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

DIETA SUPLEMENTADA COM DISSELENETO DE DIFENILA ATENUA AS ALTERAÇÕES BIOQUÍMICAS E COMPORTAMENTAIS NO MODELO DE HIPOTIREOIDISMO INDUZIDO POR METIMAZOL

TESE DE DOUTORADO

Glaecir Roseni Mundstock Dias

Santa Maria, RS, Brasil
2012

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

Orientadora: Prof. Dra. Cristina Wayne Nogueira
Co-orientadora: Prof. Dra. Nilda Berenice de Vargas Barbosa

Santa Maria, RS, Brasil
2012

Universidade Federal de Santa Maria
Centro de Ciências Naturais e Exatas
Programa de Pós-Graduação em Ciências Biológicas:
Bioquímica Toxicológica

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elaborada por
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como requisito parcial para a obtenção do grau de
Doutora em Ciências Biológicas: Bioquímica Toxicológica

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Santa Maria, 14 dezembro de 2012.

Dedico este trabalho aos meus amores, de todo o coração, Haryan, Júnior e Alyssa.

AGRADECIMENTOS

Agradeço a Universidade Federal de Santa Maria, representada por todos os seus docentes, discentes e funcionários, pela excelência em formação;

Ao programa de Pós-Graduação em Bioquímica Toxicológica, pelo crescimento e desenvolvimento profissional;

As minhas orientadoras, Prof. Dra. Cristina Wayne Nogueira e Prof. Dra. Nilda Berenice de Vargas Barbosa, pela oportunidade de desenvolver este trabalho e também pela dedicação, atenção e amizade durante este período;

Ao Prof. Dr. João Batista Teixeira da Rocha pela revisão dos manuscritos;

Ao Prof. Dr. Félix Antunes Soares, pelo uso de seu laboratório e apoio durante todos os experimentos;

A Prof. Dra. Maribel Rubin e a Prof. Dra. Marilise Burger pelo uso de suas salas de comportamento;

Ao Prof. Dr. Élgion Lucio Silva de Loreto pelo uso de seu laboratório, equipamentos e reagentes que possibilitaram a padronização da técnica de q-PCR e também pela amizade a apoio prestado sempre que solicitado;

Ao querido amigo Dr. Ronaldo Medeiros Golombieski, simplesmente, não tenho palavras suficientes para agradecer todo o apoio prestado para desenvolver a técnica de q-PCR e, principalmente a amizade, incentivo e dedicação em todos os momentos;

Aos meus queridos amigos e amigas do "Laboratório do Prof. Félix", Rodrigo, Portella, Fernando, Guilherme, Nélson, Priscila, Letícia, Jessika, Michele, Edovando e Rômulo, foi um prazer enorme dividir tantas conversas e experimentos, cercados de brincadeiras que tornavam nossos dias de trabalho muito mais felizes!!! Levo a amizade de todos com muito carinho!

As minhas queridas ICs, Tielle, Francielli, Sandra e Stephanie que se alternaram para participar deste trabalho, pela dedicação e amizade.

As queridas amigas Jéssie e Sonia e ao querido amigo Alcindo, companheiros de seminários, pela amizade, carinho e apoio;

A minha querida mãe Glaci, pelo apoio, orgulho e carinho sempre prestados... Tudo aquilo de melhor que só uma mãe amada pode oferecer, dedico e agradeço pela sua presença em nossas vidas;

Aos meus amados irmãos Joalcir, Jaldir e Jaldecir, companheiros de sempre, pelo apoio e carinho;

Ao meu querido sogro, Ary, pelo carinho, apoio e presença em nossas vidas... E a minha amada sogra, Vida, que fez muita falta durante este período porque sempre demonstrou muito orgulho e carinho... Tenho certeza que segue me apoiando;

Ao meu amado esposo, Haryan, companheiro de vida, não tenho palavras para descrever tudo àquilo que gostaria de registrar... Somos o que sonhamos e construímos com o apoio um do outro, vencendo e seguindo nosso caminho juntos com muito amor, carinho, respeito e dedicação;

Ao meu amado e querido filho, Haryan Júnior e a minha linda filha amada Alyssa, para que se orgulhem da mamãe... Pelo amor, apoio, carinho, presença, enfim por vocês existirem minha vida é completa;

A minha amiga Ieda, pelo apoio, incentivo, amizade e, principalmente por cuidar tão bem dos meus bens maiores;

“Conheça todas as teorias, domine todas as técnicas, mas ao tocar uma alma humana, seja apenas outra alma humana.”

(Carl Jung)

APRESENTAÇÃO

Nos itens **INTRODUÇÃO e DESENVOLVIMENTO** constam à revisão da literatura sobre os temas relacionados a esta tese.

A metodologia realizada e os resultados obtidos que compõem esta tese estão apresentados sob a forma de artigo e manuscritos, os quais se encontram no item **ARTIGOS CIENTÍFICOS**. Neste constam as seções: Introdução, Materiais e Métodos, Resultados, Discussão e Referências Bibliográficas.

Os itens **DISCUSSÃO E CONCLUSÕES,** encontradas no final desta tese, apresentam descrições, interpretações e comentários gerais sobre o artigo científico e os manuscritos incluídos neste trabalho.

As **REFERÊNCIAS BIBLIOGRÁFICAS** referem-se somente às citações que aparecem nos itens **INTRODUÇÃO, DESENVOLVIMENTO, DISCUSSÃO** e **CONCLUSÕES** desta tese.

Os **APÊNDICES** são resultados preliminares apresentados de forma concisa.

RESUMO

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

DIETA SUPLEMENTADA COM DISSELENETO DE DIFENILA ATENUA AS ALTERAÇÕES BIOQUÍMICAS E COMPORTAMENTAIS NO MODELO DE HIPOTIREOIDISMO INDUZIDO POR METIMAZOL

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Data e Local da Defesa: Santa Maria, 14 de dezembro de 2012.

O hipotireoidismo é uma síndrome caracterizada pela produção e secreção insuficiente dos hormônios tireóideos, triiodotironina (T3) e tiroxina (T4), frequentemente diagnosticada em mulheres. Evidências clínicas e experimentais têm relatado a associação entre redução dos hormônios tireóideos, doenças neuropsiquiátricas e estresse oxidativo. O selênio (Se) é um elemento traço essencial às funções tireóideas. Este estudo investigou o efeito da suplementação com disseleneto de difenila sobre parâmetros comportamentais e bioquímicos em um modelo de indução ao hipotireoidismo pelo metimazol, em ratas. Foram avaliados parâmetros comportamentais relacionados à memória e à aprendizagem espacial **(Artigo 1)** e ao comportamento semelhante à depressão **(Manuscrito 1)**. Parâmetros bioquímicos também foram investigados em relação ao estresse oxidativo, à atividade da enzima MAO e à expressão gênica relacionada ao sistema de defesa antioxidante **(Manuscritos 1 e 2)**. No **Artigo 1**, observamos um efeito significativo da dieta suplementada com disseleneto de difenila, ao reverter o déficit relacionando à memória e à aprendizagem espacial nos animais hipotireóideos submetidos à tarefa do Labirinto Aquático de Morris. No **Manuscrito 1** observamos que o hipotireoidismo induzido pelo metimazol causou um comportamento semelhante à depressão avaliada no Teste do Nado Forçado e que a suplementação com disseleneto de difenila provocou a reversão desse efeito. Além disso, os animais suplementados com o disseleneto de difenila apresentaram um desempenho melhor na avaliação do efeito semelhante ao antidepressivo. Em ambos **(Artigo 1 e Manuscrito 1)**, não ocorreram alterações motoras e/ou relacionadas à ansiedade, que pudessem interferir nos resultados. Os parâmetros bioquímicos avaliados nos **Manuscritos 1 e 2** mostraram que o disseleneto de difenila melhorou os marcadores de estresse oxidativo (TBARS, ROS e NP-SH) nas estruturas cerebrais e restaurou a atividade da enzima MAO B **(Manuscrito 1)**. Além disso, o **Manuscrito 2** avaliou os efeitos do hipotireoidismo e da suplementação com disseleneto de difenila sobre a expressão gênica das enzimas antioxidantes e do fator de transcrição NRF-2. Os resultados mostraram que o hipotireoidismo provocou um aumento significativo na expressão das enzimas antioxidantes, correlacionado positivamente ao fator de transcrição NRF-2. Esse efeito foi predominantemente observado no córtex cerebral e no hipocampo, sendo que a suplementação com disseleneto de difenila foi efetiva em normalizar os níveis de expressão gênica antioxidante. Apesar de o hipotireoidismo ter causado um aumento significativo nos níveis de expressão gênica das enzimas SOD, CAT e GPx não se observaram alterações nas atividades enzimáticas. A partir desse estudo pode-se concluir através dos diferentes protocolos experimentais realizados que o hipotireoidismo induzido pelo metimazol causou déficit de memória e de aprendizagem espacial, bem como o comportamento semelhante à depressão. Além disso, observaram-se as alterações relacionadas ao estresse oxidativo e à regulação da expressão gênica relacionada ao sistema de defesa antioxidante. O disseleneto de difenila apresentou efeitos significativos na reversão dos efeitos comportamentais e na restauração dos parâmetros bioquímicos avaliados, sendo que a regulação da expressão de genes relacionados ao sistema de defesa antioxidante acrescenta uma nova interpretação aos seus efeitos farmacológicos e demonstra o seu potencial para tratar as complicações causadas pelo hipotireoidismo.

Palavras-chaves: hipotireoidismo, disseleneto de difenila, comportamento, expressão gênica antioxidante, estresse oxidativo.

ABSTRACT

Thesis of Doctor's Degree
Graduation Course in Biological Sciences: Toxicological Biochemistry
Federal University of Santa Maria, RS, Brazil

DIET SUPPLEMENTED WITH DIPHENYL DISELENIDE ATTENUATES BIOCHEMICAL AND BEHAVIOURAL CHANGES IN THE METHIMAZOLE-INDUCED HYPOTHYROIDISM

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Date and Place of the Defense: Santa Maria, 14th december 2012.

Hypothyroidism is a syndrome resulting from decreased production and secretion of triiothyronine (T3) and thyroxine (T4) hormones, frequently diagnosed in women. Clinical and experimental evidence support the association between reduction in thyroid hormones, neuropsychiatric disorders and oxidative stress. Selenium (Se) is a trace element essential to thyroid functions. The present study investigated the effect of diphenyl diselenide supplementation in a model of methimazole-induced hypothyroidism in female rats. We evaluated behavioral parameters related to memory and spatial learning **(Article 1)** and depressive-like behavior **(Manuscript 1)**. Biochemical parameters were also investigated in relation to oxidative stress, MAO activity and the expression of antioxidant enzymes genes **(Manuscripts 1 and 2)**. In **Article 1**, we observed a significant effect of diet supplemented with diphenyl diselenide in improving memory and spatial learning deficits in hypothyroid animals undergoing the task of Morris Water Maze. In **Manuscript 1** we observed that methimazole-induced hypothyroidism caused a depressive-like behavior assessed in the forced swimming test and that supplementation with diphenyl diselenide caused a reversal of this effect. Furthermore, animals supplemented with diphenyl diselenide performed better in evaluating the antidepressant-like effect. In both **(Article 1 and Manuscript 1)**, there were no motor and/or anxiety alterations, which could interfere with the results. Biochemical parameters in **Manuscript 1 and 2** demonstrated that diphenyl diselenide improved analyzes related to oxidative stress (TBARS, ROS and NP-SH) in brain structures and restored the activity of MAO-B **(Manuscript 1)**. In addition, **Manuscript 2** evaluated the effects of hypothyroidism and supplementation with diphenyl diselenide on gene expression of antioxidant enzymes and transcription factor NRF-2. The results showed that hypothyroidism caused a significant increase in the expression of antioxidant enzymes, positively correlated to the transcription factor NRF-2. This effect was observed predominantly in the cerebral cortex and hippocampus, whereas supplementation with diphenyl diselenide was effective in normalizing the levels of antioxidant gene expression. Although hypothyroidism has caused a significant increase in gene expression levels of SOD, CAT and GPx were not observed changes in enzyme activity. Finally, the results of this study indicate through different experimental protocols that methimazole-induced hypothyroidism caused a deficit in spatial learning and memory, and depressive-like behavior. In addition, there were changes related to oxidative stress and the regulation of gene expression related to the antioxidant defense system. Diphenyl diselenide presented significant effects in promoting the reversal of behavioral effects and restore the biochemical parameters, and its role in regulating the expression of genes related to antioxidant defense system, adding a new interpretation to its pharmacological effects and demonstrates its potential to treat the complications caused by hypothyroidism.

Keywords: hypothyroidism, diphenyl diselenide, behavior, antioxidant gene expression, oxidative stress.

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LISTA DE ABREVIATURAS

AMPc- Adenosina Monofosfato Cíclico
ARE – Elemento de Resposta Antioxidante
CA1 - Corno de Amon 1
CA3 - Corno de Amon 3
CAT - Catalase
DCFH –DA - Diclorofluoresceína-diacetato
Dio - Iodotironina Deiodinase
EROs - Espécies Reativas de Oxigênio
GPx - Glutationa Peroxidase
HO- Heme Oxigenase
H_2O_2 - Peróxido de Hidrogênio
LTP - Potencial de Longo Prazo
MAO - Monoamino- Oxidase
Na^+/ K^+-ATPase - Sódio/Potássio Adenosina Trifosfatase
NP-SH - Tióis não Protéicos
NRF-2- Fator de transcrição nuclear 2
NQO - NADPH: Quinona Oxidoredutases
$-OH^{\bullet}$ - Radical Hidroxil
O_2^- - Radical Superóxido
PPF - Facilitação de Pulso Pareado
Prx - Peroxirredoxina
-SH - Grupos Tióis
SNC - Sistema Nervoso Central
SOD - Superóxido Dismutase
T2- Diiodotironina
T3-Triiodotironina
T4 - Tetraiodotironina
TBARS - Espécies Reativas ao Ácido Tiobarbitúrico
TBG - Globulina Ligante de Tiroxina
TBPA - Pré-Albumina Ligante de Tiroxina
TPO - Tireoperoxidase
TSH - Hormônio Estimulante da Tireóide
TRH - Hormônio Liberador de Tirotrofina
TRα - Receptor Tireóideo Alfa
TRβ - Receptor Tireóideo Beta
TrxR – Tioredoxina Redutase

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SUMÁRIO

1. INTRODUÇÃO

Considera-se desde a década de 90 que o micronutriente essencial selênio tem uma importante função relacionada à biossíntese dos hormônios tireóideos. Desde então, estudos vêm buscando compreender o papel deste elemento nas disfunções tireóideas, a fim de melhor intervir terapeuticamente e reduzir o impacto destas patologias na qualidade de vida de seus portadores. As relações observadas na clínica e em estudos experimentais entre as disfunções tireóideas, distúrbios neuropsiquiátricos e estresse oxidativo apontam a necessidade de melhor compreender os efeitos dos hormônios tireóideos na manutenção das funções do sistema nervoso central (SNC) e as possíveis contribuições do elemento selênio nesses processos.

1.1 Hormônios Tireóideos

Os hormônios tireóideos são sintetizados pela glândula tireóide, a qual se encontra aderida na parte anterior e lateral da laringe e da traquéia. A glândula possui dois lobos (direito e esquerdo) unidos por um istmo de parênquima glandular, localizados abaixo do nível da cartilagem cricóide. Do ponto de vista funcional, as unidades primárias e secretórias da tireóide são os folículos ou ácinos, sendo que as células constituintes dos folículos, denominadas células foliculares, sintetizam os hormônios tireóideos tiroxina (T4) e triidotironina (T3). Os folículos ou tireócitos são constituídos de uma camada de células epiteliais ao redor de uma cavidade constituída de um colóide espesso que contém tireoglobulina. A tireoglobulina origina os hormônios tireóideos com a ligação do iodeto aos seus resíduos tirosil, formando as iodotirosinas (monoiodotirosina e diiodotirosina) que se acoplam para formar a T4 e a T3. Além das células foliculares, o parênquima tireóideo apresenta células parafoliculares ou células C, que sintetizam a calcitonina, responsável pelo controle da homeostasia do cálcio (BIANCO, 2002; GUYTON & HALL, 2006; CAMPBEL, 2008).

O processo de biossíntese dos hormônios tireóideos requer a presença do iodeto, obtido a partir da dieta, principalmente de frutos do mar e de derivados de leite e pão. Devido à variabilidade de disponibilidade de iodo, causada pela distância do mar ou natureza dos solos, há várias décadas adiciona-se iodeto de potássio ao sal de consumo alimentar com a finalidade de diminuir os casos de bócio endêmico (BIANCO, 2002). Países menos desenvolvidos ainda enfrentam esse importante problema de saúde pública.

As complicações e o desenvolvimento de cretinismo endêmico associado à deficiência dos hormônios tireóideos são considerados de alto risco principalmente para gestantes e seus neonatos. A deficiência de iodo é apontada como a principal causa de dano cerebral e de retardo mental que pode ser prevenida em todo o mundo (DELANGE, 2001). Segundo a Organização Mundial da Saúde estima-se que 2 bilhões de pessoas de 130 países estejam expostas a esse risco (CHEN e HETZEL, 2010). As necessidades semanais de iodeto são de aproximadamente 1 mg/ semana, ou cerca de 50 mg/ ano. Para prevenir a deficiência de iodo, comumente o sal de cozinha é iodado com uma parte de iodeto de sódio para cada 100.000 partes de cloreto de sódio (GUYTON e HALL, 2006). O iodo é concentrado no interior dos tireócitos a partir de um transportador ativo, denominado NIS (co-transportador de sódio-iodo), presente na membrana basolateral. O iodo está presente no tireócito em concentrações de 20-40 vezes maiores do que aquelas encontradas no soro (STATHATOS, 2012).

Além do iodo, a atividade da enzima peroxidase (tireoperoxidase-TPO), e um suprimento de peróxido de hidrogênio (H_2O_2) são fundamentais na biossíntese dos hormônios tireóideos. Essa hemeproteína de 90 kDa oxida simultaneamente o iodeto e o radical tirosil da tireoglobulina, unindo-os permanentemente, processo denominado de organificação do iodo. As iodotirosinas permanecem na estrutura da tireoglobulina, até a liberação pela ação de proteases ácidas, peptidases e fosfatase ácida, que é estimulada pela tirotrofina (TSH) (BIANCO, 2002; CAMPBEL, 2008). A TPO é regulada pela concentração de iodo, tendo sua atividade bloqueada pelo excesso do mesmo. Este fenômeno denominado clinicamente de efeito Wolff-Chaikoff pode conduzir ao hipotireoidismo. Um quadro clínico de hipertireoidismo, fenômeno denominado de efeito Jod-Basedaw, caracteriza-se por uma biossíntese exarcebada dos hormônios tireóideos que pode ocorrer quando os tireócitos com concentrações baixas de iodo são novamente expostos a concentrações significativas de iodo (STATHATOS, 2012).

Os hormônios tireóideos T4 e T3 são transportados na circulação ligados às proteínas plasmáticas. Cerca de 80% de T4 estão ligadas à globulina ligante de tiroxina (TBG), 15% estão ligadas a pré-albumina ligante de tiroxina (TBPA) e 5% estão ligadas à albumina. A T3 está distribuída da seguinte forma: 38% com a TBG, 27% com a TBPA e 35% com a albumina. Dessa forma, os hormônios tireóideos estão 99,975% na forma ligada e 0,025% na forma livre. Como a forma livre é responsável pelo efeito biológico, a forma ligada pode ser compreendida como um reservatório hormonal que supre a fração livre, à medida que essa é consumida pelos tecidos (BIANCO, 2002).

Os hormônios tireóideos T4 e T3 são secretados na mesma proporção em que são encontrados na tireoglobulina, em média 100 μg T4/dia e 20 μg T3/dia. A T4 funciona como um pró-hormônio, sendo que os tecidos periféricos transformam 40% da T4 secretada diariamente em T3 através da ação das iodotironinas deiodinases (BIANCO, 2002; MEYER et al., 2007). Os hormônios tireóideos atravessam as membranas das células alvo por difusão passiva e, recentemente foi verificada a importância de transportadores que parecem ser críticos nesse processo, como os transportadores monocarboxilados MCT-8 e MCT-10, e o transportador ânion orgânico OATP1C1 (STATHATOS, 2012).

Os receptores dos hormônios tireóideos incluem duas famílias: TRα e TRβ, que por sua vez dividem-se em várias isoformas com funções celulares específicas, de acordo com a sua localização tecidual. A T3 atua ao interagir com estes receptores específicos, de alta afinidade, no núcleo das células-alvo, alterando a expressão gênica e a síntese de proteínas que levam as diferentes respostas, de acordo com o tecido estimulado. No fígado e musculatura, por exemplo, a modificação da expressão gênica pela T3, ativa o metabolismo energético e aumenta o consumo de oxigênio, enquanto que no tecido nervoso ocorre diferenciação celular sem ativação do metabolismo energético (BIANCO, 2002). Assim, a T3 atua de maneira a ligar-se diretamente aos receptores tireóideos nucleares ou através de fatores de transcrição por ela alterados (WEITZEL et al., 2001, 2003). Além disso, várias evidências indicam que os hormônios tireóideos, especialmente o T4, T3 reverso e o T2 (diiodotironina) podem exercer funções independentemente da interação com receptores, ou seja, ações não-genômicas, e que mesmo os receptores não ocupados por ligantes podem ter função fisiológica (CHASSANDE, 2003; MESOZI et al., 2005; DAVIS et al., 2011).

A biossíntese e a secreção dos hormônios tireóideos a partir da tireoglobulina são basicamente controladas pela tirotrofina ou hormônio estimulante da tireóide (TSH), um hormônio glicoprotéico sintetizado e liberado pela hipófise anterior. A síntese de TSH, por sua vez, é regulada pelo hipotálamo através do hormônio liberador de tirotrofina (TRH) e, também por retroalimentação negativa dos hormônios tireóideos (BIANCO, 2002; CAMPBEL, 2008; STATHATOS, 2012). O TSH liga-se a receptores específicos (receptores TSHR) localizados na membrana basolateral dos tireócitos, estimulando a síntese de AMPc e a captação de iodo, além de possuir efeito trófico sobre a glândula (STATHATOS, 2012).

1.2 Selênio e Hormônios Tireóideos

Desde a década de 90, o selênio foi reconhecido como elemento essencial na biossíntese dos hormônios tireóideos T3 e T4, responsáveis pela diferenciação, crescimento e metabolismo celular (ARTHUR et al., 1992, 1993; KÖHRLE, 1999; BECKETT & ARTHUR, 2005; KÖHRLE & GÄRTNER, 2009). A primeira evidência clínica da importância do selênio em relação às funções tireóideas foi obtida na África Central, onde se observou que somente a suplementação com iodo não era eficaz em restaurar as concentrações fisiológicas de T3 e T4 (VANDERPAS et al., 1990; CONTEMPRÉ et al., 1991). A carência de selênio foi então apresentada como a causa da persistência dos casos de cretinismo mixedematoso e da degeneração fibrótica da glândula dos pacientes (DUMONT et al., 1994; SCHWEIZER et al., 2004). Além disso, já havia também um conjunto de evidências demonstrando que a glândula tireóide possuía as maiores concentrações de selênio do organismo (DICKSON E TOMLINSON, 1967) e que acumulava esse elemento em situações de deficiência (BEHNE et al., 1988), o que levou a suposição e posterior confirmação que esta expressava várias selenoproteínas. Atualmente, as selenoproteínas já identificadas na glândula tireóide, incluem a glutationa peroxidase (GPx1 e GPx3), a tioredoxina redutase, a selenoproteína P, a selenoproteína 15 e as iodotironinas deiodinases (DIO1 e DIO2) (KÖHRLE E GÄRTNER, 2009).

As iodotironinas deiodinases são selenoproteínas essenciais na ativação e metabolismo dos hormônios tireóideos, pois convertem a forma inativa do hormônio tireóideo (T4) na forma ativa (T3) e também metabolizam o T3 na sua forma inativa. A DIO1, além de ser encontrada na tireóide, também é expressa no fígado e rins, tendo como função principal fornecer T3 para o plasma. A DIO2, além da tireóide, é encontrada no SNC, musculatura esquelética e cardíaca, tecido adiposo marrom e placenta, tendo como função principal fornecer T3 para o espaço intracelular. Já a DIO3, pode ser encontrada no útero, placenta, tecidos fetais, SNC e pele, tendo como função principal a inativação de T3 e T4 (MEYER et al., 2007).

1.3 Disfunção da Glândula Tireóide: Hipotireoidismo

O hipotireoidismo é uma síndrome clínica resultante da diminuição da produção e secreção dos hormônios da tireóide, mais comumente devido à doença dessa glândula (hipotireoidismo primário), sendo acompanhado de níveis altos de TSH. Em menos de 5% dos

pacientes, o hipotireoidismo resulta de doença hipotalâmica ou hipofisária (hipotireoidismo secundário); nesse caso, níveis baixos do hormônio da tireóide são acompanhados de níveis séricos de TSH normais ou mesmo baixos. O hipotireoidismo subclínico refere-se ao estado nos quais os níveis altos de TSH são acompanhados por níveis séricos normais de T3 e T4, e o paciente, em geral, é assintomático (CASTRO E GHARIB, 2008). No entanto, alguns pacientes podem já apresentar os sintomas característicos do hipotireoidismo e necessitarem de reposição hormonal. A freqüência de progressão para o hipotireoidismo clínico é de 2-5%/ano (JONES et al., 2009).

A prevalência e a incidência do hipotireoidismo são maiores no sexo feminino e aumentam com o passar da idade (MORGANTI et al., 2005; CASTRO E GHARIB, 2008). A Doença Auto-Imune da Tireóide ou Tireoidite de Hashimoto, a deficiência de iodo, a redução do tecido tireóideo por iodo radioativo ou a cirurgia usada no tratamento da Doença de Graves ou de câncer da tireóide são fatores envolvidos na etiologia do hipotireoidismo primário. A etiologia mais comum do hipotireoidismo subclínico é a Tireoidite de Hashimoto (NOGUEIRA, 2005). O hipotireoidismo central ou secundário resulta de doenças hipotalâmicas ou hipofisária, sendo mais raro e decorrente de tumores ou cirurgia nessa região (MOSCA FILHO, 2002; CASTRO e GHARIB, 2008).

Nos estágios iniciais da doença, os sintomas são inespecíficos, como: mialgia, artralgia, câimbras, pele seca, dores de cabeça, menorragia, unhas quebradiças, cabelos finos, palidez e sintomas da síndrome do carpo. Quando o hipotireoidismo se acentua, evidencia-se edema periférico, constipação, dispnéia e ganho de peso. Outras manifestações incluem edema pericárdico, ascite, audição diminuída e hipertensão diastólica. Em estágios avançados de hipotireoidismo primário pode ocorrer galactorréia. Os sintomas psiquiátricos incluem depressão, demência, mudança de personalidade e, raramente, psicose (NOGUEIRA, 2005; JONES et al., 2009). O bócio também pode ocorrer, pois a glândula aumenta de tamanho devido à falta dos hormônios tireóideos (BISI e ANDRADE, 2002). Dislipidemias são frequentemente descritas nos pacientes com hipotireoidismo e contribuem para quadros de aterosclerose, tendo sido relatados níveis elevados de LDL e baixos níveis de HDL (NOGUEIRA, 2005; JONES et al., 2009).

O hipotireoidismo, independente da etiologia ou gravidade, é tratado através da reposição, usando-se o hormônio tireóideo T4, a T3 ou a combinação destes. Alguns estudos sugerem que a combinação de T4 e T3 seria uma forma mais fisiológica de reposição hormonal, promovendo melhora do humor e de funções neuropsicológicas (BUNEVICIUS et al., 1999). No entanto, alguns estudos controlados não evidenciaram vantagens da

combinação dos fármacos (CLYDE et al., 2003; WALSH et al., 2003; ESCOBAR-MORREALE, 2005). O tratamento é individualizado, de acordo com a resposta clínica e os resultados dos ensaios de avaliação da glândula tireóide. É importante salientar que em muitos casos, o tratamento segue por toda a vida do paciente e que o início deve ser o mais breve possível após a detecção da deficiência de produção dos hormônios tireóideos, a fim de prevenir suas complicações (NOGUEIRA, 2005; CASTRO e GHARIB, 2008).

A Associação Americana de Tireóide recomenda o rastreamento da disfunção tireóidea por meio da avaliação do TSH em adultos, a partir dos 35 anos de idade e a cada 5 anos posteriormente, sobretudo no sexo feminino. Indivíduos com manifestações clínicas atribuíveis a disfunções tireóideas ou aqueles que possuem fatores de risco para o seu desenvolvimento devem realizar avaliações mais frequentes do TSH (SINGER et al., 1995; LADENSON et al., 2000; MAIA e VAISMAN, 2006).

1.4 Hipotireoidismo e Distúrbios Neuropsiquiátricos

Estudos clínicos têm demonstrado a associação entre hipotireoidismo e distúrbios neuropsiquiátricos como depressão, ansiedade e dificuldades de memória e aprendizagem (OSTERWEIL et al., 1992; PLACIDI et al., 1998; DEMET et al., 2002, VAN BOXTEL et al., 2004; GUIMARÃES et al., 2009). Essa correlação também tem sido evidenciada em modelos animais de hipotireoidismo induzido na vida adulta e no hipotireoidismo congênito (DARBRA et al., 1995; KULIKOV et al., 1997; SALA-ROCA et al., 2002; GERGES et al., 2004; TONG et al., 2007; REIS-LUNARDELLI, 2007, WILCOXON et al., 2007). Dentre os mecanismos que buscam explicar essa associação destacam-se as alterações morfológicas encontradas nos neurônios do hipocampo nas regiões CA3 e CA1 (SALA-ROCA et al., 2008) e modulação de rotas apoptóticas (AMBROGINI et al., 2005; DESOUZA et al., 2005; ALVA-SÁNCHEZ et al., 2009; SINHÁ et al., 2009; ZHANG et al., 2009). Além disso, estudos relacionados mostram que o hipotireoidismo reduz significativamente a LTP (Long-Term Potentiation) e a PPF (Pared-Pulse Facilitation), mecanismos ligados à formação da memória e aprendizado (SUI et al., 2006; ZHU et al., 2006).

1.5 Hipotireoidismo e Estresse Oxidativo

Em termos de mecanismos, tem sido evidenciado também que os baixos níveis de T3 e T4 estão relacionados com estresse oxidativo. Vários trabalhos demonstram que o

hipotireoidismo induz o estresse oxidativo a partir de alterações nos sistemas de defesa enzimáticos e não-enzimáticos em diversos tecidos (DAS & CHAINY, 2004; CANO-EUROPA et al., 2008; ERDAMAR et al., 2008; BHANJA & CHAINY, 2010), bem como, estimula e deprime a expressão de genes antioxidantes (CHATTOPADHYAY et al., 2007; SUBUDHI & CHAINY, 2010). Além desses efeitos, Mittag et al. (2010) evidenciou que os hormônios tireóideos podem alterar as concentrações séricas de selênio e a biossíntese de selenoproteínas. Recentemente, foi demonstrado que a suplementação com selenito de sódio, um composto inorgânico de selênio, reverte parâmetros de estresse oxidativo em rins, cérebro total e cerebelo de ratos submetidos ao hipotireoidismo neonatal (AMARA et al., 2009, 2010).

1.6 Disseleneto de Difenila

O disseleneto de difenila é um composto orgânico de selênio reconhecido pela sua potente propriedade antioxidaante, a qual está fortemente relacionada com sua atividade mimética às enzimas glutationa peroxidase (GPx) e tireodoxina redutase (TrxR) (NOGUEIRA et al., 2004; POSSER et al., 2008; NOGUEIRA & ROCHA, 2010; FREITAS & ROCHA, 2011). Um potente papel neuroprotetor vem sendo associado ao composto em diferentes modelos experimentais *in vitro* e *in vivo* (POSSER et al., 2008; NOGUEIRA & ROCHA, 2010). Estudos *in vivo* têm constatado que o composto exibe efeito semelhante ao antidepressivo (SAVEGNAGO, 2007; 2008), efeito semelhante ao ansiolítico (GHISLENI et al., 2008; SAVEGNAGO et al., 2008), e causa melhora da função cognitiva no Teste de Reconhecimento do Objeto em camundongos (ROSA et al., 2003) e da memória e aprendizagem espacial em ratos submetidos ao Labirinto Aquático de Morris e Labirinto T (STANGHERLIN et al., 2008). No entanto, cabe salientar que em doses elevadas, o disseleneto de difenila induz efeitos tóxicos como depleção de tióis, formação de espécies reativas e ação pró-convulsivante (NOGUEIRA et al, 2001, 2004; BRITO et al., 2006; NOGUEIRA & ROCHA, 2010). Além da dose, a toxicidade do composto depende da via de administração, do veículo, da espécie e da idade dos animais utilizados no protocolo experimental (NOGUEIRA et al., 2003; PRIGOL et al., 2009; 2010).

Especificamente com relação à suplementação na dieta, poucos estudos farmacológicos foram realizados com o composto. No entanto, já foi demonstrado que a ingestão prolongada de dietas suplementadas com disseleneto de difenila (1-10 ppm) reduziu alterações bioquímicas e danos oxidativos causados pela exposição ao carcinógeno N-nitroso-

metil-uréia e ao agente diabetogênico estreptozotocina em ratos, sem causar sinais de toxicidade (BARBOSA et al., 2008[a, b]).

1.7 Justificativa

O hipotireoidismo é uma disfunção endócrina predominantemente encontrada no sexo feminino que causa importantes prejuízos à qualidade de vida. As relações observadas entre o hipotireoidismo e os distúrbios neuropsiquiátricos salientam a importância dos hormônios tireóideos na manutenção das funções do SNC, que vem sendo extensivamente pesquisada ao longo dos últimos anos. A interação entre o micronutriente essencial selênio e as funções tireóideas foram reconhecidas há pouco mais de 20 anos, e contribuiram para a essencialidade deste elemento à saúde humana e animal. Desde então, o conhecimento de várias selenoproteínas, algumas até com função não bem definida, tem demonstrado a excepcional contribuição deste elemento para a manutenção das funções fisiológicas.

O disseleneto de difenila possui propriedades farmacológicas bastante exploradas em diferentes protocolos experimentais *in vitro* e *in vivo*, salientando-se o efeito antioxidante e neuroprotetor. No entanto, não existem dados disponíveis na literatura acerca dos efeitos de uma suplementação com disseleneto de difenila em modelos de hipotireoidismo. Dadas as relações estabelecidas entre selênio, função tireóidea, estresse oxidativo e distúrbios neuropsiquiátricos, este estudo foi realizado com o objetivo de investigar os possíveis benefícios oriundos da suplementação com disseleneto de difenila sobre as alterações bioquímicas e comportamentais induzidas pelo hipotireoidismo em um modelo experimental que empregou o fármaco anti-tireóideo metimazol em ratas adultas. De forma geral, a presente pesquisa visa oferecer apoio para estudos clínicos na busca de estratégias terapêuticas para o tratamento das complicações causadas pelo hipotireoidismo.

2. DESENVOLVIMENTO

2.1 Hipotireoidismo Experimental

O hipotireoidismo é a disfunção tireóidea mais comum, resultante da produção insuficiente de T3 e T4. Entre as estratégias usadas para o estudo desta disfunção endócrina, destaca-se o uso de fármacos anti-tireóideos, como o metimazol, o carbimazol (pró-droga do metimazol) e o propiltiouracil, que são comumente utilizados no tratamento do hipertireoidismo (GILMAN et al., 1996; BANDYOPADHYAY et al., 2002). Além disso, pode-se realizar a tireoidectomia total ou parcial, remoção do iodo da dieta (KULIKOV et al., 1997), bem como exposição ao iodo radioativo. A administração de metimazol provoca inibição das reações de oxidação catalisadas pela tireoperoxidase (TPO), responsáveis pela iodação dos grupos tirosil da tiroglobulina e formação das iodotirosinas; enquanto que o propiltiouracil além de promover a inibição da tireoperoxidase, também inibe a desiodação periférica da tiroxina em triiodotironina (GILMAN et al., 1996).

Os fármacos anti-tireóideos são concentrados na glândula tireóide, embora causem efeitos adversos decorrentes de ações extra-tireóideas, que incluem a inibição da lactoperoxidase, responsável pela atividade bactericida das secreções exócrinas, a estimulação das secreções ácidas gástricas e de pepsinogênio, a disfunção hematológica, a supressão do sistema imune (agranulocitose, anemia aplásica e púrpura) e perdas auditivas, olfativas e gustativas (BANDYOPADHYAY et al., 2002). Os efeitos adversos dos fármacos anti-tireóideos podem ser atribuídos à inibição de peroxidases que desempenham importantes funções fisiológicas, como a MPO (mieloperoxidase), a EPO (peroxidase eosinofílica) e a catalase. Além disso, o metabolismo do metimazol via sistema FMO (flavina monooxigenase) ou Citocromo P450 produz intermediários reativos que se ligam covalentemente a proteínas e inativam as isoenzimas do Citocromo P450, bem como provocam dano celular (MIZUTANI et al., 1999; BANDYOPADHYAY et al., 2002; SAKAMOTO et al., 2007).

O hipotireoidismo induzido pelo metimazol causou dano celular em baço, coração, fígado, pulmões e rins de ratos tratados com 60 mg metimazol/ kg de peso/dia (CANO-EUROPA et al., 2011). A suplementação com selenito de sódio restaurou parâmetros hematológicos e as atividades das enzimas antioxidantes (GPx, SOD e CAT), além de diminuir os níveis de TBARS em plasma de ratas e de sua prole submetidas a um modelo de hipotireoidismo do 14° dia gestacional até o 14° dia pós- natal (AMARA et al., 2010).

2.2 Hipotireoidismo Experimental e Memória

O processo de memória envolve a aquisição, a formação, a conservação e a evocação de informações. O aprendizado resulta do armazenamento da informação como consequência da prática que resulta em uma alteração relativamente permanente do comportamento real ou potencial. A evocação significa recordar, lembrar, sendo definida como a emissão de determinada resposta quando o sujeito experimental é recolocado na situação ambiental em que esta foi elaborada ou selecionada. Só lembramos aquilo que gravamos, ou seja, aquilo que foi aprendido (IZQUIERDO, 2002).

A avaliação da aprendizagem espacial e memória em ratos podem ser realizadas no Labirinto Aquático de Morris, que foi desenvolvido em 1981 (MORRIS, 1981, 1986; D'HOOGE & DEYN, 2001). O Labirinto Aquático de Morris consiste em um reservatório de água opaca em uma sala contendo sinalizações externas para facilitar a aprendizagem dos ratos. Os animais são submetidos a uma série de treinos visando à aquisição do aprendizado, sendo que em geral, são quatro blocos de treinos usando as quatro posições em que o reservatório é dividido e, ao encontrar a plataforma submersa o animal permanece nela por um tempo pré-determinado (por exemplo, 10 segundos). No último dia, a plataforma é retirada e o animal é colocado no aparato até encontrar o ponto onde a plataforma encontrava-se, registrando-se o tempo gasto até o encontro da plataforma, número de cruzamentos, velocidade, distância percorrida total, tempo gasto no quadrante oposto a plataforma e tempo gasto no quadrante que continha a plataforma. Animais bem treinados demonstram uma maior preferência pelo quadrante que continha a plataforma, gastando 50% ou mais de seu tempo na exploração desse espaço (D'HOOGE & DEYN, 2001).

Com relação ao hipotireoidismo, vários estudos experimentais têm demonstrado que animais hipotireóideos apresentam déficits de aprendizagem espacial e memória (REID et al., 2007; TONG et al., 2007; WILCOXON et al., 2007; REIS-LUNARDELLI, 2007). Os mecanismos envolvidos em tais déficits usualmente são atribuídos à perda das funções comumente desempenhadas pelos hormônios tireóideos no SNC: neurogênese, sinaptogênese, excitabilidade e plasticidade neuronal (SALA-ROCA et al., 2008; ALVA-SÁNCHEZ et al., 2009; ZHANG et al., 2009). Além disso, dados da literatura mostram que o hipotireoidismo reduz significativamente a LTP e a PPF, bases aceitas como responsáveis pela formação da memória e aprendizado, e a reposição hormonal melhora os déficits de LTP e PPF (SUI et al., 2006; ZHU et al., 2006).

O Teste do Campo-Aberto tem como objetivo avaliar a atividade locomotora e exploratória dos animais em um espaço novo, testando os efeitos de ambientes não familiares sobre a emocionalidade em ratos (WALSH & CUMMINS, 1976; ANNAU, 1986). Pode ser considerado um tipo de aprendizado não associativo mais elementar, onde o animal precisa habituar-se a um novo ambiente (VIANNA, 2000). O teste é realizado durante um intervalo de tempo pré-determinado avaliando-se diferentes parâmetros: o pressuposto básico é que para explorar o novo ambiente o animal deve movimentar-se, logo se avaliam número de cruzamentos (taxa de ambulação ou atividade motora) e tempo e frequência de outros comportamentos, como "rearings" (levantar-se sobre as patas traseiras), "grooming" (reflexo de auto-limpeza), "freezing" (permanecer parado) e deposição de bolos fecais na arena do teste.

Neste contexto, diferentes respostas são observadas em estudos avaliando animais hipotireóideos. Há relatos de que o hipotireoidismo induzido por propiltiouracil na vida adulta não causa alterações motoras em ratos (REIS-LUNARDELLI et al., 2007), enquanto que na fase perinatal altera a atividade motora, representada por um aumento na taxa de ambulação (DARBRA et al., 1995).

O Teste do Labirinto em Cruz Elevado vem sendo empregado com a finalidade de avaliar a ansiedade em animais, bem como o efeito de drogas sobre esse comportamento (PELLOW et al., 1985; BLATT & TAKAHASHI, 1999; BELZUNG & GRIEBEL, 2001). O aparato consiste em uma cruz com dois braços abertos e dois braços fechados, conectados por um espaço central, onde o animal é inicialmente colocado. Durante um tempo pré-determinado, o animal poderá explorar este ambiente e será avaliado o número de entradas e o tempo gasto na parte fechada e aberta do aparato. Os braços abertos representam um caráter exploratório, enquanto que os braços fechados são seguros e naturalmente atrativos para os animais, logo fármacos ansiolíticos tendem a aumentar a permanência dos animais na parte aberta do aparato (DAWSON & TRICKLEBANK, 1995; BELZUNG & GRIEBEL, 2001).

Os efeitos do hipotireoidismo no Teste do Labirinto em Cruz Elevado parecem variar de acordo com a idade do animal no momento da indução. Foi demonstrado que o hipotireoidismo perinatal induzido por metimazol causa um efeito ansiolítico nos animais expostos ao Teste do Labirinto em Cruz, enquanto que a indução na vida adulta não altera estes parâmetros (DARBRA et al, 1995; SALA-ROCA et al., 2002). Da mesma forma, a indução de hipotireoidismo por propiltiouracil na vida adulta também não modifica o comportamento relacionado à ansiedade (REIS-LUNARDELLI et al., 2007).

2.3 Hipotireoidismo Experimental e Depressão

Os hormônios tireóideos são essenciais para a manutenção das funções desempenhadas pelo SNC. A deficiência dos mesmos, durante o desenvolvimento do SNC, está associada com danos cerebrais irreversíveis, os quais causam retardo mental acentuado. No SNC de animais adultos observa-se um prejuízo acentuado nas regiões neurogênicas do giro denteado e na plasticidade neuronal do hipocampo (ALVA-SÁNCHEZ et al., 2009). Numerosos dados clínicos vêm relacionando a disfunção tireóidea com depressão (DEMET et al., 2002, VAN BOXTEL et al., 2004; GUIMARÃES et al., 2009). Neste contexto, foi recentemente demonstrado que a terapia adjuvante com hormônios tireóideos melhora o efeito antidepressivo dos fármacos tricíclicos e inibidores seletivos da recaptação de serotonina e pode ser efetiva no tratamento da depressão resistente aos fármacos disponíveis (PILHATSCH et al., 2011).

O Teste do Nado Forçado (“Desespero Comportamental”) foi desenvolvido como um modelo animal de depressão com o objetivo de realizar o “screening” de fármacos com potencial antidepressivo. Consiste basicamente na exposição do animal a uma situação extrema onde este é colocado em recipientes estreitos com água, sendo obrigado a permanecer nadando para manter-se vivo. O teste é realizado em duas etapas, sendo que a primeira (pré-teste) tem a duração de 15 minutos e a segunda, 24 horas mais tarde, de 5 minutos. Avaliam-se os tempos relacionados à imobilidade do animal, quando este executa mínimos movimentos para manter a cabeça fora da água, demonstrando a perda de motivação na luta pela vida. Em geral, os fármacos antidepressivos promovem um aumento da latência para início da imobilidade e um menor tempo total de imobilidade (PORSOLT et al., 1977).

O hipotireoidismo experimental induzido por tireoidectomia aumenta o tempo de imobilidade em ratos, demonstrando o efeito semelhante à depressão (KULIKOV et al., 1997). Além disso, experimentos com camundongos mutantes para o receptor TRα-1, que possuem reduzida afinidade para T3, mostram que o hipotireoidismo induz comportamento semelhante ao depressivo, observado no Teste do Nado Forçado (PILHATSCH et al., 2010).

2.4 Hipotireoidismo Experimental e Alterações Bioquímicas

2.4.1 Estresse Oxidativo

O estresse oxidativo pode ser definido como a perda do equilíbrio entre oxidantes e antioxidantes intracelulares. As espécies reativas de oxigênio, tais como radical superóxido (O_2^-), radical hidroxil (-$OH^\bullet$) e peróxido de hidrogênio (H_2O_2), quando em excesso podem provocar danos em biomoléculas, principalmente aos lipídios, proteínas e ácidos nucléicos, induzindo morte celular por necrose ou apoptose. Os mecanismos de defesa incluem os antioxidantes não enzimáticos, como as vitaminas A, E, C, a glutationa e elementos como o selênio, e os antioxidantes enzimáticos (superóxido dismutase (SOD), catalase (CAT), glutationa peroxidase (GPx) e tioredoxina redutase (TrxR) (SIES et al. 1997).

O SNC é particularmente sensível ao dano oxidativo devido ao seu alto consumo de oxigênio e membranas ricas em ácidos graxos poliinsaturados. Assim, o estresse oxidativo vem sendo relacionado com a gênese e/ou desenvolvimento de diversas doenças neurodegenerativas, como a Doença de Alzheimer, a Doença de Parkinson e a Doença de Huntington (VRIES et al., 2008; UTTARA et al., 2009; FREEMAN & KELLER, 2012).

Dados da literatura mostram que o hipotireoidismo induz estresse oxidativo em diferentes tecidos (AMARA et al., 2009, 2010; SAKR et al., 2011). Especificamente no SNC, danos oxidativos têm sido observados em cérebro total e cerebelo de ratos submetidos a um modelo de hipotireoidismo neonatal (AMARA et al., 2009, 2010). Similarmente, o hipotireoidismo induzido na vida adulta causa estresse oxidativo na amígdala e no hipocampo (CANO-EUROPA et al., 2008). Em termos de proteção, destaca-se a suplementação com selenito de sódio, composto inorgânico de selênio, que demonstrou foi capaz de reverter danos oxidativos e de melhorar os perfis de defesa antioxidante em rins, cérebro total, cerebelo e testículos de ratos hipotireóideos (AMARA et al., 2009, 2010; SAKR et al., 2011).

Além disso, os hormônios tireóideos podem influenciar parâmetros de estresse oxidativo através da modulação da expressão de genes relacionados com os mecanismos de defesa antioxidante enzimático (CHATTOPADHYAY et al., 2007; SUBUDHI & CHAINY, 2010).

2.5 Hipotireoidismo Experimental e Expressão Gênica

2.5.1 Expressão Gênica relacionada à Defesa Antioxidante

Estudos têm evidenciado que o hipotireoidismo além de causar estresse oxidativo (CANO-EUROPA et al., 2008; AMARA et al., 2009, 2010; SAKR et al., 2011), pode modular os processos de defesa antioxidante (DAS & CHAINY, 2001, 2004; BHANJA & CHAINY, 2010). As enzimas antioxidantes CAT, SOD, GPx e TrxR são sensíveis as concentrações de hormônios tireóideos (CHATTOPADHYAY et al., 2007; SUBUDHY & CHAINY, 2010). Além disso, a via do fator de transcrição citoprotetor denominado Nrf-2, uma importante via de sinalização ativada pelo estresse oxidativo, também pode ser regulada pela ação dos hormônios tireóideos (VENDITTI et al., 2009). A via coordenada pelo Nrf-2 atua em resposta a estímulos oxidantes, promovendo a transcrição de diversas enzimas antioxidantes e relacionadas ao metabolismo de xenobióticos, de forma a neutralizar, detoxificar e remover o estímulo nocivo (LI et al., 2008).

Todas as células possuem defesas antioxidantes enzimáticas que são estimuladas a partir da geração de radicais livres. Os genes que codificam estas proteínas têm um elemento promotor comum denominado elemento de resposta antioxidante (ARE), coordenado pelo Nrf-2. Quando o insulto oxidativo ocorre, a proteína repressora Keap-1 libera o Nrf-2 que se encontra no citoplasma. No núcleo, este se liga às regiões ARE e estimula a expressão dos genes das enzimas antioxidantes. A sinalização via Nrf-2 ativa a expressão de enzimas como CAT, SOD, GPx, TrxR, heme oxigenases (HO), peroxirredoxinas (Prx) e NADPH: quinona oxidoredutases (NQO) (JAISWAL, 2004; VRIES et al., 2008; KASPAR et al., 2009).

3. OBJETIVOS

3.1 Objetivo Geral

O presente estudo teve como objetivo avaliar os efeitos da ingestão de uma dieta suplementada com disseleneto de difenila sobre alterações bioquímicas e comportamentais induzidas pelo hipotireoidismo a partir da administração do fármaco anti-tireóideo metimazol em ratas adultas.

3.2 Objetivos Específicos

Capítulo I:

- Analisar o efeito da suplementação com disseleneto de difenila sobre as possíveis alterações de atividade locomotora e exploratória causadas por hipotireoidismo;
- Verificar o efeito da suplementação com disseleneto de difenila sobre o comportamento relacionado à ansiedade em ratas adultas com hipotireoidismo;
- Averiguar o efeito da suplementação com disseleneto de difenila sobre os déficits de aprendizagem e memória espacial induzidos por hipotireoidismo;

Capítulo II:

- Avaliar o comportamento motor e exploratório em ratas adultas expostas ao metilmazol;
- Verificar o comportamento semelhante à depressão em ratas adultas expostas ao metilmazol;
- Avaliar os efeitos da suplementação com disseleneto de difenila sobre os comportamentos motor, exploratório e semelhante à depressão em ratas adultas expostas ao metimazol;
- Analisar os efeitos da suplementação com disseleneto de difenila sobre parâmetros de estresse oxidativo em estruturas cerebrais de ratas com hipotireoidismo;
- Avaliar os efeitos da suplementação com disseleneto de difenila sobre a atividade da enzima monoamino-oxidase (MAO) em cérebro total de ratas adultas hipotireóideas.

Capítulo III:

- Avaliar os efeitos da suplementação com disseleneto de difenila sobre a atividade das enzimas antioxidantes CAT, SOD e GPx em córtex cerebral, estriado e hipocampo de ratas adultas com hipotireoidismo;
- Analisar os efeitos da suplementação com disseleneto de difenila sobre os níveis de peroxidação lipídica, espécies reativas de oxigênio e tióis não-protéicos em córtex cerebral, estriado e hipocampo de ratas adultas com hipotireoidismo;
- Averiguar o efeito da suplementação com disseleneto de difenila sobre a expressão de genes envolvidos em processos de defesa antioxidante em córtex cerebral, estriado e hipocampo de ratas adultas hipotireóideas;

4. ARTIGOS CIENTÍFICOS

Os resultados que fazem parte desta tese serão apresentados sob a forma de um artigo científico e dois manuscritos em fase de redação. Os itens Materiais e Métodos, Resultados, Discussão dos Resultados e Referências Bibliográficas, encontram-se no artigo científico e nos manuscritos.

4.1 Artigo Científico 1:

4.1.1 Consumo de dieta com disseleneto de difenila melhora déficits de memória e aprendizagem espacial em ratas com hipotireoidismo

Diphenyl diselenide diet intake improves spatial learning and memory deficits in hypothyroid female rats.

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International Journal of Developmental Neuroscience, 2012
http://dx.doi.org/10.1016/j.ijdevneu.2011.12.011

Int. J. Devl Neuroscience 30 (2012) 83–89

Contents lists available at SciVerse ScienceDirect

International Journal of Developmental Neuroscience

journal homepage: www.elsevier.com/locate/ijdevneu

Diphenyl diselenide diet intake improves spatial learning and memory deficits in hypothyroid female rats

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ARTICLE INFO

Article history:
Received 14 August 2011
Received in revised form
16 December 2011
Accepted 26 December 2011

Keywords:
Selenium
Diphenyl diselenide
Hypothyroidism
Methimazole
Behavior

ABSTRACT

Cognitive deficits have been observed in different animal models of adult-onset hypothyroidism. Thus, this study was delineated to evaluate whether diphenyl diselenide, an organoselenium compound with neuroprotective and antioxidant properties, could afford protection against the detrimental effects of hypothyroidism on behavioral parameters. Hypothyroidism condition was induced in female rats by continuous exposure to methimazole (MTZ) at 20 mg/100 ml in the drinking water, during 3 months. MTZ-induced hypothyroid rats were fed with either standard or a diet containing 5 ppm of diphenyl diselenide for 3 months. Behavioral assessments were performed monthly, in the following order: elevated plus maze, open field and Morris water maze. The levels of thyroid hormones in the animals exposed to MTZ were lower than control until the end of experimental period. The rats exposed to MTZ had a significant weight loss from the first month, which was not modified by diphenyl diselenide supplementation. In elevated plus maze test, MTZ exposure caused a reduction on the number of entries of animals in closed arms, which was avoided by diphenyl diselenide supplementation. In Morris water maze, the parameters latency to reach the platform and distance performed to find the escape platform in the test session were significantly greater in MTZ group when compared to control. These cognitive deficits observed in MTZ-induced hypothyroid rats were restored by dietary diphenyl diselenide. The group fed with diphenyl diselenide alone exhibited a better spatial learning and memory capability in some parameters of Morris water maze when compared to the control group. In summary, our data provide evidence of the effectiveness of dietary diphenyl diselenide in improving the performance of control and hypothyroid rats in the water maze test.

1. Introduction

Hypothyroidism is one of the most common thyroid disorders in the general population, especially prevalent in women (Morganti et al., 2005). Clinical observations have shown that this thyroid disease is closely related to psychiatric and cognitive disorders, such as impaired memory, anxiety and depression (Demet et al., 2002; Van Boxtel et al., 2004; Guimarães et al., 2009). In fact, the thyroid hormones are essential for maturation and normal brain functions in vertebrates and their deficiency, especially during a critical period of development, affects cognitive functions and learning (Darbra et al., 1995, 2003; Vara et al., 2002; Sala-Roca et al., 2008).

It has been postulated that the thyroid gland requires high concentrations of selenium for selenoproteins expression, which are important in maintaining the physiological levels of active hormones T3 (triiodothyronine) and T4 (thyroxine) (Köhrle, 1999; Köhrle and Gärtner, 2009); and that selenium deficiency is linked with a decrease in the levels of these hormones (Arthur et al., 1992, 1993). Really, this trace element is considered essential to the biosynthesis, activation and metabolism of the thyroid hormones (Köhrle, 1999; Köhrle and Gärtner, 2009).

The importance of selenium to the thyroid hormone biosynthesis came from myxedematous cretinism cases, predominantly observed in Central Africa, which can be caused by combined iodine and selenium deficiency with exposure to nutritional goitrogens (Vanderpas et al., 1990). In this context, experimental evidence

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doi:10.1016/j.ijdevneu.2011.12.011

have demonstrated that the supplementation with iodine alone does not promote the improvement of thyroid function (Schweizer et al., 2004) and that inadequate selenium supply associated with iodine deficiency increases the severity of hypothyroidism (Arthur et al., 1993). Additionally, recent findings in literature reported that selenium supplementation improves cerebrum and cerebellum impairments as well as alleviates oxidative stress and hematological disorders in rats with hypothyroidism induced by the antithyroid drug methimazole (MTZ) (Amara et al., 2009, 2010). Thus, the use of selenium has received a growing interest to the hypothyroidism therapy.

Numerous selenium compounds have been proposed as potential pharmacological agents mainly due their thiol-peroxidase-like and antioxidant activities (Rayman, 2000; Posser et al., 2008; Nogueira and Rocha, 2010). In fact, selenium element acts as a cofactor of the glutathione-peroxidase family that recycles glutathione, reducing lipid peroxidation by catalyzing the reduction of peroxides (Navarro-Alarcon and Cabrera-Vique, 2008). However, selenium compounds also have been found to induce toxic effects in rodents, especially when used at high doses (Nogueira et al., 2001, 2004; Brito et al., 2006; Nogueira and Rocha, 2010). Regarding the toxicity, there are several points of evidence in the literature indicating that the toxic action exhibited by some selenium compounds is highly associated with their pro-oxidant activity, which can be connected with ROS formation and thiol depletion (Nogueira et al., 2004; Nogueira and Rocha, 2010).

Diphenyl diselenide is a simple synthetic organoselenium compound, which exhibits numerous interesting effects when used in pharmacological doses. It has been reported that this compound is a potent anti-inflammatory, antioxidant, antidepressant and anxiolytic-like agent in different *in vivo* experimental models (Nogueira et al., 2004; Ghisleni et al., 2008; Savegnago et al., 2007, 2008). In addition, experimental data support the idea that diphenyl diselenide improves cognitive function and learning of mice (Rosa et al., 2003) and enhances acquisition and retention of spatial memory in rats (Stangherlin et al., 2008). On the order hand, there are also strong evidence that diphenyl diselenide may induce convulsant activity in rodents (Nogueira et al., 2003). The occurrence of seizures episodes caused by compound depends on the routes of administration, dose and animal species (Nogueira et al., 2003; Prigol et al., 2009).

Based on the evidence addressed previously, this study aims to investigate whether the intake of a diet supplemented with diphenyl diselenide could attenuate the behavioral changes related to motor and exploratory activities, anxiety, learning and memory in MTZ-induced hypothyroid female rats.

2. Material and methods

2.1. Animals and reagents

Forty adult female Wistar rats (150–200 g) purchased from our own breeding colony were acclimated for 10 days before the beginning of the experiments. The animals were housed in plastic cages and maintained at 22–24 °C, on a 12 h light/12 h dark cycle, with free access to food (Supra®, Brazil) and water. All experiments were performed in accordance with guidelines of the Committee on Care and Use of Experimental Animal Resources, Federal University of Santa Maria, Santa Maria, RS, Brazil. Methimazole was purchased from Sigma Chemical Co. (St. Louis, MO, USA). Diphenyl diselenide was synthesized according to the literature method (Paulmier, 1986). Analysis of the ^{1}H NMR and ^{13}C NMR spectra showed analytical and spectroscopic data in full agreement with its assigned structure.

2.2. Experimental protocol

2.2.1. Hypothyroidism induction

Hypothyroidism was induced by continuous exposure to the antithyroid drug MTZ at 20 mg/100 ml in the drinking water, during 3 months. After 1 and 3 months of treatment some animals were euthanized and the whole blood collected by cardiac puncture for measurement of total thyroid hormones (tT3 and tT4) levels. The work was specifically conducted in female rats in order to mimic more appropriately certain features of hypothyroidism that is an endocrine dysfunction whose prevalence and incidence is higher in females (Morganti et al., 2005).

2.2.2. Dietary treatment

The female rats were randomly divided into four experimental groups ($n = 10$): (1) control; (2) methimazole; (3) diphenyl diselenide (Se) and (4) diphenyl diselenide plus methimazole (Se + MTZ). Control and MTZ groups were supplied with a standard diet, whereas the groups Se and Se + MTZ were supplemented with a diet containing 5 ppm of the selenium compound diphenyl diselenide. The choice of this concentration was based on previous studies, which show that chronic diphenyl diselenide diet intake, in the range of 1–10 ppm, did not cause overt signals of toxicity in rats (Barbosa et al., 2008a,b). Oral MTZ exposure and diphenyl diselenide diet intake were concomitantly maintained for a period of 3 months. The food was prepared in an industrial mixer to allow the uniformity of the mixture, and the compound diphenyl diselenide was dissolved in soybean oil. After preparation, the diets were frozen (−20 °C) until they were used. The diets were offered *ad libitum* to the animals and the consumption of feed, water and MTZ solution was evaluated daily.

2.2.3. Behavioral testing

The behavioral assessments were performed monthly, in the following order: elevated plus maze, open field and Morris water maze; and conducted between 8:00 am and 2:00 pm.

2.2.3.1. Elevated plus-maze test. The apparatus consisted of four 10 cm wide, 50 cm long corridors at 90° angles and 50 cm above the floor level. Two opposite corridors were surrounded by 50 cm high wood walls (closed arms), and the other two were not (open arms). Rats were placed in the middle of the four arms facing the open arm and left to explore the apparatus for 5 min. The number of entries and time spent in open and closed arms were evaluated as a measure of anxiety of the animals (Belzung and Griebel, 2001). The apparatus was cleaned between assessments with a 20% ethanol solution.

2.2.3.2. Open-field test. This task was performed in a circular apparatus (56 cm diameter) with the surface divided into 10 areas of equal size. The five areas that had the edges bounded by walls (50 cm height) were termed peripheral, while the five remaining areas and who had no contact with the wall of the apparatus were called central. The rats were gently placed at the center of the apparatus and observed for 5 min. The following parameters were evaluated: number of central and peripheral crossings, number of rearing, number and time of grooming, defecation (number of bolus) and time of freezing. The apparatus was cleaned between assessments with a 20% ethanol solution.

2.2.3.3. Morris water-maze. This task was adapted from paradigm described by Morris (1981) in order to investigate spatial learning and memory in the laboratory rat, extensively revised by D'Hooge and Deyn (2001). The apparatus consisted in a black circular pool (180 cm diameter, 60 cm height) filled with water (depth 30 cm, 24 ± 1 °C) and located in a room that was rich in spatial cues (flags on two walls, a large door and experimenters). The pool was divided into four quadrants of equal size and one of the quadrants contained an escape platform (10 cm diameter) in the middle and submerged 1.5 cm. The escape platform was maintained in all trials sessions. Swimming activity of each animal was monitored using a video tracking system to analyze the latency to reach the escape platform, time spent in platform and opposite quadrant, number of crossings, swimming velocity and distance traveled by experimental groups. Briefly, the rats were monthly submitted to four trials sessions. The trial began by placing the rat in the water facing the wall of the pool at one of the starting points demarcated by dividing the pool into four quadrants. All four start positions were used once in a random sequence in each trial session with an interval of 10 min. If the animal did not find the platform within 60 s of the trial session, it was gently led to the escape platform by the experimenter and remained there for 10 s. After each trial session, the animals were dried and heated by a lamp before returning to their cages. Twenty-four hours after the last training, the rats were submitted to a test session without the escape platform. The test session was performed by placing the rat in water for 1 min. The time spent to find the original position of the escape platform and the swimming velocity were measured. The rats were then dried and heated by a lamp and returned to their cages.

2.2.4. Thyroid hormones determination

Plasma levels of tT4 and tT3 were measured by microparticle enzyme immunoassay (MEIA) using AxSYM® system (Abbott Laboratories, Abbott Park, Illinois, USA), according to suppliers' instructions.

2.3. Statistical analysis

Data of behavior testing were analyzed by nonparametric methods, using Kruskal–Wallis test (kw) followed by Dunn's multiple comparisons test when appropriate. Data of non-parametric analysis are represented as medians and ranges (interquartile interval) and the data of parametric analysis as means and SD. Body weight and hormones levels were analyzed by three-way ANOVA (2 MTZ × 2

Table 1
Plasma tT3 and tT4 levels at first and third month of behavioral assessments.

	Control	MTZ	Se	Se + MTZ
tT3				
tT3 (1st month)	0.38 ± 0.06	0.19 ± 0.08*	0.36 ± 0.05	0.17 ± 0.08*
tT3 (3rd month)	0.53 ± 0.11	0.19 ± 0.08*	0.42 ± 0.06	0.24 ± 0.1*
tT4				
tT4 (1st month)	2.43 ± 0.66	1.10 ± 0.51*	2.8 ± 0.52	1.23 ± 0.17*
tT4 (3rd month)	3.80 ± 0.8	1.30 ± 0.75*	3.30 ± 0.5	1.95 ± 0.65*

tT3 is expressed as ng/ml plasma and tT4 as μg/ml plasma. Values are expressed as means ± S.D. of 6–8 rats/group.
* $p < 0.05$ from control group (three-way ANOVA followed Duncan multiple range test).

Se × time). This last factor was considered as a repeated measure; and the test was followed by Duncan multiple range test when appropriate. Differences between groups were considered significant when $p < 0.05$.

3. Results

3.1. Plasma tT3 and tT4 levels

Hypothyroidism was confirmed in MTZ groups by measuring plasma tT3 and tT4 levels in first and third month of the experimental period. Three-way ANOVA revealed a significant main effect of MTZ on tT3 [$F(1,12) = 30.38$, $p = 0.0001$) and t4 [$F(1,12 = 32.57$, $p = 0.0009$] levels after 1 month of treatment. Similarly, there was a significant main effect of MTZ on tT3 [$F(1,27) = 20.22$, $p = 0.0001$] and tT4 [$F(1,27) = 42.31$, $p < 0.0001$] levels after 3 months of treatment. Statistical analysis also revealed that the main effect of Se and MTZ × Se interaction were not significant in these periods ($p > 0.05$) (Table 1).

3.2. Body weight

Three-way ANOVA with repeated measures revealed a significant main effect of MTZ [$F(1,36) = 95.60$, $p < 0.0001$], Se [$F(1,36 = 14.17$, $p = 0.0006$], time [$F(13,468) = 8.70$, $p = 0.0001$] and MTZ × time interaction [$F(13,468) = 13.79$, $p = 0.0001$] on the body weight rate. Statistical analysis also indicated that the MTZ × Se interaction was not significant [$F(1,36) = 2.52$, $p < 0.120$]. Post hoc comparisons showed that the MTZ exposure caused a significant reduction in the body weight of animals when compared to control group, which was worsened with simultaneous selenium supplementation (Table 2). Indeed, a significant difference in the body weight was observed between MTZ and Se plus MTZ groups from control group. There was no difference between Se and control group in this parameter.

Table 2
Effects of MTZ and selenium diet intake in the body weight gain (g) of experimental groups.

	Initial body weight	Final body weight	Body weight gain (g)
Control	208.7 ± 18.2	252.5 ± 19.7	+43.8
MTZ	213.8 ± 19.4	214.1 ± 23.4*,#	+0.3
Se	216.4 ± 24.6	245.3 ± 23.5	+28.9
Se + MTZ	212.5 ± 13.1	186.7 ± 10.7*,#	−25.8

Values are expressed as means ± S.D. of 10 rats/group.
* $p < 0.05$ from control group (three-way ANOVA with repeated measures followed by Duncan multiple range test).
# $p < 0.05$ as difference between groups (three-way ANOVA with repeated measures followed by Duncan multiple range test).

3.3. Elevated plus maze test

The data of Table 3 show the effects of MTZ exposure and selenium diet intake on anxiety parameters evaluated by elevated plus maze test.

Kruskal–Wallis test followed by Dunn's multiple comparisons test revealed that MTZ group had a significant decrease on the number of entries on closed arms in the first (kw = 9.55; $p = 0.022$) and third month of assessment (kw = 10.8; $p = 0.012$) when compared to the control group. No statistical difference in this parameter was observed among groups in the second month of assessment (kw = 6.21; $p = 0.1$). Likewise, there was no difference among groups on the time spent in closed arms, number of entries in open arms and time spent in open arms during all experimental period ($p > 0.05$).

Table 3
Effects of MTZ and selenium diet intake on anxiety parameters evaluated by elevated plus-maze test.

Parameters	Control	MTZ	Se	Se + MTZ
First month of treatment				
No. of entries in open arms	1 (1/2.25)	0.4 (0/1.25)	0.4 (0/1)	0.05 (0/1)
Time spent in open arms	0.15 (0.08/0.2)	0.04 (0/0.1)	0.05 (0/0.05)	0.03 (0/0.03)
No. of entries in closed arms	7 (3.5/9)	3 (1.75/4)*	5.5 (3.75/8.25)	4 (2/6)
Time spent in closed arms	4.3 (3.57/4.56)	4.3 (3.95/4.53)	4.2 (3.94/4.36)	4.3 (4.17/4.53)
Second month of treatment				
No. of entries in open arms	1 (0/2)	0.5 (0/2)	2.5 (0/3.25)	0.4 (0/1.25)
Time spent in open arms	0.06 (0/0.47)	0.06 (0/0.17)	0.27 (0/1.25)	0.05 (0/0.14)
No. of entries in closed arms	6.5 (4.75/8.5)	3.5 (2.75/6.25)	6 (4/9.25)	3.5 (2/6)
Time spent in closed arms	3.7 (3.1/4.27)	3.8 (3/4.31)	3.3 (3/4.2)	4.2 (4/4.5)
Third month of treatment				
No. of entries in open arms	0.4 (0/2)	0.5 (0/1.25)	0.5 (0/1.25)	0.3 (0/1)
Time spent in open arms	0.04 (0/0.16)	0.01 (0/0.25)	0.05 (0/0.18)	0.02 (0/0.06)
No. of entries in closed arms	6 (5/10)	2.5 (1.75/4)*	5 (1/7.25)	2.5 (1.75/6)
Time spent in closed arms	4.2 (4.16/4.35)	4.4 (4.1/4.51)	4.4 (3.95/4.53)	4.3 (4.26/4.52)

Values are expressed as median and range (interquartile interval) of 10 rats/group. Time spent in open and closed arms are expressed in minutes.
* $p < 0.05$ from control group (Kruskal–Wallis test followed Dunn' multiple comparisons test was done within each time point).

Table 4
Effects of MTZ and selenium diet intake on locomotor and exploratory activities in the open-field test.

Parameters	Control	MTZ	Se	Se + MTZ
First month of treatment				
Central crossing	7 (6.25/20.5)	5 (4.75/13.25)	13 (7/19.5)	11.5 (6/18)
Peripheral crossing	49 (33.25/53.25)	32.5 (27/33.25)*	46.5 (38.75/57)	33 (28.75/38)*
Rearing	14 (11.25/21.25)	13 (7.25/16.5)	18.5 (13.75/22.25)	13.5 (8.25/19)
Time of freezing	0.08 (0/0.66)	0.79 (0.13/1.72)	0.12 (0.01/0.5)	0.01 (0/0.06)
Second month of treatment				
Central crossing	7.5 (2/12.75)	1 (1/3.25)*	1 (1/2.25)*	1 (1/3.25)*
Peripheral crossing	25 (17/44.25)	15 (11.5/21.75)	32 (21/36.5)	14 (5/22)
Rearing	15.5 (15/23)	8 (3.75/13.5)	8 (4/11.25)	4 (0.75/12.25)
Time of freezing	0.48 (0.1/2.1)	1.4 (0.06/3.6)	0.39 (0.12/1.54)	1.2 (0.36/2.8)
Third month of treatment				
Central crossing	3.5 (1.75/5.75)	1 (1/3.25)	1.5 (1/6.5)	1.5 (1/4)
Peripheral crossing	22 (7.75/34.25)	19.5 (12.5/27.75)	25.5 (9.5/33)	20 (14/23.5)
Rearing	7.5 (3.5/14.5)	8.5 (6.5/12.5)	6.5 (3.75/15.5)	8 (5/12.25)
Time of freezing	1.5 (1.35/2.35)	0.7 (0.09/1.61)*	1.09 (0/1.61)	0.7 (0.24/1.12)*

Values are expressed as median and range (interquartile interval) of 10 rats/group. Time of freezing is expressed in minutes. Central, peripheral crossings and rearing are expressed in number of times.

* $p < 0.05$ from control group (Kruskal–Wallis test followed Dunn' multiple comparisons test was done within each time point).

3.4. Open-field test

The effects of MTZ exposure and selenium diet intake on locomotor and exploratory activities evaluated by open-field test are demonstrated in Table 4.

In the first month of assessment, Kruskal–Wallis test followed by Dunn's multiple comparisons test revealed that MTZ groups had a significant decrease in the number of peripheral crossing (kw = 15.51; $p = 0.0014$) when compared to the control group.

In the second month of assessment, Dunn's multiple comparisons test showed that MTZ, Se and Se plus MTZ groups had a significant reduction in the number of central crossing when compared to the control group (kw = 13.29; $p = 0.004$).

In the third month of assessment, Dunn's multiple comparisons test revealed that the time of freezing was decreased in MTZ and Se plus MTZ group (kw = 10.52; $p = 0.014$) when compared to the control group.

3.5. Morris water maze

3.5.1. Latency to reach the escape platform

Fig. 1 shows the effects of MTZ exposure and selenium diet intake on the latency to reach the escape platform in both trial and test sessions during the three months of evaluation.

In the first month of assessment (Fig. 1A), Dunn's multiple comparisons test revealed that the group fed with Se alone had a significant reduction in the latency to reach the platform during the trial session 3 (kw = 16.28; $p = 0.0010$) and 4 (kw = 16.28; $p = 0.0006$) when compared to the control group. No significant difference was observed among groups in the test session (kw = 3.46; $p = 0.325$). Similarly, in the second month (Fig. 1B), the group fed with Se diet alone had a marked decrease on the latency to reach the platform in trial session 3 (kw = 10.09; $p = 0.017$) and 4 (kw = 13.82; $p = 0.0032$) when compared to the control group. No significant difference was observed among groups on the test section (kw = 7.39, $p = 0.06$) in the second month of evaluation.

In the third month of assessment (Fig. 1C), Dunn's multiple comparisons test showed that the group supplemented with Se diet alone had a significant decrease in the latency to reach the platform during the trial session 3 (kw = 13.05; $p = 0.0045$) when compared to the control group. Dunn's multiple comparisons test revealed again that MTZ exposure caused a significant increase in the latency to reach the platform during the test session (kw = 10.71; $p = 0.013$)

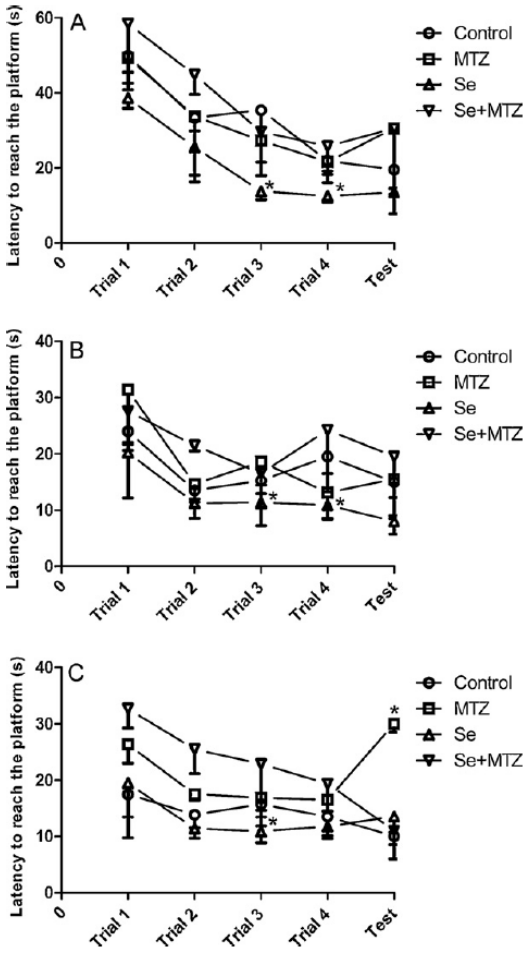

Fig. 1. Effects of MTZ exposure and selenium diet intake on the latency to reach the escape platform (expressed in seconds) in the water maze test. Graph A represents the first month of treatment. Graph B represents the second month of treatment. Graph C represents the third month of treatment. *Denoted $p < 0.05$ from control group at the same time. Values are expressed as median and range (interquartile interval) of 10 rats/group.

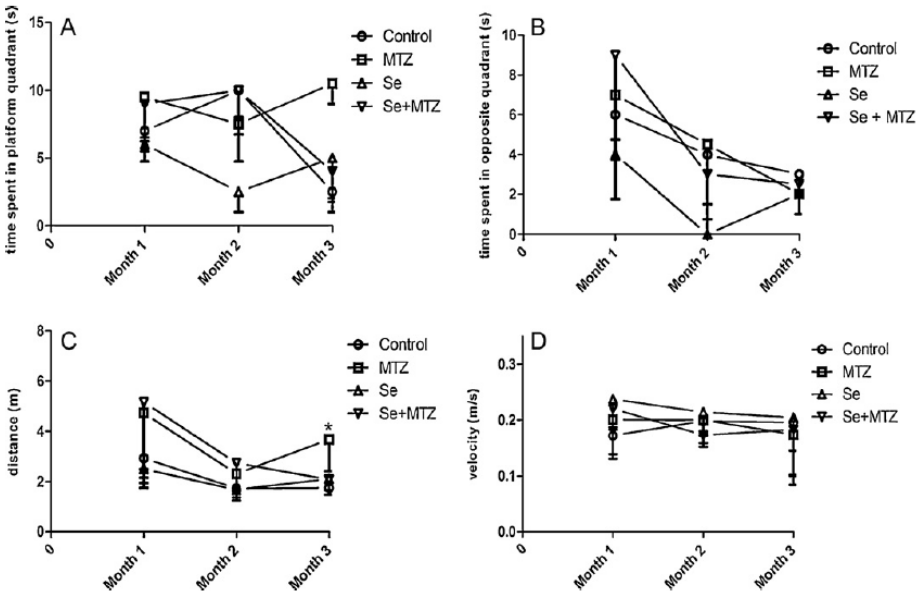

Fig. 2. (A) Effects of MTZ exposure and selenium diet intake on time spent in the platform quadrant (expressed in s) in the water maze test (test sessions). (B) Effects of MTZ exposure and selenium diet intake on time spent in the opposite quadrant (expressed in s) in the water maze test (test sessions). (C) Effects of MTZ exposure and selenium diet intake on the distance performed by experimental groups (expressed in m) in the water maze test (test sessions). (D) Effects of MTZ exposure and selenium diet intake on the swimming velocity (expressed in m/s) in the water maze test (test sessions). *Denoted $p < 0.05$ from control group at the same time. Values are expressed as median and range (interquartile interval) of 10 rats/group.

when compared to the control group. This effect of MTZ was blunted by selenium supplementation.

3.5.2. *Time spent in platform and opposite quadrant during test session*

Fig. 2A and B shows the effect of MTZ exposure and selenium diet intake on the time spent by groups in the platform and opposite quadrant during the test session, respectively.

Kruskal–Wallis test revealed that there was no significant difference among groups on the time spent on the platform quadrant during test session in the first (kw = 2.66; $p = 0.44$), second (kw = 4.53; $p = 0.20$) and third month (kw = 1.36; $p = 0.71$) of assessment. Likewise, no statistical difference was verified among groups on the time spent in the opposite quadrant during test session in first (kw = 4.73; $p = 0.192$), second (kw = 4.9; $p = 0.178$) and third month (kw = 0.56; $p = 0.90$) of evaluation.

3.5.3. *Distance and velocity performed during test session*

Fig. 2C and D shows the distance and velocity performed by experimental groups in the water maze test during the three months of assessment, respectively.

Kruskal–Wallis test showed that there was no significant difference among groups on the distance performed in the first (kw = 1.31; $p = 0.72$) and second month (kw = 7.34; $p = 0.061$) of assessment. In the third month of assessment, Dunn's multiple comparisons test revealed that the distance performed by MTZ group was significantly higher (kw = 8.38; $p = 0.038$) when compared to the control group. This effect of MTZ was abolished by selenium supplementation (Fig. 2C). No significant difference was observed among groups on the swimming velocity during test session in the first (kw = 6.00; $p = 0.11$), second (kw = 2.44; $p = 0.48$) and third month (kw = 1.67; $p = 0.64$) of evaluation (Fig. 2D).

4. Discussion

This study was primarily delineated to evaluate, through the Morris water maze test, the possible beneficial effect of diphenyl diselenide supplementation on cognitive dysfunction of hypothyroid female rats. Our results showed that the learning and memory deficits observed in MTZ-induced hypothyroid rats were ameliorated by dietary diphenyl diselenide. Interestingly, the animals fed with diphenyl diselenide alone exhibited a better performance in some parameters of Morris water maze test when compared to the control group.

In our experimental protocol, hypothyroidism induced by MTZ was associated with a significant weight loss and the long-term diphenyl diselenide diet intake did not affect the body weight of animals. However, the consumption of this diet was not effective in blunting the reduction in the body weight of MTZ-induced hypothyroid rats and the body weight of Se plus MTZ group was lower than MTZ treated rats. It is important to mention here, that diphenyl diselenide and some structural analogues may display anorexigen effect in rodents (Meotti et al., 2008; Savegnago et al., 2009; Wilhelm et al., 2009). However, in our experimental protocol, there was not a significant main effect of Se × MTZ interaction on body weight as revealed by three-way ANOVA with repeated measures. Thus, we assume that the reduction on body weight observed in MTZ and Se plus MTZ groups may be associated mainly with the low growth rate induced by hypothyroidism condition (Darbra et al., 1995; Sala-Roca et al., 2002; Zhang et al., 2009).

Elevated plus maze and open field test are widely used for evaluating anxiolytic performance and motor disorders, respectively (Belzung and Griebel, 2001). In this way, it is important to consider that the effect of hypothyroidism in these parameters may differ depending on antithyroid agent used as well as the age of induction (Darbra et al., 1995; Sala-Roca et al., 2002; Reis-Lunardelli et al., 2007). In our experimental protocol, the hypothyroid female

rats no exhibited anxiolytic performance. On the order hand, MTZ exposure caused a reduction on locomotor activity of rats represented by the number of crossings and time of freezing in the open field test. However, in the third month of evaluation, this parameter was similar among groups. This result may reflect an adaptive response of animals to the test situation. Regarding the effects of diphenyl diselenide on motor parameters, we observe that the diet intake was not effective in preventing the changes induced by MTZ. Moreover, the consumption of the diet supplemented with diphenyl diselenide caused *per se* a decrease in locomotor and exploratory activities of rats represented by the number of central crossing in the open field test during the second month. In this way, experimental evidence has shown that the effects of diphenyl diselenide in these motor parameters may be dependent on the administration route, time of exposure and animal species (Stangherlin et al., 2008; Ghisleni et al., 2008; Savegnago et al., 2008).

It has been postulated that hypothyroidism is clearly linked to cognitive dysfunction development in different animal models of adult-onset hypothyroidism (Reid et al., 2007; Tong et al., 2007; Wilcoxon et al., 2007; Reis-Lunardelli et al., 2007). In this work, the effects of hypothyroidism in memory and spatial learning were evaluated by Morris water maze test, an important task to assess these parameters in laboratory rats (Morris et al., 1986; McNamara and Skelton, 1993; D'Hooge and Deyn (2001)). We observed that in the third month of treatment, the latency to reach the platform and distance performed to find the escape platform in Morris water maze test were significantly greater in MTZ group.

It is noteworthy in Morris water maze test that the diphenyl diselenide diet intake was effective in reducing the detrimental effects of hypothyroidism related to cognitive functions. In fact, dietary diphenyl diselenide was able in restoring cognitive deficits represented by alterations on parameters as latency time to reach the platform and distance performed during test sessions in MTZ-induced hypothyroid rats. Of particular importance, the animals that received diphenyl diselenide diet alone exhibited a better performance in water maze test when compared to the control group. Really, the time of latency to reach the escape platform during the trial sessions was lower in Se group.

Although, there are no data about the effects of diphenyl diselenide supplementation on behavioral performance in hypothyroid rats; previous works from our research group have demonstrated the beneficial use of chronic diphenyl diselenide intake (1–10 ppm) on diabetes and tumorigenisis experimental (Barbosa et al., 2008a,b). Indeed, there is evidence that diphenyl diselenide as oral dietary supplement (0.3–30 ppm) was non-toxic to rabbits exposed for a long time (de Bem et al., 2007).

Here we have not determined the neuroprotective role of diphenyl diselenide and/or the deleterious action of MTZ exposure in terms of mechanism(s); and this fact could limit an analysis more detailed of our results. However, it is well established in other works that the neuroprotective action of this compound, in different animal models, possibly is associated with its potent antioxidant property, which is mediated by its glutathione peroxidase activity and also by its reduction by cerebral TrxR (thioredoxin reductase) (Nogueira and Rocha, 2010; Sausen de Freitas et al., 2010; de Freitas and Rocha, 2011). Corroborating this idea, there is evidence in the literature indicating that diphenyl diselenide molecule is able to cross the blood-brain barrier and increase the selenium levels in the brain (Maciel et al., 2003; Nogueira et al., 2004; Prigol et al., 2009). Considering that low levels of T3 and T4 may induce oxidative damage (Das and Chainy, 2004; Cano-Europa et al., 2008; Erdamar et al., 2008; Bhanja and Chainy, 2010), it is reasonable to assume that the effects observed here in diphenyl diselenide supplemented groups may be, at least in part, associated to antioxidant activity of compound.

In summary, our results provide evidence of the effectiveness of dietary diphenyl diselenide in improving spatial learning and memory deficits induced by hypothyroidism condition. However, additional investigations about the precise mechanisms involved on the neuroprotective role of diphenyl diselenide and MTZ toxicity will be needed.

Acknowledgments

The financial support by FINEP Research Grant "Rede Instituto Brasileiro de Neurociência (IBN-Net)" #01.06.0842-00, FAPERGS/Pronex, CAPES/SAUX, VITAE Fundation, INCT-CNPq-Excitotoxicity and Neuroprotection and CNPq is gratefully acknowledged. C.W.N, F.A.S and N.B.V.B are the recipients of CNPq fellowships.

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4.2 Manuscrito 1:

4.2.1 Dieta suplementada com disseleneto de difenila reduz comportamento semelhante ao depressivo em ratas com hipotireoidismo induzido por metimazol

Diphenyl diselenide supplementation reduces depressive-like behavior in methimazole-induced hypothyroid rats

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Abstract

Hypothyroidism has been associated to psychiatric disorders development and tissue oxidative damage. In this study, we evaluated the effect of diphenyl diselenide supplementation on depressive-like behavior triggered by methimazole exposure in female rats. Additionaly, thiobarbituric acid reactive substances (TBARS), reactive oxygen species (ROS) and non-protein (NP-SH) thiols levels were analyzed in cerebral cortex, hippocampus and striatum structures. Monoamino oxidase (MAO) activity was evaluated in total brain. Firstly, female rats received methimazole (MTZ) 20mg/100ml in the drinking water for 30 days and were evaluated in open-field and forced swimming tests (FST). In this set of experiments, the rats exposed to MTZ presented a depressive-like behavior, which was evidenced by a significant increase in the immobility time when compared to control group. Thereafter, MTZ-induced hypothyroid rats received either a standard or a diet containing 5 ppm of diphenyl diselenide, and then were re-evaluated monthly in open-field and FST tests during 3 months. No alteration on the locomotor performance was observed among the groups. The depressive-like behavior of hypothyroid rats was abolished by diphenyl diselenide supplementation during all experimental period. The levels of thyroid hormones remained low in MTZ exposed groups until the end of experimental period. MTZ group had an increase in TBARS and ROS levels that were restored by diphenyl diselenide supplementation. NP-SH content of cerebral structures did not modify by MTZ exposure and/or diphenyl diselenide supplementation. Diphenyl diselenide supplementation restored the MAO B activity that was decreased in MTZ group. In summary, our results show that hypothyroidism induced by methimazole triggers a depressive-like behavior in female rats and that dietary diphenyl diselenide was able to reverse this effect.

Keywords: hypothyroidism, methimazole, antidepressive-like behavior, diphenyl diselenide, oxidative stress.

1. Introduction

Depression is a common and chronic illness characterized by some “neurovegetative symptoms” such as depressed mood, anhedonia, irritability, difficulties in concentrating, and abnormalities in appetite and sleep (Nestler et al., 2002). In addition, depressed patients can present high rates of suicide behavior and are more susceptible in developing coronary artery disease and type 2 diabetes (Knol et al., 2006). Unfortunately the official diagnosis of depression is subjective and based on a documentation of a certain number of symptoms, which difficult the own diagnosis and the knowledge of neural and molecular mechanisms of disease (Nestler et al., 2002; Krishnan & Nestler, 2008).

Evidence from literature demonstrate that thyroid dysfunction is associated with psychiatric disorders including depression (Demet et al., 2002, Van Boxtel et al., 2004; Guimarães et al., 2009), and affects mainly women (Morganti et al., 2005). The mechanisms by which thyroid hormones increase the risk for depression development until remain to be established. Interestingly, thyroid hormones accelerate and improve the therapeutic response to tricyclic antidepressants and selective serotonin reuptake inhibitors in depressive patients (Aronson et al., 1996; Cooper-Kazaz et al., 2007). Moreover, thyroid hormone treatment improves antidepressant pharmacotherapy even in patients with euthyroid hormonal state (Pilhatsch et al., 2011).

Hypothyroidism is a clinical condition characterized by elevated levels of the thyroid stimulating hormone (TSH) or by low levels of triiodothyronine (T3) and thyroxin (T4). The role of thyroid hormones on the development and functions of the central nervous system are well established (Lima et al., 2001; Bernal et al., 2003). The lack of thyroid hormones affects neuronal differentiation, migration, myelination, synaptogenesis, dendritic branching and plasticity (Ambrogini et al., 2005; Alva-Sánchez et al., 2009, Zhang et al., 2009). Considering the impact of thyroid disease in people's health, American Thyroid Association recommends that adults be screened for thyroid dysfunction by measurement of the serum TSH concentration, beginning at age 35 years and every 5 years thereafter (Singer et al., 1995; Ladenson et al., 2000).

Regarding to experimental depression, recently it has been demonstrated that diphenyl diselenide, a simple organoselenium compound, exhibit potent anxiolytic-like and antidepressant-like effects, which are related to central monoaminergic and serotoninergic systems; and L-arginine/NO/cGMP pathway modulation (Savegnago et al., 2007, 2008; Ghisleni et al., 2008). In analogy, the antidepressant-like action of diphenyl diselenide was

proven in malathion-exposed rats (Acker et al., 2009). Indeed, diphenyl diselenide exhibits antioxidant and neuroprotective properties, which are closely related to its efficient mimetic activities of the enzymes glutathione peroxidase and thioredoxin reductase (Nogueira et al. 2004; Posser et al. 2008, Navarro-Alarcon & Cabrera-Vique, 2008; Nogueira & Rocha, 2010; Freitas & Rocha, 2011). Thus, the neuroprotective properties of diphenyl diselenide could be interesting to verify under hypothyroidism condition, since it is linked to oxidative stress (Das & Chainy, 2004; Erdamar et al., 2008; Cano-Europa et al., 2008, 2011; Amara et al., 2009, 2010; Bhanja & Chainy, 2010), brain damage (Ambrogini et al., 2005; Alva-Sánchez et al., 2009, Zhang et al., 2009) and depressive-like behavior (Kulikov et al., 1997). Besides, our group recently demonstrated that the intake of diets supplemented with diphenyl diselenide improves spatial learning and memory deficits in hypothyroidism (Dias et al., 2012). In view of these considerations, this study was designed to examine whether the depressive-like behavior induced by methimazole exposure in adult female rats could be prevented by dietary diphenyl diselenide. Additionally, we investigated the activity of monoamine oxidase (MAO) (E.C. 1.4.3.4) and some parameters of oxidative stress in cerebral cortex, hippocampus and striatum of rats.

2. Materials and Methods

2.1 Animals and Reagents

Forty-eight adult female Wistar rats purchased from our own breeding colony were acclimated for 10 days before the beginning of the experiments. The animals were housed in plastic cages and maintained at 22-24° C, on a 12h light/ 12 h dark cycle, with free access to food (Supra®, Brazil) and water. All experiments were performed in accordance with guidelines of the Committee on Care and Use of Experimental Animal Resources, Federal University of Santa Maria, Santa Maria, RS, Brazil. Methimazole was purchased from Sigma Chemical Co. (St. Louis, Mo, USA). Diphenyl diselenide was synthesized according to literature methods (Paulmier, 1986). Analysis of the ^{1}HNMR and ^{13}CNMR spectra showed analytical and spectroscopic data in full agreement with its assigned structure. All other reagents were of analytical grade and obtained from standard commercial suppliers.

2.2 Experimental Protocol

2.2.1 Induction of hypothyroidism: hypothyroidism was induced by administration of methimazole (MTZ) 0.02 g/100 ml in the drinking water, as previously described (Dias et al., 2012). Initially, female rats were divided into two experimental groups (n=24): control group, which received water and commercial food, and MTZ group, which received the solution of MTZ and commercial food. MTZ exposure via drinking water was maintained 30 days. After this period, both groups were evaluated in open-field test and forced swimming test.

2.2.2 Diphenyl diselenide supplementation: 7 days after the experimental period cited above, the rats were randomly redivided into 4 experimental groups (n=12): (1) control group, which received unsupplemented diet and water, MTZ group, which received unsupplemented diet and continued to receive MTZ in drinking water, Se group, which received water and the diet supplemented with diphenyl diselenide at 5 ppm and MTZ+Se group, which received the diet supplemented with diphenyl diselenide 5 ppm and continued to receive MTZ in drinking water. The diet consisted of a balanced food to meet the nutritional needs of animals (Barbosa et al., 2008[a, b]). The food was prepared in an industrial mixer to allow the uniformity of the mixture, and the diphenyl diselenide was dissolved in soybean oil. After preparation, the diets were frozen (4°C) and offered *ad libitum* to the animals. Treatments were maintained for three months and behavioral testing was performed monthly in open-field test and forced swimming test.

2.3 Behavioral Testing

The behavioral assessments were performed monthly and conducted between 8:00 a.m. and 2:00 p.m. The tasks were open-field and forced swimming test.

2.3.1 Open-Field Test: locomotor and exploratory activities were evaluated by open-field test before forced swimming test. This task was performed in a circular apparatus (56 cm diameter) with the surface divided into 10 areas of equal size. The rats were gently placed at the center of the apparatus and observed for 5 minutes to record the locomotor (number of segments crossed with the four paws- crossings) and exploratory activities (expressed by the number of time rearing on the hind limbs). The apparatus was cleaned between assessments with a 20% ethanol solution.

2.3.2 Forced Swimming Test (FST): FST was conducted using the method of Porsolt et al. (1977). Test was carried out over 2 days; of which in the first day was pre-training for the second day that was test session. Briefly, in the pre-training session, rats were individually placed for 15 minutes in open cylinders (45 cm height x 20 cm diameter) containing water, maintained at 25 ± 1°C. The rats were then dried and heated by a lamp and returned to their cages. Twenty-four hours later, rats were again placed in apparatus and the duration of immobility was recorded for 5 minutes. Each rat was recorded as immobile when floating motionless or making only those movements necessary to keep its head above water.

2.4 Tissue Preparation

At the end of the treatment period, the animals were euthanized and the whole blood collected by cardiac puncture for measurement of total thyroid hormones (tT3 and tT4) levels. Heparinized blood was centrifuged at 3500 rpm for 5 minutes. Brain was quickly removed, placed on ice and some dissected in hippocampus, striatum and cerebral cortex. Cerebral structures and total brain were homogenized in cold 10 mM Tris-HCl pH 7.4. Homogenates were centrifuged at 3500 rpm for 10 minutes to yield the low-speed supernatant fractions (S1) that were used for lipid peroxidation, reactive oxygen species (ROS) and non-protein thiols (NP-SH) levels determinations. A preparation of mitochondria from total brain was used for monoamino oxidase (MAO) assay and was obtained by differential centrifugation, as previously described (Acker et al., 2009). Protein concentration was measured by the method of Bradford, using bovine serum albumin as the standard (Bradford et al., 1976).

2.5 Thyroid Hormones Determination

Plasma levels of total T4 and total T3 were measured by microparticle enzyme immunoassay (MEIA) using AxSYM® system (Abbott Laboratories, Abbott Park, Illinois, USA), according to suppliers' instructions.

2.6 Lipid Peroxidation, ROS and NP-SH Determination

Lipid peroxidation was performed by measuring thiobarbituric acid reactive substances (TBARS) levels, according to the method of Ohkawa et al. (1979). Briefly, aliquots of the supernatant fractions from cerebral structures were incubated at 100°C for 1 hour in acid medium containing 200 µl of 8.1% sodium dodecyl sulfate, 500 µl of 500 mM acetic acid buffer pH 3.4 and 500 µl of 0.6% thiobarbituric acid (TBA). TBARS levels were

measured at 532 nm and the absorbance was compared with the standard curve using malondialdehyde (MDA). Results were expressed as nmol malondialdehyde/ mg of protein.

ROS determination was based on the deacetylation of the 2', 7'-dichlorofluorescein diacetate (DCFH-DA) and its subsequent oxidation by intracellular reactive species to DCF, a fluorescent compound (Lebel et al., 1992). The supernatant fractions (S1) from cerebral structures were added to a medium with 10 mM Tris-HCl pH 7.4 and 1 mM DCFH-DA. Incubation was performed by 1 hour in the dark until fluorescence measurement. DCF fluorescence intensity emission was recorded at 520 nm with 480 nm excitation. Results were expressed as μmol DCF/ mg of protein, using a standard curve with DCF.

NP-SH levels were determined in cerebral structures by method of Ellman (1959). Aliquots from S1 were mixed (1:1) with 10% trichloroacetic acid (TCA) and centrifuged at 3500 rpm for 10 minutes. After centrifugation, an aliquot of supernatant (200 μl) was added to a reaction medium containing 0.5 mM phosphate buffer pH 7.4, and 10 mM 5,5'-dithio-bis(2-nitrobenzoic acid) (DTNB). NP-SH levels were measured at 412 nm, using a standard curve with reduced glutathione (GSH). Results were expressed as nmol SH/ mg of protein.

2.7 Monoamino Oxidase (MAO) Activities

MAO activity was determined in total brain as described by Krajl (1965) with modifications of Matsumoto et al. (1984). Aliquots of samples were incubated at 37°C for 5 minutes in a medium containing Na_2HPO_4/KH_2PO_4 buffer pH 7.4 and specific inhibitors, selegiline (MAO B inhibitor, 250 nm) or clorgiline (MAO A inhibitor, 250 nm), at a final volume of 700 μl. Then kynuramine dihydrobromide was added to the reaction mixture (final concentration, 90 μM to MAO-A and MAO-T assays and 60μM to MAO-B assay) as substrate. Samples were incubated at 37°C for 30 minutes and reaction was stopped by adding trichloroacetic acid 10%. After centrifugation at 3000 xg for 15 minutes, an aliquot of supernatant was added to 1 ml 1M NaOH. The fluorescence intensity was detected with excitation at 315 nm and emission at 380 nm using a fluorescence spectrometer. The concentration of 4-hydroxyquinoline was estimated from a corresponding standard fluorescence curve. MAO activity was expressed as nmol of 4-hydroxyquinoline/ mg of protein.

2.8 Statistical Analysis

Data were analyzed by unpaired T Test and two-way ANOVA followed by Bonferroni multiple comparisons test when appropriated. Data are presented as mean ± S.E.M. Differences between groups were considered significant when $p< 0.05$.

3. Results

3.1 Thyroid Hormones Determination

Statistical analysis shows that MTZ exposure was associated with low plasma levels of tT3 and tT4, which were not restored by dietary diphenyl diselenide treatment ($p>0.05$) (Table 1).

3.2 Open-Field Task and Forced Swimming Test (FST) Evaluation

No significant statistical difference was verified between control and MTZ groups in the number of crossings (unpaired T Test, $p=0.73$) and rearing (unpaired T Test, $p=0.501$) before the dietary diphenyl diselenide treatment. These data exclude the motor impairments that could influence the behavior of animals in the forced swimming test (Table 2). Similarly, there was no significant difference among groups in the locomotor and exploratory activities evaluated 1, 2 and 3 months after dietary diphenyl diselenide treatment (Table 2).

Figure 1 show the immobility time of groups during the FST. Graph A reveals that the administration of MTZ in drinking water for 30 days caused a significant increase in the immobility time of animals when compared to control group (unpaired Test T, $p< 0.0001$) indicating that the experimental protocol of hypothyroidism was able in triggering a depressive-like behavior in FST.

Graph B, C and D illustrate the effects of diphenyl diselenide on depression-like behavior induced by MTZ exposure after 1, 2 and 3 months of supplementation, respectively. The results show that MTZ group had a significant increase in the immobility time ($p<0.05$) when compared to control group in all periods of assessment. This effect antidepressive-like induced by MTZ exposure was completely abolished by diphenyl diselenide supplementation in all periods analyzed ($p<0.05$; Graphs B, C and D). Indeed, diphenyl diselenide caused *per se* a reduction in the immobility time of animals when compared to control group after 3 months of supplementation.

3.3 Lipid Peroxidation, ROS and NP-SH Determination

The data of Table 3 show that MTZ exposure increased TBARS and ROS levels in hippocampus when compared to control group. These effects of MTZ were restored to control levels by diphenyl diselenide supplementation ($p<0.05$). In contrast, no statistical difference was verified in the cerebral cortex and in the striatum among the groups. MTZ exposure and/or diphenyl diselenide supplementation did not induce any change in the NP-SH content of cerebral structures (Table 3).

3.4 MAO Activities

Figure 2 illustrates the MAO activities in total brain. Statistical analysis showed that MAO-total and MAO-A activities were not altered by MTZ exposure and/or diphenyl diselenide supplementation. On the order hand, MAO-B activity was significantly decreased in the MTZ group when compared to control group ($p<0.05$). Dietary diphenyl diselenide treatment was effective in restoring MAO-B activity to the control levels ($p<0.05$). Indeed, diphenyl diselenide did not modify *per se* MAO activities.

4. Discussion

In the current study we demonstrated that hypothyroidism induced by methimazole produced depressive-like behavior in female rats and that diphenyl diselenide supplementation in the diet was able to reverse this effect. Moreover diphenyl diselenide reversed depressive-like behavior during the three months evaluated demonstrating its pharmacological potential as antidepressive-like compound. Antidepressant-like effect of diphenyl diselenide was obtained independently of thyroid hormones levels, which remained lower even in supplemented group with diphenyl diselenide.

The depressive-like behavior evaluation was performed by the FST that is widely employed as a behavioral tool for screening of antidepressant drugs (Porsolt et al., 1977). The test is based on the observation that rodents, after initial escape-oriented movements, develop an immobile posture when exposed to an inescapable stressful event. In this way, when some antidepressant drug is administered prior to the test, the experimental subject will develop an escape-directed behavior for longer periods of time when compared to control group. This task is quite sensitive to all major classes of antidepressant drugs including tricyclics, serotonin-specific reuptake inhibitors, monoamine oxidase inhibitors, and atypicals (Cryan et al., 2002). We also conduct an evaluation of locomotor and exploratory activities by open-

field test in order to exclude mismatch factors to the forced swimming test. In this parameter, we observed that the reduction in the immobility time in the FST elicited by dietary diphenyl diselenide was not accompanied by alterations on locomotor activity, demonstrating that the antidepressant-like effect of diphenyl diselenide treatment cannot be attributable to a psychostimulant action. Similarly, hypothyroidism condition did not change the locomotor and exploratory activities that could be harmful to animal performance in FST. It is important to mention here that the chronic intake of diphenyl diselenide supplied in the diet did not cause apparent signs of toxicity. Accordingly, previous works of our research group have shown that oral supplementation with this selenium compound (1-10 ppm) during similar periods may be considered relatively secure in terms of toxicity (Bem et al., 2007; Barbosa et al, 2008[a,b]; Dias et al., 2012).

Hypothyroidism is an endocrine dysfunction linked to an increased susceptibility to depression and reduction in human health-related quality of life (Krishnan & Nestler, 2008). Hypothyroid patients presented a significant reduction in platelet serotonin concentrations demonstrating a possible interaction between serotoninergic system and hypothalamic-pituitary-thyroid (HPT) axis (Stipcevic et al., 2009). However neuropharmacological basis and the functional pathways for the modulatory effects of thyroid hormones on mood are yet to be understood, even though several studies revealed interactions with different neurotransmitter systems, such as monoaminergic system (Pilhatsch et al., 2011).

In accordance with epidemiological studies (Van Boxtel et al., 2004; Guimarães et al., 2009), Kulikov et al (1997) have demonstrated that severe hypothyroidism induced by thyroidectomy and mild hypothyroidism induced by iodine-free diet increased immobility time in the forced swimming paradigm. Accordingly, our findings confirm these results and also show that hypothyroidism triggered by the methimazole exposure may be considered a consistent model to study this neurological disorder. Furthemore, we demonstrated previously that hypothyroid rats had spatial learning and memory deficits (Dias et al., 2012).

The pharmacological role of diphenyl diselenide is strongly linked to its antioxidant potential (Nogueira et al. 2004; Posser et al. 2008, Navarro-Alarcon & Cabrera-Vique, 2008; Nogueira & Rocha, 2010; Freitas & Rocha, 2011). Important, literatures data also show that the compound exhibits antidepressant property in different experimental models and that this effect seems involve the serotoninergic, noradrenergic and dopaminergic systems as well as the cerebral MAO activity (Savegnago et al.; 2007, 2008; Ghisleni et al., 2008; Acker et al., 2009). Indeed, the antidepressant-like effect of diphenyl diselenide also verified by Acker et al. (2009) on malathion-exposed rats was related with cerebral cortex Na^+-K^+-ATPase activity

restoration. On the order hand, no correlation of this effect and oxidative stress and/or MAO activity was found in this study (Acker et al., 2009). In our experimental protocol, the antidepressant-like action of compound observed on hypothyroid rats was followed by a reduction in hippocampal TBARS and ROS levels. It is possible assume here that the antioxidant activity of diphenyl diselenide had contributed to diminish the depression symptoms under hypothyroidism condition. Consistent with this finding, some studies have demonstrated the efficacy of antioxidant therapies in reducing stress oxidative parameters in experimental models of hypothyroidism (Das & Chainy, 2004; Erdamar et al., 2008; Cano-Europa et al., 2008, 2011; Bhanja & Chainy, 2010; Amara et al., 2009, 2010).

In the current study we also found a significant inhibition of MAO B activity in hypothyroid group. MAO is a mitochondrial bound enzyme, which catalyzes the oxidative deamination of dietary amines, monoamine neurotransmitters and hormones. Two different types of MAO, named A and B, have been characterized on the basis of substrate and inhibitor sensivity. MAO A displays a higher affinity for serotonin and norepinefhrine, while MAO B prefers beta-phenylethylamine (Bortolato et al., 2008). MAO B knockout mice show increased brain levels of beta-phenylethylamine, but not serotonin, norepinefhrine and dopamine. Behavioral changes include a reduction in immobility time in the forced swimming test, upon single or repeated presentation, without significant changes in locomotor patterns in the open-field and elevated plus maze tests (Grimsby et al., 1997). However, in our work the inhibition of MAO B caused by hypothyroidism no improved the depressive-like behavior. We hypothesized that MAO B inhibition may be a compensatory mechanism to a depressive disorder induced by hypothyroidism, since could elevate beta-phenylethylamine levels, which is assumed to act as an endogenous amphetamine (Janssen et al., 1999). Additionally, inhibition of MAO contributes to reduce oxidative stress and redox unbalance due to lower formation of hydrogen peroxide in monoamine degradation (Bortolato et al., 2008).

Taken together, our results show the efficacy of dietary diphenyl diselenide in declining the depressive-like behavior associated to methimazole-induced hypothyroidism in female rats. However, additional studies are necessary to better understanding both the mechanisms involved in this effect of diphenyl diselenide and how thyroid hormones can trigger depression symptoms.

Acknowledgements

The financial support by FINEP Research Grant ‘‘Rede Instituto Brasileiro de Neurociência (IBN-Net)’’ #01.06.0842-00, FAPERGS/Pronex, Pronem, CAPES/SAUX, VITAE Fundation, INCT-CNPq-Excitotoxicity and Neuroprotection and CNPq is gratefully acknowledged. C.W.N, F.A.S and N.B.V.B are the recipients of CNPq fellowships.

Legends of figures

Figure 1: Effects of MTZ and diphenyl diselenide supplementation on the immobility time in the FST. Graph A represents the period of hypothyroidism induction, Graph B represents the first month of diphenyl diselenide treatment, Graph C represents the second month of diphenyl diselenide treatment and Graph D represents the third month of diphenyl diselenide treatment. Immobility time is expressed in seconds and represents the mean±S.E.M of 9-11 rats/group. * $p < 0.0001$ from control group.

Figure 2: Effects of MTZ and diphenyl diselenide supplementation on MAO activity. Graph A represents the MAO total activity, Graph B represents the MAO A activity and Graph C represents the MAO B activity. MAO activities were expressed as ηmol of 4-hydroxyquinoline formed/ mg of protein and represent the mean ±S.E.M of 5-6 rats/group.*$p < 0.05$ from control group.

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Figure 1

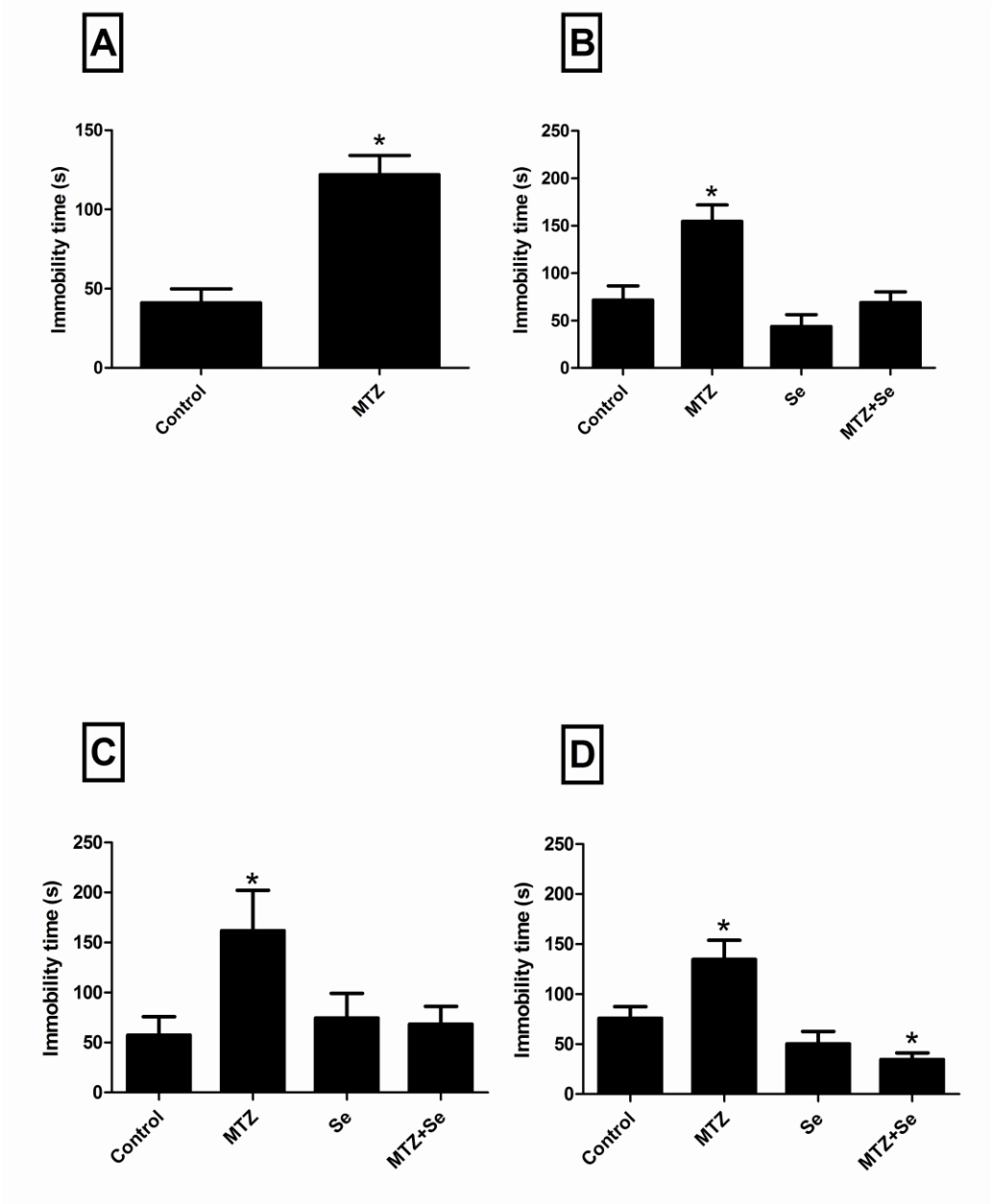
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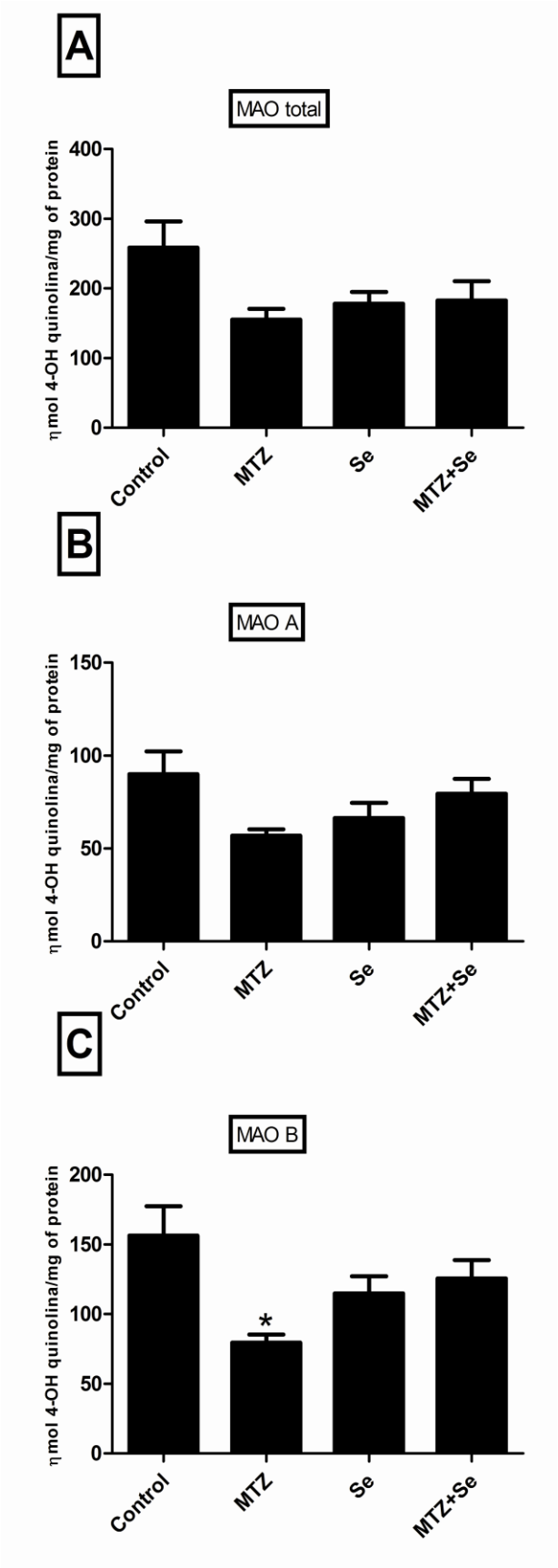
Figure 2


A
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ηmol 4-OH quinolina/mg of protein
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Control
MTZ
Se
MTZ+Se
B
MAO A
ηmol 4-OH quinolina/mg of protein
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Control
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ηmol 4-OH quinolina/mg of protein
200
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Control
MTZ
Se
MTZ+Se

Table 1: Plasma tT3 and tT4 levels

	Control	MTZ	Se	MTZ+Se
tT3	0.68 ± 0.05	0.29 ± 0.03*	0.58 ± 0.05	0.48 ± 0.03*
tT4	3.01 ± 0.25	0.53 ± 0.17*	1.90 ± 0.39	1.47 ± 0.20*

tT3 is expressed as ng/ml plasma and tT4 as pg/ ml plasma. Values are expressed as means ± S.E.M. of 9-11 rats/group. * Denoted $p<0.0001$ from control group (Two-way ANOVA followed Bonferroni Test).

Table 2: Effects of MTZ and diphenyl diselenide supplementation on locomotor and exploratory activities in the open-field test.

Hypothyroidism induction	Groups			
Parameters	Control		MTZ	
Crossings	46.17 ± 2.47		44.88 ± 2.86	
Rearing	14.5 ± 1.39		16.09 ± 1.85	
First Month of Treatment				
Parameters	Control	MTZ	Se	MTZ+Se
Crossings	43.07 ± 3.88	44.11 ± 4.28	47.33 ± 4.02	38.53 ± 2.62
Rearing	19.6 ± 2.1	24.22 ± 3.4	18.33 ± 1.85	20.33 ± 2.14
Second Month of Treatment				
Crossings	45.93 ± 3.43	35.11 ± 3.07	41.44 ± 3.6	38.27 ± 1.77
Rearing	13.4 ± 1.69	9.33 ± 1.87	8.0 ± 1.36	8.78 ± 1.08
Third Month of Treatment				
Crossings	29.87 ± 2.71	24.89 ± 3.38	35.67 ± 2.38	28.8 ± 4.69
Rearing	11.67 ± 1.67	10.56 ± 1.38	14.33 ± 1.92	10.29 ± 2.98

Values are expressed as means ± S.E.M. of 9-11 rats/group. Crossings and rearing are expressed as number of times.

Table 3: Effects of MTZ and diphenyl diselenide supplementation on TBARS, ROS and NP-SH levels in cerebral cortex, hippocampus and striatum.

		Control	MTZ	Se	MTZ+Se
Cerebral cortex	TBARS	0.29±0.04	0.24±0.02	0.24±0.03	0.19±0.01
	ROS	7.27±1.08	10.09±3.18	8.98±1.38	7.61±1.52
	NP-SH	16.43±1.32	13.13±0.94	15.35±0.87	16.11±1.62
Hippocampus	TBARS	0.08±0.02	0.26±0.05*	0.11±0.01	0.17±0.06
	ROS	5.52±1.19	10.61±0.93*	6.59±0.69	5.75±1.01
	NP-SH	16.02±1.02	12.69±0.63	12.28±0.49	15.44±1.34
Striatum	TBARS	0.29±0.03	0.27±0.01	0.36±0.05	0.11±0.03**
	ROS	26.47±6.32	24.63±6.51	21.68±3.67	21.24±2.4
	NP-SH	16.33±1.02	11.98±2.48	14.13±1.86	16.01±1.26

Values are expressed as means ± S.E.M. of 5-6 rats/ group. TBARS are expressed as nmol malondialdehyde/ mg of protein, ROS are expressed as μmol DCF/ mg of protein and NP-SH are expressed as nmol SH/ mg of protein. * Denoted $p<0.05$ from control group, ** Denoted $p< 0.01$ from MTZ group (Two-way ANOVA followed Bonferroni Test).

4.3 Manuscrito 2:

4.3.1 Disseneto de difenila na dieta modula a expressão dos genes de enzimas antioxidantes no hipotireoidismo induzido por metimazol

Dietary diphenyl diselenide modulates the expression of antioxidant enzymes genes in methimazole-induced hypothyroidism

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Abstract

This study was primarily aimed to investigate the potential use of dietary diphenyl diselenide in regulating the brain antioxidant enzyme genes expression under hypothyroidism condition. Besides, some oxidative stress parameters were simultaneously evaluated. Female rats were rendered hypothyroid by continuous exposure to methimazole (MTZ) at 20 mg/100 ml in the drinking water, during 3 months. Concomitantly, MTZ-induced hypothyroid rats were fed or not with a diet supplemented with diphenyl diselenide at 5 ppm. At the end of trials, mRNA levels and activities of antioxidant enzymes as well as lipid peroxidation, reactive oxygen species (ROS) generation and non-protein thiol (NPSH) content were determined in cerebral cortex, hippocampus and striatum. MTZ exposure was associated with a significant reduction in both thyroid hormones levels and body weight gain of animals. These parameters were not modified by diphenyl diselenide supplementation. Concerning to genes expression, hypothyroidism caused a marked up-regulation on mRNA expression of antioxidant enzymes catalase (CAT) and superoxide dismutase (SOD1, SOD2 and SOD3), glutathione peroxidase (GPx-1 and GPx-4), thioredoxin-reductase (TrxR1 and TrxR-2) isoforms in most of cerebral structures analyzed. The increase in the mRNA expression of all enzymes was positively correlated with the Nrf-2 transcription in cerebral cortex and hippocampus. Hypothyroid rats also exhibited clear signals of oxidative stress, which were verified by lipid peroxidation and ROS levels augmented in hippocampus and striatum and by NPSH levels decreased in cerebral cortex. These effects were not accompanied by changes in CAT, SOD and GPx activities. Dietary diphenyl diselenide was effective in reducing brain oxidative damage and normalizing most of changes on antioxidant genes expression associated to hypothyroidism. Indeed, dietary diphenyl diselenide elevated *per se* the NPSH content in cerebral cortex and hippocampus. In summary, the data of the present work confirm that hypothyroidism affects the brain oxidative stress status and the expression of antioxidant enzymes as well as indicate that diphenyl diselenide may be considered a promising molecule in counteracting these effects under hypothyroidism state.

Keywords: hypothyroidism, diphenyl diselenide, antioxidant genes expression, oxidative stress.

1. Introduction

Hypothyroidism is a syndrome resulting from decreased production and secretion of T3 (triiothyronine) and T4 (thyroxine) hormones, frequently diagnosed in women (Morganti et al., 2005). Clinical and experimental evidence has reported a correlation between reduction in thyroid hormones and brain disorders such as impaired memory, anxiety and depression (Demet et al., 2002, van Boxtel et al., 2004; Guimarães et al., 2009; Dias et al., 2012). Regarding to molecular and cellular mechanisms, it is known that thyroid hormones modulate gene activity via binding to thyroid hormone receptors, which are regulated by transcription factors or by additional transcription factors-mediated pathways (Weitzel et al., 2001, 2003). T3 can interact with nuclear receptors to regulate patterns of gene expression related to antioxidant defense systems and modulate specific cell cycle regulators (Ambrogini et al., 2005; Desouza et al., 2005; Puzianowska-Kuznicka et al., 2006; Subudhy & Chainy, 2010).

In conformity, neuronal damage induced by adult hypothyroidism has been linked to deficits on the regulation of cellular events as proliferation, differentiation and oxidative stress (Rahaman et al., 2001; Cano-Europa et al., 2008, Erdamar et al., 2008; Alva-Sánchez et al., 2009). Indeed, hypothyroidism may cause oxidative stress by altering antioxidant defense systems status (Das & Chainy, 2001, 2004; Bhanja & Chainy, 2010; Jena et al., 2012[a, b]). This fact is supported by experimental studies showing that antioxidant enzymes as catalase (CAT), superoxide dismutase (SOD), glutathione peroxidase (GPx) and thioredoxin reductase (TrxR) isoforms are regulated by thyroid hormones and selenium status (Wu et al., 2003; Chattopadhyay et al., 2007; Bhanja et al., 2008; Subudhy & Chainy, 2010). Besides, thyroid hormones may influence Nrf2-antioxidant signaling, an important cytoprotective transcription factor, that induces the production of a battery of endogenous enzymes, including SOD, CAT, GPx, peroxiredoxins (Prx), heme oxygenases (HOs) and TrxR (Jaiswal, 2004; Vries et al., 2008; Kaspar et al., 2009; Venditti et al., 2009).

Selenium (Se) is a trace element essential to human health. It is well established that an adequate Se supply is required to maintain physiological processes as normal thyroid (Arthur et al., 1992, 1993; Köhrle, 1999; Beckett & Arthur, 2005) and immune system functions (Baun et al., 1997) as well as to prevent cancer, heart disease and neurological pathologies (Fleet et al., 1997; Clement et al., 1998; Letavayová et al., 2006; Schweizer et al., 2004). This element is found in the active site of at least 30 Se-containing enzymes coded by 25 selenoprotein genes (Rayman, 2000; Kryukov et al., 2003), including GPx, TrxR and iodothyronine deiodinases families (Schomburg et al., 2004; Papp et al., 2007; Köhrle and

Gärtner, 2009). Although Se functions as an antioxidant, high dietary levels have been linked with toxic symptoms occurrence (Oldfield, 1987).

Diphenyl diselenide is a simple synthetic organic selenium compound that exhibits a series of pharmacological properties, including antioxidant and neuroprotective activities (Nogueira et al., 2004; Ghisleni et al., 2008; Savegnago et al., 2008). Similar to other selenium forms, diphenyl diselenide plays toxicological effects when used at high concentrations (Nogueira et al., 2003, Nogueira et al., 2004; Weis et al., 2006; Nogueira & Rocha, 2010).

With respect to use of organic Se compounds as therapeutic strategy against the neuropathologic symptoms induced by hypothyroidism, our group recently demonstrated that dietary diphenyl diselenide was effective in improving spatial learning and memory deficits in hypothyroid rats (Dias et al., 2012). Although the neuroprotective action of diphenyl diselenide has already been observed in different *in vivo* disease models, it is still necessary researches to investigate the cerebral molecular mechanisms involved in this effect. Thus, keeping in mind that thyroid disturbances may trigger brain pathways related to oxidative damage, we evaluated the effects of hypothyroidism condition on cerebral expression of antioxidant genes; and verified the role of diphenyl diselenide supplementation on the regulation of these cellular events. Besides, some antioxidant/oxidant parameters were simultaneously assayed.

2. Materials and Methods

2.1 Animals and Reagents

Forty-four adult female Wistar rats purchased from our own breeding colony were acclimated for 10 days before the beginning of the experiments. The animals were housed in plastic cages and maintained at 22-24° C, on a 12h light/ 12 h dark cycle, with free access to food (Supra®, Brazil) and water. All experiments were performed in accordance with guidelines of the Committee on Care and Use of Experimental Animal Resources, Federal University of Santa Maria, Santa Maria, RS, Brazil. Methimazole was purchased from Sigma Chemical Co. (St. Louis, Mo, USA). Diphenyl diselenide was synthesized according to literature methods (Paulmier, 1986). Analysis of the ^{1}HNMR and ^{13}CNMR spectra showed analytical and spectroscopic data in full agreement with its assigned structure. Reagents employed in total RNA isolation and analyses of mRNA expression by qPCR were purchased

from Invitrogen®. All other reagents and kits were of analytical grade and obtained from standard commercial suppliers.

2.2 Hypothyroidism Induction and Dietary Diphenyl Diselenide

Adult female rats were rendered hypothyroid by administration of methimazole (MTZ) 0.02 g/100 ml in the drinking water. The diet offered to animals consisted of a balanced food (Barbosa et al., 2008[a, b]); supplemented or not with diphenyl diselenide at 5 ppm. The food was prepared in an industrial mixer to allow the uniformity of the mixture, and the diphenyl diselenide added to supplemented diet was dissolved in soybean oil. After preparation, the diets were frozen (4°C) and offered *ad libitum* to the animals. The rats were randomly divided into four experimental groups (n=10): control group, received unsupplemented diet and water; MTZ group, received unsupplemented diet and MTZ in drinking water; Se group, received the diet supplemented with diphenyl diselenide and water; and Se+MTZ group, received both diet supplemented with diphenyl diselenide and MTZ in drinking water. The treatments were maintained concomitantly for three months. The amount of feed, water and MTZ solution consumed by groups were evaluated daily.

2.3 Tissue Preparation and RNA Isolation

At the end of the experimental period, animals were euthanized and the whole blood collected by cardiac puncture for measurement of total thyroid hormones (tT3 and tT4) levels. Brain was quickly removed, placed on ice and dissected in cerebral cortex, hippocampus and striatum. For analysis of mRNA expression, the total RNA was isolated from brain structures using Trizol® reagent (Invitrogen®) accordingly to the manufacturer´s suggested protocol. For others *ex vivo* assays, the respective tissues were homogenized in cold 10 mM Tris-HCl pH 7.4 and centrifuged at 3500 rpm for 10 minutes to yield the low-speed supernatant fractions (S1).

2.4 Thyroid Hormones Determination

Plasma levels of total T4 and total T3 were measured by microparticle enzyme immunoassay (MEIA) using AxSYM® system (Abbott Laboratories, Abbott Park, Illinois, USA), according to suppliers' instructions.

2.5 Antioxidant Enzyme Activities

CAT activity was determined spectrophotometrically according to the method proposed by Aebi (1984), based in the H_2O_2 decomposition at 240 nm during 120 seconds. Enzymatic reaction was initiated by adding 50 µl of S1 and the substrate (H_2O_2) to a concentration of 0.3 mM in a medium containing 50 mM potassium phosphate buffer, pH 7.0. One unit of CAT was considered as the amount of enzyme which decomposes 1 µmol H_2O_2/ minute at pH 7.0 at 25°C. The enzymatic activity was expressed as µmol H_2O_2/min/mg of protein.

SOD activity was determined spectrophotometrically according to the method proposed by Misra and Fridovich (1972), based in the formation of adrenochrome. Briefly, S1 fractions (20, 40 and 60 µl) were added to a medium containing 50 mM glycine buffer, pH 10.8 and adrenaline 1 mM. The kinetic analysis of SOD was started after adrenaline addiction and observed at 480 nm. One unit of SOD is defined as the amount of enzyme that inhibits 50% the speed of oxidation of adrenalin. The enzymatic activity was expressed as units of SOD/mg of protein.

GPx activity was determined spectrophotometrically by the method of Wendel (1981), through the GSH/NADPH/glutathione reductase system in 100 mM potassium phosphate buffer pH 7.0, by the dismutation of H_2O_2 at 340 nm. Briefly, S1 fractions were added in GSH/NADPH/glutathione reductase system and the enzymatic reaction was started after H_2O_2 addition. The enzymatic activity was expressed as nmol NADPH/min/mg of protein.

2.6 Lipid Peroxidation Determination

Lipid peroxidation was performed by measuring thiobarbituric acid reactive substances (TBARS) levels, according to the method of Ohkawa et al (1979). Briefly, aliquots of S1 from cerebral structures were incubated at 100°C for 1 hour in acid medium containing 200 µl of 8.1% sodium dodecyl sulfate, 500 µl of 500 mM acetic acid buffer pH 3.4 and 500 µl of 0.6% thiobarbituric acid (TBA). TBARS levels were measured at 532 nm and the absorbance was compared with the standard curve using malondialdehyde (MDA). Results were expressed as nmol malondialdehyde/mg of protein.

2.7 Reactive oxygen species (ROS) Determination

ROS determination was based on the deacetylation of the 2', 7'-dichlorofluorescein diacetato (DCFH-DA) and its subsequent oxidation by intracellular reactive species to DCF, a high fluorescence compound (Lebel et al., 1992). The supernatant fraction (S1) from cerebral structures was added to a medium with 10 mM Tris-HCl pH 7.4 and 1 mM DCFH-DA. Incubation was performed by 1 hour in the dark until fluorescence measurement. DCF fluorescence intensity emission was recorded at 520 nm with 480 nm excitation. Results were expressed as μmol DCF/ mg of protein, using a standard curve with DCF.

2.8 Non Protein Thiols (NPSH) Determination

NPSH levels of cerebral structures were determined by method of Ellman (1959). Aliquots from S1 were mixed (1:1) with 10% trichloroacetic acid (TCA) and centrifuged at 3500 rpm for 10 minutes. After centrifugation, an aliquot of supernatant (200 μl) was added to a reaction medium containing 0.5 mM phosphate buffer pH 7.4, and 10 mM 5,5'-dithio-bis(2-nitrobenzoic acid) (DTNB). NPSH levels were measured at 412 nm, using a standard curve with reduced glutathione (GSH).

2.9 Analysis of mRNA expression by q-PCR (Quantitative Real-Time PCR)

Gene specific primer sequences were based on published sequences in GenBank Overview (http://www.ncbi.nlm.nih.gov/genbank/) designed with Primer3 program version 0.4.0 (http://frodo.wi.mit.edu/primer3/) and custom made by Invitrogen® (Table 1). Tubulin served as reference gene. Total RNA samples were treated with DNase I (Invitrogen®) to remove genomic DNA contamination in the presence of RNase inhibitor. Reverse transcription (RT) of approximately 1 μg total RNA was performed using random primer, RNase inhibitor, dNTPs and M-MLV reverse transcriptase enzyme (Invitrogen®), accordingly to the manufacturer's suggested protocol. RT products (cDNAs) were maintained at -20°C. Quantitative real-time PCR were performed in 20 μl PCR mixture containing 1 μl RT product (cDNAs) as template, 1x PCR Buffer, 25 μM dNTPs, 0.2 μM of each primer, 1.5-2.5 mM $MgCl_2$ (Table 1), 0.1x SYBR Green I (Molecular Probes®) and 1U Taq DNA Polymerase (Invitrogen®). PCR mixtures were subjected to PCR at 95°C for 5 min followed of 40 cycles of 15 s at 95°C, 15 s at annealing temperature appropriated to each primer sequence (Table 1) and 25 s at 72°C for extension in a Thermocycler StepOne Plus (Applied Biosystems, Foster City, CA, USA). All samples were analyzed as technical triplicates with a no-template control also included. SYBR Green fluorescence was analyzed by StepOne Plus

Software version 2.0 (Applied Biosystems, Foster City, CA, USA) and Cq value (ΔCq) for each sample was calculated and reported using ΔΔCq method (Livak & Schmittgen, 2001). Briefly, for each well, a ΔCq value was obtained by the difference in Cq values (ΔCq) between the target gene and the reference gene. The ΔCq mean value obtained from the control group of each gene was used to calculate the ΔΔCq of the respective gene ($2^{-\Delta\Delta Cq}$).

2.10 Protein Determination

Protein concentration was measured by method of Bradford et al. (1976), using bovine serum albumin as standard.

2.11 Statistical Analysis

As there was no homogeneity of variances, the data of mRNA expression by q-PCR were analyzed by non parametric methods using Kruskal–Wallis test (kw) followed by Dunn's multiple comparisons test when appropriate. Correlation analysis (Coefficient of Spearman) was performed between mRNA expression of Nrf-2 and antioxidant enzymes expression. All others data were analyzed by Two-way ANOVA followed by Duncan's Multiple Range Test when appropriate. Non parametric data are represented as medians and ranges and parametric data as means and S.E.M. Differences among groups were considered significant when $p< 0.05$.

3. Results

3.1 Body weight gain and tT3 and tT4 levels evaluation

Two-way ANOVA with repeated measures showed that MTZ exposure caused a significant reduction on the body weight gain of animals when compared to the control group and that this effect was not restored by dietary diphenyl diselenide ($p<0.05$) (Table 2). Statistical analysis also revealed that the rats exposed to MTZ had a marked decrease in the levels of tT3 and tT4 (Table 3). Likewise, tT3 and tT4 levels were significantly reduced in hypothyroid rats supplemented with diphenyl diselenide ($p<0.05$).

3.2 mRNA Expression of Antioxidant Genes

In order to investigate the effects of MTZ exposure and diphenyl diselenide intake on mRNA expression of antioxidant genes, qPCR analysis was performed using primers sequences to CAT, SOD-1, SOD-2, SOD-3, GPx-1, GPx-4, TrxR-1, TrxR-2 and to the

transcripion factor Nrf-2. All values for each qPCR product for antioxidant genes were normalized and expressed as a ratio in relation to the reference gene (tubulin).

The data of Figure 1 show the effects of MTZ exposure and diphenyl diselenide on mRNA expression of CAT in brain structures. Kruskal-Wallis test followed by Dunn's Multiple Comparisons Test revealed that MTZ exposure was associated with an up-regulation of CAT mRNA expression in cerebral cortex [Kw=10.02, p=0.018], hippocampus [Kw=8.51, p=0.036] and striatum [Kw=11.21, p=0.01] when compared to control group and that these effects induced by MTZ were normalized by dietary diphenyl diselenide (Figure 1A, 1B and 1C , respectively).

The results of figure 2 illustrate the effects of MTZ exposure and diphenyl diselenide on mRNA expression of SOD enzymes in brain structures. Kruskal-Wallis test followed by Dunn's Multiple Comparisons Test revealed that MTZ exposed group had an increase on mRNA expression of SOD-1 in cerebral cortex [Kw=14.44, p=0.002], hippocampus [Kw=14.65, p=0.002] and striatum [Kw=11.63, p=0.008] (Figure 2A, 2B and 2C respectively). Similarly, MTZ exposure caused an increase on mRNA expression of SOD-2 in cerebral cortex [Kw=11.59, p=0.0089] and in striatum [Kw=9.21, p=0.026] when compared to control. Hippocampal SOD-2 expression was not modified by any treatment. The mRNA expression of SOD-3 in cerebral cortex [Kw=14.06, p=0.002], hippocampus [Kw=13.71, p=0.0033] and striatum [Kw=12.48, p=0.0059] was also significantly increased by MTZ exposure (Figure 2A, 2B and 2C). Indeed, diphenyl diselenide augmented *per se* the striatal SOD-3 expression (Figure 2C).

The Figure 3 shows the effects of MTZ exposure and diphenyl diselenide on mRNA expression of GPx enzymes in the different brain structures. Kruskal-Wallis followed by Dunn's Multiple Comparisons Test revealed that the mRNA expression of GPx-1 was significantly increased by MTZ exposure in cerebral cortex [Kw=13.18, p=0.0043], hippocampus [Kw=11.60, p=0.008] and striatum [Kw=12.33, p=0.0063] when compared to the control group and that dietary diphenyl diselenide was effective in reversing these alterations (Figure 3A, 3B and 3C). Indeed, the Figure 3C shows that diphenyl diselenide caused *per se* an increase on striatal GPx-1 mRNA expression compared to control group (Figure 3C). Regarding to GPx-4, mRNA expression was significantly increased in cerebral cortex [Kw=12.37, p=0.0062] and striatum [Kw=13.63, p=0.0034] of MTZ exposed rats when compared to control group. These effects were restored by diphenyl disselenide supplementation (Figure 3A and 3C). In hippocampus there was a tendency of MTZ in increasing GPx-4 expression; however, this difference was not statistically significant.

The results of figure 4 illustrate the effects of MTZ exposure and diphenyl diselenide on mRNA expression of TrxR enzymes in brain structures. Kruskal-Wallis analysis followed by Dunn's Multiple Comparisons Test revealed that MTZ exposure caused an significant increase on mRNA expression of TrxR-1 in cerebral cortex [Kw=12.78, p=0.0051] and striatum [Kw=11.18, p=0.01] when compared to the control and that these effects were restored by diphenyl diselenide intake (Figure 4A and 4C). In hippocampus, there was significant difference only between MTZ and MTZ+Se groups [Kw=9.68, p=0.021]. MTZ exposure also enhanced the mRNA TrxR-2 expression in cerebral cortex [Kw=12.73, p=0.005] when compared to the control group (Figure 4A). No statistical difference among the groups was observed on mRNA expression of TrxR-2 in hippocampus [Kw=5.44, p=0.14] and striatum [Kw=6.94, p=0.12]. To note, diphenyl diselenide supplementation reduced significantly the changes on mRNA expression in both TrxR-1 and TrxR-2 induced by MTZ (p<0.05).

Figure 5 shows the effects of MTZ exposure and diphenyl diselenide on mRNA expression of transcription factor Nrf-2. Kruskal-Wallis analysis followed by Dunn's Multiple Comparisons Test revealed that MTZ exposure was associated with an increase on mRNA expression of Nrf-2 in cerebral cortex [Kw=13.01, p=0.0046] and hippocampus [Kw=11.02, p=0.0048] when compared to control group (Figure 5A). In both structures, the elevated expression of Nrf-2 was normalized by diphenyl diselenide supplementation. No difference among the groups was observed on striatal mRNA expression of Nrf-2 (Figure 5C).

Correlation analysis (Coefficient of Spearman) was performed between mRNA expression of Nrf-2 and antioxidant enzymes expression. The data of Table 5 show that there was a positive and significant correlation between mRNA expression of transcription factor Nrf-2 and mRNA expression of CAT, SOD-1, SOD-2, SOD-3, GPx-1, GPx-4, TrxR-1 and TrxR-2 in cerebral cortex and hippocampus.

3.3 Antioxidant Enzyme Activities

Two-way ANOVA followed by Post hoc comparisons showed that no alteration on CAT activity was observed among groups in cerebral cortex and hippocampus (Table 4). Different, striatal CAT activity was significantly decreased in all groups when compared to the control (p<0.05). SOD activity was increased in hippocampus of hypothyroid rats supplemented with diphenyl diselenide when compared to control group (p<0.05). GPx activity was not significantly affected by MTZ exposure and/or dietary diphenyl diselenide in any tissue analyzed (Table 4).

3.4 TBARS and ROS levels determination

The results of Table 6 show the effects of MTZ exposure and diphenyl diselenide on TBARS and ROS levels determinations. Two-way ANOVA followed by Post hoc comparisons showed that MTZ exposure induced a significant increase on the levels of TBARS in hippocampus and striatum, which were restored by diphenyl diselenide supplementation ($p<0.05$). In these parameters, statistical analysis indicated that MTZ x Se interaction was not significant in both hippocampus [F (1, 25=3.26, $p=0.084$)] and striatum [F(1, 26=3.42, $p=0.075$)]. No statistical difference was observed in cortical TBARS levels among the groups [F (1, 28=0.0, $p=0.99$)].

Similarly, ROS formation was increased in hippocampus and striatum of MTZ treated rats. However, only the increase verified in hippocampus was reduced by diphenyl diselenide supplementation ($p<0.05$). In these parameters, Two-way ANOVA revealed that there was not significant MTZ x Se interaction in both hippocampus [F (1, 20=0.94, $p=0.34$)] and striatum [F (1, 21=0.38, $p=0.54$)]. In contrast, MTZ exposure did not induce any change on cerebral cortex ROS levels when compared to other groups [F (1, 28=0.0, $p=0.98$)].

3.5 NPSH Levels Determination

Table 6 shows the effects of MTZ exposure and diphenyl diselenide on NPSH levels determination. Two-way ANOVA followed by Post hoc comparisons showed that MTZ exposure decreased the NPSH content in cortex, which was avoided by diphenyl diselenide intake ($p<0.05$).This effect of MTZ was not observed in hippocampus and striatum. Of note, the groups supplemented with diphenyl diselenide alone had an increase on the NPSH levels in cortex and hippocampus ($p<0.05$) (Table 6). No MTZ x Se interaction on NPSH levels was observed in both cortex [F (1, 17=0.83)] and hippocampus [F (1, 16=0.09)].

4 Discussion

The advantageous use of dietary diphenyl diselenide has been evidenced in different experimental models of human pathologies (Barbosa et al., 2008[a,b]; Bem et al., 2009). With emphasis in hypothyroidism, we recently demonstrated the effectiveness of this selenium compound in reducing cognitive deficits induced by MTZ exposure (Dias et al., 2012). In this way, there are several studies linking the neuroprotective role of diphenyl diselenide with its antioxidant property (Posser et al., 2008; Nogueira et al., 2008; Nogueira & Rocha, 2010). However, most of them are restricted to biochemical analysis in terms of mechanisms.

Considering that antioxidant defense parameters are influenced by thyroid hormones, the present work was primarily aimed to investigate whether the neuroprotection offered by diphenyl diselenide intake under hypothyroidism state could be interrelated to antioxidant enzymes expression modulation. Our results showed that hypothyroidism caused a marked up-regulation on cerebral mRNA expression of antioxidant enzymes CAT, SOD-1, SOD-2, SOD-3, GPx-1, GPx-4, TrxR-1, TrxR-2 and transcription factor Nrf-2, which were normalized to basal values by diphenyl diselenide supplementation. Concomitantly, dietary diphenyl diselenide alleviated oxidative stress induced by hypothyroidism in some brain structures. These effects were not accompanied by changes in the activity of antioxidant enzymes.

According to previous studies, in our experimental protocol hypothyroidism resulted in a significant decrease of plasma tT3 and tT4 levels and body weight of rats (Zhang et al., 2009; Dias et al., 2012). Although, diphenyl diselenide supplementation has not been effective in blunting these alterations, the long-term of diphenyl diselenide diet consumption did not affect "*per se*" the body weight gain of animals.

Many experimental and clinical researches have investigated the involvement of oxidative stress under altered thyroid status since the antioxidant defense parameters are considerably influenced by thyroid hormones (Bhanja and Chainy, 2010; Subudhi and Chainy, 2010, 2012; ; Jena et al., 2012[a,b,c]). In this sense, exacerbated ROS formation and deteriorated antioxidant defense systems are among the harmful effects provoked by hypothyroidism in different tissues (Bhanja and Chainy, 2010; Jena et al., 2012[a,b,c]). With particular emphasis in brain, there is evidence that hypothyroidism induces lipid peroxidation, ROS overproduction and changes in gene expression and activities of antioxidant enzymes like SOD, CAT and GPx in structures as cerebellum, hippocampus, amygdala and cerebral cortex (Das and Chainy, 2004; Cano-Europa et al., 2008; Bhanja and Chainy, 2010; Jena et al., 2012[a,b]). In line with this, our data show that hypothyroid rats exhibited clear signals of oxidative stress, which were verified by lipid peroxidation and ROS levels augmented in hippocampus and striatum and by NPSH content decreased in cerebral cortex. In general, these detrimental effects were attenuated by diphenyl diselenide supplementation. Indeed, dietary diphenyl diselenide elevated *per se* the NPSH levels in cerebral cortex and hippocampus.

Although a growing body of evidence confirms that neurological disturbances induced by hypothyroidism have a close relationship with oxidative damage, there are few experimental works investigating the regulatory role of hypothyroidism on cerebral

expression of antioxidant systems. Likewise, there are no available data in the literature about the effects of diphenyl diselenide on antioxidant genes expression. Here, we found that hypothyroid rats had elevated transcripts of CAT and SOD, GPx and TrxR families in most of brain regions analyzed. Regarding to CAT, it was verified that hypothyroidism increased the expression of this enzyme in cerebral cortex, hippocampus and striatum. In most cases, mRNAs of SOD-s, including SOD-1, SOD-2 and SOD-3 were also up-regulated by hypothyroidism. However, this effect was not observed to SOD-2 in hippocampus. Exception GPx-4 (hippocampus), TrxR-1 (hippocampus) and TrxR-2 (hippocampus and striatum); mRNA expression of GPxs and TrxRs families were similarly enhanced under hypothyroidism condition. It is important mention here that the up-regulation of mRNA expression of all enzymes was positively correlated with the mRNA expression of Nrf-2 in cerebral cortex and hippocampus. It is probable that the increase observed in NrF-2 transcription is a compensatory response to oxidative stress induced by hypothyroidism condition, since upon exposure to ROS, Nrf-2 can dissociate from cytosolic Keap-1 and translocates to the nucleus, where it binds to the antioxidant response element (ARE) in the promoter region of genes encoding antioxidant enzymes (Jaiswal, 2004; Vries et al., 2008; Kaspar et al., 2009). Of note, the augments in mRNAs levels induced by hypothyroidism were not followed by modifications in enzymes activities, suggesting a post- transcriptional control by thyroid hormones.

In relation to regulatory role of hypothyroidism on brain genes expression, recently an elegant study demonstrated that the expression of SOD1 and GPx genes were down regulated and that CAT gene expression was not modified in cerebellum of PTU-treated adult rats. In contrast, in PTU challenged neonates the transcript product of SOD1 in developing cerebellum was increased and no alteration occurred in the expression of CAT and GPx genes (Bhanja and Chainy, 2010). Divergent responses are also observed among different tissues in hypothyroid rats. For example, in liver there is evidence that PTU-induced hypothyroidism enhanced the transcripts of CAT, GPx1 and GR enzymes and did not modify the expression of SOD (Subudhi and Chainy, 2012). Unlike, mRNA levels of SOD1 and SOD2 in kidney cortex were significantly decreased after PTU exposure (Jena et al., 2012[c]). Altogether, these data suggest that hypothyroidism induces selective pattern of responses, which may be differentially modulated by factors as age and specific tissues.

It is noteworthy here that the intake of a diet supplemented with diphenyl diselenide was effective in reducing brain oxidative damage and normalizing most of changes in antioxidant genes expression associated to hypothyroidism. It is plausible suppose that the

neuroprotection linked to diphenyl diselenide consumption is derived, at least part, from the antioxidant potential of compound, which may be attributed to its peroxidase-like activity and ability in improving the NPSH levels in brain (Navarro-Alarcon & Cabrera-Vique, 2008; Nogueira & Rocha, 2010). Supporting these proposal, current literature findings showed that indeed to glutathione peroxidase-like activity, diphenyl diselenide is able in stimulating NADPH oxidation in the presence of rat brain TrxR, indicating that it is substrate of brain TrxR (Freitas and Rocha, 2011). In addition, the results mentioned above suggest that diphenyl diselenide plays a modulatory role on antioxidant gene expression under hypothyroidism. In agreement, the beneficial effects of other antioxidant compounds such vitamin E and curcumin against oxidative stress and alterations on expression of antioxidant defense enzymes has been pointed in experimental models of hyper and hypothyroidism (Subudhy and Chainy 2010, 2012; Jena et al., 2012[a,b,c]).

Despite the regulation of selenoenzymes by selenium, there are several studies demonstrating the regulatory effect of selenium compounds in different tissues and species; however, the knowledge concerning the cerebral selenoproteins is scarce. Different from our expectancies, dietary diphenyl diselenide did not influence "*per se*" the expression of selenoproteins GPxs and TrxRs in the distinct brain regions analyzed. Different, it has been verified in some *in vivo* studies that the consumption of diets supplemented with Se from sodium selenite, Se-enriched yeast and/or selenomethionine increased the mRNA levels of GPx (GPx-1, GPx-4) and TrxRs (TrxR-1) genes in tissues like liver, kidney, thyroid and testis (Qin et al., 2009; Zhou et al., 2009; Wu et al., 2010; Yuan et al., 2012).

In summary, the data of the present work confirm that hypothyroidism affects the brain oxidative stress status and the expression of antioxidant enzymes as well as indicate that diphenyl diselenide may be considered a promising molecule in counteracting these effects under hypothyroidism condition. However, further studies are needed to investigate the molecular mechanism(s) involved in the neuroprotective role of compound since thyroid hormones may influence selectively the transcription and translation of antioxidant enzymes in different tissues.

Acknowledgements

The financial support by FINEP Research Grant ‘‘Rede Instituto Brasileiro de Neurociência (IBN-Net)’’#01.06.0842-00, FAPERGS/Pronex, CAPES/SAUX, VITAE Fundation, INCT-CNPq-Excitotoxicity and Neuroprotection and CNPq is gratefully acknowledged. J.B.T.R, C.W.N, F.A.S and N.B.V.B are the recipients of CNPq fellowships.

Legends of figures

Figure 1: Effects of MTZ exposure and dietary diphenyl diselenide on mRNA expression of CAT in cerebral cortex (graph A), hippocampus (graph B) and striatum (graph C). * Denoted $p<0.05$ from control group. All values are expressed as median and range (n=3).

Figure 2: Effects of MTZ exposure and dietary diphenyl diselenide on mRNA expression of SOD-1, SOD-2 and SOD-3 in cerebral cortex (graphs A), hippocampus (graphs B) and striatum (graphs C). * Denoted $p<0.05$ from control group, $^{\#}$ Denoted $p< 0.05$ between MTZ and Se+MTZ groups. All values are expressed as median and range (n=3).

Figure 3: Effects of MTZ exposure and dietary diphenyl diselenide on mRNA expression of GPx-1 and GPx-4 in cerebral cortex (graphs A), hippocampus (graphs B) and striatum (graphs C). * Denoted $p<0.05$ from control group. All values are expressed as median and range (n=3).

Figure 4: Effects of MTZ exposure and dietary diphenyl diselenide on mRNA expression of Thioredoxin-reductase-1 and Thioredoxin-reductase-2 in cerebral cortex (graphs A), hippocampus (graphs B) and striatum (graphs C). * Denoted $p<0.05$ from control group. All values are expressed as median and range (n=3).

Figure 5: Effects of MTZ exposure and dietary diphenyl diselenide on mRNA expression of Nrf-2 in cerebral cortex (graph A), hippocampus (graph B) and striatum (graph C). * Denoted $p<0.01$ from control group. All values are expressed as median and range (n=3).

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Figure 1

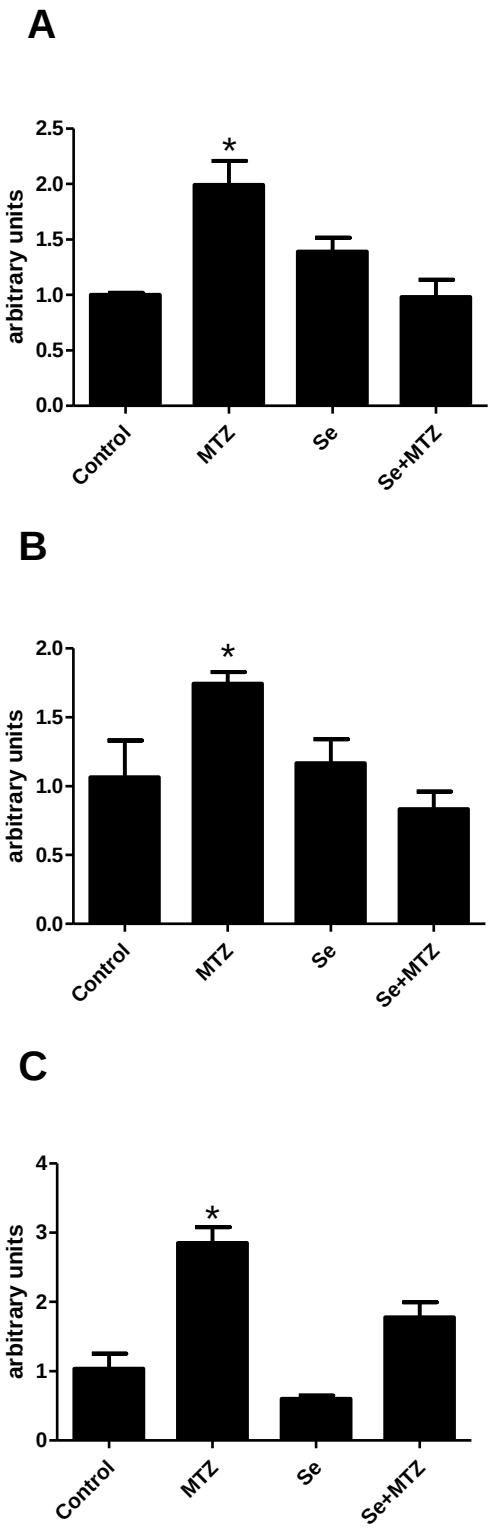
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Figure 2

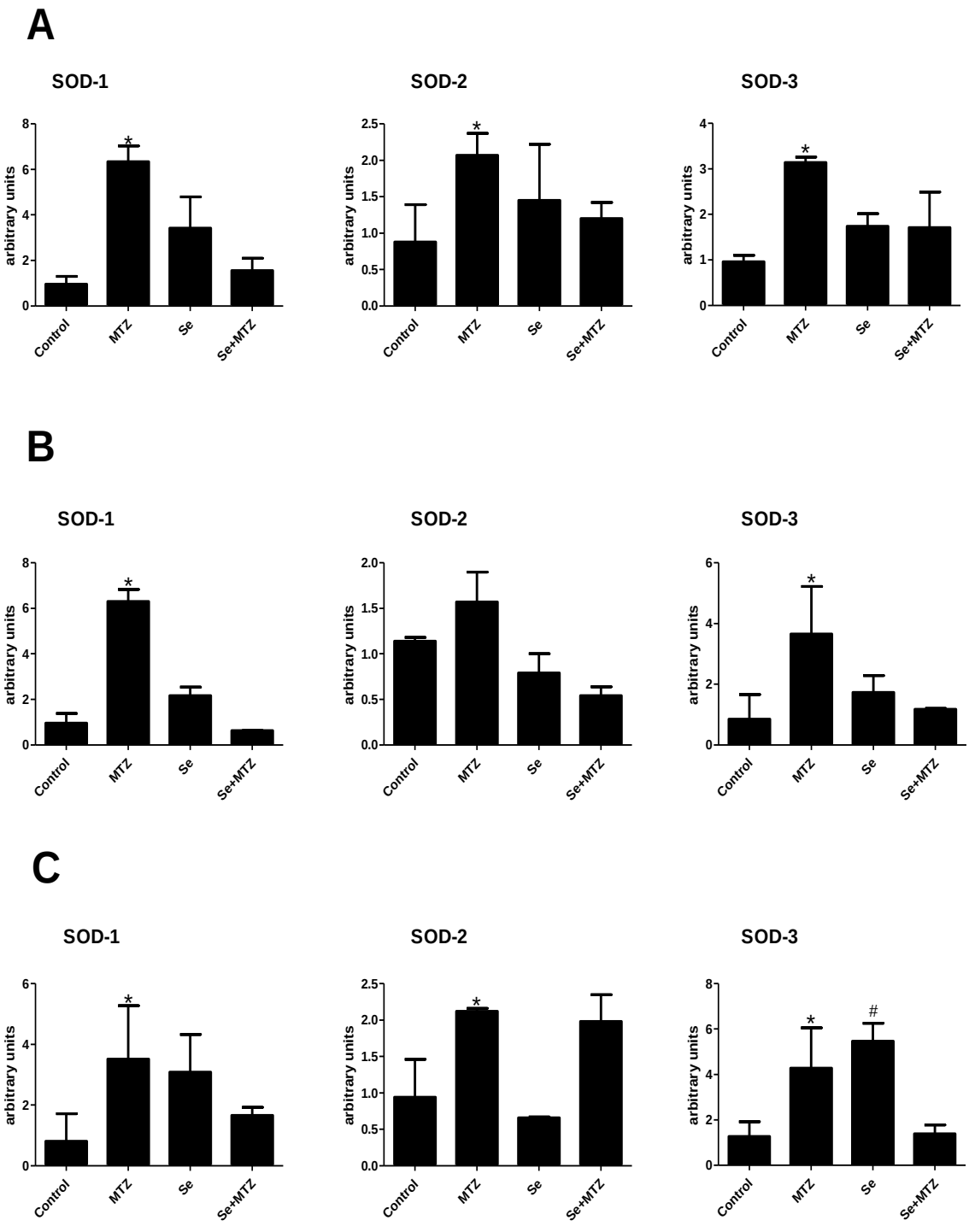
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Figure 3

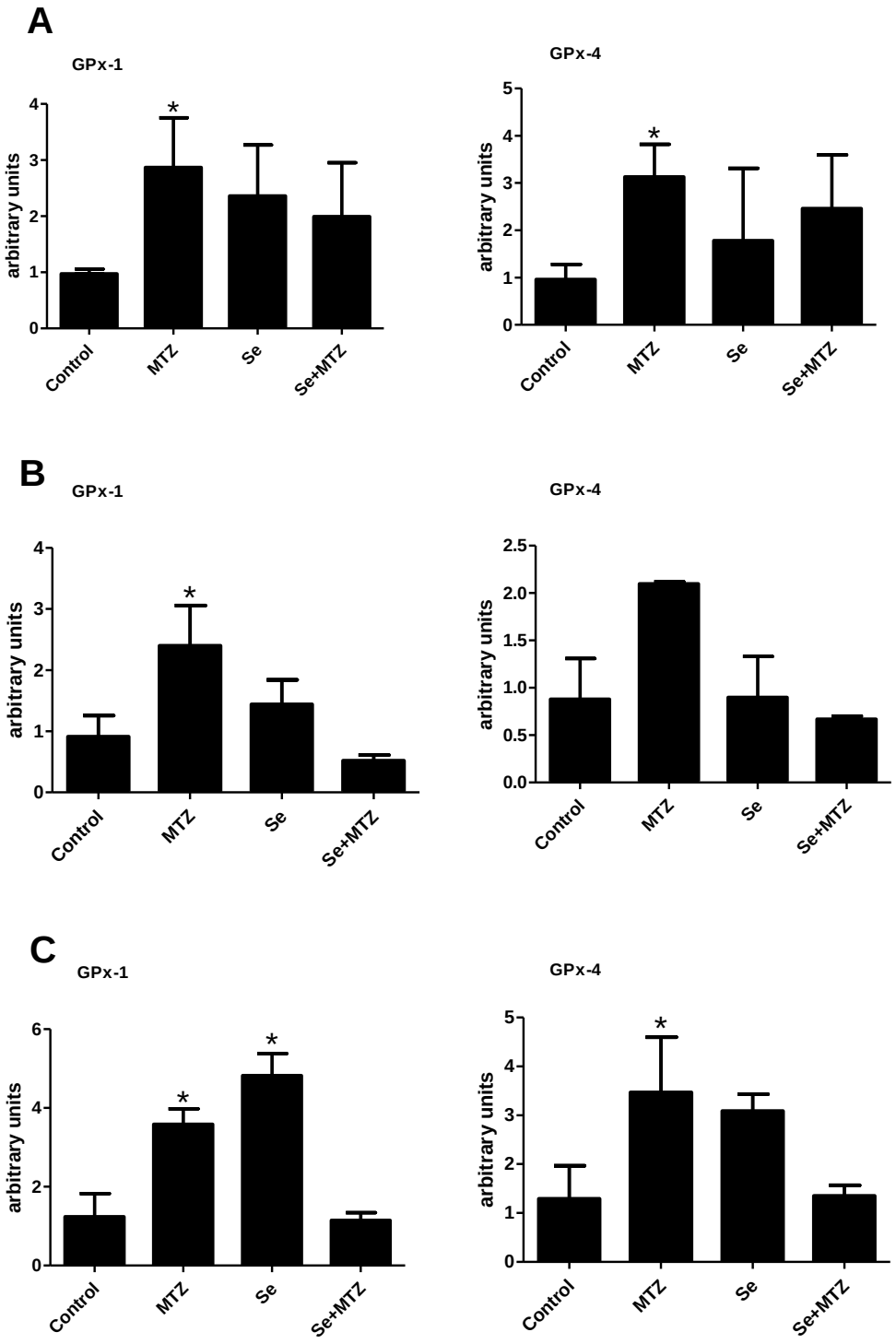
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Figure 4

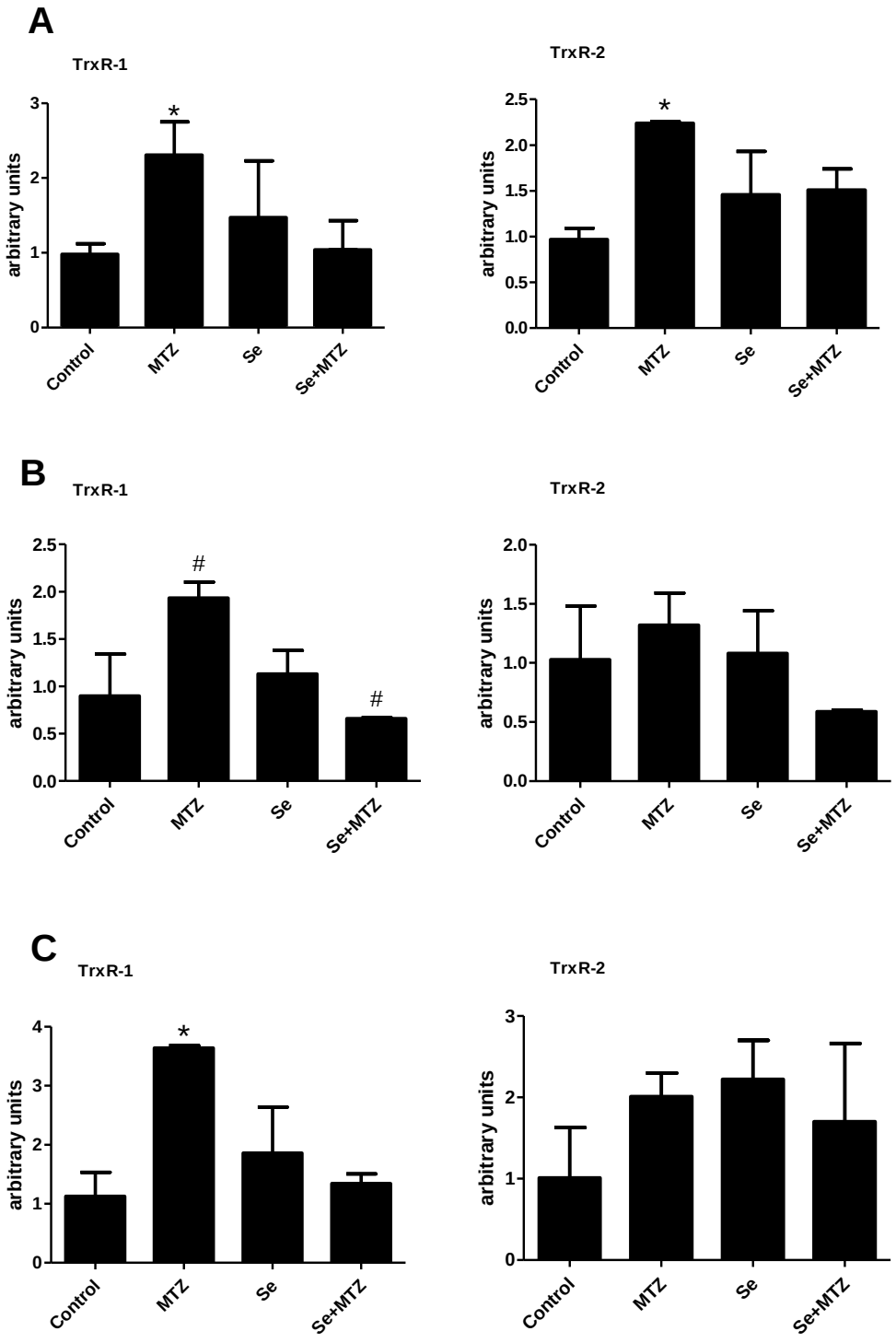
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Figure 5

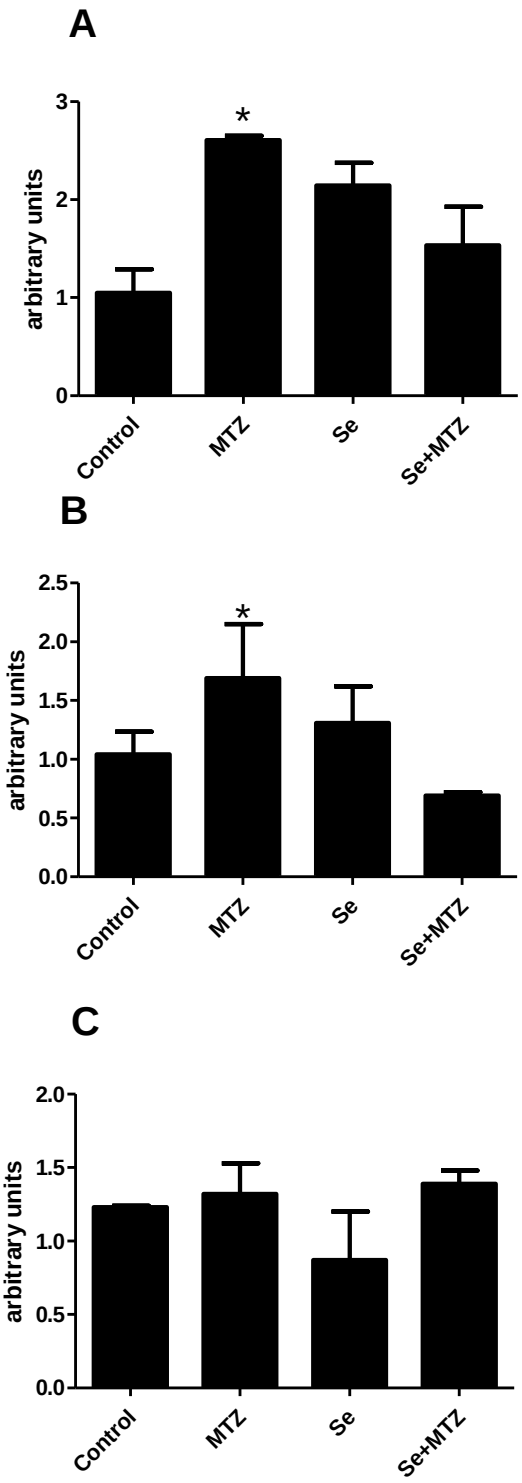
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Table 1: Accession number, nucleotide sequence, binding position, annealing temperature and products of qPCR primers (S=sense and AS=antisense)

Gene	Accession no.	Primer sequence (5'-3')	Binding position	Annealing temperature	qPCR product
CAT	BC081853.1	S- ACA TGG TCT GGG ACT TCT GG	539-558	55°C	188 bp
		AS- GAT GCC CTG GTC AGT CTT GT	726-707		
SOD-1	Y00404.1	S- CCA CTG CAG GAC CTC ATT TT	176-195	62°C	190 bp
		AS- TCG TGG ACC ACC ATA GTA CG	365-346		
SOD-2	BC070913.1	S- ATC TGA ACG TCA CCG AGG AG	182-201	60°C	186 bp
		AS- GCT TGA TAG CCT CCA GCA AC	367-348		
SOD-3	Z24721.1	S- ATG GTG GCC TTC TTG TTC TG	1-20	63°C	177 bp
		AS- CCG TTG TTT TCC TAG CTC CA	177-158		
GPx-1	NM_030826.3	S- TGA GAA GTG CGA GGT GAA TG	330-349	60°C	187 bp
		AS- AAC ACC GTC TGG ACC TAC CA	516-497		
GPx-4	NM_017165.2	S- GCC GAG TGT GGT TTA CGA AT	271-290	60°C	182 bp
		AS- GGC TGG ACT TTC ATC CAT TT	452-433		
Thiored-1	BC085726.1	S- GCA GAC CAA TGT GCC TTA CA	1209-1228	60°C	185 bp
		AS- CAC AGC AGC CAT ACT CCA AA	1393-1374		
Thiored-2	BC085734.1	S- CAG TTA TGT GGC CCT GGA GT	684-703	63°C	188 bp
		AS- TCG GGA GTT TTC TGA TGA GG	871-852		

NRF2	AF037350.1	S- GAG ACG GCC ATG ACT GAT TT AS- CAG TGA GGG GAT CGA TGA GT	487- 506 684- 665	57°C	198 bp
Tubulin	BC070957.1	S- CAT GAA CAA CGA CCT CAT CG AS- TGT GGA CAC CAT CAC GTT CT	744-763 921- 902	57°C	178 bp

Table 2: Effects of MTZ and dietary diphenyl diselenide on the body weight gain (g) of experimental groups.

	Initial body weight	Final body weight	Body weight gain (g)
Control	229.3 ± 8.1	256.9 ± 5.7	+ 27.6
MTZ	228.7 ± 7.4	223.4 ± 6.1*	- 5.3
Se	229.6 ± 7.2	254.3 ± 6.2	+ 24.7
Se+MTZ	219.8 ± 4.1	213.1 ± 9.9*	- 6.7

Values are expressed as means ± S.E.M. of 11 rats/ group. *Denoted $p<0.05$ as compared to control group (Two-way ANOVA followed Duncan's Multiple Range Test).

Table 3: Plasma tT3 and tT4 levels of experimental groups

	Control	MTZ	Se	Se+MTZ
tT3	0.46 ± 0.05	0.13 ± 0.04*	0.39 ± 0.02	0.21 ± 0.06*
tT4	3.44 ± 0.28	1.07 ± 0.27*	3.06 ± 0.22	1.38 ± 0.41*

tT3 is expressed as ng/ml plasma and tT4 as pg/ ml plasma. Values are expressed as means ± S.E.M. of 9-11 rats/group. * Denoted $p>0.05$ from control group (Two-way ANOVA followed Duncan's Multiple Range Test).

Table 4: Effects of hypothyroidism and dietary diphenyl diselenide on antioxidant enzyme activities

		Control	MTZ	Se	Se+MTZ
CAT	Cerebral Cortex	16.1±2.2	13.3±3.1	12.1±2.2	11.8±3.1
	Hippocampus	5.1±1.1	8.1±2.1	14.7±3.9	14.2±2.9
	Striatum	12.7±1.7	7.1±0.9*	4.7±0.8*	4.7±1.0*
SOD	Cerebral Cortex	23.2±1.5	20.7±4.1	18.5±0.9	18.6±1.4
	Hippocampus	21.1±3.6	30.5±1.9	29.3±2.6	50.4±4.7*
	Striatum	32.9±4.5	27±2.8	26.1±2.6	33.2±2.9
GPx	Cerebral Cortex	17±1.5	11.5±0.9	14±1.7	13.9±0.8
	Hippocampus	10.1±2.9	14.3±1.8	11.5±1.3	12.2±2.1
	Striatum	13.5±2.8	15.2±2.7	15.4±2.4	12.9±0.8

Values are expressed as means ± S.E.M. of 6-8 rats/ group. CAT activity is expressed as μmol H_2O_2/ min/ mg of protein, SOD activity is expressed as units of SOD/ mg of protein and GPx activity is expressed as nmol NADPH/ min/ mg of protein.*Denoted $p<0.05$ as compared to control group (Two-way ANOVA followed by Duncan's Multiple Range Test).

Table 5: Correlation between mRNA expression of transcription factor NRF-2 and mRNAs expression of antioxidant enzymes in cerebral cortex and hippocampus

		mRNA NRF-2		
		r (Coefficient of Spearman)	r^2	*p* value
mRNA CAT	Cerebral cortex	0.61	0.37	0.03*
	Hippocampus	0.84	0.71	0.0005*
	Striatum	0.45	0.16	0.16
mRNA SOD-1	Cerebral cortex	0.68	0.47	0.01*
	Hippocampus	0.83	0.69	0.001*
	Striatum	-0.16	0.02	0.6
mRNA SOD-2	Cerebral cortex	0.74	0.55	0.008*
	Hippocampus	0.79	0.63	0.001*
	Striatum	0.52	0.27	0.09
mRNA SOD-3	Cerebral cortex	0.81	0.67	0.001*
	Hippocampus	0.81	0.65	0.001*
	Striatum	-0.16	0.02	0.6
mRNA GPx-1	Cerebral cortex	0.85	0.73	0.0004*
	Hippocampus	0.92	0.84	0.0001*
	Striatum	-0.27	0.07	0.41
mRNA GPx-4	Cerebral cortex	0.8	0.65	0.001*
	Hippocampus	0.82	0.67	0.001*
	Striatum	0.09	0.008	0.78
mRNA TrxR-1	Cerebral cortex	0.79	0.63	0.001*
	Hippocampus	0.88	0.79	0.0001*
	Striatum	0.15	0.02	0.63
mRNA TrxR-2	Cerebral cortex	0.66	0.44	0.01*
	Hippocampus	0.61	0.37	0.03*
	Striatum	0.09	0.008	0.8

* Significant *p*-value

Table 6: Effects of hypothyroidism and dietary diphenyl diselenide on TBARS, ROS and NPSH levels determinations

		Control	MTZ	Se	Se+MTZ
TBARS	Cerebral Cortex	2.94±0.37	2.35±0.25	2.94±0.54	2.35±0.23
	Hippocampus	1.94±0.09	3.38±0.47*	1.85±0.24	2.29±0.2
	Striatum	1.83±0.22	3.44±0.67*	1.35±0.11	1.7±0.3
ROS	Cerebral Cortex	2.87±0.19	3.19±0.36	3.45±0.33	3.76±0.31
	Hippocampus	3.05±0.27	4.74±0.72*	2.62±0.08	3.38±0.24
	Striatum	3.33±0.17	4.45±0.45*	3.24±0.29	4.68±0.19*
NPSH	Cerebral Cortex	22.75±1.19	13.15±0.89*	27.34±3.77#	23.85±1.93
	Hippocampus	15.35±1.48	15.47±2	22.31±2.7#	23.71±2.66
	Striatum	14.24±1.32	14.05±0.99	12.3±1.08	13.9±1.58

Values are expressed as means ± S.E.M. of 6-8 rats/ group. TBARS are expressed as nmol malondialdehyde/ mg of protein, ROS are expressed as μmol DCF/ mg of protein and NP-SH are expressed as nmol SH/ mg of protein. *Denoted $p<0.05$ as compared to control group, # Denoted $p<0.05$ as compared to MTZ group (Two-way ANOVA followed by Duncan Multiple Range Test).

5. DISCUSSÃO

O hipotireoidismo clínico ou subclínico é a doença endócrina mais comum, com elevada prevalência e incidência no sexo feminino, sendo cada vez mais diagnosticado e associado como fator de risco para diversas patologias que afetam diferentes funções fisiológicas. O hipotireoidismo pode comprometer as funções cardiovasculares, músculo-esqueléticas e neurológicas, trazendo enormes prejuízos aos seus portadores. Como explorado neste trabalho, salientamos o comprometimento das funções neurológicas, as quais são têm sido frequentemente abordadas em estudos epidemiológicos e experimentais.

Neste trabalho, utilizamos um modelo experimental de hipotireoidismo a partir da administração do fármaco anti-tireóideo metimazol. Este fármaco é comercializado no Brasil, sendo comumente empregado no tratamento da Doença de Graves. Seu mecanismo de ação é baseado no bloqueio da tireoperoxidase, inibindo assim a biossíntese dos hormônios tireóideos. Vários estudos experimentais que empregam o metimazol na indução de hipotireoidismo consideram o método efetivo principalmente devido a fácil aceitação pelos animais de experimentação e a forma de administração através da dissolução na água de beber. Outros métodos de indução de hipotireoidismo incluem a tireoidectomia, método cirúrgico e definitivo que gera um quadro de hipotireoidismo severo; a administração de iodo radioativo e o uso do fármaco anti-tireóideo propiltiouracil, que também bloqueia a tireoperoxidase e possui como mecanismo de ação adicional o bloqueio da conversão periférica de T4 em T3. No entanto, diferente do metimazol, este fármaco possui sabor desagradável e não é bem aceito pelos animais de experimentação.

Como exposto anteriormente, o conhecimento sobre as funções da glândula tireóide são relativamente recentes e foram originados principalmente a partir de observações clínicas que mostravam que em áreas carentes de iodo, havia altos índices de casos de cretinismo endêmico. No entanto, foi constatado que somente a suplementação com iodo não era capaz de reverter os danos teciduais localizados na glândula e consequentemente, os danos sistêmicos provocados pelo hipotireoidismo. Posteriormente, o selênio foi reconhecido como elemento essencial para a biossíntese dos hormônios tireóideos. Esse achado foi responsável pela inclusão do selênio junto aos micronutrientes essenciais, pois até então somente as propriedades toxicológicas deste elemento eram conhecidas (ARTHUR et al., 1992, 1993; KÖHRLE, 1999; BECKETT & ARTHUR, 2005; KÖHRLE & GÄRTNER, 2009).

Desde então, muitos estudos vêm sendo desenvolvidos com o objetivo de elucidar os efeitos farmacológico-toxicológicos de compostos contendo selênio sobre diversas patologias. Com relação aos trabalhos que visam investigar as funções tireóideas, observa-se principalmente a utilização do composto inorgânico de selênio, selenito de sódio. No que diz respeito ao uso de compostos orgânicos, os relatos são escassos.

O disseleneto de difenila é um composto orgânico de selênio com reconhecidas propriedades farmacológicas, as quais estão estreitamente relacionadas com seu potencial antioxidante, derivado de sua eficiente atividade mimética à enzima glutationa peroxidase e do seu papel como substrato do sistema da tioredoxina redutase (NOGUEIRA et al., 2004, NOGUEIRA & ROCHA, 2010; FREITAS & ROCHA, 2011). Suas propriedades toxicológicas também são amplamente pesquisadas em diferentes modelos experimentais e incluem entre outras, a depleção de tióis endógenos e ação pró-convulsivante (NOGUEIRA et al, 2001; NOGUEIRA et al., 2004; BRITO et al., 2006; NOGUEIRA & ROCHA, 2010).

Com base nas relações estabelecidas entre disfunções tireóideas, distúrbios neuropsiquiátricos, estresse oxidativo e promissores agentes terapêuticos, este estudo procurou investigar se o consumo prolongado de uma dieta suplementada com disseleneto de difenila poderia reduzir as alterações neurológicas (comportamentais e bioquímicas) associadas ao hipotireoidismo. Cabe salientar, que o uso do disseleneto de difenila como complemento alimentar já foi previamente testado em modelos de diabetes mellitus e tumorigênese experimentais (1-10 ppm), onde não foram evidenciados sinais de toxicidade associados ao tempo (1-3 meses) e a forma de tratamento (BARBOSA et al., 2008[a, b]).

Estudos epidemiológicos relatam a correlação existente entre hipotireoidismo e déficits de funções cognitivas relacionadas à aprendizagem e memória, bem como ao desenvolvimento de distúrbios como depressão e ansiedade (OSTERWEIL et al., 1992; PLACIDI et al., 1998; DEMET et al., 2002, VAN BOXTEL et al., 2004; GUIMARÃES et al., 2009). Em linhas gerais, os pacientes hipotireóideos apresentam dificuldades na memorização e aprendizagem, no processamento de informações e menor eficiência em executar as funções propostas (DAVIS & TREMONT, 2007). Neste contexto, o **ARTIGO 1** teve como objetivo principal avaliar se a suplementação com disseleneto de difenila na dieta poderia reduzir os déficits cognitivos induzidos pela exposição ao metimazol em três tarefas comportamentais: campo-aberto para avaliar a atividade locomotora, labirinto-em-cruz elevado para avaliar o comportamento relacionado à ansiedade e o labirinto aquático de Morris a fim de avaliar a aprendizagem espacial e memória. O protocolo experimental que constitui o **MANUSCRITO 1,** foi basicamente desenvolvido para investigar se o modelo de indução de hipotireoidismo

com metimazol poderia causar um comportamento semelhante à depressão, uma vez que, dados da literatura indicam a correlação do hipotireoidismo com esta desordem neurológica e mostram que o hipotireoidismo via tireoidectomia ou dieta com restrição de iodo induz comportamento semelhante a depressão (KULIKOV et al., 1997). A partir desta verificação, a dieta suplementada com disseleneto de difenila foi empregada como opção terapêutica para o tratamento desta condição Adicionalmente, avaliaram-se parâmetros bioquímicos associados a eventos oxidativos.

O **MANUSCRITO 2**, contempla os dados referentes a investigações direcionadas para verificar se o efeito neuroprotetor do disseleneto de difenila observado nos protocolos comportamentais acima citados poderia, além da ação antioxidante, envolver a modulação da expressão gênica de enzimas antioxidantes em áreas cerebrais específicas.

Os resultados verificados no **ARTIGO 1** e no **MANUSCRITO 1** mostram que a suplementação com disseleneto de difenila aos animais hipotireóideos causou uma melhora nos parâmetros de aprendizagem espacial e memória, que foram avaliadas através no Labirinto Aquático de Morris **(Figuras 1 e 2- Artigo 1)** e reverteu o comportamento semelhante à depressão, diminuindo o tempo de imobilidade dos animais no Teste do Nado Forçado durante todo o período experimental **(Figura 1- Manuscrito 1)**. É importante ressaltar que o grupo suplementado com o composto também apresentou um desempenho melhor que o grupo controle no tempo gasto para encontrar a plataforma durante os treinos do labirinto aquático de Morris **(Figura 1 - Artigo 1)** e também um tempo de imobilidade menor do que o grupo controle no último mês de avaliação no Nado Forçado **(Figura 1- Manuscrito 1)**. Os efeitos do tratamento com disseleneto de difenila e/ou o hipotireoidismo não envolveram alterações motoras verificadas no Teste do Campo-Aberto que pudessem influenciar nos resultados obtidos no Labirinto Aquático de Morris **(Artigo 1 - Tabela 4)** e no Teste do Nado Forçado **(Manuscrito 1 – Tabela 1)**. Esses dados confirmam resultados de estudos prévios, que mostram que o composto disseleneto de difenila causa uma melhora em parâmetros de memória e de aprendizagem avaliados no Labirinto Aquático de Morris, Labirinto em T e Teste do Reconhecimento do Objeto, (ROSA et al., 2003; STANGERLHIN et al., 2008) bem como um efeito semelhante ao antidepressivo no Teste da Suspensão da Cauda e Teste do Nado Forçado (SAVEGNAGO et al., 2007, 2008; ACKER et al., 2009) em diferentes modelos experimentais.

Usualmente a recuperação das funções cognitivas associadas ao tratamento com disseleneto de difenila é atribuída, pelo menos em parte, às propriedades antioxidantes do composto; enquanto o efeito semelhante ao antidepressivo às interações com o sistema

monoaminérgico, nitrérgico e a atividade da enzima Na^+-K^+-ATPase (SAVEGNAGO et al., 2007, 2008; ACKER et al., 2009). No entanto, cabe salientar aqui que a maior parte desses estudos são referentes a protocolos de experimentação de curta duração e que, em geral, utilizam o composto por via oral (gavagem), subcutânea e/ou intra-peritoneal. Os efeitos da suplementação com disseleneto de difenila na dieta ainda não haviam sido testados em tarefas comportamentais. Assim, nossos resultados são os primeiros a evidenciar a ação neuroprotetora do composto nesses parâmetros quando ingerido através da dieta.

De forma geral, as análises bioquímicas desenvolvidas (**MANUSCRITOS 1 e 2)** indicam que o hipotireoidismo induziu estresse oxidativo em estruturas cerebrais específicas, achados que estão de acordo com protocolos experimentais relacionados (CANO-EUROPA et al., 2008; AMARA et al., 2009, 2010; SAKR et al., 2011). Através da análise dos níveis de TBARS, NPSH e ROS **(MANUSCRITO 1 (tabela 3)** e **MANUSCRITO 2 (tabela 6))**, foi verificado que o tecido cerebral dos animais hipotireóideos foi susceptível a peroxidação lipídica, a depleção de tióis e a formação de espécies reativas de oxigênio, sendo o córtex cerebral e o hipocampo as estruturas mais afetadas na condição hipotireóidea. Importante ressaltar, que nos animais hipotireóideos suplementados com disseleneto de difenila, esses marcadores de danos oxidativos foram significativamente reduzidos.

Alteração na atividade da MAO, responsável por regular os níveis de monoaminas na fenda sináptica, tem sido previamente sugerida como um dos mecanismos pelo qual o disseleneto de difenila exerce efeito semelhante ao antidepressivo (SAVEGNAGO et al., 2007). No entanto, aqui não foi constatado efeito "per se" do disseleneto de difenila sobre a atividade desta enzima. Por outro lado, foi observado que o hipotireoidismo causou uma inibição significativa na atividade da MAO B **(Manuscrito 1 - figura 2)**, isoforma responsável principalmente pelo metabolismo da beta-feniletilamina, que foi restaurada pelo tratamento com disseleneto de difenila. Tem sido sugerido que a MAO B em ratos não exerce um efeito pronunciado no metabolismo da dopamina, ao contrário do que é observado em seres humanos (BORTOLATO et al., 2008). No entanto, a inibição da MAO B no hipotireoidismo poderia refletir uma resposta tecidual adaptativa para promover o aumento dos níveis de beta-feniletilamina, anfetamina endógena e estimulante das funções cerebrais (JANSSEN et al., 1999).

As análises de expressão gênica representadas no **MANUSCRITO 2** mostram que a condição hipotireóidea dos animais causou um aumento significativo na expressão do RNA mensageiro do fator de transcrição NRF-2 e das enzimas antioxidantes (CAT, SOD-1, SOD-2, SOD-3, GPx-1, Gpx-4, Thiored-1 e Thiored-2) na maioria das estruturas cerebrais avaliadas

(manuscrito 2- figura 1, 2, 3, 4 e 5). Estatisticamente foi encontrada uma correlação positiva entre os níveis de NRF-2 e a expressão das enzimas antioxidantes **(manuscrito 2 - tabela 5)**. O NRF-2 é um importante regulador da resposta celular contra o estresse oxidativo e pode sofrer modulação a partir do status tireóideo (JAISWAL, 2004; VRIES et al., 2008; KASPAR et al., 2009; VENDITTI et al., 2009). De acordo com esses resultados, trabalhos recentes indicam que disfunções na glândula tireóide afetam a expressão gênica de diversas enzimas antioxidantes em diferentes tecidos e que terapias antioxidantes (vitamina E, curcumina) têm sido eficazes em modular esses efeitos (BHANJA et al., 2008; BHANJA & CHAINY, 2010; SUBUDHI & CHAINY, 2010, 2012; JENA et al., 2012[a, b, c]). O aumento da expressão dos mRNAs não foi acompanhado pelo aumento na atividade das enzimas, uma vez que as atividades da CAT, SOD e GPx não foram significativamente alteradas pelo hipotireoidismo **(Manuscrito 2 - tabela 4)**. É importante enfatizar aqui que os pontos de regulação até que o mRNA seja transcrito na proteína (ou no caso, nas enzimas) são bastante complexos e não totalmente compreendidos. No entanto, sabe-se que os hormônios tireóideos podem atuar em diferentes níveis de regulação e o seu déficit pode inviabilizar a transcrição da enzima ou a sua ativação. Recentemente, também foi lançada a hipótese de que a regulação da transcrição gênica pelos hormônios tireóideos pode envolver a expressão de micro-RNAs, pequenos RNAs não codificantes que modulam a transcrição ao se ligar de forma específica nos genes-alvo (DONG et al., 2010).

Com relação aos parâmetros de expressão avaliados, nossos resultados mostram ainda que as alterações induzidas pelo hipotireoidismo foram normalizadas pela suplementação com disseleneto de difenila. Esse conjunto de resultados mostra pela primeira vez, que a neuroproteção oferecida pelo disseleneto de difenila pode estar relacionada com um possível efeito modulador do composto sobre a expressão gênica de enzimas com papel antioxidante, que não somente selenoproteínas. No entanto, mais pesquisas são necessárias para esclarecer os mecanismos moleculares envolvidos tanto nos efeitos causados pelo hipotireoidismo como pelo disseleneto de difenila sobre a regulação da expressão gênica relacionada às defesas antioxidantes em áreas específicas do cérebro.

No geral, os dados obtidos nos diferentes protocolos experimentais realizados, indicam que o hipotireoidismo possui uma estreita ligação com o desenvolvimento de distúrbios neurológicos, particularmente o déficit cognitivo e a depressão. Além disso, ficaram evidentes também as relações entre o hipotireoidismo, o estresse oxidativo e a regulação da expressão gênica relacionada às defesas antioxidantes. Em todos estes resultados os efeitos da suplementação com disseleneto de difenila na dieta mostraram um perfil terapêutico

favorável, revertendo os parâmetros desfavoráveis e melhorando os perfis de resposta aos níveis do controle. Assim, este trabalho contribuiu para que os efeitos farmacológicos do disseleneto de difenila, principalmente os relacionados à melhora da função cognitiva **(Artigo 1)**, efeito semelhante ao antidepressivo **(Manuscrito 2)** e de regulador da expressão gênica relacionada as defesas antioxidantes **(Manuscrito 3)** fossem amplamente explorados. Com certeza, estudos adicionais são necessários para esclarecer os mecanismos que envolvem a ocorrência destes efeitos farmacológicos. Nesses efeitos, destaca-se a eficácia do tratamento usando a suplementação com o disseleneto de difenila na dieta em melhorar os perfis de respostas dos animais hipotireóideos e ressalta-se, além da propriedade antioxidante, o possível papel modulador do composto de selênio sobre a expressão de genes antioxidantes no SNC. De forma geral, a realização deste trabalho contribuiu para demonstrar o efeito promissor do disseleneto de difenila no tratamento das complicações causadas pelo hipotireoidismo.

6. CONCLUSÕES

De acordo com os objetivos apresentados nesta tese podemos concluir que:

Capítulo I

- A atividade motora e exploratória não foi alterada pela suplementação com disseleneto de difenila em ratas adultas com hipotireoidismo induzido pelo metimazol;

- O comportamento relacionado à ansiedade não foi alterado pela suplementação com disseleneto de difenila em ratas adultas com hipotireoidismo induzido pelo metimazol;

- A aprendizagem espacial e memória das ratas induzidas ao hipotireoidismo pelo metimazol melhoraram significativamente com a suplementação de disseleneto de difenila na dieta, revertendo o déficit cognitivo causado por esta disfunção endócrina.

Capítulo II

- O modelo de indução ao hipotireoidismo pelo metimazol não alterou o comportamento motor e exploratório em ratas adultas;

- O modelo de indução ao hipotireoidismo pelo metimazol provocou o comportamento semelhante à depressão em ratas adultas;

- A suplementação com disseleneto de difenila não provocou alterações motoras e exploratórias. Além disso, a suplementação com disseleneto de difenila reverteu o efeito semelhante à depressão induzido por hipotireoidismo em ratas adultas durante todo o período experimental;

- Os níveis de TBARS e ROS foram significativamente aumentados no hipocampo das ratas hipotireóideas, sendo que a suplementação com disseleneto de difenila reverteu esses parâmetros. Os níveis de NP-SH não foram alterados;

- A atividade da enzima MAO- B foi inibida no hipotireoidismo, sendo que a suplementação com disseleneto de difenila reverteu esse parâmetro.

Capítulo III

- As atividades das enzimas CAT, SOD e GPx nas estruturas cerebrais analisadas não foram significativamente alteradas pela condição hipotireóidea dos animais e/ou pelo tratamento com disseleneto de difenila na dieta;

- O hipotireoidismo causou um aumento nos níveis de peroxidação lipídica e formação de ROS nas estruturas cerebrais estudadas, os quais foram reduzidos pela suplementação com disseleneto de difenila. O tratamento com disseleneto de difenila elevou *"per se"* os níveis de tióis-não protéicos;

- As alterações induzidas pelo hipotireoidismo na expressão gênica do fator de transcrição NRF-2 e das enzimas antioxidantes CAT, SOD-1, SOD-2, SOD-3, GPx-1, GPx-4, Tiored-1 e Tiored-2 em córtex cerebral, hipocampo e estriado foram normalizadas pela suplementação com disseleneto de difenila na dieta;

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APÊNDICE

Os apêndices A e B são resultados preliminares obtidos a partir da investigação do efeito do hipotireoidismo e suplementação com disseleneto de difenila sobre a expressão de genes relacionados a apoptose: caspases 3, 6 e 9; e Família Bcl-2 (membros pró-apoptóticos: BAX, BAD e BAK; e membros anti-apoptóticos: Bcl-2 e Bcl-XL) nas estruturas cerebrais.

Apêndice A: Efeito do metimazol e da suplementação com disseleneto de difenila sobre a expressão gênica das caspases 3, 6 e 9 em córtex cerebral, hipocampo e estriado de ratas.

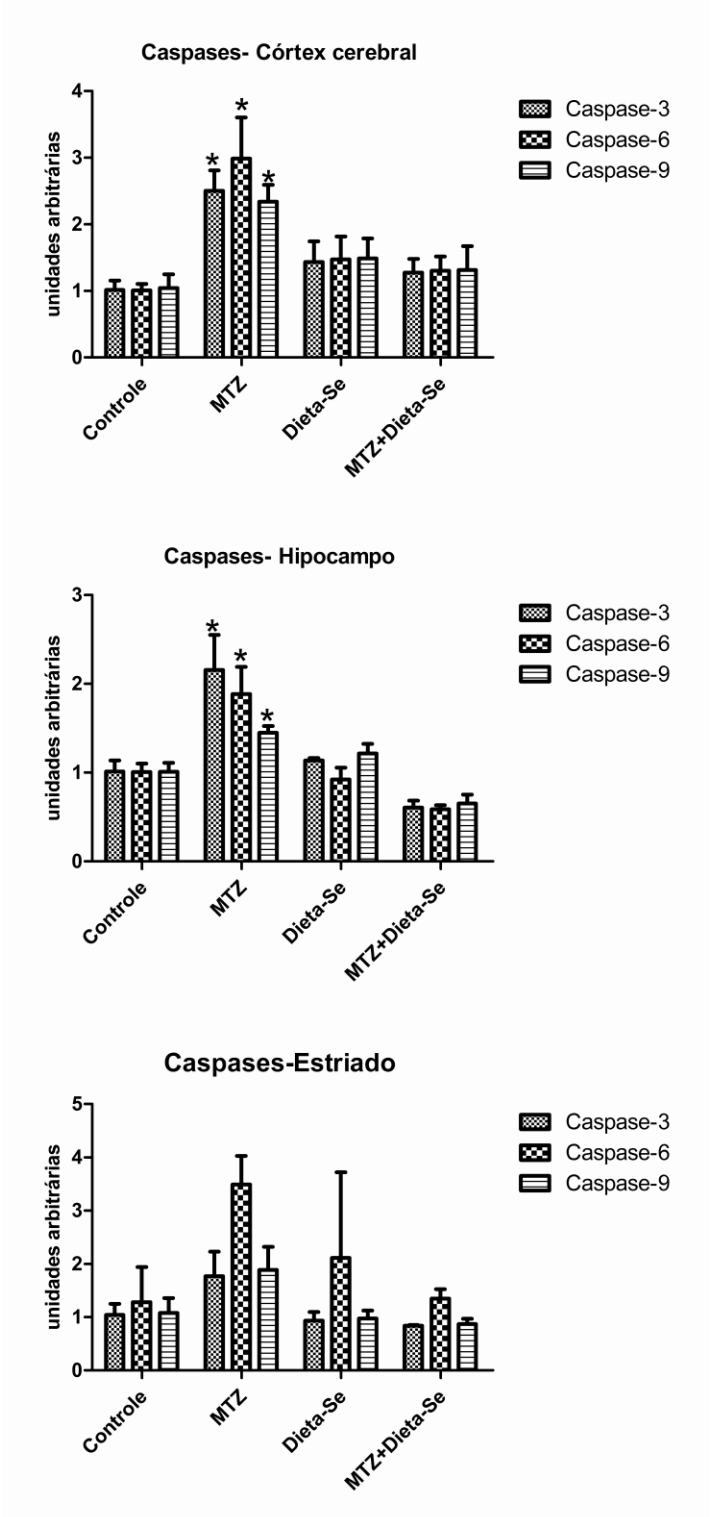

Figura 1: Efeito do metimazol e da suplementação com disseleneto de difenila sobre a expressão gênica das caspases 3, 6 e 9 em córtex cerebral, hipocampo e estriado de ratas. *diferente do grupo controle (p<0.0001, ANOVA seguida do teste de Bonferroni), média ±S.E.M, n=3.

Apêndice B: Efeito do metimazol e da suplementação com disseleneto de difenila sobre a expressão gênica da Família Bcl-2 em córtex cerebral, hipocampo e estriado de ratas.

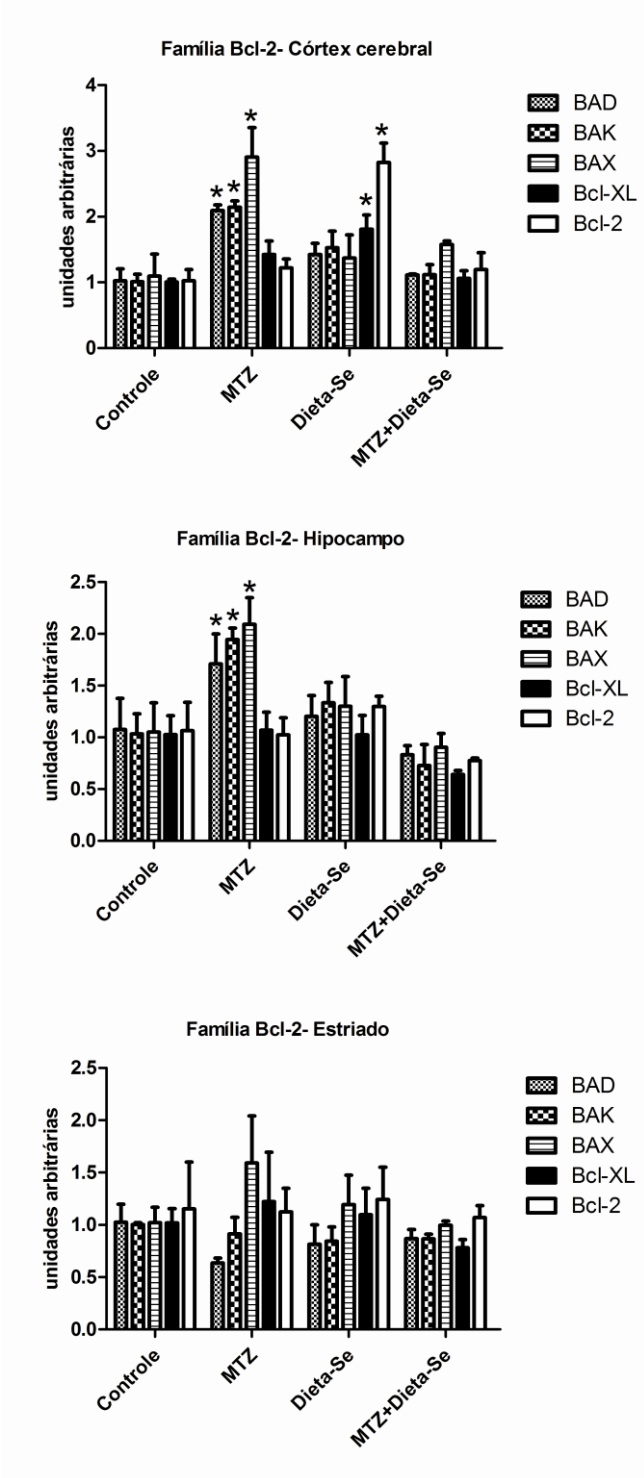

Figura 2: Efeito do metimazol e da suplementação com disseleneto de difenila sobre a expressão gênica da Família Bcl-2 em córtex cerebral, hipocampo e estriado de ratas. *diferente do grupo controle (p<0.0001, ANOVA seguida do teste de Bonferroni), média ± S.E.M, n=3.