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EFEITO DE COMPOSTOS ORGÂNICOS DE SELÊNIO EM MODELOS EXPERIMENTAIS DE CÂNCER E DIABETES MELLITUS

Nilda B. Vargas Barbosa

Santa Maria, RS, Brasil

2006

EFEITO DE COMPOSTOS ORGÂNICOS DE SELÊNIO EM MODELOS EXPERIMENTAIS DE CÂNCER E DIABETES MELLITUS

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por

Nilda B. Vargas Barbosa

Tese apresentada ao Programa de Pós-Graduação em Bioquímica Toxicológica, Área de Concentração em Bioquímica Toxicológica, da Universidade Federal de Santa Maria (UFSM, RS), como requisito parcial para obtenção do grau de
Doutor em Bioquímica Toxicológica.

Santa Maria, RS, Brasil

Santa Maria, 16 de março de 2006

AGRADECIMENTOS

Primeiramente agradeço a Deus pela vida e pelas oportunidades concedidas.

Agradeço a minha mãe, pelo exemplo de força, amor, bondade e fé e pela grande dedicação para comigo.

A “Nurinha” e a “Ciça”, por toda ajuda que me deram com os “ratinhos”. Adoro vocês!

Cristina e GZ, não existe palavras que expressem a tamanha gratidão que sinto por tudo que vocês fizeram por mim e pelo curso para que pudéssemos chegar até aqui. Admiro muito a personalidade batalhadora de vocês e a forma de pensar de que é sempre possível. Isso faz com que sejam diferenciados e eu só tenho que agradecer a Deus por ter colocado no meu caminho pessoas como vocês. Como amigos, a companhia de vocês sempre me deixa muito bem! Acredito que seja uma terapia contra o “mau humor e o baixo astral”. Amo vocês!

Ao JÃO, por “EXISTIR” na minha vida e por ser o grande responsável pela minha história na Bioquímica desde a Graduação.

A Daninha, que foi mais que uma bolsista, mas sim uma “amiga e companheirona” de bancada.

À Vanessa e ao Lysandro, pela ajuda constante em “todos” os meus trabalhos. Espero um dia retribuir tamanha boa vontade. Tudo de bom para vocês!

A Nati por toda ajuda no período que esteve no Lab.

A turma do Lab do João, em especial ao Juliano, Vanderlei e Verônica, Marinei, Rose, Gustavo, Carol e Andreza. Muito obrigada por toda ajuda nas intermináveis “matanças”.

A Marilise e ao Júlio por toda a ajuda durante os experimentos com diabetes. Obrigada pela força e o incentivo!

A Fran, a Eluza, a Lú, ao Ricardo e ao Alexandre e Simone. Obrigada pela parceria e colaboração nos meus trabalhos. Foi muito bom trabalhar na companhia de vcs!

Ao pessoal antigo do Lab que mudou: ao Elisandro, a Dionéia, ao Fabrício, ao Carlos Eduardo, ao Márcio. Foi uma época muito boa!

A turma nova que acabou de chegar: Cristiane, Marina, Cristiano, Daniele, Simone P., Gabrielli, Ethel, Ana e Larissa.

Ao pessoal do Lab do GZ: ao Rodrigo, ao Schumaker, a Angélica e ao Joel. Obrigada pela parceria e colaboração.

Ao grupo da Prof. Rosinha, em especial a Jamile e o Alexssandro.

A Terimar, pelo companheirismo, desabafos e incentivo. Obrigada amiga!

Agradeço a todos os funcionários da UFSM, especialmente ao Rinaldo pelo cuidado com os animais.

Agradeço também a CAPES, pelo auxílio financeiro durante a realização deste trabalho.

RESUMO

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

EFEITO DE COMPOSTOS ORGÂNICOS DE SELÊNIO EM MODELOS EXPERIMENTAIS DE DIABETES MELLITUS E CÂNCER

AUTOR: Nilda B. Vargas Barbosa
ORIENTADORA: Cristina Wayne Nogueira
DATA E LOCAL DA DEFESA: Santa Maria, março de 2006.

O presente estudo foi delineado para avaliar o efeito de compostos orgânicos de selênio em modelos experimentais de câncer e diabetes. No modelo de carcinogênese mamária induzida por N-nitroso-N-metilurea (NMU, 3 doses de 50 mg/Kg, i.p.) observou-se que o consumo de uma dieta suplementada com disseleneto de difenila (1 ppm, 7 meses) foi eficaz em aumentar a latência para o aparecimento dos tumores e em reduzir a incidência e o número total de tumores induzidos pelo carcinógeno. O consumo da dieta não ocasionou efeitos tóxicos aos animais, como a perda de peso e alterações em marcadores de dano hepático e renal. Esses dados sugerem que o composto exibiu uma baixa toxicidade mesmo quando suplementado por períodos prolongados. A capacidade antioxidante e pró-oxidante do selênio; as quais dependem diretamente da sua concentração, podem estar associadas à sua atividade anticarcinogênica. Nossos resultados relacionados com tais aspectos indicam que a atividade antioxidante exibida pelo composto pode ter contribuído para o seu efeito protetor neste modelo experimental de carcinogênese mamária. De fato, o consumo da dieta suplementada com disseleneto de difenila normalizou a atividade da enzima superóxido dismutase (SOD) e elevou os níveis de vitamina C no sangue, fígado e baço dos animais tratados com NMU. Nos modelos experimentais de Diabetes Mellitus dois tipos de tratamento com selênio foram utilizados: (1) os animais foram tratados com disseleneto de difenila e ebselen (1mg/Kg, s.c.) durante 3 meses após a indução de diabetes; (2) os animais foram tratados com uma dieta suplementada com disseleneto de difenila (10 ppm) desde a fase de desmame até o final do período experimental. Em ambos os modelos, a indução de diabetes foi realizada pela administração de uma dose de streptozotocina (STZ) (50 mg/Kg, i.p.). No modelo 1 evidenciou-se que somente o tratamento com disseleneto de difenila causou uma significante redução na hiperglicemia induzida por STZ. Este efeito foi acompanhado por uma redução significativa nos níveis de proteínas glicadas; os quais foram elevados nos animais diabéticos. O tratamento com disseleneto de difenila causou um aumento na atividade da enzima SOD e nos níveis de vitamina C, os quais foram diminuídos nos animais tratados com STZ. De particular importância, o tratamento com este composto promoveu *per se* um aumento nos níveis de glutationa reduzida (GSH) de fígado, rim e sangue e na atividade da enzima SOD de rim. De maneira similar, o tratamento com o

disseleneto de difenila aumentou os níveis de GSH hepático e renal nos animais tratados com STZ. O tratamento com STZ causou uma redução na atividade da enzima aminolevulinato desidratase (δ-ALA-D) hepática, a qual não foi revertida pelos compostos orgânicos de selênio. Esta redução de atividade causada pela STZ não foi observada na enzima renal. No modelo 2 evidenciou-se que o consumo da dieta suplementada com 10 ppm de disseleneto de difenila, não causou efeitos tóxicos aos animais e reduziu de forma significativa o índice de mortalidade induzido pela administração de STZ. A atividade antioxidante do disseleneto de difenila, mais uma vez pode estar relacionada com tal efeito, uma vez que a ação pró-oxidante da STZ está envolvida na sua capacidade de causar a destruição das células β pancreáticas. Assim como observado para o modelo 1, o consumo da dieta suplementada com disseleneto de difenila reduziu as alterações nas defesas antioxidantes nos animais tratados com STZ e causou *per se* um aumento nos níveis de -SH hepático e sanguíneo dos animais. Novamente, o tratamento com STZ causou um decréscimo na atividade da enzima δ-ALA-D hepática, a qual não foi revertida pelo consumo com a dieta suplementada com disseleneto de difenila. A atividade da enzima δ-ALA-D renal não foi modificada nos animais diabéticos. Em resumo, nossos dados apontam o disseleneto de difenila como um composto de valor terapêutico significativo para o tratamento de câncer e Diabetes Mellitus. No entanto, mais estudos se fazem necessários para comprovarem a eficácia do composto e seu provável mecanismo de ação.

Palavras chave: disseleneto de difenila, ebselen, selênio, diabetes e câncer.

ABSTRACT

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

EFFECT OF ORGANOSELENIUM COMPOUNDS ON EXPERIMENTAL MODELS OF DIABETES MELLITUS AND CANCER

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DATE AND PLACE OF THE DEFENSE: Santa Maria, 2006

This study was designed to determine the effect of organoselenium compounds on experimental models of cancer and diabetes. In mammary carcinogenesis induced by N-nitroso-N-methylurea (NMU, 3 doses of 50 mg/kg, i.p.), it was observed that the use of diet supplemented with diphenyl diselenide (1ppm, 7 months) was efficient in increase the latency to tumor onset and in reduce mammary tumor incidence and total number of tumors induced by NMU. The use of diet supplemented with diphenyl diselenide did not cause toxic effects in animals such as loss of body weight and alterations in hepatic and renal markers. These results suggest that diphenyl diselenide compound exhibited low toxicity even when supplemented by long time period. The both antioxidant and pro-oxidant properties of selenium may be linked to its anti-carcinogenic activity. In this context, our results indicated that antioxidant property exhibited by diphenyl diselenide can contribute for its protective effect against mammary carcinogenesis. In fact, the diet supplemented with diphenyl diselenide normalized superoxide dismutase (SOD) activity and elevated blood, hepatic and spleenic vitamin C levels in NMU treated animals. On experimental models of diabetes mellitus two treatments using organo selenium were carried out: (1) animals were treated with diphenyl diselenide and ebselen (1 mg/kg, s.c.) by 3 months after diabetes induction; (2) animals were treated with a diet supplemented with diphenyl diselenide (10 ppm) after the wean phase at the end of experimental period. Diabetes was induced with a single dose streptozotcin (STZ) (50 mg/Kg, i.p.). In model 1, it was observed that only diphenyl diselenide treatment caused significant reduction in hyperglycemia induced by STZ. This effect of diphenyl diselenide was accompanied by a reduction in the levels of glycated proteins, which were elevated in diabetic rats. Treatment with diphenyl diselenide increased SOD activity and vitamin C levels that were decreased in STZ treated rats. Of particular importance, diphenyl diselenide treatment promoted *per se* an increase in hepatic, renal and blood reduced glutathione (GSH) levels in animals. Similary, diphenyl diselenide caused an increase in hepatic and renal GSH levels in STZ treated rats. The STZ treatment caused a decrease in hepatic δ-ALA-D activity, which was normalized by diphenyl diselenide and ebselen treatments. This reduction in δ-ALA-D activity was not observed in renal enzyme. In model 2, it was observed that the use of diet supplemented with 10 ppm of diphenyl diselenide did not produce significant

toxicity and reduced significantly the mortality index caused by STZ administration. The antioxidant property of diphenyl diselenide can be associated with this protective effect, since pro-oxidative action of STZ is linked to destruction process of cells β pancreatics. As observed in model 1, the use of diet supplemented with diphenyl diselenide reduced the alterations in antioxidant defenses induced by STZ and caused *per se* an increase in hepatic and blood -SH levels of animals. STZ treatment caused a decrease in hepatic δ-ALA-D activity, which was restored by the use of diet supplemented with diphenyl diselenide. The activity of renal δ-ALA-D enzyme was not modified in diabetic rats. In summary, our findings suggest that diphenyl diselenide can be considered a compound with significant therapeutic value on treatment of cancer and diabetes. However, further studies are needed to elucidate the mechanism(s) of action and the efficacy of compound as anti-diabetogenic and anticarcinogenic agent.

Keywords: diphenyl diselenide, ebselen, selenium, diabetes e cancer.

LISTA DE FIGURAS

LISTA DE TABELAS

Artigo 1

Artigo 2

Artigo 3

LISTA DE ABREVIATURAS

ADA - American Diabetes Association

δ-ALA-D - Delta-aminolevulinato desidratase ou porfobilinogênio sintase

ALT- Alanina aminotransferase

ANOVA- Análise de variância

AST - Aspartato aminotransferase

CAT - Catalase

DM – Diabetes mellitus

EROs - Espécies reativas de oxigênio

GPx - Glutationa peroxidase

GSH - Glutationa reduzida

MDA - Malondialdeído

NMU - N-nitroso-N-metilurea

NPSH – Tióis não-protéicos

OMS – Organização Mundial da Saúde

PBG – Porfibilinogênio

PHGPx- Fosfolipídeo hidroperóxido glutationa peroxidase

PTGs- Produtos de glicação avançada

SOD - Superóxido dismutase

STZ - Streptozotocina

TBARS - Espécies reativas ao ácido tiobarbitúrico

SUMÁRIO

APRESENTAÇÃO

Os resultados que fazem parte desta dissertação estão apresentados sob a forma de artigo, o qual encontra-se no item **ARTIGO CIENTÍFICO**. As seções Materiais e Métodos, Resultados, Discussão dos Resultados e Referências Bibliográficas, encontram-se no próprio artigo e representam à íntegra deste estudo.

Os itens **DISCUSSÃO E CONCLUSÕES**, encontrados no final desta dissertação, apresentam interpretações e comentários gerais sobre o artigo científico contido neste trabalho.

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

1. INTRODUÇÃO

O selênio suplementado em baixas concentrações é reconhecido como um elemento nutricionalmente essencial aos mamíferos. Na forma de selenocisteína este micronutriente encontra-se presente no sítio ativo de diversas enzimas com papel antioxidante como a glutationa peroxidase e a fosfolipídeo hidroperóxido glutationa peroxidase (Flohe et al., 1973; Rotruck et al., 1973; Ursini et al., 1982).

Estudos epidemiológicos têm demonstrado que a deficiência de selênio na dieta está relacionada com a gênese e ou progressão de diversas patologias como desordens cardiovasculares e neurológicas, câncer e diabetes (Wilber, 1980; Salonen et al., 1982; Oldifield, 1987; Clark et al., 1991; El-Bayoumy, 1991; Salonen, et al., 1991; Combs and Gray, 1998; Armstrong et al., 1996; Navarro-Alárcon e Lopez-Martinez, 2000; Reddi et al., 2001). A atividade antioxidante exibida pelo elemento parece ser responsável pela sua eficácia no tratamento de doenças que tem o estresse oxidativo como processo central no seu desenvolvimento. Além da propriedade antioxidante, determinados compostos de selênio exibem ação antinociceptiva, neuroprotetora, anti-inflamatória, anticarcinogênica, hepatoprotetora e insulina-like (El-Bayoumy, 1991; Stapleton et al., 2000; Rossato et al., 2002; Porciuncula et al., 2003; Nogueira et al., 2003, Burguer et al., 2005; Zasso et al., 2005, Borges, et al., 2006). Em vista disto, tem crescido muito nas últimas décadas o interesse em investigar o papel de compostos de selênio como possíveis agentes terapêuticos no tratamento de diversas patologias.

O Diabetes Mellitus (DM) é uma desordem metabólica crônica caracterizada por um estado de hiperglicemia persistente; o qual parece ser responsável por desencadear um aumento na gênese do estresse oxidativo presente nesta condição (Wolff et al., 1987; Sensi et al., 1995; Hunt et al., 1997; Morgan et al., 2002). Em diferentes modelos experimentais de hiperglicemia têm-se observado que compostos de selênio protegem determinados tecidos contra danos oxidativos como a peroxidação lipídica e de alterações em sistemas de defesas antioxidantes (Mc Neill et al., 1991; Becker et al., 1996; Kimura, 1996; Mukherjee et al.,1998; Naziroglu et al., 2001). De especial importância, são as evidências de que o selênio pode mimetizar a ação da insulina no controle de vários processos biológicos tanto *in vitro* como *in vivo* (Ezaki, 1990; Stapleton, 1997; 2000) e proteger as células pancreáticas contra danos oxidativos (Błasiak, et al., 2004 et al., 2004).

Embora o selênio seja bem reconhecido como elemento traço essencial e apresente uma variedade de efeitos protetores para humanos e animais contra determinadas doenças (Combs and Gray, 1998; Navarro-Alárcon e Lopez-Martinez, 2000), sua toxicidade foi descrita em 1941 (Painter, 1941). Apesar, do mecanismo pelo qual este elemento exerce sua toxicidade não encontrar-se totalmente elucidado, vários estudos sugerem que os efeitos tóxicos do mesmo estão associados à sua habilidade em catalisar a oxidação de tióis endógenos e com a gênese de radicais livres (Seko et al., 1989, Spallhoz et al., 1994; Barbosa et al., 1998; Nogueira et al., 2003; 2004). Por outro lado, estes efeitos pró-oxidantes estão envolvidos, em parte, no mecanismo de proteção que este elemento oferece contra doenças como o câncer (Spallhoz, et al., 2001a; Spallhoz

et al., 2004). De fato, diversos estudos relacionados com a atividade anticarcinogênica do selênio mostram que a suplementação dietética com selênio em doses acima das nutricionalmente requeridas, apesar da toxicidade, inibe o processo de carcinogênese experimental em vários tipos de câncer (Bayoumy, 1991; Spallhoz, 1994; Fleming et al., 2001).

Considerando os aspectos acima mencionados, o presente estudo visa investigar o efeito de diferentes tratamentos com compostos orgânicos de selênio em modelos experimentais de Diabetes Mellitus tipo 1 e de Câncer; afim de verificar se tais compostos são capazes de prevenir e/ou atenuar o desenvolvimento dessas doenças.

2. REVISÂO BIBLIOGRÁFICA

2.1. Selênio

O Selênio foi descoberto em 1817, pelo químico sueco J.J Berzelius. Este elemento está localizado no grupo VI da tabela periódica e pode encontrar-se sob quatro estados de oxidação: Se^{0}, Se^{+6}, Se^{+4}, Se^{-2}. O selênio compartilha propriedades físicas e químicas com o enxofre. Esta similaridade permite uma substituição do Se por S em diversas reações químicas que ocorrem nos sistemas biológicos. Por outro lado, as diferenças nas propriedades físicas e químicas entre esses elementos constituem a base específica de seus respectivos papéis biológicos. A química do selênio sugere que nos sistema biológicos este encontre-se sob a forma de selenol (R-SeH). Selenóis são compostos correspondentes à forma de tióis (R-SH), onde o átomo de Se é substituído pelo átomo de S. A principal diferença química entre selenóis e tióis está relacionada com as suas respectivas constantes de dissociação e seus caráteres nucleofílicos (Klayman e Gunther, 1973). Devido ao maior caráter nucleofílico os selenóis são capazes de reduzir dissulfetos e sulfóxidos e ainda na presença de agentes oxidantes serem convertidos a seus respectivos disselenetos (Klayman e Gunther, 1973).

2.1.1. Atividade Biológica e Toxicologia

O papel do selênio como elemento traço essencial na dieta foi demonstrado experimentalmente em 1957 por Klaus Schwarz na Alemanha. Em 1980 a Junta de Alimentação e Nutrição da Academia de Ciências dos Estados Unidos propôs a ingestão diária entre 50 e 200μg de selênio para homens adultos; aporte este considerado adequado e isento de efeitos tóxicos. Atualmente, o conhecimento sobre as necessidades dietéticas de selênio, tanto para homens como para animais, aumentou notavelmente. Existem várias evidências de que a deficiência deste micronutriente está diretamente relacionada com o desenvolvimento de doenças como: desordens cardiovasculares, cirrose, esclerose, câncer e diabetes (El-Bayoumy, 1991; Salonen, et al., 1991; Combs and Gray, 1998; Armstrong, 1996; Navarro-Alárcon e Lopez-Martinez, 2000; Reddi et al., 2001). O valor preventivo e terapêutico do selênio contra tais patologias parece estar fortemente associado com a propriedade antioxidante que o mesmo exibe. Tal propriedade se deve ao fato do mesmo fazer parte do sítio ativo de enzimas que desempenham papel antioxidante nos sistemas biológicos como a glutationa peroxidase (GPx) e a fosfolipídeo hidroperóxido glutationa peroxidase (PHGPx) (Flohe et al.,1973, Rotruck et al.,1973; Ursini et al.,1985). No entanto, a dose de selênio a ser administrada e/ou ingerida também constitui um fator crítico na atividade biológica do elemento, uma vez que a quantidade requerida nutricionalmente de selênio é muito próxima da quantidade considerada tóxica.

Apesar do rápido progresso no entendimento do metabolismo do selênio, os mecanismos de sua toxicidade ainda não estão totalmente definidos. No entanto, muitos dos achados sobre os efeitos tóxicos de determinados compostos de selênio estão associados à sua capacidade de catalisar a oxidação de tióis e de gerar espécies reativas de oxigênio (Barbosa et al., 2000; Spallhoz et al., 2001a; Nogueira et al., 2004). De acordo, dados do nosso grupo mostram que compostos orgânicos de selênio, como o diseleneto de difenila, quando em altas concentrações, inibem a enzima δ-ALA-D *in vitro* e *in vivo* por oxidarem seus grupamentos -SH (Barbosa et al., 1998; Maciel et al., 2003; Nogueira et al., 2004).

2.1.2. Disseleneto de Difenila $(PhSe)_2$ e Ebselen

O Disseleneto de difenila e o ebselen são compostos orgânicos sintéticos de selênio que compartilham algumas propriedades químicas como por exemplo a atividade tiol-peroxidase (Wendel, 1984; Parnham, 1991; Engman, et al., 1994; Nogueira et al., 2004). De importância particular, estes organocalcogênios também exibem outras propriedades farmacológicas em comum como: atividades antioxidante; anti-inflamatória; analgésica; neuroprotetora e hepatoprotetora (Meotti, et al., 2003; Porciúncula et al., 2003; Borges et al., 2004; Nogueira, et al., 2004; Borges et al., 2005; Bürger, et al., 2005; Zasso et al, 2005). Parte destes efeitos protetores estão ligados com a capacidade dos mesmos em decompor

peróxidos na presença de tióis e de reduzir a peroxidação lipídica em diversos modelos experimentais.

Da mesma forma, a toxicologia destes compostos é bastante similar. Em doses farmacológicas ambos os compostos apresentam baixa toxicidade tanto para ratos como para camundongos (Perottoni et al., 2005; Fachineto et al., 2006). No entanto, a DL_{50} do disseleneto de difenila (i.p. e s.c.) para estes animais é maior que a do ebselen (Meotti et al., 2003). Por outro lado, o tratamento crônico (14-21 dias) ou agudo com altas doses de disseleneto de difenila, acarreta na inibição da atividade da enzima δ-ALA-D e na depleção de grupos tióis de moléculas endógenas de baixo peso molecular (Maciel et al., 2003). Tais efeitos também parecem estar correlacionados com a atividade pró-oxidante do composto.

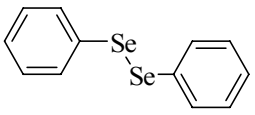

Figure 1. Disseleneto de Difenila $(PhSe)_2$

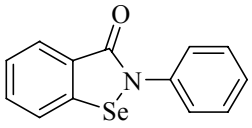

Figure 2. Ebselen

2.2. Diabetes Mellitus

2.2.1. Histórico

Na história da medicina o Diabetes Mellitus (DM) é o único distúrbio metabólico cuja existência e sintomatologia foram relatadas há mais de 20 séculos (1500 AC). Ainda no ano 70 (D.C) a denominação “DIABETES” para a doença e seus sintomas mais evidentes como a polidipsia, poliúria e polifagia foram descritos pelo médico Areteu da Capadócia. Entretanto, devido ao pequeno avanço da ciência na época, somente em 1670 foi descoberto que a urina dos pacientes diabéticos apresentava sabor adocicado (Dinsmoor, 1996). Quinze anos após, o médico M. Chevreul confirmou que o açúcar presente na urina de pacientes com DM tratava-se da glicose. A partir daí a doença passou a chamar-se de “Diabetes Açucarada” ou “Diabetes Mellitus”. Em 1869, Paul Langerhans observou que o pâncreas continha um tipo diferenciado de células, agrupadas em ilhotas, cuja função poderia ser endócrina. O papel endócrino destas células foi confirmado no final do século XIX, em experimentos com cães pancreactomizados, pelos pesquisadores Oskar Minkowski e Joseph Von Mering. Entre 1916 e 1920 foi demonstrado que extratos pancreáticos tinham a capacidade de diminuir a glicemia. Um ano após, o cirurgião Frederic G. Banting descobriu a insulina (Minkowski, 1989). Este achado rendeu ao mesmo o Prêmio Nobel de Medicina e acima de tudo promoveu uma melhor qualidade de vida aos pacientes com Diabetes Mellitus.

2.2.2. Fisiopatologia

Como conseqüência da hiperglicemia e dos distúrbios que esta condição ocasiona no metabolismo de carboidratos, proteínas e lipídeos a maioria dos pacientes com DM manifesta a curto-prazo quadro de glicosúria, cetose e cetonúria, polifagia, polidipsia e poliúria. Estes sintomas freqüentes na população diabética são conhecidos como clássicos na história da doença. No entanto, a ausência dos mesmos é comum em muitos pacientes com DM e não descarta a possibilidade de que exista um grau de hiperglicemia suficiente para causar alterações funcionais ou patológicas antes que o diagnóstico seja estabelecido.

As conseqüências do DM a longo-prazo incluem danos, disfunção e falência de vários órgãos, especialmente rins, olhos, nervos, coração e vasos sanguíneos. Entre estas, o desenvolvimento de doenças cardiovasculares têm sido considerado a principal causa da redução da sobrevida e da mortalidade dos pacientes diabéticos.

O DM aparece como a sexta causa mais freqüente de internação hospitalar e representa cerca de 30% dos pacientes que internam em Unidades Coronarianas Intensivas e 26% dos pacientes que ingressam em tratamento de diálise. Esta síndrome além de contribuir de forma significativa para o desenvolvimento de acidente vascular cerebral e de hipertensão arterial, também constitui a principal causa de amputações de membros inferiores e de cegueira adquirida (Bach, 1994).

2.2.3. Classificação e Etiologia

O DM é uma síndrome metabólica hereditária de etiologia múltipla, caracterizada por um estado de hiperglicemia persistente; o qual resulta de uma deficiência na produção de insulina ou da resistência dos tecidos a ação deste hormônio. O caráter hereditário da DM está relacionado como a expressão de um gene regulador da produção de anticorpos anti-células β; o qual localiza-se no braço curto do cromossoma 6. Foi evidenciado que fatores ambientais estimulam sua expressão gênica de forma variável, o que justifica as diferentes faixas etárias de manifestação da sintomatologia da doença. A interação de tais fatores parece também estar envolvida na potencialização da expressão patológica da doença.

De acordo com o Comitê Executivo para Diagnóstico e Classificação do DM da " American Diabetes Association" (ADA, 1997) a síndrome pode surgir de forma secundária a alguma doença que cause destruição das ilhotas pancreáticas como: tumores, patologias endócrinas, defeitos genéticos e funcionais das células β ou na ação da insulina. No entanto, as formas mais comuns de DM resultam de desordens primárias no sistema sinalizador da insulina. Tais desordens podem ser divididas em duas categorias: DM tipo 1 e DM tipo 2.

- Diabetes Mellitus tipo 1: também conhecido como insulino-dependente (IDDM) corresponde a aproximadamente 10% dos casos de DM primária. Manifesta-se geralmente durante a infância e caracteriza-se por uma severa ou total inibição da produção de insulina pelas células β do pâncreas. Fatores

genéticos, ambientais e imunológicos são considerados os principais responsáveis pela gênese do processo de destruição das células β do pâncreas e conseqüentemente pela manifestação da doença. A reação imunológica pode ser de origem genética somente ou ainda decorrer de um evento ambiental que afete as características imunogênicas das células pancreáticas. Estudos epidemiológicos sugerem que infecções virais também podem desencadear o processo auto-imune característico do DM tipo 1 (Bach, 1994).

- Diabetes mellitus tipo 2: também conhecido como não-insulino-dependente (NIDDM) é considerado a forma mais comum da doença, a qual afeta aproximadamente 90% da população diabética. Caracteriza-se por ser um distúrbio multifatorial que geralmente resulta de graus variáveis de resistência tecidual à insulina e de uma deficiência relativa na secreção do hormônio pelas células pancreáticas. Na realidade o DM tipo 2 tem demonstrado ser a manifestação de uma desordem muito mais ampla conhecida atualmente como "Síndrome X". Esta representa um grupo de fatores de risco para doenças cardiovasculares que predispõem a manifestação de DM tipo 2 (Hansen, 1999). Estes fatores incluem hipertensão arterial, lipidemias, hiperinsulinemia, intolerância à glicose e obesidade visceral. Fatores genéticos, ambientais e comportamentais (sedentarismo e dieta) também contribuem de forma significativa para o surgimento do DM tipo 2 (Zimmet, 1992).

2.2.4. Estresse Oxidativo e Diabetes Mellitus

O exato mecanismo celular e molecular envolvido na etiologia e progressão do DM ainda não encontra-se totalmente elucidado. No entanto, uma série de evidências mostram que o estresse oxidativo tem um papel central no desenvolvimento de muitas complicações crônicas características do estado diabético (Oberley, 1988; Baynes et al., 1991; Mohamed et al., 1999; Packer et al., 2001 Faure, 2003; Maritim, et al., 2003). A hiperglicemia de longa duração parece ser o principal fator envolvido na gênese e no aumento do estresse oxidativo evidenciado no DM (Wolff et al., 1987; Sensi et al., 1995; Hunt et al., 1997; Morgan et al., 2002). De fato, eventos conseqüentes da hiperglicemia como auto-oxidação da glicose; glicação não-enzimática de proteínas e consequente formação de PTGAs (produtos terminais de glicação avançada) culminam com a produção de radicais livres, os quais causam alterações na estrutura de proteínas, inativação de enzimas, alterações nos sistemas de defesas antioxidantes e acentuado aumento nos níveis de peroxidação lipídica em vários tecidos (Mukherjee et al., 1998; Jain et al., 2001; Rosen et al., 2001; Folmer et al., 2002; Morgan et al., 2002). Além disso, vários tipos de células, como as endoteliais, neuronais, bem como monócitos e macrófagos expressam receptores para os PTGAs. A ligação destes com seus receptores pode levar a prejuízos no funcionamento celular, e conseqüentemente no desenvolvimento dos problemas angiopáticos existentes no DM (Brownlee et al., 1981).

2.2.5. Diabetes Mellitus e Selênio

Como conseqüência da hiperglicemia e do estresse oxidativo presentes no DM, uma intensificação na busca por compostos naturais ou sintéticos que exibam propriedades antioxidantes e/ou anti-hiperglicêmicas tem sido feita nas últimas décadas. Devido à atividade antioxidante, o selênio tem sido usado com relativo sucesso no combate ao estresse oxidativo em vários modelos experimentais de DM. A suplementação com compostos inorgânicos de selênio, como selenito e selenato de sódio, a animais com DM aumenta a atividade de enzimas antioxidantes e diminui os níveis de peroxidação lipídica em diferentes tecidos (Faure, 2003). A suplementação com formas inorgânicas de selênio demonstrou ser efetiva em reduzir a atividade do fator de transcrição NF-kB (sensível a ação de oxidantes) em sujeitos com DM tipo 2 (Faure et al., 2004).

De especial importância são as evidências de que o selênio, além da atividade antioxidante também contribui para atenuar os efeitos deletérios da hiperglicemia por mimetizar a ação da insulina no controle de vários processos biológicos (Ezaki, 1990; Stapleton, et al., 1997; Stapleton, 2000). Como a insulina, formas inorgânicas de selênio são efetivas em normalizar os níveis de glicose no sangue e de aumentar a captação de glicose em tecidos insulino-dependentes em ratos diabéticos. A exposição ao selenato de sódio causa um aumento significativo no número de receptores para a glicose e na expressão e atividade de enzimas envolvidas no metabolismo da glicose e dos ácidos graxos em culturas de adipócitos e hepatócitos (Ezaki, 1990; Berg et al., 1995; O'Brien and Granner,

1996; Stapleton et al., 1997; Stapleton, 2000). Além disso, essa forma inorgânica de selênio também promove a fosforilação de resíduos tirosil de diversas proteínas, as quais são fosforiladas durante a cascata de eventos desencadeada pela ligação da insulina ao seu receptor (Ezaki, 1990; Berg et al., 1995; Stapleton et al., 1997; Stapleton, 2000).

2.3. Câncer

O câncer é considerado um problema de saúde pública mundial, o qual é responsável por 12.5% da taxa de mortalidade no mundo a cada ano (7 milhões de morte/ano). Segundo a OMS (Organização Mundial da Saúde) mais de 11 milhões de pessoas são diagnosticadas com câncer anualmente e estima-se que este valor aumente para 16 milhões até o ano de 2020. O câncer de pulmão, cólon e estômago estão entre os cinco tipos mais comuns que acometem homens e mulheres. A carcinogênese mamária é considerada a forma mais comum de câncer em mulheres e constitui a segunda causa de mortalidade por câncer na América (Greenlee, 2001).

Há cerca de trinta anos, a suscetibilidade ao câncer era principalmente atribuída aos níveis de exposição aos diferentes tipos de carcinógenos. Atualmente, embora essa correlação seja efetiva, estima-se que a interação entre fatores genéticos e ambientais seja responsável por grande parte da incidência da neoplasia. A contribuição exclusivamente genética parece ser responsável por apenas 5% de todos os tumores. A fração restante é atribuída a fatores

ambientais "externos" que atuam em conjunto com a suscetibilidade genética (Greenlee, et al., 2001). Assim a incidência e a mortalidade por câncer estão relacionadas com múltiplos fatores, como a idade, o sexo, a raça, o status nutricional, a saúde, a predisposição genética e a exposição a carcinógenos ambientais. Conseqüentemente, a hipótese de que o processo carcinogênico é composto por múltiplas etapas, nas quais um conjunto de eventos contribui para a transformação celular e subseqüente estágios malignos, é hoje amplamente aceita.

2.3.1. Carcinogênese

A carcinogênese caracteriza-se por ser um processo de crescimento anormal e difuso das células, o qual pode iniciar-se de forma espontânea ou ser provocado pela ação de agentes carcinogênicos (químicos, físicos e biológicos). Em ambos os casos verificam-se a indução de alterações mutagênicas e não mutagênicas ou epigenéticas nas células.

2.3.1.1. Carcinogênese Espontânea

É resultante de mutações espontâneas, as quais não alteram o desenvolvimento normal da população celular como um todo. Estes fenômenos incluem danos oxidativos, erros de ação de polimerases e de recombinases à nível de DNA ou ainda de alterações cromossômicas e imunológicas. Tais eventos

podem condicionar uma maior ou menor instabilidade genômica, a qual pode ser crucial nos processos iniciais da carcinogênese.

2.3.1.2. Carcinogênese Induzida

È resultante da exposição do organismo a múltiplos fatores carcinogênicos. Pode ser classificada como biológica, física ou química.

- **Carcinogênese biológica:** inclui aquelas causadas por vírus capazes de produzirem câncer por provocarem mutações no DNA ou no RNA (retro-vírus) e diversos outros agentes biológicos tais como báctérias e parasitas. Acredita-se que os agentes carcinogênicos biológicos atuem criando condições propícias para mutações por erros de transcrição do DNA.

- **Carcinogênese Física:** é causada pela exposição exporádica ou seqüencial a radiação ionizante e não ionizante. O mecanismo da carcinogênese pela radiação reside na sua capacidade de induzir mutações, as quais podem resultar de efeito direto da radiação ou via produção de espécies reativas de oxigênio.

- **Carcinogênese Química:** é um processo seqüencial dividido em duas fases: a iniciação e a promoção. A primeira (Iniciação) consiste de um fator iniciador ou carcinógeno que causa dano ou mutação celular. A mutação do DNA

é o fenômeno central da etapa de iniciação. As células “iniciadas” permanecem latentes até que sobre elas atuem agentes promotores.

A segunda etapa (Promoção) estimula o crescimento da célula que sofreu mutação e pode acontecer a qualquer momento, após a transformação celular inicial. Os fatores de promoção podem ser agentes químicos, processo inflamatório, hormônios e fatores que atuam no crescimento celular normal. È importante destacar que o agente promotor não tem ação mutagênica nem carcinogênica e que para exercer seu efeito biológico deve persistir no ambiente.

Nos processos de iniciação e promoção a célula pode ainda encontrar-se sob a ação dos fatores de inibição de crescimento e o resultado final dependerá do balanço obtido entre estes fatores e a intensidade das alterações provocadas nas células pela ação dos agentes iniciadores e promotores.

2.3.2. Câncer e Estresse oxidativo

As espécies reativas de oxigênio (EROs) e de nitrogênio (ERNs) são conhecidas como mutagênicas por causarem danos oxidativos ao DNA, favorecendo o processo de transformação celular e contribuindo para o desenvolvimento de doenças malignas como o câncer (Mattes et al., 2000; Prasad et al., 2002; Kinulla et al., 2004). Evidências de um aumento significativo na produção de radicais livres e uma diminuição na expressão e atividade de enzimas antioxidantes pelas células cancerosas, fazem com que o estresse oxidativo seja considerado um evento molecular fundamental no processo de carcinogênese (Prasad et al., 2002; Zang et al., 2002). No entanto, inúmeros

mecanismos associados com a gênese do estresse oxidativo podem culminar nas alterações celulares necessárias para desencadear ou acelerar o processo de carcinogênese.

Sabe-se que a modificação oxidativa do DNA acarreta na formação do produto 8-oxo-2'deoxiguanosina, o qual é altamente mutagênico (Floyd et al., 1990; Mills et al., 1998). Além disso, radicais livres são conhecidos por modular vários eventos que culminam na ativação de oncogenes e na inativação de genes supressores de tumor (Tanaka et al., 1998; Mattes et al., 2000). Tais eventos incluem modificação na comunicação celular, na estrutura de membranas, na atividade de quinases específicas e na modulação de uma série de fatores de crescimento (Manna et al., 1998; Mattes et al., 2000). A capacidade de mutação, invasão e metástase dos tumores também estão relacionadas com a ação de radicais livres (Mattes et al., 2000). Assim a terapia com antioxidantes tem sido usada com bastante eficácia no tratamento de diferentes tipos de câncer em humanos e animais experimentais (Manna et al., 1998; Mates e Sánches-Jiménez, 1999; 2000; Kinulla et al., 2004).

2.3.3. Câncer e Selênio

A atividade anticarcinogênica do selênio foi relatada somente 12 anos após a sua descoberta como elemento traço essencial, onde relacionou-se a deficiência do elemento com o aumento no risco de desenvolvimento de câncer (Shamberger and Frost, 1969). A partir daí, inúmeros estudos demonstraram que a suplementação com selênio era capaz de prevenir, reduzir ou inibir o processo de

carcinogênese experimental em diferentes fases de vários tipos de câncer (Nayini et l., 1991; El-Bayomi, 1994; El-Bayoumy et al., 1995; Combs and Gray, 1998). Em humanos também foi constatada a efetividade do selênio em reduzir a incidência de alguns tipos de câncer (Hunter et al., 1990; Combs and Gray, 1997, 1998).

Nas últimas décadas, uma série de compostos orgânicos de selênio vêm sendo sintetizados e testados como agentes anticarcinogênicos (Nayini et al., 1989; El-Bayoumy, 1991; Nayini, et al., 1991; El-Bayoumy, et al., 1995; El-Bayoumy et al., 1996; Reddy et al., 1996; Spallhoz et al., 2001a). Grande parte, dos compostos sintetizados com este objetivo, como por exemplo: o *p*-metoxibenzenoselenol, o benzilselenocianato e o fenilenebis(metil)selenocianato, são efetivos em inibir o desenvolvimento de tumor causado por diferentes carcinógenos (Nayini et al., 1989; Nayini, et al., 1991; El-Bayoumy, 1991; El-Bayoumy et al., 1995; 1996; Reddy et al., 1996). No entanto, a atividade anticarcinogênica de tais compostos tem sido obtida com doses acima das nutricionalmente requeridas. Como conseqüência da toxicidade dessas altas doses os animais experimentais geralmente desenvolvem injúria hepática e perda de peso. Assim, a busca por compostos que apresentem alta efetividade e baixa toxicidade passou a ser alvo de muitas pesquisas nas últimas décadas.

Os efeitos protetores do selênio contra o câncer parecem ser oriundos de diferentes mecanismos. Em doses farmacológicas tais efeitos parecem estar associados ao fato do selênio ser componente de enzimas antioxidantes como a GPx, a qual protege o DNA e outros componentes celulares de danos oxidativos. As selenoproteínas também são conhecidas por terem papel importante no controle de processos como divisão celular, detoxificação, apoptose, sistema auto-

imune e inativação de oncogenes (Ramakrishnan, et al., 1996; Combs e Gray, 1998; Fleming et al., 2001). Por outro lado, sabe-se também que a citotoxicidade de altas doses de selênio, via produção de radicais livres e oxidação de tióis, é responsável por grande parte dos seus efeitos como agente anticarcinogênico (Fleming et al., 2001; Spalhoz, 2001b; 2004).

3. OBJETIVOS

O uso do selênio como agente preventivo e terapêutico tem sido bastante comum e promissor no tratamento de doenças como o câncer e a diabetes. No entanto, a grande maioria das pesquisas relacionando selênio e diabetes experimental fazem uso de formas inorgânicas de selênio. Já em modelos de câncer experimental a forma de selênio mais testada é a orgânica, porém em dosagens acima das nutricionalmente requeridas. Considerando estas abordagens, o presente estudo tem como objetivo investigar os seguintes aspectos: (1) estudar o efeito da ingestão de uma dieta suplementada com baixas concentrações de disseleneto de difenila sobre o modelo experimental de carcinogênese mamária; afim de verificar se este composto exibe atividade anticarcinogênica (2) investigar o efeito dos compostos disseleneto de difenila e ebselen, administrados por via subcutânea, no tratamento de diabetes tipo 1 e (3) analisar o efeito do consumo de uma dieta suplementada com disseleneto de difenila no tratamento de diabetes tipo 1; afim de verificar se tais compostos exibem propiedades anti-diabetogênicas nesses modelos experimentais de DM.

4. ARTIGOS CIENTÍFICOS

Os resultados que fazem parte desta tese estão apresentados sob a forma de artigos científicos, os quais encontram-se aqui organizados. Os itens Materiais e Métodos, Resultados, Discussão dos resultados e Referências Bibliográficas, encontram-se nos próprios artigos. Os artigos estão dispostos da mesma forma que foram submetidos na edição das revistas científicas (**Artigos 1 e 2**). O artigo 3 encontra-se em fase de redação.

4.1. Efeito do consumo de uma dieta suplementada com disseleneto de difenila $(PhSe)_2$ em um modelo experimental de carcinogênese mamária induzida por (NMU)

DIPHENYL DISELENIDE SUPPLEMENTATION DELAYS THE DEVELOPMENT OF NMU-INDUCED MAMMARY TUMORS

Submetido a *Food and Chemical Toxicology*

Diphenyl Diselenide Supplementation Delays the Development of NMU-Induced Mammary Tumors

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Abstract

The effect of dietary diphenyl diselenide (1ppm) on N-nitroso-N-methylurea (NMU)-induced mammary carcinogenesis was examined in female Wistar rats. Beginning at 5 week of age, the animals were supplied with both control and diphenyl diselenide supplied diets. Mammary tumor was induced by the administration of three doses of NMU (50 mg/kg) at 50, 60 and 70 days of life. In experimental trials, latency to tumor onset was extended in rats fed diet supplemented with diphenyl diselenide. A significant reduction in mammary tumor incidence and in the total number of tumors was observed in rats treated with diphenyl diselenide in comparison to the control group. Diphenyl diselenide supplementation restored superoxide dismutase (SOD) activity and vitamin C levels altered in the NMU group. Our findings suggest that diphenyl diselenide can be considered a chemopreventive agent, even when supplemented at a relatively low concentration.

Keywords: Mammary tumors, Chemoprophyilaxis, diphenyl diselenide, N-Nitroso-N-Methylurea

1. Introduction

Breast cancer is the second most frequent cause of cancer-related deaths in women (Greenlee et al., 2001), however, its etiology remains obscure and primary prevention strategies are yet not available. Moreover, advances in therapy are limited and, consequently, alternatives need to be developed for breast cancer control. Thus, the search for synthetic or natural chemical agents, that inhibit and/or delay the preneoplasic events, has received a growing interest in the cancer therapy.

Selenium intake at low concentrations is recognized as essential in animal and human nutrition (Combs and Gray, 1998). In the form of selenocysteine, selenium is a component of a number of antioxidant enzymes, e.g. glutathione peroxidase and thioredoxin redutase (Arner et al., 2000; Rotruck et al., 1973). Conversely, high doses of selenium can be cytotoxic via its ability to catalyze the oxidation of thiols and to generate free radicals (Barbosa et al., 1998; Nogueira et al., 2004; Spallholz, 1994; Spallholz et al., 2001a). Of particular importance, this element has received considerable attention for its possible role as an effective, naturally occurring, anticarcinogenic agent, when used at supranutritional concentrations (Combs and Gray, 1998; Shamberger and Frost, 1969; Spallholz, 1994).

In accordance, human epidemiological studies have clearly indicated that low selenium status is invariably associated with increased cancer risk and that selenium supplementation is associated with reduction in the incidence of several cancers (Combs and Gray, 1998; Clark et al., 1996; El-Bayoumy, 1985, 1991; Nayini et al., 1991; Spallholz, 2001b; Willett et al., 1983), including breast cancer (Nayini et al., 1989; Hunter et al., 1990). In experimental in vitro and in vivo models**,** organic and inorganic selenium supplementation has been shown to suppress

carcinogenesis in initiation and/or post-initiation phases in different types of cancer (El-Bayoumy, 1985, 1991, 1994; Milner and Hsu, 1981; Milner et al., 1985; Reddy et al., 1996; Thompson et al., 1984). Similarly, selenomethionine and/or sodium selenite, at doses well above the dietary requirement, inhibit mammary carcinogenesis but can induce severe liver necrosis (El-Bayoumy, 1991; Fan and Kizer, 1990). Consequently, in the last few years, attempts have being made to develop or search for chemicals with high efficacy against cancer but with low toxicity to mammals. Of particular importance, a number of novel synthetic organoselenium compounds inhibit tumor development caused by a variety of chemical carcinogens (El-Bayoumy, 1991; Reddy et al., 1996). However, the majority of the studies have used synthetic organoselenium compounds at concentrations considerably greater than the nutritional requirement of selenium (El-Bayoumy, 1991; Nayini et al., 1991).

Diphenyl diselenide is a synthetic organoselenium compound, which possesses glutathione peroxidase-like activity and exhibits antioxidant and anti-inflammatory properties (Meotti et al., 2004; Nogueira et al., 2003; Zasso et al., 2005). Of particular importance, diphenyl diselenide has low toxicity to rodents, when used in pharmacological doses (Fachineto et al., 2006; Perottoni et al., 2005). In contrast, diphenyl diselenide at high doses or concentrations has pro-oxidative and toxic effects in yeast, bacteria and rodents (Maciel et al., 2000; Rosa et al., 2003, 2005). Recently, studies from our group have indicated the protective role of diphenyl diselenide against acute liver damage induced by 2-nitropropane (Borges et al., 2005, 2006). However, data about the possible anti-carcinogenic action of diphenyl diselenide on chemically-induced cancer models is lacking in the literature.

In the current study, we investigated the effect of dietary diphenyl diselenide on N-nitroso-N-methylurea (NMU)-induced mammary carcinogenesis in order to delineate the possible

potential of this compound as a chemopreventive agent when used at low concentrations in the diet.

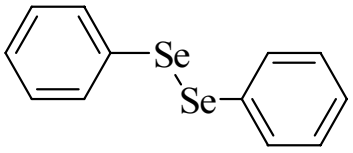
2. Materials and methods

2.1. Chemicals

NMU (N-nitroso-N-methylurea) was obtained from Sigma. 2',7'- Dichlorofluorescein diacetate (DCHF-DA) and dichlorofluorescein (DCF) were obtained from Sigma Chemical. Low melting point (LMP) agarose and normal agarose (electrophoresis grade) were obtained from Gibco-BRL (Grand Island, NY).

Diphenyl diselenide (Figure 1) was synthesized by the method described by 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. The chemical purity of diphenyl diselenide (99.9%) was determined by GC/HPLC. All other chemicals were of analytical grade and obtained from standard commercial suppliers.

Figure 1.



2.2. Animals

Sixty virgin female Wistar rats (150-200g) were obtained at 45 days of age from our own breeding colony. Rats were housed in plastic cages with water and food *ad libitum,* temperature at 22-23^{0}C, humidity at roughly 56% and 12hs light cycle. The animals were used accordingly

to guidelines of the Committee on Care and use of Experimental Animal Resources, Federal University of Santa Maria, Brazil.

2.3. Tumor Induction

After 1 week of acclimatization, rats were separated in 4 groups of 15 rats each as follow: (1) Control; (2) Diphenyl diselenide-supplemented (Se); (3) NMU and (4) NMU-diphenyl diselenide supplemented (NMU/Se). Mammary tumor was induced by three doses of NMU administration at 50, 60 and 70 days of life. NMU was dissolved in 0.9% NaCl solution pH 4.0 (acidified with acetic acid) and administered by intraperitoneal injection within 30 min of preparation at the dose of 50 mg/kg body weight. After the first NMU injection, all animals were weekly weighed and palpated up to 210 days for tumor detection. The parameters recorded were: (a) latency period (the number of days between the first NMU injection and the appearance of first tumor); (b) tumor incidence (% of animals that develop at least one tumor), (c) tumor multiplicity (average number of tumor per animal), (d) tumor frequency (number of tumor per group).

At the end of the experimental period (dated from the first NMU injection), animals were sacrificed under ether anesthesia, and mammary tumors were excised, fixed in 10% formaldehyde and prepared for histological analysis. The samples of tissues (uterus and spleen) with macroscopic alterations were also harvested for histological examination. The weight gain of all animals was measured weekly until the end of the experimental period.

To evaluate the short-time effect of NMU the animals were divided in 4 groups and submitted to an identical treatment to that described above for cancer study. Rats received three NMU injections at 50, 60 and 70 days of life. Twenty-four hours after the last NMU administration, animals were killed.

2.4. Diets

Beginning at 5 week of age, groups 1 and 3 were supplied with the control diet, whereas groups

2 and 4 were fed with diet containing 1 ppm of diphenyl diselenide. The selenium compound was dissolved in soybean oil and mixed in the diet in a food mixer to insure uniform distribution. The diet provided approximately 1 μg selenium/g of diet/per day, which is considered an acceptable amount for this element. The two diets were continued until the end of the study. Control and selenium supplemented diets were prepared weekly and stored at 20^0C. Table 1 outlines the composition of control and selenium supplemented diets.

2.5. Tissue preparation

After the sacrifice, blood was colleted by cardiac puncture in heparinized tube. Tissues samples were quickly removed, placed on ice and homogenized in cold 50 mM Tris-HCl pH 7.4. The homogenate was centrifuged at 4,000 x g for 10 min to yield low-speed supernatant fraction that was used for biochemical assays. For ex vivo assays, a group of six to ten animals was usually tested in each experiment.

2.6. Biochemical analysis

AST (aspartate aminotransferase), ALT (alanine aminotransferase), LDH (lactate deydrogenase), γ-GGT (γ-glutamyl transferase) enzymes, as well as urea and creatinine levels were determined in plasma by using commercial Kits (Labtest, Minas Gerais, Brazil).

2.7. Superoxide dismutase (SOD) activity

The measurement of SOD activity in liver was determined by the capacity of enzyme to inhibit the epinephrine autooxidation at alkaline pH at 480 nm as described by Misra and Fridovich (1973).

2.8. Vitamin C analysis

Ascorbic acid determination was performed as described by Jacques-Silva et al. (2001). Protein (liver, spleen and blood) was precipitated in 10 volumes of a cold 4 % trichloroacetic acid solution. An aliquot of the supernatants (300 μL; in a final volume of 1mL) was incubated at 38°C for 3 hours, then 1 mL H_2SO_4 65 % (v/v) was added to the medium. The reaction product was determined using a color reagent containing 4.5 mg/mL dinitrophenyl hydrazine and $CuSO_4$ (0.075 mg/mL). A standard curve was constructed using ascorbic acid.

2. 9. Lipid peroxidation

Lipid peroxidation was performed as described by Draper and Hadley (1990). Briefly, the samples were mixed with 1 ml of 10% TCA and 1ml of 0.67% thiobarbituric acid and incubated at 95^0C for 60 min. TBARS (thiobarbituric acid reactive species) were determined by the absorbance at 535 nm and were expressed as nmol malondialdehyde (MDA)/g tissue.

2.10. RS Measurement

To estimate the level of total blood reactive species (RS) production, heparinized samples were diluted (1:10) in phosphate buffer-saline (pH 7.4) and incubated with 1.6 μM of 2',7'-dichlorofluorescein diacetate (DCHF-DA) in the presence or the absence of either a pro-oxidant (10 mM sodium azida) or antioxidant (40 μM ebselen) agents. The oxidation of DCHF-DA to

fluorescent dichlorofluorescein (DCF) was measured for the detection of DFC reactive species (DCF-RS). The DCF fluorescence intensity emission was recorded at 520 nm (with 480 nm excitation) 20 min after the addition of DCHF-DA to the medium.

2.11. Comet Assay

The alkaline comet assay was performed as described by Singh *et al*., 1988. Briefly, 5 μl of blood sample were mixed with 100 μl 0.5% LMP, spread on normal agarose-precoated microscope slide and placed at 4^{o}C for 5 minute to allow for solidification. The cells were lysed in high salt and detergent, and placed in a horizontal electrophoresis box. Subsequently, the cells were exposed to alkali (300 mM NaOH /1 mM Na_2EDTA, pH 13) for 30 minutes at 4^{o}C, to allow for DNA unwinding and expression of alkali-labile sites. Electrophoresis (0.86V/cm) was carried out for 30 min at 4^{o}C. Positive controls treated with H_2O_2 (10 μM) for 5 min on ice, were included for each experiment. After electrophoresis the slides were neutralized, silver stained, and analyzed at 200 x magnification. One hundred randomly selected cells per sample were scored visually according to tail intensity into five classes (from undamaged, 0, to maximally damaged, 4). Thus, the damage score for each sample can range from 0 (completely undamaged – 100 cells x 0) to 400 (maximum damaged – 100 cells x 4). Differences in the extent of DNA strand breakage between the controls and the treatments were tested for significance using ANOVA.

2.12. Selenium determination

Elemental selenium (Se^0) analysis in diet was determined using a Perkin–Elmer (Norwalk, CT, USA) model 3030 atomic absorption spectrometer equipped with a MHS-10 hydride generation system.

2.13. **Statistical Analysis**.

Data were analyzed by analysis of variance (ANOVA), followed by Duncan's Multiple Range Test when appropriate. Tumor incidence and multiplicity were analyzed statistically by the X^2 method and Fisher's exact test and tumor latency by Student's *t*-test. Differences between groups were considered significant when $p< 0.05$.

3. Results

No significant differences in biochemical parameters analyzed were found between group treated with NMU by short-time period and control groups (data not shown).

3.1. Body and organs weight

The body weight gain of animals fed experimental and control diets are shown in Table 2. NMU administration produced a significant decrease in the body weight gain compared to the control group and diphenyl diselenide supplementation (NMU/Se group) did not prevent this effect. Diphenyl diselenide supplementation, given alone, did not affect the body weight gain of animals.

No significant difference in liver, kidney and spleen weights was observed between NMU and control group. However, NMU administration caused an increase in the mean uterine weight of animals. Diphenyl diselenide supplementation (NMU/Se group) did not modify the weight of this organ when compared to the NMU group (Table 2).

3.1.2. TGO, TGP, γ-GGT and LDH activities

There is no difference in AST, ALT and γ-GGT activities between experimental groups. NMU-treated rats exhibited a significant increase (2 fold higher than the control group) in plasma LDH activity and supplementation with diphenyl diselenide (NMU/Se group) significantly reduced the NMU-induced increase in plasma LDH activity (Table 3).

3.1.3. Urea and creatinine levels

The renal markers were not altered by NMU and/or diphenyl diselenide treatments (Table 3).

3.1.4. Tumor development parameters

Histopathological evaluation confirmed the presence of mammary carcinoma in each of tumors, in accordance with previous studies using this model of tumor induction (Cocca et al., 1998; Kubatka et al., 2003).

The results presented in table 4 show a marked increase in the incidence of mammary tumors and in the total number of palpable tumors per group in the NMU group when compared to control group. Conversely, (NMU/Se) group showed significant reduction in the mammary tumor incidence and in the total number of tumors as evidenced by the fact that NMU/Se group did not differ from the control group, when tumor incidence and total number of tumors were considered.

Tumor multiplicity and animal survival index in NMU and NMU/Se groups were not significantly different from the control group (Table 4).

Tumor latency (expressed as the number of weeks for first tumor detection), indicated that the latency period was extended in rats fed diet supplemented with diphenyl diselenide. Tumors began to develop in NMU group at 8th week following NMU administration. In rats fed with diphenyl diselenide (NMU/Se), tumor development was delayed by 9 weeks compared to the NMU group (Figure 2a). The latency for the appearance of tumor was 16 and 25 weeks in NMU and NMU/Se groups, respectively (Figure 2b).

Morphological analysis of uterus of the animals revealed that NMU administration induced the development of uterine inflammatory process in both NMU and NMU/Se groups (data not shown).

3.1.5. RS production and lipid peroxidation

TBARS levels and DFC-RS production are shown in table 5. In NMU group TBARS levels of liver and spleen were reduced when compared to the control group. There was no difference in basal TBARS levels in liver and spleen between NMU/Se and control group.

NMU administration did not induce change in the levels of DFC reactive species in total blood when compared to the control group, even when evaluated with or without sodium azide or ebselen. Diphenyl diselenide supplementation (NMU/Se) did not modify DFC-RS production when compared to the control group (Table 5).

3.1.6. Antioxidants defenses

Data in table 6 show that diphenyl diselenide supplementation, given alone, tended to increase the levels of vitamin C in liver, spleen and blood of the animals. Vitamin C levels were significantly decreased in spleen, liver and blood of NMU group as compared to the levels found

in diphenyl diselenide group. On the other hand, this reduction was normalized by diphenyl diselenide supplementation (NMU/Se) (Table 6).

SOD activity in liver of NMU group was significantly increased when compared to the control group. Diphenyl diselenide supplementation (NMU/Se) restored enzyme activity to the control levels (Table 6).

3.1.7. Comet assay

As observed in the Figure 3 NMU administration did not increase cometa formation at this experimental period. Furthermore, the supplementation with diphenyl diselenide did not change the cometa formation, regardless of the NMU treatment.

4. Discussion

It has been demonstrated that the dietary supplementation with organic or inorganic selenium compounds can inhibit both initiation and post-initiation phases of spontaneous or chemically-induced mammary tumorigenesis (El-Bayoumy et al., 1996). However, the antitumorigenic effect of selenium compounds has been consistently associated with supranutritional levels of exposure to this element. In fact, the anticancer activity of organic and inorganic selenium is obtained only at toxic levels of the element (El-Bayoumy, 1991; El-Bayoumy et al., 1995; Spallholz, 1994; Spallholz et al., 2001b).

In the current study, focusing on tumorigenisis process, dietary diphenyl diselenide promoted pronounced increase in the latency to the onset of tumor development, as indicated by prolonged mean latency period in the diphenyl diselenide supplemented group when compared to the NMU group. The incidence and total number of tumors were also small in animals supplemented with diphenyl diselenide. The results indicated that this compound presents a

protective effect against the tumor development, even when supplemented at a relatively low concentration. This attribute may represent an advantage over the other selenium compounds, since most of them when tested at low concentrations have provided small protective effect on the development of mammary tumors. Accordingly, studies have likewise showed that benzyl selenocyanate (BSC) and 1,4-phenylenebis (methylene) selenocyanate (p-XSC), structurally modified synthetic organoselenium compounds, were much more effective in inhibiting the development of DMBA-induced mammary tumors only when supplemented at levels above 5 ppm (El-Bayoumy et al., 1996; Nayini et al., 1989).

Similarly, at doses above the physiological requirement, inorganic selenium is an established chemopreventive agent. In fact, supplementing the diet or drinking water with inorganic selenium forms protects against cancer of mammary gland, colon, lung, pancreas, liver and skin (El-Bayoumy, 1991; Tanaka et al., 1995). However, chronic feeding of inorganic selenium compounds at levels ≥ 5 ppm is usually hepatotoxic to animals (El-Bayoumy, 1991). Some naturally occurring selenium containing amino acids, such as selenomethionine and selenocysteine, were equally effective chemopreventive agents and had comparable toxicity to that of inorganic selenium (Ip, 1998; Thompson, 1984).

In this study, diphenyl diselenide supplemented at relative low concentrations (1 ppm) delayed the initiation stage of chemically-induced mammary carcinogenesis and did not produce significant toxicity. In fact, diphenyl diselenide supplementation did not affect the body weight gain of animals and did not cause hepatotoxicity as indicated by the activities of plasma AST, ALT and γ-GGT enzymes. Furthermore, dietary diphenyl diselenide protected against NMU-induced cytotoxicity by reducing the accentuated increase in plasmatic LDH activity caused by NMU administration.

However, the molecular mechanism(s) by which selenium delayed the initiation stage of chemically-induced carcinogenesis remains to be elucidated. The protective effects of selenium, at the physiological dosage range, seem to be primarily associated with its presence in the glutathione peroxidases, which are known to protect DNA and other cellular components from damage by oxygen radicals. Selenium is also known to play a role in the control of cell division, in the oxygen metabolism, in detoxification processes, in apoptosis induction and in the functioning of the immune system (Combs and Gray, 1998; Faure et al., 2004; Fleming et al., 2001; Ramakrishnan et al., 1996; Spallholz, 2001a; Spallholz et al., 2004). Thus, it is possible that selenium compound acts at several stages of the multi step carcinogenesis processes.

The participation of DNA damage on NMU-induced carcinogenesis was investigated by using the comet assay. Results indicated that NMU, diphenyl diselenide and their combination did not induce changes in comet assay. These results can be explained by the fact that the DNA-toxicity induced by NMU is transitory and occurs just after the cancer induction. In line with this, literature data indicate that the DNA damage caused by NMU is only seen shortly after NMU exposure (Buschfort et al., 1997; Uhl et al., 1999).

Even though, DNA alkylation products induced by NMU were not observed in this experimental protocol, the risk of cells to accumulate potentially oncogenic mutations does not depend only of carcinogen exposure but also of the cellular capacity for protective DNA repair, such as the antioxidant defenses. Moreover, it has been shown that reactive oxygen species production in cellular processes results in the modulation of proto-oncogenes and/or the inactivation of some tumor-suppressor genes (Matés and Sánches-Jiménez, 2000; Tanaka et al., 1998), therefore antioxidants might prevent this damage and in turn protect against cancer (El-Bayoumy, 1994; Ip, 1998; Kinnula and Crapo, 2004).

The parameters related to oxidative stress, such as lipid peroxidation and reactive oxygen species production were not altered in NMU group. However, the SOD activity was increased in NMU group and vitamin C tended to be decreased in NMU treated group. One unexpected finding of the present study was a small decrease in TBARS levels in liver and spleen of NMU treated animals. These results, such as the SOD activity, may be related to a compensatory response of the tissues to a previous insult. Thus, the hypothesis that the delay of the tumor development by diphenyl diselenide selenium could be linked to its antioxidant properties can not be discarded, since diphenyl diselenide supplementation restored the activity of superoxide dismutase, an antioxidant enzyme, increased in NMU group.

In conclusion, our findings suggest that diphenyl diselenide can retard cancer development and may be considered a potential chemopreventive agent. Further studies are needed to elucidate its mechanism of action as well as the efficacy of this compound as an anticancer agent.

Table 1- Composition of the control and diphenyl diselenide-supplemented diets (weight dry) (g/Kg)

Ingredients	Quantity/kg of diet	Protein	Carbohydrate	Lipid
Wheat meat	300	20.27	154.00	1.80
Corn Starch	66	4.50	34.54	0.36
Soybean meal	133	43.24	13.51	0.90
Sucrose	86	-	58.55	-
Soybean oil	33	-	-	23.52
Lard	33	-	-	22.52
Eggs	266	17.70	-	12.28
Mineral mixture[a]	80	[a]	[a]	[a]
Vitamin mixture[b]	6	[b]	[b]	[b]

[a]The salt mixture has the following composition (g/Kg): NaCl, 152; KCl, 96.3; $MgSO_4$, 56.7; $ZnCl_2$. $7H_2O$, 0.4; $CuSO_4$. 5 H_2O, 0.7; $MnSO_4$, 1.2 and $FeSO_4$. 7 H_2O, 2.0. [b]The vitamin mixture (mg/IU g) was composed of Vitamin A, 5000 UI; Vitamin D 400 UI; thiamin,1.5mg; riboflavin, 1.7mg; pyridoxine, 2mg; ascorbic acid, 60 mg; Vitamin E, 30 UI; Vitamin K1, 0.025mg; nicotinamide, 0.02mg; folic acid, 0.4mg; calcium D-panthotenate, 10mg; biotin, 0.03mg, metiocolinB_{12}. Selenium supplemented diet containing 1 ppm of diphenyl diselenide $(PhSe)_2$.

Table 2- Body weight gain (g) and organ weight (g) of female rats

Treatment	B W gain	Liver	Kidney	Uterus	Spleen
Control	117	6.2±0.25	1.5±0.07	0.73±0.02	0.54±0.24
Selenium	126	7.7±0.80	1.7±0.08	0.70±0.05	0.58±0.07
NMU	80*	7.0±0.52	1.5±0.19	1.48±0.09*	0.74±0.13
NMU/Se	79*	6.8±0.38	1.4±0.06	2.00±0.35*	0.92±0.23

Data are expressed as means± SEM of six animals. *Denoted $p<0.05$ as compared to the control group (ANOVA/Duncan).

Table 3- Effect of diphenyl diselenide supplementation on biochemical parameters in NMU- administrated rats.

	Control	Selenium	NMU	NMU/Se
TGP[a]	51±13.4	24± 4.60	46±13.8	35±13.8
TGO[a]	196±19.2	130±9.80	271±46.4	176 ±11.1
GGT[a]	3.8±0.35	4.0±0.44	4.5±0.76	5.2±0.35
LDH[a]	962±25.0	940±16.9	2054± 230*	1080 ± 210
Urea[b]	43±0.93	37±4.24	43±1.60	30±3.30
Creatinine[b]	0.29±0.17	0.36±0.22	0.26±0.04	0.21±3.9

Data are expressed as means± SEM of six animals. *Denoted $p<0.05$ as compared to the control group (ANOVA/Duncan). [a]Data of enzyme activities are presented as U/l. [b] Data of renal markers levels are presented as mg/dl.

Table 4- Effect of dietary diphenyl diselenide on NMU-induced mammary tumors in female rats

	Control	Selenium	NMU	NMU/Se
Tumor incidence (%)	0/15	0/15	56.3 (8/15)*	26.6 (4/15)
Tumor multiplicity	0/15	0/15	1.25	1.00
Total tumor number	0/15	0/15	10*	04
Survival (%)	100 (15/15)	100 (15/15)	80 (12/15)	53 (08/15)

Tumorigenicity parameters are calculated as % from the control group (100%). *Significant different as compared to the control group (Fischer's exact probability test, $p < 0.05$). Means indicate the number of animals with mammary tumors.

Table 5- Effect of diphenyl diselenide supplementation on oxidative stress parameters in NMU- administered rats

	TBARS[a]		DFC-RS Spectrofluorometric assay[b]		
Groups	Liver	Spleen	Blood	Blood+Eb	Blood+Az
Control	13.0±0.57	11.2±0.38	84.3± 5.72	27.3±2.48	161.2±4.84
Selenium	12.6±0.43	11.4±0.55	82.4±3.66	24.1±0.80	145.1±4.72
NMU	10.3±0.69*#	9.4±0.4*#	89.2±8.87	34.2±7.51	143.8±7.83
NMU/Se	11.6±0.28	9.9± 0.73	83.8± 7.25	31.4±5.54	134.7±13.6

Data are expressed as means± SEM of ten animals. [a]Data of TBARS levels are presented as nmol MDA/g tissue. [b]Data of reactive species (RS) levels are presented as fluorescence intensity emission (UAF). *Significantly different as compared to the control group (ANOVA/Duncan, $p< 0.05$). # Significantly different as compared to the dyphenyl diselenide group (ANOVA/Duncan, $p< 0.05$).

Table 6- Effects of diphenyl diselenide supplementation on levels of antioxidants defenses in NMU-administered rats.

	SOD	Vitamin C		
Groups	Liver	Spleen	Liver	Blood
Control	15.81±1.34	223±3.26	379±17.8	224±3.81
Selenium	18.61±1.06	257±16.3	448±28.1	258±15.9
NMU	22.74±0.77*	199±8.98[#]	355±20.4[#]	195±9.79[#]
NMU/Se	16.97±1.96	222±27.3	418±23.6	222±24.0

Data are expressed as means± SEM of ten animals. [a]Data of SOD activity are presented as U/mg protein. [b]Data of vitamin C levels are presented as μ /g tissue. *Significantly different as compared to the control group (ANOVA/Duncan, $p< 0.05$). [#]Significantly different as compared to the diphenyl diselenide group (ANOVA/Duncan, $p< 0.05$).

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Legends

Figure 1. Chemical structure of diphenyl diselenide

Figure 2. Effect of dietary selenium on mean latency period of NMU-induced mammary tumors in female rats (2a). The mean latency to tumor is expressed as the time interval (in weeks) from NMU administration to the appearance of the first palpable tumor (2b). The groups were significantly different ($p < 0.05$) by analysis of variance.

Figure 3. Effect of NMU administration on Cometa assay. Differences among experimental groups were not statistically significant.

Figures

Figure 2a

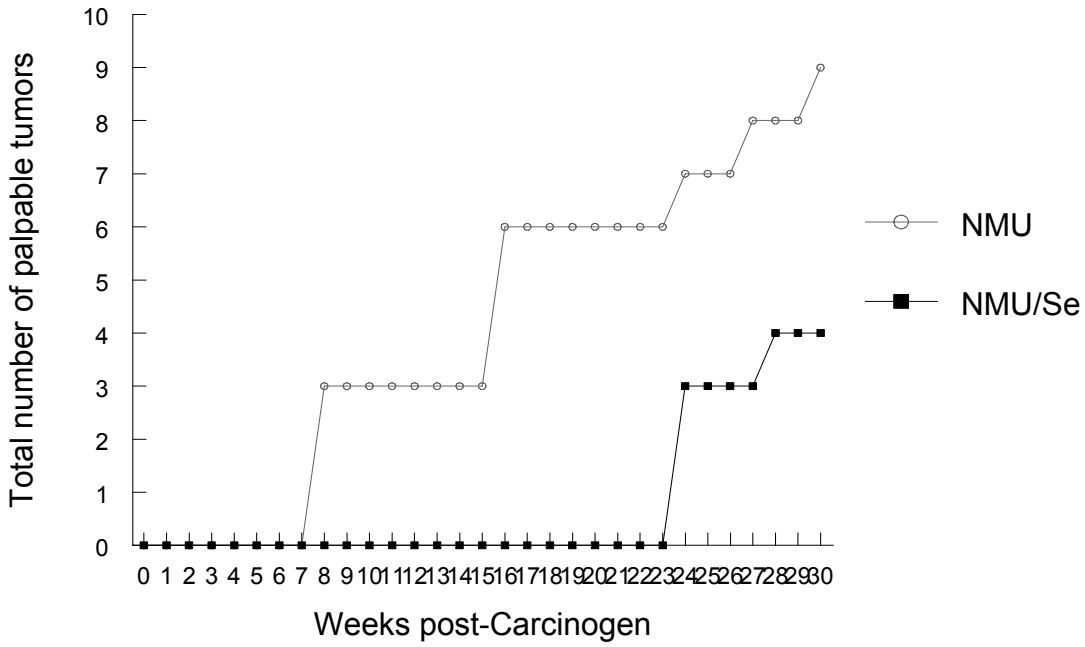


Figure 2b

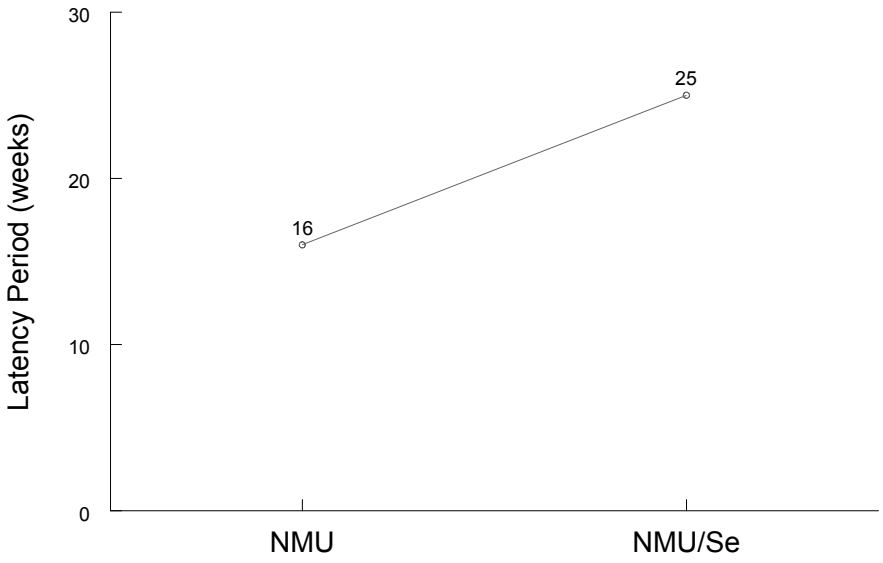
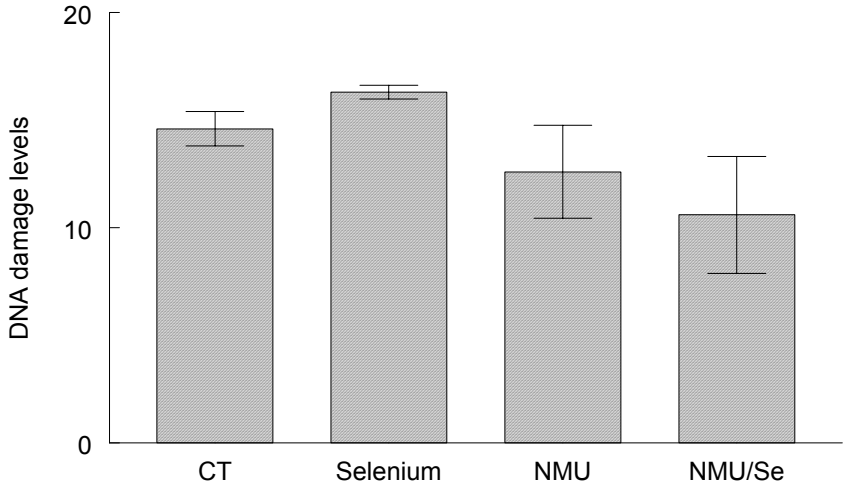


Figure 3



4.2. Efeito dos compostos orgânicos de selênio, disseleneto de difenila e ebselen, em um modelo experimental de Diabetes Mellitus induzida por streptozotocina (STZ)

DIPHENYL DISELENIDE REDUCES TEMPORARILY HYPERGLYCEMIA: POSSIBLE RELATIONSHIP WITH OXIDATIVE STRESS

Submetido a *Diabetes Research*

Diphenyl Diselenide Reduces Temporarily Hyperglycemia: Possible Relationship with Oxidative Stress

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Abstract

This study was designed to determine the effect of diphenyl diselenide and ebselen, synthetic organoselenium compounds with antioxidant properties, in diabetic rats. Diabetes was induced by the administration of streptozotocin (STZ) (45 mg/kg, i.p). In experimental trials, diphenyl diselenide, but not ebselen, caused a significant reduction on blood glucose levels of STZ treated rats. This effect of diphenyl diselenide was accompanied by a reduction in the levels of glycated proteins. Diphenyl diselenide treatment increased SOD activity and vitamin C levels what were decreased in STZ-treated rats. In normal rats, diphenyl diselenide promoted *per se* an increase in hepatic, renal and blood GSH levels. Similary, diphenyl diselenide caused an increase in hepatic and renal GSH levels in STZ treated rats. TBARS and protein carbonyl levels were not modified by STZ and/or diphenyl diselenide and ebselen treatments. Our findings suggest that diphenyl diselenide can be considered an anti-diabetogenic agent by exhibiting anti-hyperglycemic and antioxidant properties.

Keywords: Diabetes; oxidative stress; diphenyl diselenide; ebselen.

1. Introduction

Diabetes mellitus is a group of metabolic diseases characterized by hyperglycemia resulting from defects in both insulin secretion and/or insulin action. The precise cellular and molecular mechanism(s) which underlie(s) the etiology and progression of diabetes are still not fully understood. However, oxidative stress is thought to play a central role on the development of many diabetic complications [4,21,26,28,37,48,51].

In line with this, an increase in lipid peroxidation and deficits in the antioxidant defense systems have been observed in a variety of experimental models of hyperglycemia [2,14,24,30,32,38,41,43,58,68,74]. In addition, protein glycation and AGES formation are enhanced under oxidative stress [28,29,32,41,51,63,74]. Thus, compounds that present anti-hyperglycemic and/or antioxidant effects are of potential therapeutic interest for the treatment of human and animal diabetics [15,16,18,19,21,39,43,45,50,67].

Selenium plays a crucial role as an integral component of several antioxidant enzymes involved in peroxide decomposition [6,23,34,59,70]. The antioxidant properties of selenium certainly contributes to preserve health conditions and its deficiency has been linked to an increase in the incidency of cardiovascular disease, immune functions, cancer, seizures and diabetes have been linked to selenium deficiency [15,44]. Conversely, high doses of selenium have pro-oxidative and toxic effects to mammal, yeast and bacteria [3,47,65,36,57]. Of particular significance for the prevention of diabetics onset and/or progression, inorganic selenium supplementation can reduce the oxidative stress in experimental animals [1,16,21,22,32,43,45,56,58,67] and reduce over activity of NF-kB in peripheral blood mononuclear cells of type II diabetic subjects [22]. The exact mechanism(s) via which inorganic selenium has protective effects against diabetes complications is yet-still not known, but its antioxidant role in cell physiology is thought to

be of central important to it as anti-diabetogenic properties [17,21,43,45,56,60,64]. In addition, inorganic selenium can attenuate diabetic complications via insulin-mimetic properties [5,18,19,27,33,39,49,67,66].

Diphenyl diselenide and ebselen are synthetic organoselenium compounds that have been considered potentential pharmacological agents [47]. In fact, these compounds exhibit antioxidant, anti-nociceptive, neuroprotective and anti-inflammatory properties in different experimental models [40,46,55,76]. Indeed, ebselen reduces the extent of DNA damage evoked by streptozotocin (STZ) in normal and cancer cells [8].

Of particular importance, these compounds have low toxicity to rodents, even when used at supra pharmacological doses [20,46,53,76]. Therefore, the aim of this study was to examine whether diphenyl diselenide and ebselen could prevent and/or reduce biochemical alterations associated with streptozotocin (STZ)-induced diabetes in rats.

2.0. Materials and Methods

2. 1. Chemicals

δ-Aminolevulinic acid (δ-ALA) and *p*-dimethylaminobenzaldehyde were purchased from Sigma (St. Louis, MO, U.S.A), Streptozotocin (STZ), reduced glutathione (GSH), 5.5'-dithio-bis-(2-nitrobenzoic acid) (DTNB), thiobarbituric acid (TBA), DL-dithiothreitol (DTT), DCHF-DA and DCF were obtained from Sigma. Diphenyl diselenide and ebselen compounds were synthesized by the method described by [17,46] . Analysis of the ^{1}H NMR and ^{13}C NMR spectra showed analytical and spectroscopic data in full agreement with its assigned structure. The chemical purity of diphenyl diselenide and ebselen (99.9%)

was determined by GC/HPLC. All other chemicals were of analytical grade and obtained from standard commercial suppliers.

2. 2. Animals

Adult male Wistar rats weighing 180-200g were used for the experiments. All rats received food (Guabi, Ribeirao Preto, SP, Brazil) and water *ad libitum* and were kept on a 12-h ligth/12-h dark cycle, in a room with the temperature regulated to 21- 25^0 C and humidity at roughly 56%. The animals were used accordingly to guidelines of the Committee on Care and use of Experimental Animal Resources of the Federal University of Santa Maria, Brazil.

2. 3. Diabetes induction

Diabetes was induced by a single intravenous injection of Streptozotocin (STZ) (45 mg/kg), diluted in 0.1 M citrate-buffer (pH 4.5). Control rats received an equivalent amount of the buffer. STZ-treated received a 5% of glucose instead of water. Blood samples were taken 48 hs after STZ or vehicle injection from the tail vein**.** Glucose levels were measured with an automatic auto analyzer (GLUCOTREND®). Animals were considered diabetic when blood glucose levels exceeded above 250 mg/dL.

2. 4. Treatment

The animals were randomly divided into the following groups: (1) control; (2) Diphenyl diselenide (Ph) (3) Ebselen-(Ebs); (4) STZ; (5) STZ+$(PhSe)_2$ (STZ/Ph) and (6) STZ+Ebselen (STZ/Ebs). Selenium groups were subcutaneously administered with diphenyl diselenide or ebselen at the dose of 1 mg/kg (once a day) for 90 days after the

administration of STZ. The organoselenium compounds were dissolved in 25% Tween 80. Control rats were similarly treated with Tween. At the end of the experimental period, diabetic rats and the corresponding control animals were anesthetized with ether and sacrificed by decapitation. Rats were fasted 12h prior to the sacrifice.

2.5. Tissue preparation

Blood of anesthetized rats was colleted by cardiac puncture in heparinized tube. Tissue samples were quickly removed, placed on ice and homogenized in cold 50 mM Tris-HCL pH 7.4. The homogenate was centrifuged at 4,000 x g for 10 min to yield the low-speed supernatant fraction that was used for biochemical assays. The protein content was determined by the method of [9], using bovine serum albumin as the standard.

2.6. Biochemical analysis

Plasma AST (aspartate aminotransferase), ALT (alanine aminotransferase) and LDH (lactate dehydrogenase) enzymes, urea, creatinine, cholesterol, triglyceride and uric acid levels were determined using commercial Kit (Labtest, Minas Gerais, Brazil).

2.7. Glycated proteins

The levels of glycated-albumin (frutosamine) and glycated-hemoglobin were determined in plasma by using a commercial Kit (Labtest, Minas Gerais, Brazil).

2.8. Antioxidant defense systems

2.8.1. Superoxide dismutase (SOD) activity

Hepatic and renal SOD activity was determined by the capacity of enzyme to inhibit the epinephrine autooxidation at alkaline pH, as described by [69].

2.8.2. Vitamin C content

Blood, hepatic renal and cerebral vitamin C (ascorbic acid) levels were determined colorimetrically as described by [31].

2.8.3. GSH levels

Blood (erythrocytes), hepatic renal and cerebral reduced glutathione (GSH) content was estimated using Ellman's reagent after desproteinization with TCA (5% in 1 mmol/EDTA).

2.9. Enzymes activity

2.9.1. δ-ALA-D

Hepatic, renal and cerebral δ-ALA-D activity was assayed according to the method of [61] by measuring the rate of product porphobilinogen (PBG) formation except that 84 mM potassium phosphate buffer, pH 6.4 and 2.4 mM ALA were used. The reaction was started 10 min after the addition of the enzyme preparation by adding the substrate. The incubation was carried out for 1h (liver), 2hs (kidney) and 2hs (brain) at 37^0 C. The reaction product was determined using modified Ehrlich's reagent at 555 nm, with a molar absorption coefficient of 6.1×10^4 M^{-1} for the Ehrlich-PBG salt.

2.9.2. Na^+/K^+-ATPase

Cerebral Na^+/K^+-ATPase activity was measured as described by [9].

3.0. Statistical analysis

All values obtained are expressed as mean±standard error. The data were analyzed by one-way or two-way ANOVA and MANOVA analyses of variance followed by Duncan's multiple range test when appropriate. Differences between groups were considered to be significant when $p< 0.05$.

4.0. Results

4.1. Body weight and organ weight ratio

Body weight gain was reduced in diabetic rats in comparison to the control group (Figure 1). The reduction in body weight of diabetic rats was not prevented by diphenyl diselenide or ebselen (Figure 1). These compounds, when given alone, did not change the body weight gain of rats.

STZ-induced diabetes increased the organ to body ratio for liver and kidney. These changes were not prevented by diphenyl diselenide and ebselen (Table 1). The brain to body weight ratio was increased in diabetic rats and treatment with ebselen and $(PhSe)_2$ restored this to the control value.

4.2. Plasma glucose levels

Blood glucose levels were significantly higher (approximately 5-fold) in diabetic rats than in control groups. Diphenyl diselenide caused a significant decrease in glucose levels.

This anti-hyperglycemic effect of dyphenyl diselenide was evident at 30 and 45 days after STZ administration and the blood glucose levels of STZ/Ph was about 50% lower than the of the STZ group. However, the blood glucose levels of STZ/Ph group returned to the levels found in STZ group. Ebselen did not modify significantly the toxic effect of STZ (Table 2).

4.3. Frutosamine and glycated-hemoglobin levels

Total glycated-hemoglobin and glycated-albumin concentrations in diabetic rats were significantly higher than in the control groups (Figures 2A and 2B). Diphenyl diselenide treatment caused a significant reduction in glycated proteins levels in diabetic rats. Ebselen did not change glycated hemoglobin and frutosamine in diabetic rats (Figures 2A and 2B).

4.4. Biochemical analysis

Plasma urea was increased in diabetic rats when compared to control groups (Table 3). Diphenyl diselenide and ebselen treatments restored only urea levels in STZ treated rats. Plasma triglyceride levels were also decreased in diabetic rats, but ebeselen and $(PhSe)_2$ treatments did not change these levels. AST, ALT, LDH, uric acid, creatinine and cholesterol were not modified by STZ and/or $(PhSe)_2$ and ebselen treatments (Table 3).

4.5. Antioxidant defenses

4.5.1. Vitamin C levels

Blood, hepatic and renal levels of vitamin C were decreased in STZ-treated rats. Diphenyl diselenide treatment normalized vitamin C levels in all tissues of STZ treated rats. In normal rats diphenyl diselenide caused *per se* a significant elevation on vitamin C

levels in blood tissue (Table 4). Ebselen treatment to diabetic rats caused an increase in vitamin C levels in renal and blood tissue when compared to the control group.

In the brain, no significant differences in vitamin C levels were found between control and diabetic rats (Table 4).

4.5.2. GSH levels

Hepatic, renal and blood GSH levels were not modified significantly by STZ treatment. Diphenyl diselenide treatment, given alone, promoted *per se* a significant increase on hepatic, renal and blood GSH levels compared to control group. Similary, in STZ treated rats diphenyl diselenide elevated GSH levels in liver and kidney (Table 4). Ebselen treatment to STZ treated rats tended to increase GSH levels in these tissues.

Cerebral GSH levels did nor changed by STZ and/or diphenyl diselenide and ebselen treatments.

4.5.3. SOD activity

STZ treatment caused a significant decreased in hepatic SOD activity, when compared to control groups. Treatment of diabetic rats with both diphenyl diselenide and ebselen restored SOD activity to control levels (Figure 3B). STZ did not change the renal SOD activity and diphenyl diselenide treatment caused *per se* an increase in SOD activity (Figure 3A).

4.6. Enzymes activity

4.6.1. δ-ALA-D

Hepatic δ-ALA-D activity was significantly reduced in diabetic rats when compared to the control group. Diphenyl diselenide and ebselen abolished the decrease in hepatic δ-ALA-D activity caused by diabetes (Table 5). Cerebral and renal δ-ALA-D were not modified by STZ-induced diabetes or selenium treatments (Table 5).

4.6.2. Na^+K^+ATPase

Cerebral Na^+K^+ATPase activity was significantly stimulated in diabetic rats when compared to the control group. Diphenyl diselenide and ebselen abolished the increase in cerebral Na^+K^+ATPase activity caused by STZ treatment (Table 5).

6.0. Discussion

Literature data have indicated the potential usefulness of inorganic selenium as a therapeutic agent for the prevention and treatment of diabetes mellitus [7,43,45,66,67]. However, data about the usefulness of organic synthetic selenium in animal models of diabetes are not available in the literature. The current study examined the potential effect of synthetic organoselenium compounds diphenyl diselenide and ebselen in attenuating hyperglycemia and other biochemical alterations in diabetic rats. Diphenyl diselenide treatment caused a transitory decrease in plasma glucose levels in STZ treated rats. In contrast to diphenyl diselenide, ebselen did not have a consistent anti-hyperglycemic effect in this experimental protocol.

The dose of organoselenium compounds chosen for treating animals did not cause overt signals of toxicity. Indeed, chronic administration of diphenyl diselenide or ebselen at

1mg/Kg did not affect the body weight gain of animals and did not cause evident signs of liver injury as indicated by the activities of plasma AST, ALT and LDH enzymes.

In diabetic condition, the long term period of exposure proteins to hyperglycemia causes an excessive non-enzymatic glycation of its their structure(s), which can result in inactivation of enzymes; increased lipid peroxidation and changes in the antioxidant defense systems [12,13,24,42]. In this context, our results showed that the anti-hyperglycemic effect of diphenyl diselenide was associated with a reduction in the levels of glycated proteins. In fact, both glycated hemoglobin and glycated albumin levels, which were elevated in diabetic rats, were significantly reduced by diphenyl diselenide treatment. This anti-glycating effect of diphenyl diselenide may be directly related to its anti-hyperglycemic properties and/or linked to its antioxidant activity; since the effect of hyperglycemia on proteins such as hemoglobin and albumin can be reduced by antioxidant treatments [25,29,62,71,75].

The exact mechanisms via which inorganic selenium compounds exhibit *in vitro* anti-hyperglycemic action are still unclear, but appear to involve their insulin-like properties [7,19,66,67]. In fact, inorganic selenium treatment to diabetic rats reduces blood glucose concentration and stimulates glucose uptake in insulin-responsive tissues; translocates glucose transporter to the plasma membrane; stimulates the tyrosyl phosphorilation of proteins phosphorilated by insulin [7,19,39,66] and also positively affects the expression and activity of enzymes associated with both carbohydrate and fatty acid metabolism *in vitro* [7,49].

In addition, *in vivo* supplementation or administration of inorganic selenium can promote an increase in cell antioxidant defenses, which could counteract the toxic effects associated with diabetes state. In accordance, we demonstrated that antioxidant defenses

which were significantly decreased in STZ-treated rats were restored to normal levels after treatment with organoselenium compound, diphenyl diselenide. In fact, SOD activity and vitamin C levels were normalized by diphenyl diselenide treatment. This compound also increased *per se* renal SOD activity and GSH levels, indicating that diphenyl diselenide besides to act as an antioxidant may promote an increase in antioxidant systems. Different from diphenyl diselenide, ebselen treatment to diabetic rats was not effective in modify the majority of these antioxidant parameters.

As stated previously, the hyperglycemia condition can cause glycation of proteins and free radical generation, which in turn can lead to inhibition of enzymes [12,24,42,54]. In line with this hepatic δ-ALA-D activity was inhibited in diabetic rats and restored by diphenyl diselenide and ebselen treatments. Conversely, cerebral Na^+K^+ATPase activity was increased in this study. Literature data have indicated that erytrhrocyte Na^+K^+ATPase can be inhibited after in vitro exposure to high glucose concentrations [30]. However, data regarding the effects of experimental models of diabetes on brain Na^+K^+ATPase are rare in literature. Thus, the increase in brain Na^+K^+ATPase from STZ-treated rats can be related to an adaptative response to oxidative stress. Althought less probable, glycation could also promote an increase in ATPase activity. In fact, recent data from literature indicate that diabetes both in humans and rats are associated with an enhanced platelet ATP/ADPase [35].

In summary, our findings suggest that diphenyl diselenide was effective in controlling hyperglycemia to a significant extent in STZ-diabetic rats. Indeed, these data may imply that organoselenium compounds, functioning as antioxidants, can be beneficial for reducing diabetic complications linked to oxidative stress. Hence, further studies are

needed to elucidate the mechanism(s) of action, as well as, the efficacy of these compounds as anti-diabetogenic agents.

Acknowledgements

The financial support by FAPERGS, CAPES and CNPq is gratefully acknowledged. C.W.N., G.Z. and J.B.T.R are the recipients of CNPq fellowships.

Tables

Table 1. Organ-to-body weight ratio (mg/g) in STZ-induced diabetic rats treated with diphenyl diselenide and ebselen.

Groups	Liver	Kidney	Brain
Control	29.5±12.0	6.96±0.52	5.89±0.17
$(PhSe)_2$	29.7±12.1	7.03±0.30	5.62±0.17
Ebs	29.3±13.1	7.28±0.52	6.18±0.15
STZ	40.9±16.7*	11.7±1.18*	8.60±0.77*
STZ/Ph	42.4±16.0*	10.6±0.40*	6.80±0.17*[a]
STZ/Ebs	38.6±19.3*	9.83±0.31*	7.10±0.14*[a]

Data are expressed as means± SEM of six animals. *Denoted $p<0.05$ as compared to the control group (ANOVA/Duncan). [a] Significantly different from the diabetic group (ANOVA/Duncan, $p< 0.05$).

Table 2. Effect of diphenyl diselenide and ebselen on blood glucose of STZ-treated rats.

	Glucose levels (mg/dl)				
	Time after STZ-diabetes induction (days)				
	2	30	45	60	90
Control	83±11.7	89±5.27	90±5.81	88±2.93	94±3.20
$(PhSe)_2$	77±2.60	80±32.1	88±3.48	93±4.89	86±5.20
Ebs	84±6.12	85±5.81	91±3.13	92±2.28	89±3.00
STZ	502±29.1*	431±37.0*	408±43.8*	504±37.4*	518±39.0*
STZ/Ph	446±37.7*	223±35.9*[a]	189±44.5[a]	471±38.5*	459±37.0*
STZ/Ebs	499±29.1*	442±32.1*	296±56.6*	558±29.5*	570±29.0*

Data are expressed as means±SEM of six animals. *Significantly different from the control group (ANOVA/Duncan, $p < 0.05$). [a]Significantly different from the diabetic group (ANOVA/Duncan, $p < 0.05$).

Table 3. Effect of diphenyl diselenide and ebselen on biochemical parameters of STZ-treated rats.

	Control	$(PhSe)_2$	Ebs	STZ	STZ/Ph	STZ/Ebs
Uric acid	3.1±1.38	1.5±0.16	1.3±0.34	1.8±0.18	1.7±0.27	1.8±0.21
Urea[b]	48±2.58	50±7.44	46±2.40	90±12.8*	73±11.2	73±4.02
Creatinine[b]	0.3±0.02	0.3±0.02	0.3±0.02	0.4±0.06	0.3±0.02	1.0±1.12
TGP[a]	69±12.7	88±29.5	60±4.50	71±5.86	89±17.6	95±14.6
TGO[a]	172±12.0	216±46.4	165±9.13	152±11.3	190±31.2	189±33.7
LDH[a]	343±24.9	358±27.3	386±24.5	389±35.6	354±63.0	421±90.5
TG[b]	90±14.7	75±5.78	68±5.42	60±6.02*	53±11.0*	58±4.26*
Cholesterol[b]	73±5.60	72±2.89	65±3.15	74±5.98	67±8.83	68±3.72

Data are expressed as means± SEM six animals. *Denoted $p<0.05$ as compared to the control group (ANOVA/Duncan). [a]Data of enzyme activities are presented as U/l. [b]Data of renal markers and lipid levels are presented as mg/dl (ANOVA/Duncan).

Table 4. Effect of diphenyl diselenide and ebselen on non-enzymatic antioxidant defenses of STZ-treated rats.

	Control	$(PhSe)_2$	Ebs	STZ	STZ/Ph	STZ/Ebs
VIT C Levels						
Liver	239±29	246±26	225±13	164±3.60*	265±17.5	177±14.7*
Kidney	193±23.4	172±14.2	172±14.3	107±9.63*	160±6.54	180±6.42
Brain	291±4.03	279±14.6	259±19.5	273±30.6	288±26.8	292±39.6
Blood	170±13.8[a]	223±18.3*	158±23.7[a]	91±21.9*[a]	152±13.0a	141±10.7[a]
GSH Levels						
Liver	2985±234	3828±221*	2810±540	2043±454[a]	3622±461[b]	2445±600[a]
Kidney	1614±400	2349±606*	1499±305[a]	1405±370[a]	2099±581[b]	1981±741[a]
Brain	1796±325	2208±281	1861±313	1814±286	1807±295	1975±254
Erythrocytes	1346±107	1937±133*	1642±154	1250±119[a]	1101±103[a]	1393±169[a]

Data are expressed as means±SEM of six animals. *Denoted $p<0.05$ as compared to the control group (ANOVA/Duncan). [a]Significantly different from the $(PhSe)_2$ group (ANOVA/Duncan, $p< 0.05$). [b]Significantly different from the diabetic group (ANOVA/Duncan, $p< 0.05$).

Table 5. Effect of diphenyl diselenide and ebselen on δ-ALA-D and Na^+K^+ATPase activity of STZ-induced diabetic rats.

	δ-ALA-D activity[a]			Na^+K^+ATPase[b]
	Liver	Kidney	Brain	Brain
Control	11.20±2.2	2.09±0.2	1.24±0.2	1257±142
$(PhSe)_2$	10.51±1.6	2.39±0.2	1.18±0.2	1014±217
Ebs	11.05±1.6	2.24±0.1	1.27±0.2	1471±200
STZ	6.02±0.7*	1.55±0.1	1.42±0.2	2165±360*
STZ/Ph	7.00±0.6	1.82±0.2	1.31±0.2	1207±252
STZ/Ebs	7.19±1.0	1.77±0.2	1.10±2.0	1013±388

Data are expressed as means± SEM of six animals. [a]Data of δ-ALA-D activity are presented as nmol PBG (mg/protein/h). [b]Data of Na^+K^+ATPase activity are presented as nmol Pi (mg/protein/min). *Significantly different from the control group (ANOVA/Duncan, $p< 0.05$).

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Legends

Figure 1. Body weight (means±SEM, n=6) of STZ-induced diabetic rats treated with diphenyl diselenide and ebselen.

Figure 2. Plasma concentration of glycated-hemoglobin (A) and (B) glycated- albumin (frutosamine) in STZ-induced diabetic rats treated with diphenyl diselenide and ebselen. Values are means± SEM of six animals. *Denoted $p<0.05$ as compared to the control group (ANOVA/Duncan).

Figure 3. Renal (A) and Hepatic (B) SOD activity from STZ-induced diabetic rats treated with diphenyl diselenide and ebselen. Date are expressed ± SEM of five to six animals. Denoted *$P< 0.05$ as compared to the control group.

Figure 1

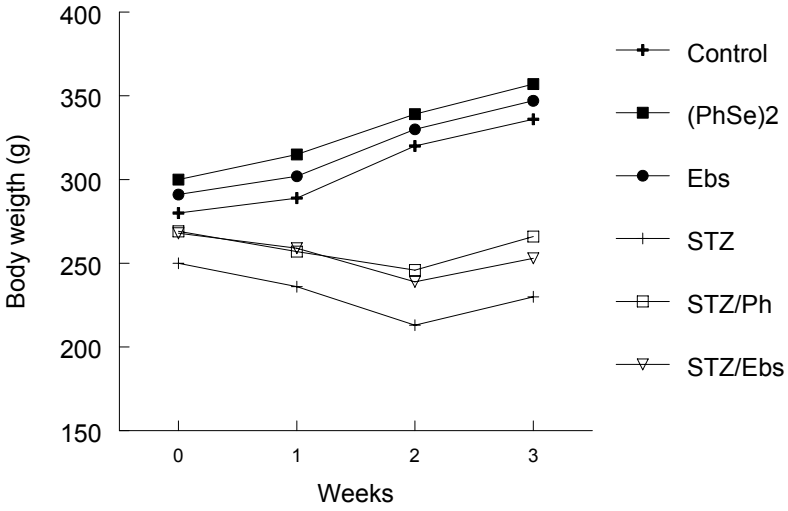
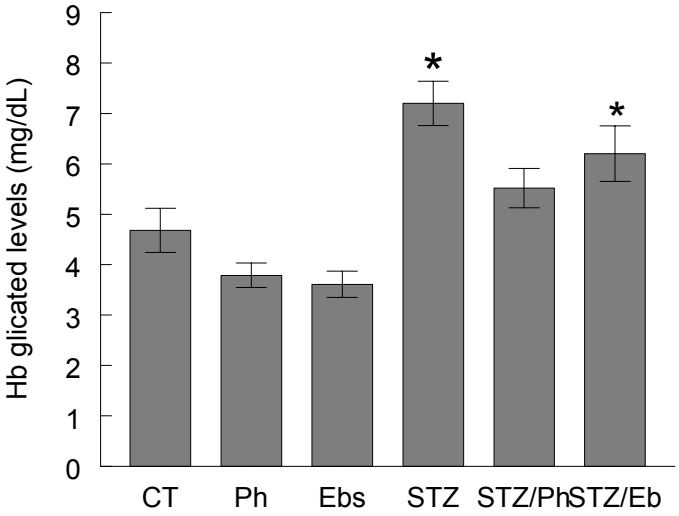
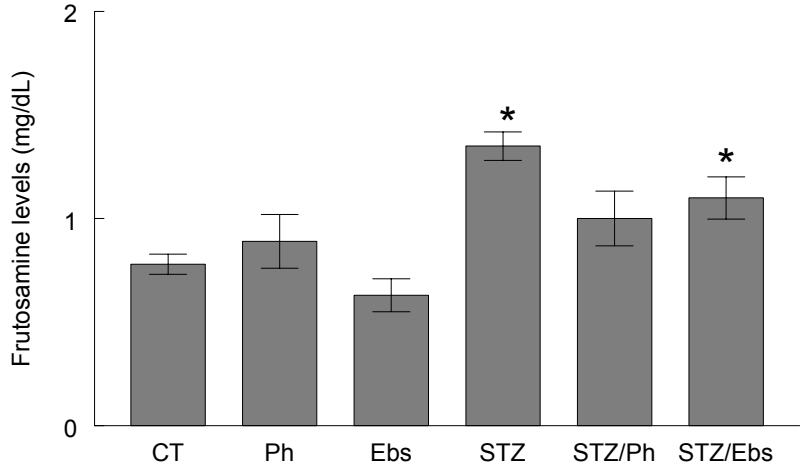

400
350
300
250
200
150
Body weigth (g)
0
1
2
3
Weeks
Control
(PhSe)2
Ebs
STZ
STZ/Ph
STZ/Ebs

Figure 2

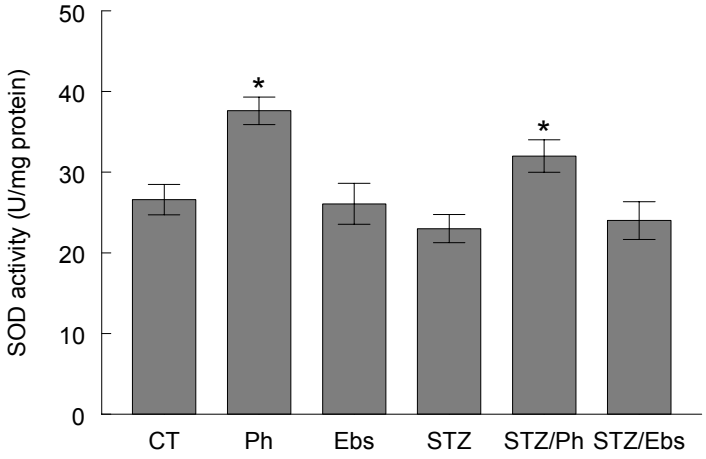

Hb glicated levels (mg/dL)
0
1
2
3
4
5
6
7
8
9
*
*
CT
Ph
Ebs
STZ
STZ/Ph
STZ/Eb

(A)

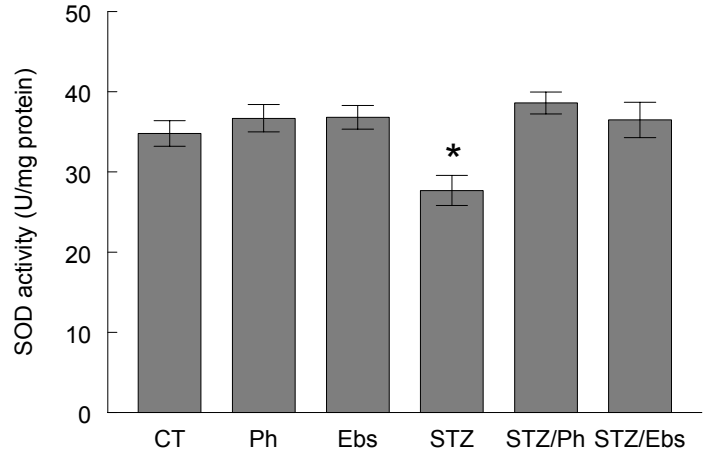

Frutosamine levels (mg/dL)
0
1
2
*
*
CT
Ph
Ebs
STZ
STZ/Ph
STZ/Ebs

(B)

Figure 3


SOD activity (U/mg protein)
50
40
30
20
10
0
*
*
CT
Ph
Ebs
STZ
STZ/Ph
STZ/Ebs

(A)


SOD activity (U/mg protein)
50
40
30
20
10
0
*
CT
Ph
Ebs
STZ
STZ/Ph
STZ/Ebs

(B)

4.3. Efeito do consumo de uma dieta suplementada com disseleneto de difenila em ratos tratados com STZ

EFFECT OF DIETARY DIPHENYL DISELENIDE ON STZ-DIABETIC RATS

Em fase de redação

Effect of dietary diphenyl diselenide on STZ-induced diabetic rats

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1. Introduction

Diabetes Mellitus is a metabolic disorder associated with impairment of glucose utilization caused by a derangement in insulin function. Oxidative stress resulting of hyperglycemia condition appears to be an integral and possibly causative part of the pathogenesis of diabetes (Wolff and Dean, 1987, Jain and Palmer, 1997, Jain and Kannan, 2001, Rosen et al., 2001, Maritim, 2003). In fact, studies have suggested that the free radicals generation may be crucial effectors in beta cell damage (Gille et al., 2000). In addition, the activation of NF-kB (a ROS-sensitive transcription factor) may be one of the key cellular process that bridges oxidative stress and death of beta cells (Ho, et al., 2001). In line with this, an increase in lipid peroxidation and deficits in the antioxidant defense systems have been observed in a variety of experimental models of hyperglycemia (Jennings et al., 1987; Collier et al, 1990; McLennan et al., 1991; Mukherjee et al., 1998; Armistrong et al., 1996; Folmer et al., 2002; Jain et al., 2001; Mohamed et al., 1999; Rosen et al., 2001; Strain et al., 1991; Wolff et al., 1987, 1993).

Selenium intake at low concentrations has been shown to be a nutritionally essential trace element to mammals (Combs and Gray, 1998; Navarro-Alarcón, and Lopes-Martinez, 2000). Selenium is an integral component of several antioxidant enzymes involved in peroxide decomposition (Flohe et al., 1973; Rotruck et al., 1973; Ursini et al., 1982; Behne et al., 1990; Linder, et al., 1990). However, high levels of exposure to selenium have been found to induce pro-oxidative and toxic effects to mammal, yeast and bacteria (Spallholz, 1994; Barbosa et al., 2000; Maciel et al., 2003; Spallholz et al., 2001; Nogueira et al., 2004; Rosa et al., 2003, 2005).

This element, because its physiological antioxidant properties, has been reported to exhibit a number of potentially beneficial effects against the development of different

diseases, including diabetes (Adachi et al., 1991; Dincer et al., 2002; Ezaki, 1990; Faure, 2003; Faure et al., 2004; Jennings et al., 1987; Mukherjee et al., 1998; Naziroglu et al., 2001; Reddi, et al., 2001; Rosen et al., 2001; Stapleton 2000). Inorganic selenium can decrease or inhibit lipid peroxidation and overcome abnormalities in endogenous damage antioxidant defense systems in diabetic patients and in animal models of the disease (Jennings et al., 1987; Mukherjee et al., 1998; Naziroglu et al., 2001; Reddi, et al., 2001; Rosen et al., 2001; Stapleton 2000; Ruiz C et al., 1999, Faure P, 2003, Mukherjee, B. et al., 1998). Accordingly, inorganic selenium supplementation decreased NF-kB over activity in peripheral blood mononuclear cells of type II diabetic subjects (Faure, et al., 2004).

Additionally to its antioxidant effect, inorganic selenium can attenuate the development of diabets via its insulin-mimetic properties (Stapleton, 2000, Berg et al., 1995, Becker et al, 1996; Kimura, 1996, Glosh et al., 1994; McNeill et al., 1991; Ezaki, 1990; Berg et al., 1995; Stapleton et al., 1996, 2000).

Although literature data indicate that inorganic selenium forms can be important in the prevention and progression of a variety of human disease, no detailed studies are avaliable about the effect of synthetic organic forms of selenium on diabetes. Of particular importance, ebselen, a synthetic organoselenium compound with antioxidant properties, reduces the genotoxicity induced by streptozotocin (STZ) in normal and cancer cells (Błasiak, et al., 2004). However, the potential protective effect of organoselenium compounds in animal models of diabetes has yet not been tested.

Diphenyl diselenide is the simplest of the synthetic diaryl diselenides and in the last decade our group has obtained persuasive points of evidence that it exhibits antioxidant, anti-nociceptive, neuroprotective, hepatoprotective and anti-inflammatory properties in different *in vitro* and *in vivo* experimental models (Porciúncula, et al., 2003; Meotti et al.,

2004; Zasso, et al., 2005; Burguer, et al., 2005; Borges, et al., 2006; Ghisleni et al. 2003). Importantly, this compound has low toxicity to rodents, even when used at supra pharmacological doses (Fachineto et al., 2006; Perottoni, et al., 2005; Rocha et al. 2005). However, the possible protective effect of diphenyl diselenide in rodent models of diabetes is not avaliable in the literature. Hence, the aim of the present study was to investigate whether diphenyl diselenide could prevent or delay the biochemical alterations in streptozotocin-induced diabetes in rats.

2.0. Materials and Methods

2. 1. Chemicals

δ-Aminolevulinic acid (δ-ALA) and p-dimethylaminobenzaldehyde were purchased from Sigma (St. Louis, MO, U.S.A), Streptozotocin (STZ), reduced glutathione (GSH), 5.5'-dithio-bis-(2-nitrobenzoic acid) (DTNB), DL-dithiothreitol (DTT) were obtained from Sigma. Diphenyl diselenide compound were synthesized by the method described by 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. The chemical purity of diphenyl diselenide (99.9%) was determined by GC/HPLC. All other chemicals were of analytical grade and obtained from standard commercial suppliers.

2. 2. Animals

Adult male Wistar rats weighing 180-200g were used for the experiments. All rats received food (Guabi, Ribeirao Preto, SP, Brazil) and water *ad libitum* and were kept on a 12-h ligth/12-h dark cycle, in a room with the temperature regulated to 21- 25^{0} C and

humidity at roughly 56%. The animals were used accordingly to guidelines of the Committee on Care and use of Experimental Animal Resources of the Federal University of Santa Maria, Brazil.

2. 3. Diabetes induction

Diabetes was induced by a single intravenous injection of Streptozotocin (STZ) (45 mg/kg), diluted in 0.1 M citrate-buffer (pH 4.5). Control rats received an equivalent amount of the buffer. STZ-treated received 5% of glucose instead of water. Blood samples were taken 48 hs after STZ or vehicle injection from the tail vein. Glucose levels were measured with an automatic auto analyzer (GLUCOTREND®). Animals were considered diabetic when blood glucose levels exceeded above 250 mg/dL.

2.4. Diet Composition and Treatment

Beginning at 5 week of age, the rats were divided in two groups supplied with the control diet or with diet containing 10 ppm of diphenyl diselenide. The selenium compound was dissolved in soybean oil and mixed in the diet in a food mixer to insure uniform distribution. The diet provided approximately 10 μg selenium/g of diet/per day, which is considered an acceptable amount for this element. The two diets were continued until the end of the study. Control and selenium supplemented diets were weekly prepared and stored at 20^0C. Table 1 outlines the composition of control and selenium supplemented diets.

After the induction of diabetes by STZ, two groups were randomly subdivided into: (1) control; (2) diphenyl diselenide (Ph); (3) diabetes (db) and (4) diabetes+diphenyl

diselenide (db/Ph). Groups 1 and 3 were supplied with the control diet, whereas groups 2 and 4 received diet supplemented with 10 ppm of diphenyl diselenide. At the end of the experimental period, diabetic rats and the corresponding control animals were anesthetized with ether and sacrificed by decapitation. Rats were fasted 12h prior to the sacrifice.

2.5. Tissue preparation

Blood of anesthetized rats was collected by cardiac puncture in heparinized tube. Tissue samples were quickly removed, placed on ice and homogenized in cold 50 mM Tris-HCL pH 7.4. The homogenate was centrifuged at 4,000 x g for 10 min to yield the low-speed supernatant fraction that was used for biochemical assays. The protein content was determined by the method of Bradford (1976), using bovine serum albumin as the standard.

2.6. Biochemical analysis

Plasmatic AST (aspartate aminotransferase), ALT (alanine aminotransferase) and LDH (lactate dehydrogenase) enzymes, urea and creatinine levels were determined using commercial Kit (Labtest, Minas Gerais, Brazil).

2.8. Antioxidant defense systems

2.8.1. Vitamin C content

Blood, hepatic renal and cerebral vitamin C (ascorbic acid) levels were determined colorimetrically as described by Jacques-Silva et al (2001).

2.8.3. -SH levels

-SH content from erythrocytes and liver was determined as described by Ellman (1959)

2.8.3. Catalase (CAT) activity

The measurement of CAT activity from liver, kidney and erythrocytes was determined as described by Nelson and Kiesow (1972).

2.8.4. Superoxide dismutase (SOD) activity

The measurement of SOD activity in liver, kidney and erythrocytes was determined by capacity of enzyme to inhibit the epinephrine autooxidation at pH alkaline, observed at 480 nm, as described by Sun and Zigman (1978).

3.0. Enzymes activity

3.1. δ-ALA-D

Hepatic, renal and cerebral δ-ALA-D activity was assayed according to the method of Sassa (1982) by measuring the rate of product porphobilinogen (PBG) formation except that 84 mM potassium phosphate buffer, pH 6.4 and 2.4 mM ALA were used. The reaction was started 10 min after the addition of the enzyme preparation by adding the substrate. The incubation was carried out for 1h (liver), 2hs (kidney) at 37^0 C. The reaction product was determined using modified Ehrlich's reagent at 555 nm, with a molar absorption coefficient of $6.1 \times 10^4 M^{-1}$ for the Ehrlich-PBG salt.

3.2. Na^+/K^+-ATPase

Cerebral Na^+/K^+-ATPase activity was measured as described by Borges et al. (2005).

3.3. Apirase (NTPDase) and Ecto 5'-nucleotidase

Platelets Apirase (NTPDase) and Ecto 5'-nucleotidase activity were measured as described by Pilla et al (1996),

4.0. Statistical analysis

All values obtained are expressed as mean±standard error. The data were analyzed by one-way or two-way ANOVA and MANOVA analyses of variance followed by Duncan's multiple range test when appropriate. Differences between groups were considered to be significant when $p < 0.05$.

5.0. Results

5.1-Diphenyl Diselenide Intake Reduces the STZ-induced Toxicity

STZ administration to rats maintained on the basal diet were associated with a high percentage of death (Figure 1). In fact, only about 20% of the STZ-treated animals survived. In contrast, STZ administration practically did not cause death in rats maintained in the diet supplemented with 10ppm of diphenyl diselenide (Figure 1).

5.1. Body weight and organ weight ratio

Diphenyl diselenide supplementation did not modifiy the body weight gain of control animals (Figure 2; Table 1). STZ treatment caused a significant reduction in body weight gain of rats and diphenyl diselenide supplementation did not modify this effect (Figure 2; Table 1).

STZ administration, regardless of the diphenyl diselenide intake, caused a significant increase in the organ-to-body weight ratio for liver, kidney and brain (Table 1).

5.2. Blood glucose levels and Biochemical Parameters

STZ treatment caused a long-lasting increase of about 5 times in blood glucose levels that were not modified by diphenyl diselenide intake (Table 2). Plasma AST, LDH, urea and creatinine levels were not modified by STZ treatment (Table 3). In contrast, STZ caused a significant increase of about 70% in ALT and diphenyl diselenide hindered this increase in ALT activity (Table 3).

5.3. Antioxidant defenses

5.3.1. Vitamin C levels

STZ administration caused a significant decrease in vitamin of 20, 10 and 23% in liver, kidney and blood, respectively (Figures 3A, 3B and 3C). Renal, hepatic and blood vitamin C contents in STZ treated rats maintained on the diet supplemented with diphenyl diselenide were similar to that of control groups. Diphenyl diselenide intake did not change the vitamin C in animals that were not treated with STZ.

5.3.2. –SH levels

Total hepatic –SH groups content was slightly decreased after STZ treatment, but the decrease was statistically significant (Table 4). Diphenyl diselenide had no effect on total –SH in control rats. However, diphenyl diselenide caused a significant incrase in total hepatic –SH groups in rats treated with STZ. STZ treatment caused a reduction of about 23% in the hepatic NPSH levels. Ingestion of diphenyl diselenide supplemented diet caused

a small but significant increase in hepatic NPSH both in control and in STZ-treated rats. In erythrocytes, STZ treatment did not modify the–SH levels; in contrast, diphenyl diselenide (regardless of STZ treatment) caused a significant increase in –SH levels (Table 4).

5.3.3. CAT activity

STZ treatment caused a significant decrease in hepatic and renal catalase activity and ingestion of diphenyl diselenide prevented the decrease in enzyme activity in kidney (Figure 3A) and liver (Figure 3B). Catalase activity was also reduced after STZ treatment in erythrocytes/blood; however, diphenyl diselenide did not prevent the enzyme decrease (Figure 3C).

5.4. δ-ALA-D and Na^+K^+ATPase

STZ treatment caused a significant reduction in the hepatic and renal ALA-D activity and diphenyl diselenide did not change the enzyme activity in control and STZ treated rats (Table 5). Cerebral Na^+K^+ATPase was not modified by STZ or diphenyl diselenide treatment (Table 5).

Tables

Table 1. Body weight gain (g) and relative organ weight (mg/g) changes in STZ-induced diabetic rats supplemented with diphenyl diselenide.

Groups	B W gain	Liver	Kidney	Spleen	Brain
Control	143	37.7±2.41	6.06±0.28	1.71±0.06	2.70±0.04
Se	98	41.3±2.34	5.78± 0.22	1.51±0.07	2.76±0.04
Db	24*	48.8±3.04*	9.26±0.42*	1.97±0.21	4.08±0.09*
Db/Se	29*	58.4±4.41*	9.08±0.42*	2.04±0.11	3.56±0.04*

Data are expressed as means± SEM of 7 to 10 animals. *Denoted $p<0.05$ as compared to the control group (ANOVA/Duncan).

Table 2. Glucose levels of STZ-induced diabetic rats supplemented with diphenyl diselenide.

	Glucose levels (mg/dl)			
	Time after STZ-diabetes induction (days)			
	2	30	60	90
Control	92±3.10	97±5.36	90±3.01	88±3.01
Se	86±3.58	81±4.33	88±3.47	90±4.89
Db	469±45.3*	500±44.2*	483±40.0*	496±49.8*
Db/Se	429±27.0*	466±26.2*	521±30.0*	532±48.8*

Data are expressed as means±SEM of 7 to 10 animals. * Significantly different as compared to the control group (ANOVA/Duncan, $p < 0.05$).

Table 3. Effect of diphenyl diselenide supplementation on biochemical parameters of STZ-induced diabetic in rats.

Groups	ALT[a]	AST[a]	LDH[a]	Urea[b]	Creatinine[b]
Control	43.6±5.40	107±16.1	295±44.9	40.5±3.83	0.40±0.02
Se	42.0±4.52	106±18.5	335±57.6	52.6±6.01	0.43±0.01
Db	72.7±18.3*	99±12.6	339±37.4	44.2±3.40	0.38±0.02
Db/Se	57.7±7.40	127±19.8	396±67.6	50.5±2.64	0.38±0.03

Data are expressed as means± SEM of 6 to 8 animals. *Denoted $p<0.05$ as compared to the control group (ANOVA/Duncan). [a]Data of enzyme activities are presented as U/l. [b]Data of renal markers are presented as mg/dl.

Table 4. Effect of dietary diphenyl diselenide on –SH levels in STZ-induced diabetic rats.

Groups	Control	Se	Db	Db/Se
-SH Levels				
Liver (T)	9938±206	10052±315	9139±356*	10824±302*
Liver (NP)	4995±117	5348±131*	3834±123*[a]	5629±98.0*
Erythrocytes	2582±125	2987±84.6*	2558±130*[a]	2975±174*

Data are expressed as means±SEM of 7 to 8 animals. *Denoted $p<0.05$ as compared to the control group (ANOVA/Duncan). [a]Significantly different from the Se group (ANOVA/Duncan, $p< 0.05$). Total (T) and non-protein (NP) -SH levels.

Table 5. Effect of dietary diphenyl diselenide on δ-ALA-D and Na^+K^+ATPase activity of STZ-induced diabetic rats treated with.

	δ-ALA-D activity[a]		Na^+K^+ATPase[b]
	Liver	Kidney	Brain
Control	3.70±2.64	1.64±0.12	100±0.01
Se	3.10±0.27	1.30±0.10	109±0.01
Db	2.20±0.27*	1.18±0.12*	111±0.01
Db/Se	1.80±0.13*	1.09±0.11*	105±0.03

Data are expressed as means± SEM of six animals. [a]Data of δ-ALA-D activity are expressed as nmol PBG (mg/protein/h). [b]Data of Na^+K^+ATPase activity are expressed as % from control group (100%) (ANOVA/Duncan, $p< 0.05$). *Denoted $p<0.05$ as compared to the control group (ANOVA/Duncan).

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Legends

Figure 1. Effect of dietary diphenyl diselenide on survival of diabetic rats 48hs after administration of STZ. The index of survival is expressed as % from control group (100 %). *Significant different as compared to the control group (Fischer's exact probability test, $p< 0.05$).

Figure 2. Effect of dietary diphenyl diselenide on body weight of normal (A) and STZ-induced diabetic rats (B). Date are expressed ± SEM of 6 to 8 animals.

Figure 3. Renal (A), Hepatic (B) and Blood (C) vitamin C levels of diabetic rats supplemented with dyphenyl diselenide. Date are expressed ± SEM of 6 to 8 animals. Denoted *$P< 0.05$ as compared to the control group.

Figure 4. Renal (A); Hepatic (B) and (C) Erythrocytes CAT activity from diabetic rats supplemented with diphenyl diselenide. Date are expressed ± SEM of 6 to 8 animals. Denoted *$P< 0.05$ as compared to the control group.

Figure 1

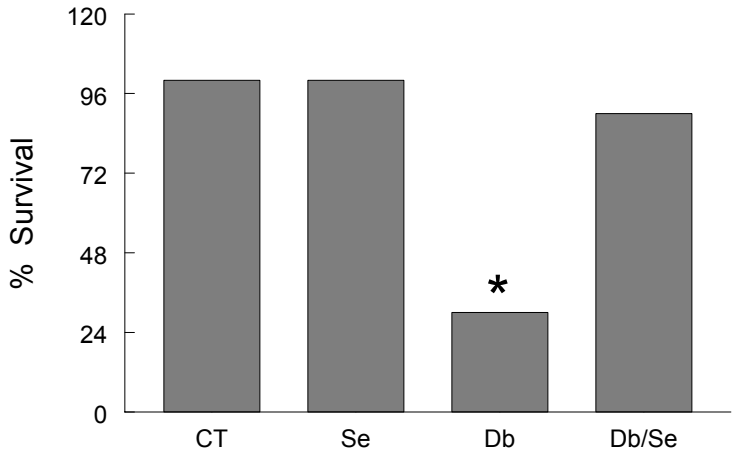

Figure 2.

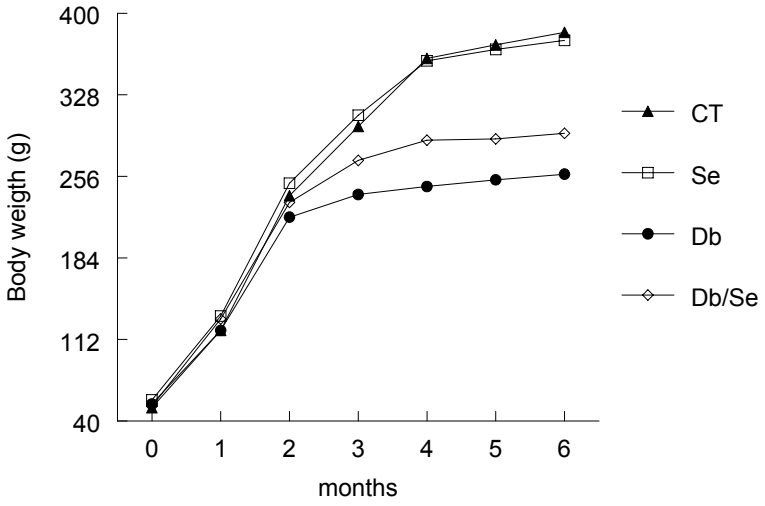
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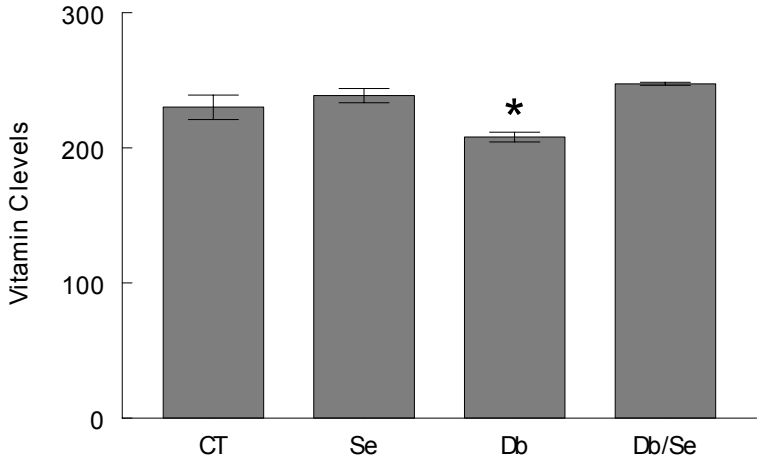
Figure 3



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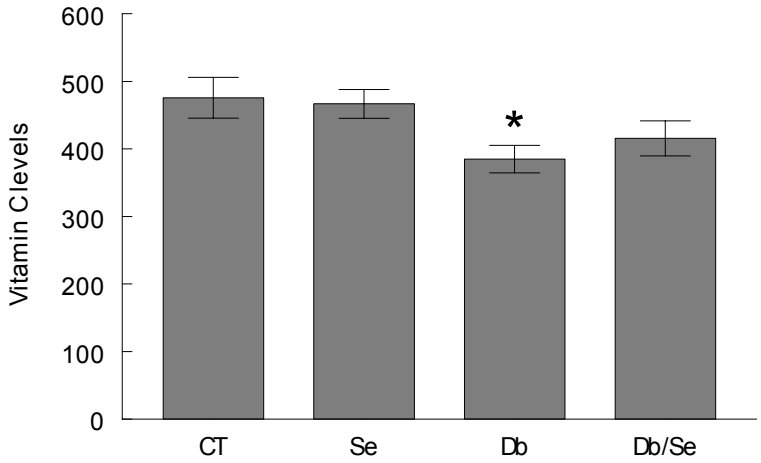
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Vitamin C levels
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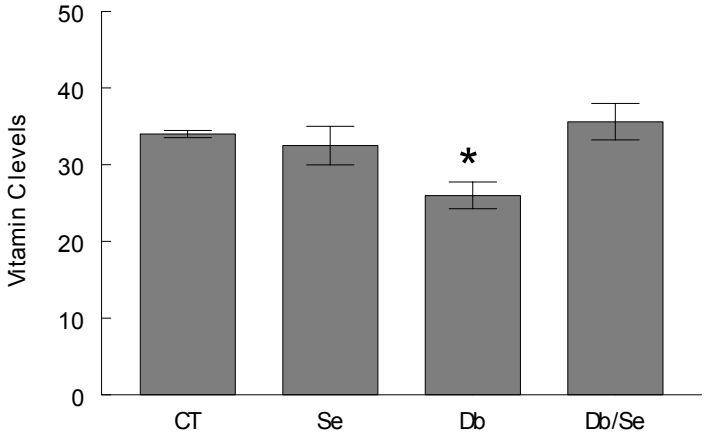
(B)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Vitamin C levels
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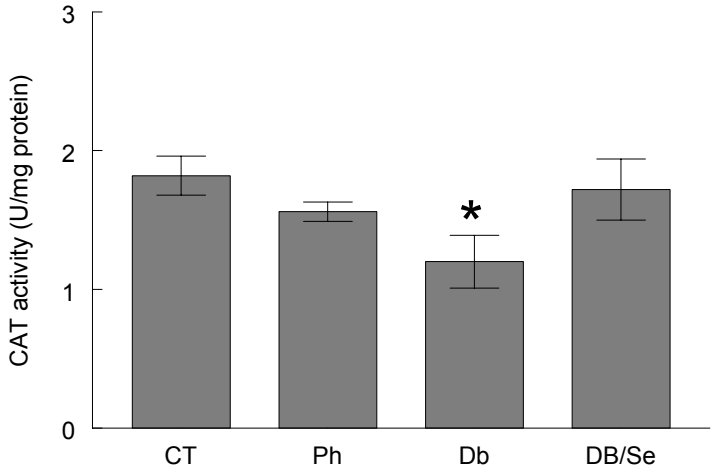
Figure 4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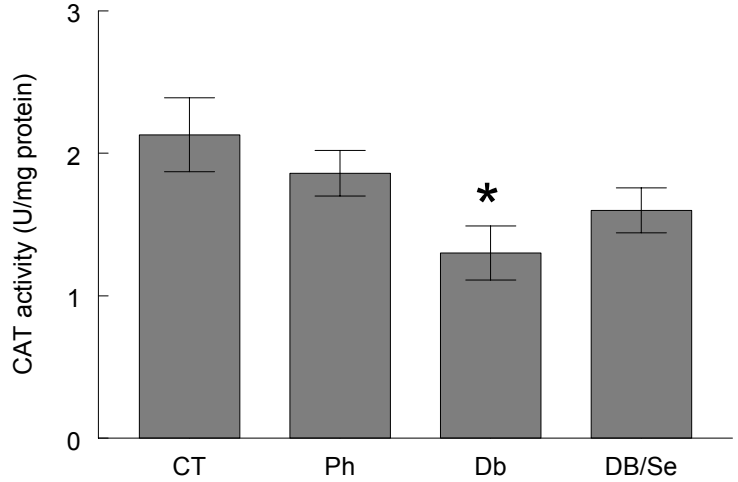
(A)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CAT activity (U/mg protein)
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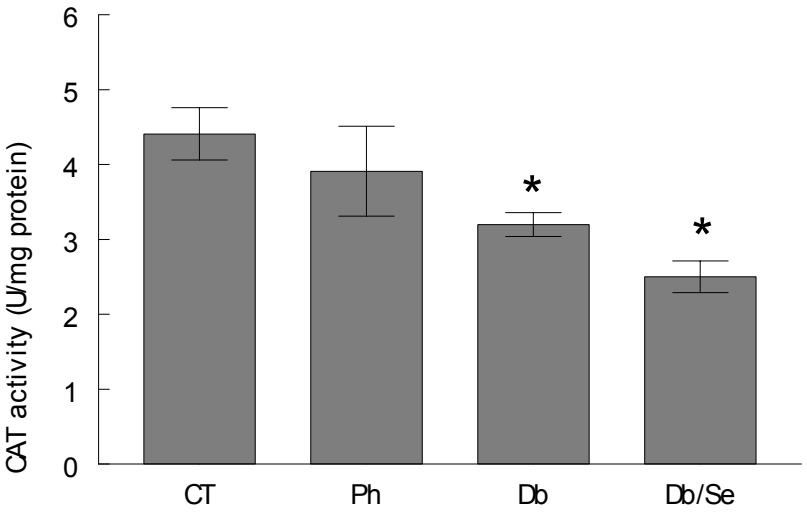

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5. DISCUSSÂO

A deficiência de selênio está associada com uma série de patologias como doenças cardiovasculares, imunológicas, neurológicas, câncer e diabetes (Salonen et al., 1982; Strain et al., 1991; Combs and Gray, 1998; Spallhoz, 2001; Navarro-Alárcon e Lopez-Martinez, 2000; Ip et al., 2002). A suplementação com formas orgânicas de selênio é bem conhecida como eficaz em inibir o processo de carcinogênese induzida por diferentes carcinógenos (El-Bayoumy et al., 1991; Tanaka et al., 1995; El-Bayoumy, 1996; Reddy et al., 1996; Spalhoz, et al., 2004). No entanto, este efeito está geralmente associado com altos níveis de exposição ao selênio (El-Bayoumy, 1991; El-Bayoumy et al., 1995; Spallholz et al., 2001a), os quais causam significante toxicidade aos animais. No presente estudo, focando o processo de carcinogênese mamária induzida por NMU (**Artigo 1**), a dieta suplementada com baixas concentrações de disseleneto de difenila promoveu um aumento no período de latência para o aparecimento dos tumores. Outros parâmetros como a incidência e o número total de tumores também foram reduzidos pelo tratamento dietético com disseleneto de difenila. Estes resultados indicam que o composto apresenta efeitos protetores contra o desenvolvimento de câncer de mama mesmo quando suplementado em baixas concentrações. Esta propriedade representa uma vantagem sobre a maioria dos compostos sintéticos cuja atividade carcinogênica só é constatada com altas doses de selênio. De fato, a efetividade de compostos como o benzilselenocianato (BSC) e o 1,4-*para*-fenil-bis-metil-selenocianato (p-XSC) em inibir o desenvolvimento de câncer mamário

induzido por DMBA é obtida somente com dietas que contenham níveis iguais ou superiores a 5ppm de selênio (El-Bayoumy, 1991; El-Bayoumy et al., 1996; Nayini et al., 1989). Outra vantagem encontrada foi a baixa toxicidade do composto mesmo quando suplementado por períodos prolongados. A ingestão da dieta por aproximadamente 7meses não causou efeitos tóxicos, como a perda de peso ou danos hepáticos (**Artigo 1**).

A atividade anticarcinogênica do selênio parece envolver uma série de eventos ao nível molecular e celular. O efeito pró-oxidante está associado com a capacidade do selênio em inibir a carcinogênese quando usado em doses consideradas tóxicas. Já o efeito da suplementação de baixas concentrações de selênio contra o desenvolvimento de câncer parece estar relacionado com sua atividade antioxidante, uma vez que selenoproteínas como a GPx são conhecidas por proteger o DNA de danos oxidativos e participarem no controle de vários eventos fisiológicos envolvidos no processo de carcinogênese (Tanaka et al., 1998; Matés and Sánches-Jiménez, 1999, 2000; Schaurzer, 2000).

Neste contexto, nossos resultados sugerem que há possibilidade de que a propriedade antioxidante exibida pelo disseleneto de difenila esteja relacionada com o efeito protetor que o mesmo apresentou no modelo de carcinogênese mamária estudado. De fato, observou-se que o consumo da dieta suplementada com disseleneto de difenila foi eficaz em reduzir as alterações nas defesas antioxidantes testadas causadas pela administração do carcinógeno NMU. No entanto, esses dados são escassos para confirmar a hipótese de que uma diminuição no estresse oxidativo tenha sido o mecanismo pelo qual o composto protegeu os animais do aparecimento, incidência e desenvolvimento de tumores.

Está hipótese seria mais evidente se o NMU tivesse causado um aumento na peroxidação lipídica ou na produção de EROs ou ainda danos no DNA no período avaliado (**Artigo 1**). No entanto, essas alterações não foram obtidas e conseqüentemente a provável ação antioxidante do composto não foi totalmente confirmada. A aplicação de outras técnicas que possam verificar danos ao nível de DNA ou o uso de outro carcinógeno seriam importantes para estabelecer o mecanismo de ação do disseleneto de difenila neste modelo de câncer.

Atualmente, a procura por compostos naturais ou sintéticos efetivos no tratamento do DM também tem sido intensa. Compostos com propriedades antioxidantes e hipoglicêmicas são usados com relativo sucesso no controle da hiperglicemia e do estresse oxidativo, o qual pode ser resultante desta condição. A suplementação com formas inorgânicas de selênio, como o selenito e selenato de sódio, são bastante usadas no tratamento de pacientes e de animais com diabetes por exibirem tais propriedades (Berg et al., 1995; Mukherjee, 1998; Stapleton, 2000; Reddi et l., 2001; Faure, 2004). Nosso estudo com Diabetes Mellitus tipo1 avaliou o efeito de compostos orgânicos de selênio sobre a hiperglicemia e determinadas alterações conseqüentes desta condição (**Artigos 2 e 3**).

No modelo experimental de diabetes em que os animais foram tratados com disseleneto de difenila ou ebselen (s.c.) por 3 meses após a indução, observou-se que os elevados níveis de glicose induzidos pela administração de STZ foram diminuídos de forma significativa pelo tratamento com o disseleneto de difenila **(Artigo 2**). De acordo com estes resultados, dados recentes em nosso laboratório demonstraram que o tratamento sub-crônico com disseleneto de difenila na dose de 10 mg/Kg (s.c) reduziu a hiperglicemia induzida por aloxano, mas causou

hepatotoxicidade nos animais. Neste modelo de tratamento (**Artigo 2**) a administração crônica de disseleneto de difenila e ebselen a doses relativamente baixas não causou efeitos tóxicos como a perda de peso e dano hepático. Evidenciou-se também que o disseleneto de difenila causou uma redução significativa nos níveis de proteínas glicadas dos animais com DM. Este efeito provavelmente esteja associado ao o efeito antihiperglicêmico exibido pelo composto.

A proteção que o selênio oferece contra algumas complicações agudas e/ou crônicas do DM parece envolver suas propriedades antioxidantes e miméticas a insulina. De acordo com tais constatações, observou-se neste trabalho que as defesas antioxidantes diminúidas nos animais diabéticos foram aumentadas pelo tratamento com o disseleneto de difenila. De particular importância, o mesmo causou *per se* uma elevação nos níveis das defesas antioxidantes em alguns tecidos. De fato, houve um evidente aumento na atividade da enzima renal SOD e nos níveis de GSH renal, hepático e sanguíneo dos animais tratados com disseleneto de difenila (**Artigo 2**). A maioria dos parâmetros citados até aqui não foram modificados pelo ebselen. Os resultados obtidos neste trabalho apontam o disseleneto de difenila como um composto de valor terapêutico considerável para o tratamento do DM, principalmente devido às suas propriedades antioxidantes e antihiperglicêmicas. No entanto, devido à amplitude das complicações que surgem em decorrência DM é provável que outras propriedades farmacológicas do disseleneto de difenila, como a antiinflamatória, a antinociceptiva, a hepatoprotetora e a neuroprotetora (Porciúncula et al., 2003; Nogueira, et al., 2004; Borges et al., 2005; Bürger, et al., 2005, Zasso et al, 2005. Borges et al.,

2006) estejam, em parte, relacionadas ao efeito protetor que o mesmo oferece contra o DM. Sendo assim, de importância especial para o estudo do mecanismo de ação do composto seria a investigação destas propriedades em modelos experimentais de diabetes. O estudo dos efeitos do composto sobre a captação de glicose em tecidos responsivos a insulina, *in vitro* ou *in situ,* também seria necessário para ajudar a definir se o composto apresenta atividade insulina-like.

Em um outro modelo de tratamento do DM com disseleneto de difenila, verificamos o efeito do composto quando suplementado na dieta dos animais (**Artigo 3**).

Neste estudo (Artigo 3) observou-se que a concentração de disseleneto de difenila suplementada na dieta (10 ppm/kg) dos animais não causou efeitos tóxicos aos mesmos. De fato, nenhuma alteração ocorreu no ganho de peso dos animais suplementados com a dieta durante os 6 meses de tratamento. Os marcadores de dano hepático tais como a atividade das enzimas TGO, TGP, bem como, a atividade da enzima LDH (marcador de dano tecidual) também não foram modificados pelo consumo prolongado da dieta.

Um resultado importante obtido neste trabalho foi a evidência de que o consumo da dieta suplementada com disseleneto de difenila, desde o desmame até a fase adulta, protegeu os animais da mortalidade causada pela administração de STZ. O mecanismo de destruição das células β do pâncreas induzida pela STZ parece envolver processos como a alquilação do DNA e a produção de EROs (Oberley, 1998; Gille et al., 2002; Błasiak, et al., 2004). A alquilação está relacionada com o grupamento nitrosouréia, presente na estrutura da droga, o

qual pode promover além da metilação de bases específicas do DNA, o cross-link do mesmo. Os efeitos pró-oxidantes da droga também podem causar a fragmentação do DNA nestas células e outros danos oxidativos (Oberley, 1998; Gille et al., 2002). Neste contexto torna-se razoável supor que os efeitos antioxidantes do disseleneto de difenila participem deste mecanismo de proteção. Estes resultados estão de acordo com estudos que mostram que a atividade antioxidante do ebselen protege células normais e cancerosas da ação genotóxica da STZ (Błasiak, et al., 2004).

Assim como o observado no trabalho anterior (**Artigo 2**), o consumo da dieta suplementada com o disseleneto de difenila causou um aumento nos níveis de vitamina C e de -SH nos animais diabéticos e elevou *per se* a concentração de -SH nos tecidos analisados. O tratamento com STZ também causou uma redução na atividade da CAT renal e hepática; a qual foi revertida pela suplementação com disseleneto de difenila. Estes achados intensificam a hipótese de que a ação antioxidante do composto tenha protegido as células pancreáticas de danos oxidativos, como por exemplo da depleção de GSH; a qual pode ser causada pela exposição a STZ (Oberley, 1998).

Neste trabalho (**Artigo 3**) constatou-se que o disseleneto de difenila quando suplementado na dieta não foi efetivo em diminuir a hiperglicemia dos animais tratados com STZ. Estes achados sugerem que a via de administração e conseqüentemente a forma de metabolização do composto estão diretamente relacionadas com a sua atividade anti-hiperglicêmica. Portanto, outras investigações como a identificação de metabólitos e a dosagem de selênio em amostras teciduais seriam de especial importância para a complementação e

continuação destes estudos envolvendo compostos orgânicos de selênio e diabetes.

6. CONCLUSÕES

De acordo com os resultados apresentados nesta tese podemos inferir que:

⇒ As concentrações dos compostos orgânicos usadas no tratamento dos animais com Câncer ou DM não causaram efeitos tóxicos.

⇒ O consumo de uma dieta suplementada com baixas concentrações de disseleneto de difenila retardou o processo de carcinogênese mamária. A suplementação com disseleneto de difenila foi eficaz em aumentar o período de latência para o aparecimento dos tumores mamários e em diminuir a incidência e o número total dos mesmos nos animais tratados com NMU.

⇒ O disseleneto de difenila apresentou propriedades antioxidantes e anti-hiperglicêmicas que podem contribuir para o tratamento do DM.

O tratamento crônico com baixas doses de disseleneto reduziu a hiperglicemia dos animais diabéticos e complicações associadas como a glicação de proteínas e o aumento do estresse oxidativo. A suplementação de uma dieta com disseleneto de difenila também protegeu os animais da mortalidade causada pela ação pró-oxidante do agente diabetogênico STZ e de alterações provocadas nas defesas antioxidantes. No entanto, sob esta forma de tratamento o disseleneto de difenila não foi efetivo em modificar os elevados níveis de glicose dos animais diabéticos.

⇒ A atividade antioxidante do disseleneto de difenila associada a sua capacidade de elevar *per se* os níveis de defesas antioxidantes, contribuiu para a efetividade do composto no tratamento de ambas as patologias: Câncer e DM.

⇒ O disseleneto de difenila foi mais eficiente que o ebselen em reduzir as alterações conseqüentes do DM no modelo experimental testado.

7. PERSPECTIVAS

Tendo em vista os efeitos benéficos que o tratamento com os compostos orgânicos de selênio apresentaram contra o Diabetes Mellitus e o Câncer; faz-se importante para a continuidade do trabalho e conseqüente estabelecimento da eficácia dos compostos de selênio como agentes anti-carcinogênicos e anti-diabetogênicos a avaliação dos seguintes aspectos:

♦ Investigar se outras propriedades farmacológicas (anti-inflamatória, neuroprotetora e antinociceptiva), já descritas para os compostos, podem contribuir para atenuar outras complicações oriundas das doenças;

♦ Testar os mesmos compostos em concentrações, via de administração e tempo de tratamento diferentes, afim de comparar a efetividade destes e estabelecer uma relação dose/resposta;

♦ Procurar identificar o metabólito responsável pelo efeito farmacológico dos compostos, uma vez que a via de administração modifica a eficácia dos tratamentos;

♦ Testar outros compostos orgânicos de selênio no tratamento de tais doenças; afim de comparar a efetividade destes e de buscar novos tratamentos

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9. ANEXO

9.1. Highly stereoselective one-pot procedure to prepare bis- and tris-chalcogenide alkenes via addition of disulfides and diselenides to terminal alkynes