)_2$ apresentados no **Manuscrito 1**.

Estudos publicados na última década têm estabelecido uma considerável associação entre o *status* oxidativo e os hormônios sexuais (Kaya et al., 2004; Prediger et al., 2004; Kumru et al., 2005; Ha et al., 2006; Kankofer et al., 2007; Oztekin et al., 2007; Topçuoglu et al., 2009). De fato, de acordo com os dados mostrados no **Artigo 1**, 30 dias após a ovariectomia as ratas Wistar apresentam uma redução nos níveis hepáticos de GSH e na atividade da enzima catalase, embora outros parâmetros característicos de estresse oxidativo, como níveis de espécies reativas ao ácido tiobarbitúrico (TBARS) e atividade de outras enzimas antioxidantes não tenham sido alterados. O tratamento com $(PhSe)_2$ na dose de 5 mg/kg uma vez ao dia durante esse período foi efetivo em aumentar os níveis hepáticos de GSH e de ácido ascórbico, bem como aumentar a atividade da enzima GST e restaurar a atividade da catalase no fígado das ratas ovariectomizadas.

Evidências clínicas sugerem que a redução na produção de hormônios ovarianos contribui para o declínio cognitivo nas mulheres (Sherwin, 1988; Phillips e Sherwin, 1992; Farrag et al., 2002; Bimonte-Nelson et al., 2010). Corroborando com esses achados clínicos, estudos conduzidos em roedores, primatas não humanos e seres humanos têm demonstrado que os hormônios gonadais são benéficos para a manutenção das habilidades cognitivas (Sandstrom e Williams, 2001; Rapp et al., 2003; Gibbs et al., 2004; Leuner et al., 2004; Talboom et al.,2008, 2010; Acosta et al., 2010; Bimonte-Nelson et al., 2010; Gibbs, 2010).

O teste do Labirinto Aquático de Morris é um dos mais utilizados para avaliação da memória espacial dependente do hipocampo (Lee e Silva, 2009). Nesse teste, os animais são colocados para nadar em uma piscina na qual existe uma plataforma submersa logo abaixo da superfície da água. Para escapar da água, os animais são treinados diariamente para nadar até encontrarem a plataforma, orientando-se por pontos de referência existentes na sala. O tempo (latência) que os animais demoram a encontrar a plataforma é usado como um índice de memória. Uma medida mais sensível do aprendizado espacial é fornecida pelo desempenho dos animais no dia do teste, durante o qual a plataforma é removida da piscina, sendo dado aos animais um curto período de tempo (60 segundos, por exemplo) para nadar até o local em que a plataforma ficava nos dias de treino (Morris, 1984; Lee e Silva, 2009). De acordo com isso, os dados apresentados no **Artigo 2** evidenciam que ratas avaliadas no teste do Labirinto Aquático de Morris 30 dias após a realização da ovariectomia apresentam uma latência maior para encontrar a plataforma quando comparadas às ratas controle (não ovariectomizadas). Além disso, o tratamento com $(PhSe)_2$ na dose de 5 mg/kg uma vez ao dia durante 30 dias foi capaz de reduzir essa latência nas fêmeas ovariectomizadas, sugerindo um papel benéfico desse composto orgânico de Se em melhorar a memória dos animais ovariectomizados.

Ainda nesse contexto, dados da literatura têm revelado a habilidade do estradiol em modular o funcionamento do sistema colinérgico (Luine, 1985; Gibbs et al., 2004; Daniel e Bohacek, 2010; Gibbs, 2010; Daniel, 2012) e trabalhos têm evidenciado a existência de uma interação entre ovariectomia e sistema colinérgico (Talboom et al., 2008; Acosta et al., 2009; Bimonte-Nelson et al., 2010; Craig et al., 2010). Em conformidade a isso, os resultados contidos no **Artigo 2** mostram que as ratas ovariectomizadas apresentam um aumento significativo na atividade da enzima acetilcolinesterase cerebral quando comparadas às não ovariectomizadas. Um resultado bastante interessante apresentado na presente tese diz respeito ao fato de que o tratamento com $(PhSe)_2$ na dose de 5 mg/kg uma vez ao dia durante 30 dias foi efetivo em impedir o aumento na atividade da acetilcolinesterase em cérebro total

(**Artigo 2**), sugerindo o uso do $(PhSe)_2$ como uma possível terapia para prevenir ou mesmo retardar o aparecimento de prejuízos cognitivos em mulheres após a menopausa.

Interessantemente, estudos têm relatado que a privação de hormônios estrogênicos por um longo período de tempo após a ovariectomia reduz a capacidade do estradiol (administrado exogenamente) em exercer efeitos benéficos sobre a cognição e o funcionamento cerebral (Gibbs, 2000; Daniel, 2012). Dessa forma tem sido proposto que a terapia com estrógenos só é benéfica se a administração desse hormônio for iniciada logo após o início do declínio da função ovariana (Resnick e Henderson, 2002; Maki et al., 2011; Daniel, 2012). Nesse sentido, ainda se faz necessário determinar se a terapia empregando $(PhSe)_2$ também é afetada pelo tempo entre a cessação da função ovariana e o início de sua administração, uma vez que os resultados contidos no **Artigo 2** apenas demonstram um efeito benéfico do $(PhSe)_2$ quando administrado imediatamente após a realização da ovariectomia.

De uma maneira geral, as mulheres apresentam uma maior incidência de depressão quando comparadas aos homens (Seeman, 1997). Além disso, existem evidências sugestivas de que a redução na produção de estrógenos observada durante a menopausa pode aumentar a susceptibilidade a episódios depressivos (Birkhäuser, 2002; Freeman, 2010). Por essa razão, a associação entre desordens depressivas e menopausa é um importante problema de saúde pública que necessita de atenção (Llaneza et a., 2012). Ainda nesse sentido, considerando que o estresse favorece o surgimento de inúmeras psicopatologias, principalmente em mulheres, torna-se importante entender de que forma o estresse e os hormônios gonadais interagem para a regulação do humor (Solomon e Herman, 2009). Nesse contexto, evidências obtidas a partir de estudos observacionais e clínicos têm sugerido que a terapia com estrogênios após o início da menopausa melhora não apenas a cognição mas também o estado de humor nas mulheres (Birkhäuser, 2002).

Em meio a isso, os dados apresentados no **Artigo 3** evidenciam que fêmeas ovariectomizadas de camundongos Swiss submetidas a um protocolo de estresse sub-crônico apresentam um aumento no tempo de imobilidade nos testes de Suspensão da Cauda e do Nado Forçado, ambos preditivos de comportamento do tipo depressivo, corroborando com achados publicados por Nakagawasai et al. (2009). Também foi observado que esse prolongamento no tempo de imobilidade apresentado pelas fêmeas ovariectomizadas foi prevenido pelo tratamento com $(PhSe)_2$ na dose de 10 mg/kg administrado 30 minutos antes de cada exposição ao protocolo de estresse. Além disso, evidenciou-se o envolvimento dos receptores de serotonina do tipo 5-$HT_{2A/2C}$ e 5-HT_3 no efeito do tipo antidepressivo apresentado pelo $(PhSe)_2$. Um fato bastante interessante demonstrado no **Artigo 3** diz respeito

a inibição da atividade das enzimas monoamino oxidase (MAO) A e B pelo $(PhSe)_2$. Embora tenha sido observado que o $(PhSe)_2$ inibe *in vitro* a atividade da MAO-A e da MAO-B em córtex e hipocampo de camundongos, tal inibição não foi observada quando o ensaio da atividade da MAO foi realizado *ex vivo*. Dessa forma, esses resultados poderiam sugerir que o $(PhSe)_2$ enquadra-se como um inibidor da MAO de "quarta geração" por combinar reversibilidade e atividade inibitória mista para as duas isoformas da MAO (Aubin et al., 2004).

Em conclusão, o conjunto de resultados apresentado nesta tese (esquematizados na **Figura 3**) aponta o uso do $(PhSe)_2$ como uma terapia alternativa bastante promissora para o tratamento de algumas das principais conseqüências da menopausa, a saber aumento no ganho de peso, dislipidemia, prejuízos cognitivos e ocorrência de episódios depressivos. Entretanto, faz-se importante mencionar que os efeitos globais do $(PhSe)_2$ ainda precisam ser melhor caracterizados no intuito de verificar a existência de possíveis efeitos adversos.

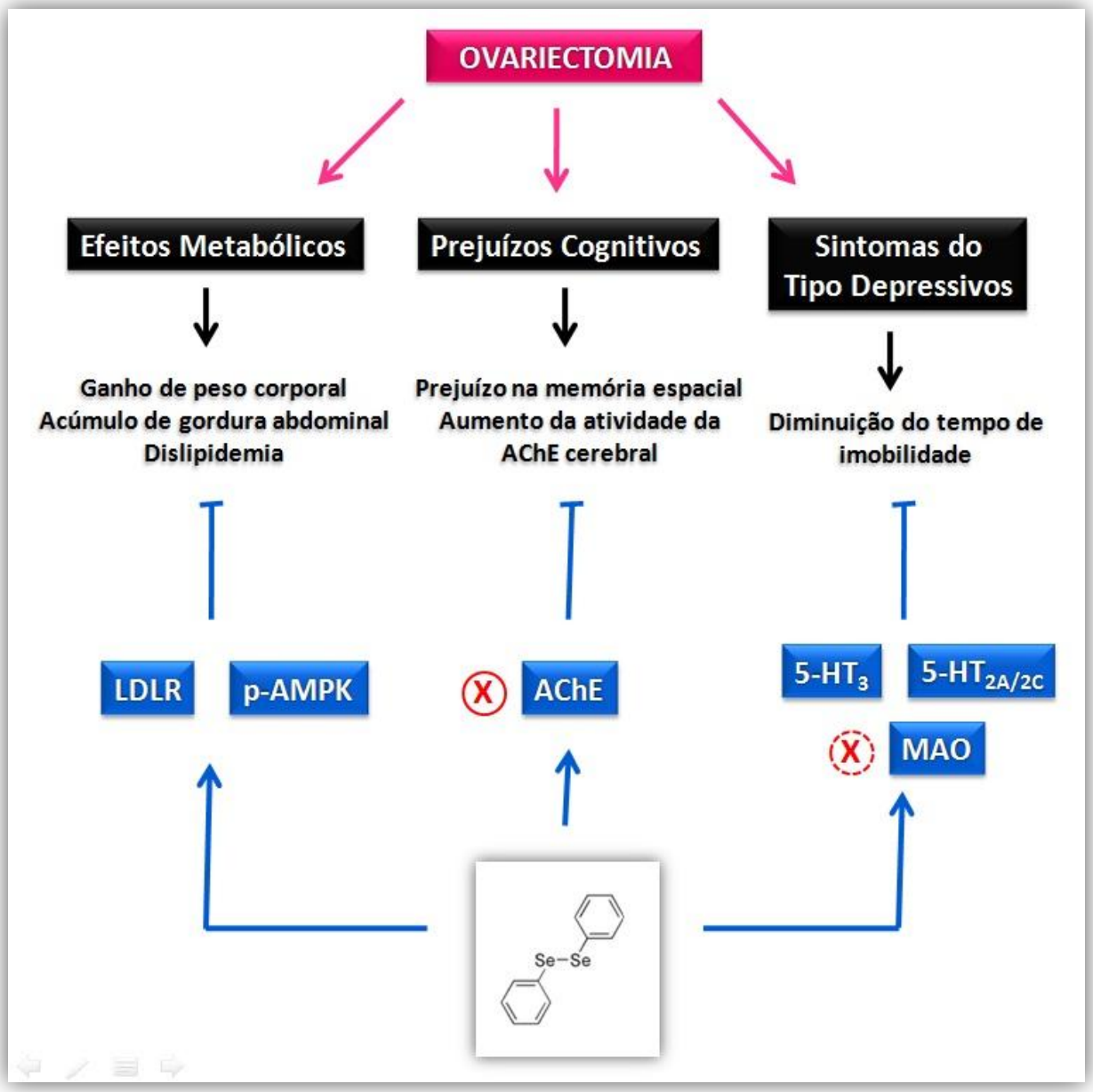


Figura 3 Esquema geral dos efeitos do $(PhSe)_2$ apresentados nessa tese. LDLR, receptor de LDL; p-AMPK, AMP quinase fosforilada; AChE, acetilcolinesterase; MAO, monoamino oxidase; $5\text{-}HT_{2A/2C}$, receptor serotonérgico do tipo 2A/2C; $5\text{-}HT_3$, receptor serotonérgico do tipo 3; ⓧ, inibição de atividade enzimática comprovada nessa tese; ⓧ, inibição de atividade enzimática sugerida nessa tese.

5 CONCLUSÃO

De acordo com os dados apresentados na presente tese, é possível concluir que:

- A administração de $(PhSe)_2$ em ratas Wistar ovariectomizadas reduz o ganho de peso corporal e o acúmulo de gordura intra-abdominal, diminui os níveis plasmáticos de triglicerídeos e aumenta os HDL, bem como melhora parâmetros hepáticos relacionados a estresse oxidativo;
- O tratamento com $(PhSe)_2$ melhora o desempenho de ratas Wistar ovariectomizadas no teste do Labirinto Aquático de Morris, possivelmente por impedir o aumento da atividade da enzima acetilcolinesterase cerebral;
- O prolongamento no tempo de imobilidade apresentado pelas fêmeas de camundongos Swiss ovariectomizadas foi prevenido pelo tratamento com $(PhSe)_2$ administrado 30 minutos antes de cada exposição ao protocolo de estresse. Além disso, evidenciou-se o envolvimento dos receptores de serotonina do tipo 5-$HT_{2A/2C}$ e 5-HT_3 no efeito do tipo antidepressivo apresentado pelo $(PhSe)_2$, além de um provável envolvimento da inibição da atividade da monoamina oxidase cerebral por este composto orgânico de Se;
- Conforme observado em cultura de células HepG2, o efeito hipocolesterolêmico do $(PhSe)_2$ é mediado pelo aumento na expressão dos receptores de LDL e pelo aumento no estado de ativação da AMPK, sem inibir diretamente a atividade da HMG-CoA redutase. Além disso, em culturas de células musculares L6, o $(PhSe)_2$ aumenta a translocação do GLUT4 para a membrana dessas células musculares, o que poderia explicar a ação hipoglicemiante do $(PhSe)_2$.

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