Universidade Federal do Rio Grande do Sul

Instituto de Ciências Básicas da Saúde

Departamento de Bioquímica

Programa de Pós-Graduação em Ciências Biológicas: Bioquímica

EFEITOS DO SULFORAFANO NA RESISTÊNCIA À INSULINA, NO DIABETES INDUZIDO POR ESTREPTOZOTOCINA E NO SISTEMA NERVOSO CENTRAL DE RATOS

TESE DE DOUTORADO

Carolina Guerini de Souza

Porto Alegre, agosto de 2012

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APRESENTAÇÃO

Os resultados desta tese de doutorado estão apresentados sob a forma de artigos científicos, sendo que as informações técnicas mais precisas sobre cada metodologia utilizada poderão ser encontradas em cada um dos trabalhos correspondentes.

Os itens Introdução, Discussão e Conclusão apresentam interpretações e comentários gerais, relacionando todos os trabalhos desenvolvidos.

SUMÁRIO

Parte I

RESUMO

O diabetes mellitus (DM) é um importante problema de saúde pública em todo o mundo, já tendo atingido 12 milhões de brasileiros, podendo ser classificado como tipo 1 ou tipo 2, de acordo com a sua etiologia. Em ambos os casos o resultado final é a hiperglicemia¸ que por sua toxicidade promove o surgimento de comorbidades, como doenças cardiovasculares, retinopatia, nefropatia e neuropatia periférica. A toxicidade da hiperglicemia é mediada pela produção de espécies reativas, promovendo aumento do estresse oxidativo. Embora a retina, os rins e os nervos periféricos sejam os tecidos mais sensíveis à hiperglicemia, inúmeros outros órgãos e tecidos também são afetados pela mesma, tais como fígado¸ músculo esquelético, pâncreas, vasos sanguíneos e até o mesmo o cérebro. Uma vez que os maiores efeitos deletérios do excesso de glicose são mediados por estresse oxidativo a busca por novos compostos e moléculas antioxidantes que sirvam como opção terapêutica ou adjuvante para o DM é foco de inúmeros trabalhos científicos da atualidade. Grande parte direciona-se à compostos bioativos presentes em plantas e alimentos, chamados de fitoquímicos. Alguns fitoquímicos já demonstraram atenuar alterações do DM, retardá-lo e até mesmo preveni-lo enquanto outros ainda estão sendo avaliados. O sulforafano (SFN) é um destes fitoquímicos; trata-se de um glicosídeo sulfurado presente em vegetais crucíferos, com alta capacidade antioxidante, indutor de enzimas de fase 2. Em razão de suas características, o objetivo desta tese foi avaliar os efeitos do SFN em dois modelos de indução de DM (por dieta e por estreptozotocina) e também sobre parâmetros metabólicos do sistema nervoso central de ratos. No modelo por dieta hiperpalatável os animais foram tratados concomitantemente com 1mg/kg de SFN, via gavagem diária durante 4 meses, para testar seu efeito na prevenção da resistência à insulina. Os animais tratados com o fitoquímico tornaram-se mais hiperglicêmicos e houve uma tendência à diminuição da expressão do transportador de glicose 3 (GLUT-3) no córtex e no hipotálamo dos mesmos, embora perfil lipídico, provas de função hepática e renal não tenham sido afetados. Em decorrência das alterações na glicemia e na expressão do GLUT 3 dois novos estudos foram desenhados. Um deles objetivou caracterizar os efeitos de uma curva de concentração de SFN (0 a 10 μM) sobre a viabilidade celular, produção de CO_2, síntese proteica e lipídica em córtex cerebral de ratos jovens e adultos. Nenhuma alteração na viabilidade foi encontrada, entretanto os valores mais baixos da curva (0,25 and 0,5 μM) aumentaram a oxidação de leucina e a síntese proteica nos animais jovens, enquanto as concentrações mais altas (5 e 10 μM) diminuíram estes mesmos parâmetros. Nenhuma alteração pode foi observada no córtex cerebral de animais adultos. A partir das alterações apresentadas pelos animais jovens também avaliamos neste trabalho de caracterização a atividade das enzimas Na^+,K^+-ATPase e glutamina sintetase, além da produção de radicais livres por oxidação de DCFH e pudemos constatar que as concentrações de SFN 5 e 10 μM aumentaram a atividade da Na^+,K^+-ATPase e tem uma tendência a aumentar a glutamina sintetase e a produção de radicais livres, o que não ocorre nas concentrações 0.25 and 0.5 μM. Concluímos desta forma que, de acordo com a concentração, o SFN pode estimular o consumo de ATP via Na^+,K^+-ATPase, diminuindo a liberação de CO2, e diminuir a conversão de glutamato a glutamina, o que associado à uma menor síntese proteica aumenta a possibilidade de uso de aminoácidos para produção de glutamato e posterior acúmulo do mesmo, promovendo excitotoxicidade. O outro estudo desenhado avaliou diferentes doses de SFN (0,1, 0,25 e 0,5 mg/kg) na prevenção da indução do DM por estreptozotocina. Todas as doses tiveram sucesso na prevenção da indução e na preservação do glicogênio hepático comparadas com o grupo diabético que não recebeu SFN. Entretanto, foram observadas elevação do colesterol total e diminuição da ureia plasmática. Para ampliar o espectro de efeitos, decidimos testar uma das doses utilizadas neste trabalho (5 mg/kg) pós indução de DM por estreptozotocina, administrada via intraperitoneal por 21 dias sobre glicemia, lipídios e defesas antioxidantes. Observamos que a sensibilidade à insulina é maior nos

animais diabéticos que receberam SFN, além do perfil lipídico ser similar ao de animais não diabéticos. Entretanto, o SFN não exerceu nenhum efeito sobre a atividade das enzimas antioxidantes superóxido dismutase e catalase, tampouco sobre os grupamentos sulfidril. Concluímos com este conjunto de dados que o fitoquímico SFN pode exercer efeitos de prevenção do DM induzido por estreptozotocina e da dislipidemia secundária a ele, bem como ser estimulador do metabolismo energético e da síntese proteica em córtex de ratos jovens, de acordo com a dose/concentração utilizada. Corrobora com isso os efeitos deletérios observados na glicemia, expressão de GLUT3, diminuição do metabolismo energético e síntese proteica observados com a doses/concentrações mais altas. Consideramos como maior contribuição deste trabalho a confirmação do o tênue limite existente entre benefício e malefício de compostos antioxidantes, determinado apenas pela dose utilizada.

ABSTRACT

Diabetes mellitus (DM) is an important health problem around the world, that already reached 12 million brazillian, and according the etiology can be classified as type 1 or type 2. In both cases the result is hyperglycemia and its toxicity promotes comorbidities such as cardiovascular disease, retinopathy, nephropathy and peripheral neuropathy. Hyperglycemia toxicity is mediated by production of reactive species and increase oxidative stress. Although eyes lens, kidneys and peripheral nerves are the tissues most sensitive to hyperglycemia, several organs and tissues also are affected by high glucose, like liver, muscle, pancreas, blood vessels and even the brain. Because major deleterious effects of excessive glucose are mediated by oxidative stress the search for new antioxidant compounds and molecules that acts as terapeuthical or adjuvant options are the aim of recent scientific works. Most of them focus on bioactive compounds present in plants and food, called phytochemicals. Some phytochemicals have been shown to ameliorate, delay and even prevent some DM complications, while others still are under evaluation. Sulforaphane (SFN) is one of them; it is a glycoside sulphide found in cruciferous vegetables, which have antioxidant properties by induce phase 2 detoxification enzymes. Due this, the aim of this thesis was to evaluate SFN effects on two models of DM induction (by diet and by streptozotocin) and also on metabolic parameters of rats central nervous system. In the model of induction with a highly palatable diet, the animals were concomitantly treated with SFN 1mg/kg by daily gavage during 4 months, to test the compound effects on prevention of insulin resistance. Animals treated with SFN became more hyperglicemic and also had a trend to decrease glucose transporter 3 (GLUT3) expression on brain cortex and hypothalamus. However lipid profile, hepatic and kidney markers were not affected. Because of glycemic and GLUT 3 alterations observed we decided to design two other studies. One of the aimed to characterize the effects of dose response curve of SFN (0 a 10 μM) on cellular viability, CO_2 production, protein and lipid synthesis in brain cortex of young and adult rats. No alteration in cellular viability was found, however lower SFN concentrations (0,25 and 0,5 μM) increased CO_2 production and protein synthesis in young animals, while higher concentrations (5 e 10 μM) decreased same parameters. None of this alterations were observed in brain cortex of adult animals. Considering the results of young animals we also evaluated in this characterization work the activity of Na^+,K^+-ATPase and glutamine syntethase enzymes, besides free radical production by DCFH oxidation. We could inferred that SFN 5 e 10 μM increased Na^+,K^+- ATPase activity and also there is a trend to increase glutamine syntethase activity and free radicals production. But the same results do not occur under SFN 0.25 and 0.5 μM. we conclude that SFN can stimulate ATP consumption by Na^+,K^+-ATPase, decreasing CO_2 production and reduce glutamate conversion to glutamine, which associated to a lower protein synthesis drives more aminoacids to glutamate production and its subsequent accumulation, promoting excitotoxicity. The other designed study tested different SFN doses (0.1, 0.25 e 0.5 mg/kg) on prevention of DM induction by streptozotocin. All tested doses succeed on the prevention and preservation of hepatic glycogen levels compared to the diabetic group who not received SFN. However, we observed elevation of total cholesterol and decrease of serum urea in the same animals. To expand the spectrum of effects, we decided to evaluate one of the doses used in this work (5 mg/kg) injected intraperitoneally during 21 days post-induction of DM by streptozotocin, on glycemia, lipids levels and antioxidant defenses. We noted that insulin responsiveness is better on diabetic animals who received SFN besides they showed lipid profile similar to non-diabetic. Unfortunately, SFN did not exert any effect on antioxidant enzymes superoxide dismutase and catalase, neither on sulfydril groups. With this set of information we conclude that the phytochemical SFN can act on DM prevention induced bu streptozotocin and the consequent dyslipidemia, the same way that can stimulate energetic metabolism and protein synthesis on brain cortex of young rats according to the dose/concentration

used. Corroborates with this the deleterious effects found in glycemia, GLUT 3 expression, decrease in energetic metabolism and protein synthesis observed under the higher doses/concentrations. We consider as major contribution of this work the confirmation of the subtle limit between beneficial and harmfulness of antioxidant compounds, determined only by the dose used.

LISTA DE ABREVIATURAS

ADA= American Diabetes Association

AGE= produto final de glicação avançada

ARE= elemento de resposta antioxidante

DCNT= doenças crônicas não transmissíveis

DM= diabetes melittus

DM1= diabetes melittus do tipo 1

DM2= diabetes melittus do tipo 2

DNA= ácido desoxiribonucléico

GAPDH= gliceraldeído-3 fosfato desidrogenase

GLUT3= transportador de glicose 3

GLUT4= transportador de glicose 4

HDL= lipoproteína de alta densidade

HO-1 = hemeoxigenase-1

IDF= International Diabetes Federation

Keap1= proteína 1 associada ao ECH- tipo Kelch

NADPH= nicotinamida adenina dinucleotídeo fosfato

NQO1= NADPH quinona redutase

Nrf2= fator nuclear E2 relacionado ao fator 2

PARP= poli ADP-ribose polimerase

PKC= proteína quinase C

SBD= Sociedade Brasileira de Diabetes

SFN= sulforafano

SOD= superóxido dismutase

SNC= sistema nervoso central

STZ= estreptozotocina

WHO= World Health Organization

γ-GCL= glutamilcisteina ligase

1. INTRODUÇÃO

1.1. Diabetes Mellitus

Evidências atuais apontam o Diabetes Mellitus (DM) como um problema de saúde pública, cuja prevalência é crescente e com estimativas de atingir 300 milhões de pessoas em todo o mundo até 2025 (SBD, 2009), sendo que mais de 12 milhões destas já foram diagnosticadas no Brasil (IDF, 2010). Estes números elevados têm como causa principal o aumento da obesidade, do sedentarismo e o consumo de alimentos ricos em açúcar e gordura, bem como crescimento e envelhecimento populacional, além da maior sobrevida de indivíduos diabéticos (WHO, 2002, Odermatt, 2011).

O DM não é uma única doença, mas sim um conjunto de alterações metabólicas que culmina em hiperglicemia, estando envolvidas nestas alterações as desregulações no tecido adiposo, no metabolismo de ácidos graxos, na produção de insulina pelos pâncreas e na ação periférica deste hormônio. Em função disso, a alimentação saudável e a atividade física são consideradas bases para prevenção do DM, da mesma forma que para o seu tratamento (ADAa, 2008).

Sendo a hiperglicemia a principal característica do DM, o equilíbrio alimentar é fundamental para estabilização dos níveis glicêmicos, da mesma forma que para prevenção de complicações micro e macrovasculares que podem advir da sua descompensação, bem como do aumento do risco de infecções e prejuízos na cicatrização de feridas (Ministério da Saúde, 2006). Outros fatores importantes relacionados à essencialidade do controle glicêmico no DM são os custos gerados para a rede de saúde pública, não só com o tratamento, mas também com medicamentos, hospitalização e cirurgias. Segundo estimativas, os custos diretos com diabetes variam de 2,5% a 15% do orçamento anual da rede de saúde, oscilando em torno de 3,9 bilhões de dólares (Barceló et al., 2003). A estes custos somam-se ainda os custos intangíveis como dor, ansiedade, inconveniências e diminuição da qualidade de vida, além da incapacidade produtiva que ocorre nos casos mais extremos.

Por todos estes motivos, estratégias que promovam melhor controle glicêmico são de extrema importância para mudança de comportamento e encorajamento de estilo de vida saudáveis.

1.2. Fisiopatologia do DM e suas consequências

De acordo com sua fisiopatologia, o DM pode ser dividido em dois tipos. O DM do tipo 1 (DM1) constitui-se pela destruição das células β- pancreáticas, usualmente levando à deficiência absoluta na produção endógena de insulina, gerando assim hiperglicemia (ADAb, 2008). Ocorre principalmente em crianças e adolescentes, mas pode manifestar-se em qualquer estágio da vida, até mesmo com 80 a 90 anos de idade, perfazendo de 5 a 10% da prevalência total de DM (ADAc, 2008). Na maioria dos casos, a destruição das células β- pancreáticas é mediada por reação auto-imune, havendo uma minoria de casos considerados idiopáticos. No momento do diagnóstico em torno de 70 a 80% da massa de células β já está reduzida, o que ocorre lentamente ao longo dos anos (Cnop et al., 2005).

O momento de início e a progressão do DM1 é variável, começando a partir de um evento desencadeador (em indivíduos pré-dispostos geneticamente para a reação auto-imune contra as células β), a partir do qual alterações imunológicas já são encontradas, embora ainda haja produção normal de insulina. No período inicial de diminuição da produção de insulina a glicemia ainda mantém-se normal. Entretanto, com a diminuição progressiva de células β e, consequentemente da produção deste hormônio, a hiperglicemia prevalece e culmina na instalação do DM1, podendo chegar na ausência total de produção de insulina (Eisenbarth and Jeffrey, 2008) (Figura 1).

A destruição das células β ocorre num processo lento e progressivo, com a invasão das ilhotas de Langerhans por células mononuclerares, em uma reação inflamatória chamada “insulite”, a qual leva à apoptose destas células. A morte celular no decurso da insulite é provavelmente causada pela exposição à mediadores tóxicos, como citocinas, óxido nítrico e espécies reativas de oxigênio, secretados por macrófagos e células T ativas (In't Veld, 2011).

Os fatores mais cogitados como eventos desencadeadores da ativação desta reação auto-imune incluem algumas infecções virais (enterovírus, rubéola, caxumba, rotavírus, parvovírus e citomegalovírus), fatores dietéticos e nutricionais durante a infância (consumo de leite de vaca e, mais recentemente, excesso de peso), vacinação, toxinas e o *turnover* fisiológico de células β que ocorre em recém-nascidos (van der Werf et al., 2007).

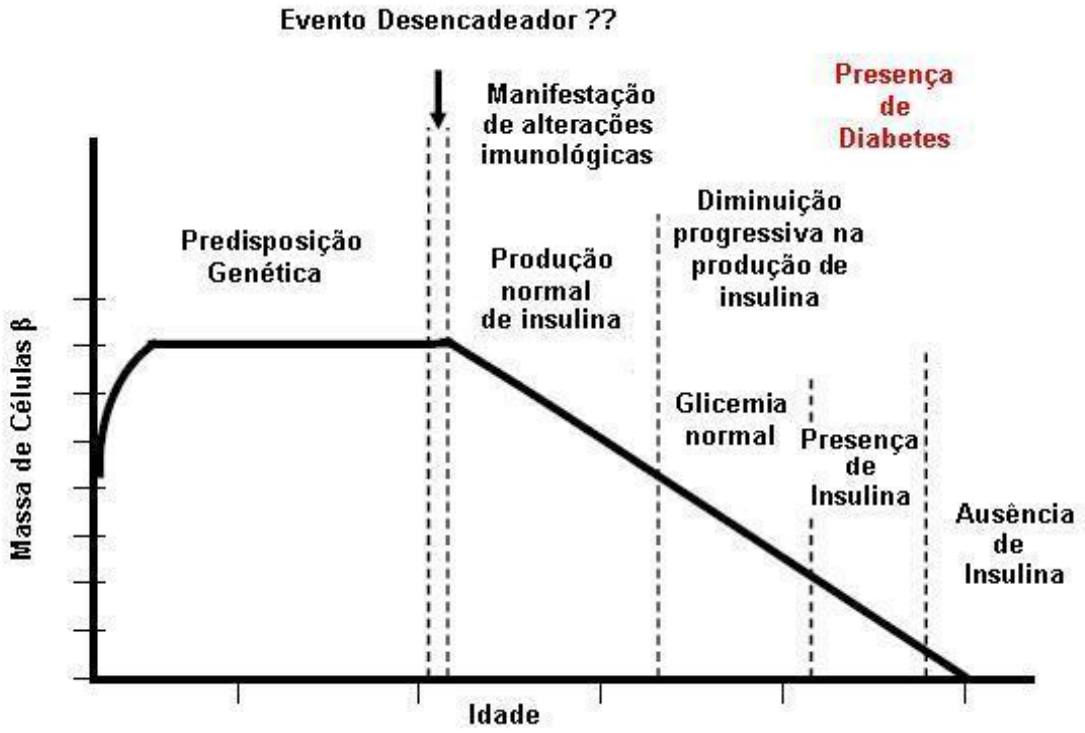


Figura 1. Estágios do desenvolvimento do Diabetes Tipo 1.*Adaptado de Eisenbarth e Jeffrey, 2008.*

Neste tipo de DM a administração de insulina exógena é indispensável e deve ser iniciada assim que for feito o diagnóstico, pois a prioridade é retomar o equilíbrio metabólico, propiciando um estado o mais próximo possível da fisiologia normal do organismo. Entretanto, a dieta equilibrada segue como segundo componente indispensável para que este equilíbrio seja alcançado, principalmente o controle glicêmico, prevenindo as comorbidades associadas ao DM descompensado.

Embora o DM1 seja uma das principais doenças crônicas da infância e adolescência, a maior prevalência de DM na população é do tipo 2 (DM2) - cerca de 90 a 95% dos casos diagnosticados. O DM2 se caracteriza por reunir várias alterações endócrinas, promotoras de hiperglicemia persistente e secundária a resistência à insulina. Neste tipo de diabete, as alterações estão associadas ao defeito de ação celular da insulina, defeitos na secreção da mesma ou ambos (ADA[c], 2008).

Embora o fator genético esteja presente no desenvolvimento do DM2, o fator ambiental é seu principal determinante, uma vez que a maior parte dos indivíduos o desenvolve a partir dos quarenta anos de idade (Montecucco et al., 2008). As mudanças no estilo de vida moderno, que diminuíram o consumo de alimentos nutricionalmente adequados e o gasto energético diário, promoveram um aumento no consumo de alimentos industrializados (ricos em gordura e açúcar) e um padrão de vida sedentário, sendo estes considerados os principais responsáveis pelo aumento populacional do DM2, principalmente por também desencadearem obesidade (Chakravarthy and Booth, 2004; Haslam and James, 2005). Considera-se essa uma das explicações para o desenvolvimento de DM2 também por adolescentes e adultos jovens (Peters et al., 2011).

A obesidade está associada ao aparecimento do DM2 devido ao aumento do tecido adiposo e seus efeitos na sensibilidade periférica à insulina. Independente da causa, o aumento de gordura corporal, especialmente da gordura visceral, promove o aumento de ácidos graxos livres no sangue e acúmulo dos mesmos em tecidos que não sejam o adiposo, como fígado, músculo esquelético e vasos sanguíneos (Arner, 2005; Yudkin et al., 2006). Ao acúmulo de ácidos graxos livres em tecidos não adiposos foi atribuído o termo de lipotoxicidade e acredita-se que esta é a causa comum das complicações da obesidade, resistência à insulina/DM2 e doenças cardiovasculares (van de Weijer et al., 2011).

Esses ácidos graxos livres aumentam a secreção de citocinas inflamatórias pelos macrófagos (células de defesa mediadoras de inflamação), que se ligam à células de diversos tecidos, modificando a sensibilidade do receptor de insulina e da via de sinalização insulínica pós-receptor,

causando resistência periférica à mesma e promovendo hiperglicemia. Os ácidos graxos depositados em tecidos não adiposos também podem ativar vias intracelulares responsáveis pela alteração da sinalização da insulina (Shoelson et al., 2006). À hiperglicemia promovida e as suas consequências foi dado o nome de glicotoxicidade, caracterizada pela alta capacidade de produção de radicais livres e demais substância reativas, como produtos finais de glicação avançada (AGEs), e pela alteração na estrutura e função de proteínas, lipídios e até mesmo do DNA das células lesadas (Brownlee, 2005).

A resistência à insulina ocorre desde o estágio inicial do DM, chamado pré-diabetes, ocorrendo antes mesmo da hiperglicemia diagnóstica. Com o aparecimento da resistência à insulina, a glicose sanguínea se mantém elevada, estimulando o pâncreas a secretar mais e mais insulina, caracterizando um estado de hiperinsulinismo, o que pode manter a glicemia sem alterações diagnósticas (DeFronzo, 2004). O quadro de hiperinsulinismo é comum somente na fase inicial de resistência à insulina, pois as células β-pancreáticas não conseguem sustentar uma produção continuamente elevada com a permanência excessiva de ácidos graxos no plasma, voltando aos níveis de produção normais ou menores que o normal, de acordo com tempo de resistência. A secreção deficiente de insulina é resultado da exposição crônica ao excesso de ácidos graxos plasmáticos, os quais inibem a expressão do gene insulínico, atuando como verdadeiros agentes tóxicos para as células β-pancreáticas (Lyssenko et al., 2005, Poitout et al., 2006). Quando a secreção aumentada de insulina não é mais suficiente e a glicemia permanece elevada, o diagnóstico de DM2 aparece e neste momento, é estimado que o indivíduo já tenha perdido aproximadamente 80% da função de suas células β-pancreáticas (DeFronzo, 2009). Um resumo destas alterações pode ser visto na Figura 2.

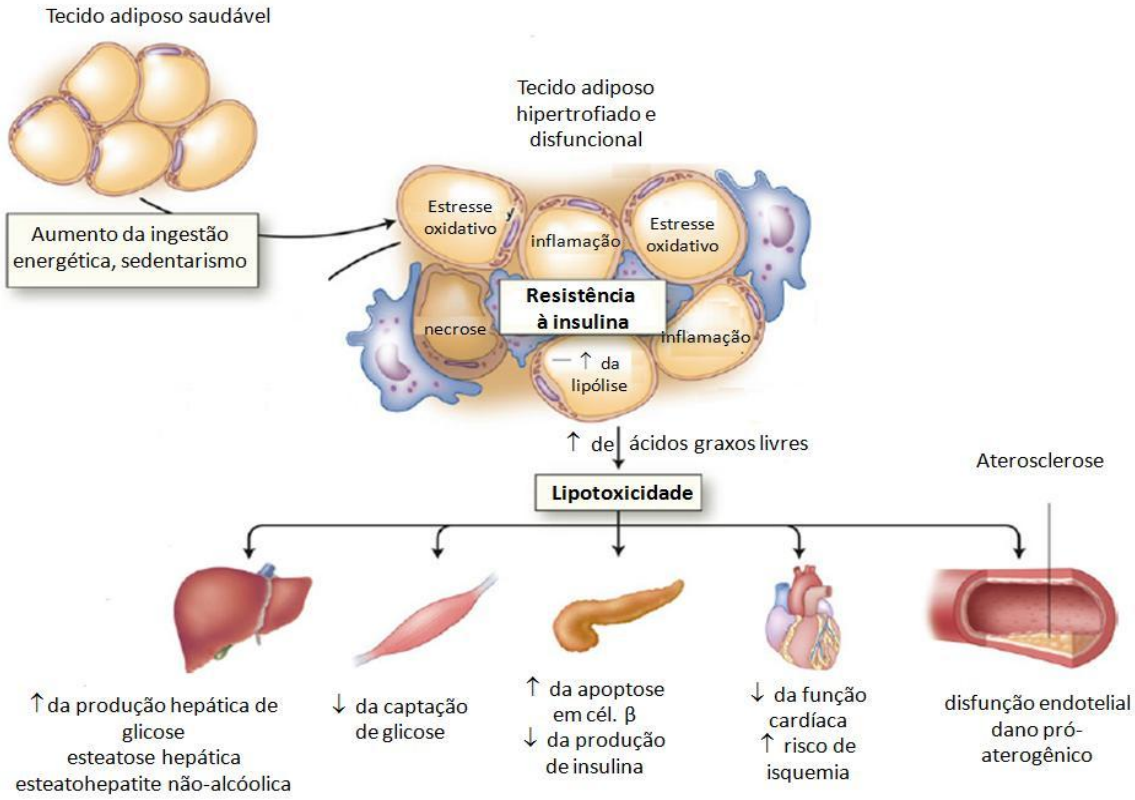


Figura 2. Fisiopatologia da resistência à insulina e DM2. *Adaptado de Cusi, 2010.*

Embora os mecanismos do DM2 ainda não estejam totalmente esclarecidos, existe unanimidade em afirmar que as alterações no metabolismo lipídico culminam na resistência à insulina e posteriormente no DM, sendo a base da fisiopatologia diabética. As alterações no metabolismo da glicose e suas consequências deletérias nos sistemas biológicos são considerados efeitos e não causas desta patologia (Shafrir and Raz, 2003). Porém é importante ressaltar que as disfunções lipídicas iniciam a partir do aumento de gordura corporal e plasmática, que pode ser proveniente tanto de uma dieta rica em carboidratos (especialmente sacarose) quanto rica em lipídios, geralmente em associação com a falta de exercício físico e a baixa atividade física diária (James, 2008).

As complicações do DM são comuns à ambos os tipos, uma vez que advém da hiperglicemia recorrente não compensada; esta hiperglicemia atinge todas as células corporais, porém as células dos capilares endoteliais da retina, células mesangiais dos glomérulos renais e neurônios e células

de Schwann dos nervos periféricos são as mais danificadas (Brownlee, 2005). Isto ocorre porque a maior parte das células corporais consegue reduzir o transporte de glicose do meio extracelular para o meio intracelular, mantendo sua concentração interna de glicose constante, mesmo durante a hiperglicemia, o que não ocorre com as células supracitadas. As altas concentrações de glicose dentro destas células geram aumento de radiciais livres, glicação de proteínas, ativação de vias inflamatórias que promovem uma série de alterações funcionais, desde a alteração na estrutura e função de proteínas e lipídios, até alterações no próprio DNA (Giacco and Brownlee, 2009).

As principais comorbidades decorrentes do DM podem ser divididas em microvasculares (retinopatia diabética, a nefropatia diabética e a neuropatia diabética) e macrovasculares (doenças arteriais coronarianas, doenças periféricas vasculares e as doenças cerebrovasculares) (Tripathi and Srivastava, 2006). Entretanto, é importante citar que disfunções cognitivas também afetam indivíduos diabéticos, especialmente retardo nas funções executivas (motoras), desaceleração neural, atrofia cortical aumentada e anomalias microestruturais do cérebro (McCrimmon et al., 2012).

1.3. Estresse oxidativo: o foco do problema

O estresse oxidativo é considerado a base das complicações do diabetes, em função de alguns tecidos terem menos habilidade em lidar com a hiperglicemia e por isso lesionarem-se mais facilmente (retina, rins e nervos periféricos), sendo a hiperglicemia uma grande produtora de espécies reativas (Brownlee, 2005). Além disso, a maior parte de óbitos em diabéticos é por doença cardiovascular, sendo ¾ destas decorrentes da aterosclerose, a qual é um processo também mediado por aumento de radicais livres (Vazzana et al., 2012).

O estresse oxidativo pode ser definido como um estado de desbalanço entre fatores que geram espécies reativas, como ânion superóxido (O_2^-), peróxido de hidrogênio (H_2O_2), radical hidroxil (OH^-), e fatores que protegem as estruturas celulares destes mesmas espécies, como as

enzimas superóxido dismutase (SOD), catalase e glutationa peroxidase, chamadas de antioxidantes (Styskal et al., 2012). Além destas enzimas, que são as principais, existem outras que também são antioxidantes além de compostos antioxidantes não-enzimáticos, como por exemplo vitaminas e fitoquímicos (Balsano and Alisi, 2009).

No diabetes, a hiperglicemia recorrente promove um contrafluxo na cadeia transportadora de elétrons, mais exatamente a partir do complexo III, fazendo com que estes elétrons retornem para a coenzima Q e sejam despejados diretamente no oxigênio molecular, aumentando assim a produção de ânion superóxido. A isoforma mitocondrial da enzima SOD degrada este ânion superóxido a peróxido de hidrogênio, que podem então ser convertido a água e oxigênio (H_2O e O_2) por ação da enzima catalase ou da glutationa peroxidase, tornando-se inócuo (Brownlee, 2005). Entretanto, o peróxido de hidrogênio é uma espécie reativa permeável à membrana mitocondrial e a plasmática, podendo transitar entre diferentes espaços celulares e produzir novas espécies reativas, por meio da reação de Fenton ao interagir com metais de transição (Fe^{++} ou Cu^{+}) (Smith et al., 2007).

Independente disso, a produção aumentada de ânion superóxido na cadeia transportadora de elétrons desencadeia um ciclo de eventos interligados, que embasam os efeitos lesivos e complicações secundárias ao diabetes. Com o aumento de superóxido, ocorrem quebras no DNA celular que ativam uma enzima chamada PARP (poli ADP-ribose polimerase), responsável pelo reparo desta estrutura. Entretanto, a ativação da PARP faz com que a enzima gliceraldeído-3 fosfato desidrogenase (GAPDH) tenha sua estrutura modificada, o que diminui sua atividade. Esta enzima da via glicolítica é responsável pela oxidação e fosforilação do gliceraldeído-3 fosfato em 1,3 difosfoglicerato, e como esta reação está diminuída todos os substratos anteriores a ela se acumulam no meio intracelular. O acúmulo de gliceraldeído-3 fosfato aumenta a produção de AGEs e a ativação da proteína quinase C (PKC), responsáveis pela alteração de proteínas da matriz celular + diversas vias de transdução e da produção de citocinas inflamatórias, respectivamente. O acúmulo dos substratos anteriores ao gliceraldeído-3 fosfato, como a frutose-6 fosfato e a própria glicose,

aumentam as vias das hexosaminas e do poliol, respectivamente, gerando fatores de transcrição pró-vasoconstrição e agregação plaquetária, além de depletarem cofatores de enzimas antioxidantes (Brownlee 2005; 2010). A Figura 3 mostra resumidamente o mecanismo unificador deste ciclo de eventos.

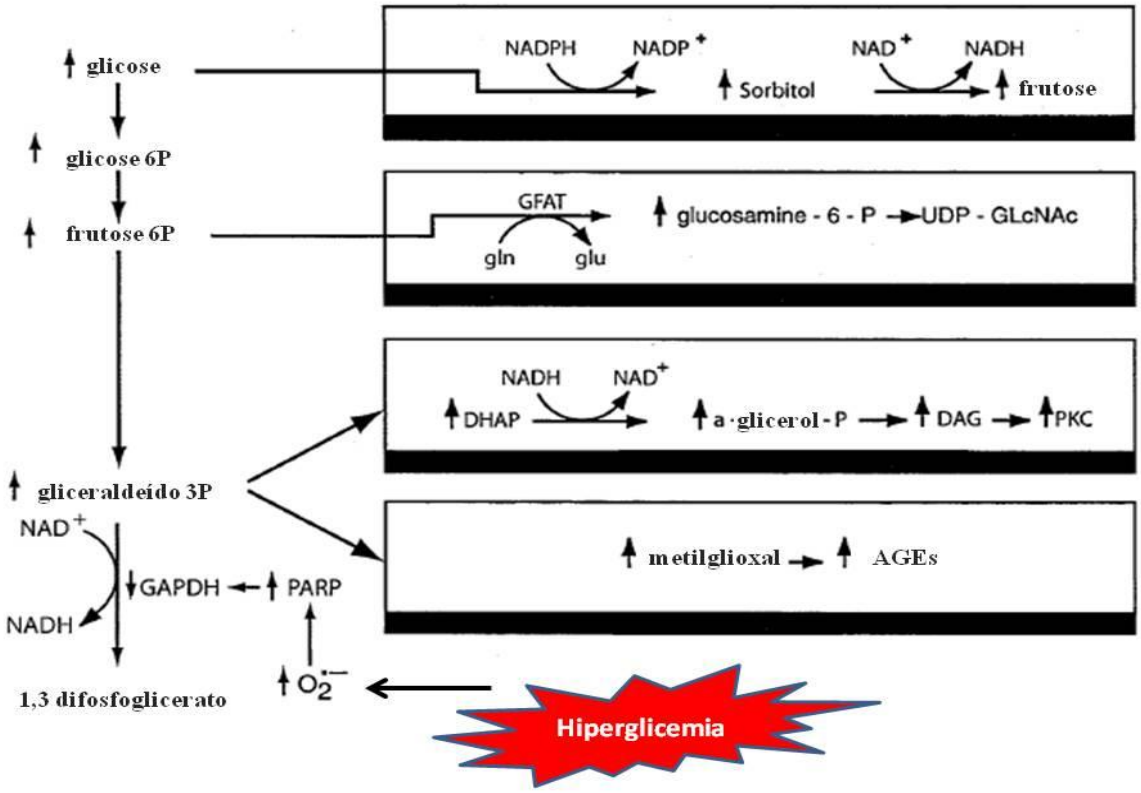


Figura 3. Mecanismo unificador das complicações do diabetes. *Adaptado de Brownlee, 2005.*

Em decorrência da própria fisiopatologia do DM ocorre diminuição das defesas antioxidantes, o que se acentua se o controle glicêmico for mal executado. Em razão disso, é cada vez maior a pesquisa e busca por moléculas antioxidantes que possam previnir,atenuar e/ou tratar as alterações supracitadas.

1.4. Alimentos funcionais, nutracêuticos, fitoquímicos e, mais propriamente, sulforafano: relação com o diabetes.

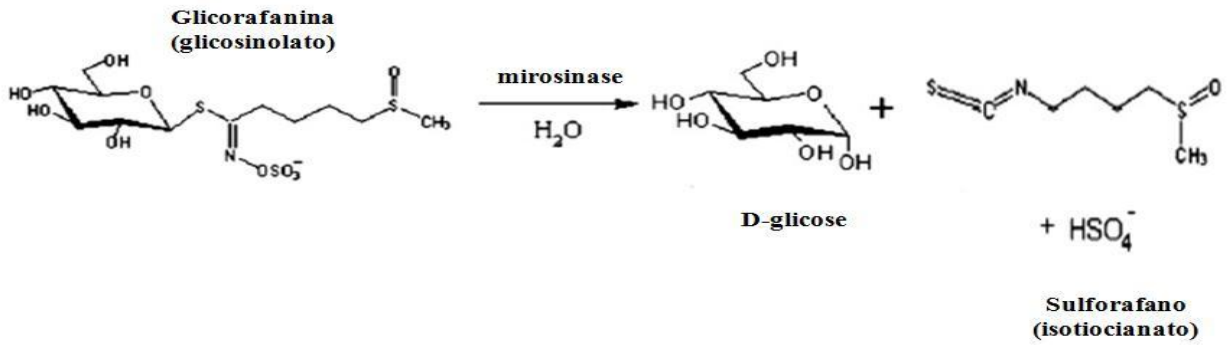
A elevada prevalência de doenças crônicas não transmissíveis (DCNT), dentre elas o DM, no atual cenário de saúde do Brasil e do mundo é um dos principais impulsionadores da ciência na busca por novos compostos que possam prevenir, atenuar ou tratar as mesmas. Neste sentido, grande ênfase tem sido dada aos alimentos funcionais, nutracêuticos e fitoquímicos, uma vez que estudos epidemiológicos associam o consumo destes com menores índices de DCNTs.

Alimento funcional é todo alimento que pode afetar beneficamente uma ou mais funções alvo no corpo, além de nutrir (fornecer calorias, carboidratos, proteínas, lipídios, vitaminas e/ou minerais), de maneira que seja tanto relevante para o bem-estar e a saúde quanto para a redução do risco de uma doença (Roberfroid, 2002). Nutracêutico é o alimento ou parte de um alimento que proporciona benefícios à saúde, incluindo a prevenção e/ou tratamento da doença, sendo que tais produtos podem abranger desde os alimentos em si até compostos isolados, suplementos dietéticos na forma de cápsulas e até mesmo produtos geneticamente modificados (Andlauer and Fürst, 2002). Já os fitoquímicos são compostos não-nutrientes, derivados do metabolismo secundário de plantas, que tem demonstrado exercer uma grande variedade de atividades biológicas, dentre elas reduzir o risco de doenças crônicas. Esta capacidade, na maioria das vezes, é decorrente de sua capacidade antioxidante (Espín et al., 2007). Existem milhares de fitoquímicos na natureza (estima-se que mais de 80000), sendo que os grupos mais comuns são os compostos fenólicos (resveratrol, quercetina, isoflavonas, etc), os organosulfurados (sulforafano, dialil-sulfito, etc) e os terpenóides (licopeno, luteína, etc) (Acamovic and Brooker, 2005). Embora alimentos funcionais, nutracêuticos e fitoquímicos tenham definições diferentes, inúmeros trabalhos mostram que todos estes exercem funções de prevenção e/ou tratamento de doenças, de acordo com a forma, quantidade e tempo que são administrados. A característica comum a todos é o efeito na redução do estresse oxidativo, que na maioria das vezes também é acompanhado por diminuição de processos inflamatórios, sendo que

estresse oxidativo e inflamação constituem a base fisiopatológica de grande parte das DCNTs (Gonzalez-Castejon and Rodriguez-Casado, 2011).

Dentre os inúmeros fitoquímicos existentes alguns já tiveram seus efeitos investigados em relação ao diabetes, como por exemplo, resveratrol (Jing et al., 2010), rutina (Fernandes et al., 2010), catequinas (Yan et al., 2012) e o sulforafano (Song et al., 2009), sendo este último o objeto de estudo desta tese.

O sulforafano (SFN) é um glicosídeo sulfurado, um isotiocianato derivado da hidrólise do glicosinolato glicorafanina pela enzima mirosinase, a qual é encontrada no intestino humano e nos próprios vegetais-fonte deste composto. O SFN está presente em vegetais crucíferos (brócolis, couve-flor, couve verde, repolho, dentre outros) e quando ingerido age como antioxidante indireto, por promover a indução de enzimas de fase 2, relacionadas à processos de detoxificação. Seu mecanismo de ação é por ativação do fator nuclear E2 relacionado ao fator 2 (Nrf2), que por intermédio do SFN se rompe do complexo formado junto à proteína 1 associada ao ECH- tipo Kelch (Keap1). Ao se romper do complexo, o SFN age no núcleo celular via elemento de resposta antioxidante (ARE), modulando a expressão gênica das enzimas antioxidantes NADPH quinona oxideredutase (NQO1), hemeoxigenase-1(HO-1) e γ-glutamilcisteina ligase (γGCL), além de interações com a via da proteína quinase ativada por mitógeno (MAPK) (Figura 4)(Guerrero-Beltrán et al., 2012).



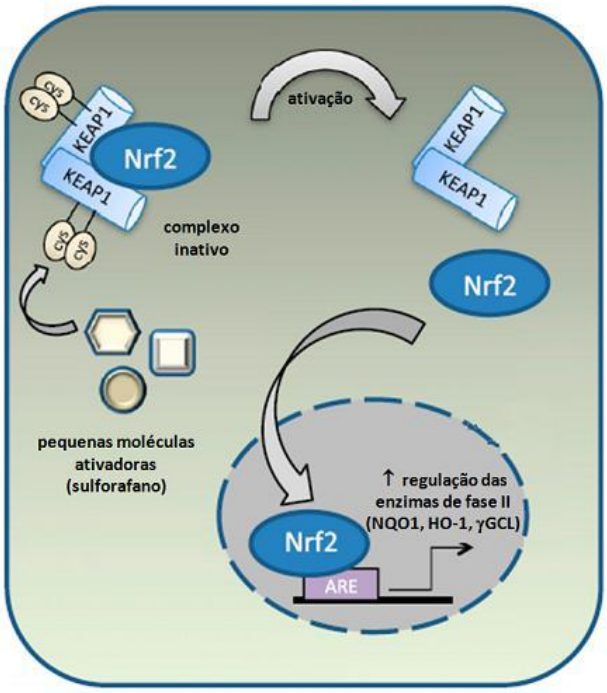


Figura 4. Hidrólise da glicorafanina em sulforafano e mecanismo de ação do sulforafano via Nrf2. *Adaptado de Haan, 2011.*

Devido ao seu grande potencial antioxidante, o SFN foi primeiro e extensivamente estudado como agente anticarcinogênico, demonstrando efetiva inibição da proliferação e crescimento de células tumorais (Zhang et a., 1992, Zhang et al., 1994, Fimognari and Hrelia, 2007). Mais recentemente, tem sido foco de estudos de sobrevivência celular, envolvendo mecanismos antiinflamação e antiapoptose, além de trabalhos mostrando melhora do perfil lipídico, cardio e neuroproteção (Murashima et al., 2004, Xue et al., 2008, Danilov et al., 2009). Como estas são alterações comuns ao DM, especulam-se quais seriam os efeitos deste fitoquímico na sua prevenção e tratamento, tanto em aspectos do metabolismo intermediário quanto no sistema nervoso central, os quais tenham sua funcionalidade afetada pela hiperglicemia.

OBJETIVOS

Principal

Em função das evidências atuais sobre o uso de fitoquímicos antioxidantes na tentativa de prevenção e tratamento do diabetes, buscamos:

- analisar os efeitos do tratamento oral crônico com SFN em um modelo de obesidade e resistência à insulina induzido por dieta hiperpalatável;
- caracterizar os efeitos *in vitro* de diferentes concentrações de SFN no sistema nervoso central de ratos jovens e adultos;
- testar o efeito protetor do tratamento oral agudo com SFN pré-indução de diabetes com estreptozotocina;
- testar a ação terapêutica do tratamento intraperitoneal agudo com SFN pós-indução de diabetes com estreptozotocina;

Específicos

- avaliar a massa corporal, massa adiposa, glicemia, perfil lipídico, provas de função hepática e renal e expressão dos transportadores de glicose no cérebro de ratos adultos submetidos à dieta hiperpalatável e ao tratamento oral crônico com SFN;
- caracterizar a viabilidade celular, a produção de energia, a síntese proteica e lipídica *in vitro* no córtex cerebral de ratos jovens e adultos expostos a diferentes concentrações de SFN;
- avaliar a massa corporal, massa adiposa, responsividade à insulina, perfil lipídico, provas de função hepática e renal, captação de glicose em músculo e tecido adiposo e concentração de glicogênio hepático e muscular de ratos submetidos ao tratamento oral agudo com SFN pré-indução de DM;

- avaliar a massa corporal, responsividade à insulina, perfil lipídico, provas de função hepática e renal, captação de glicose em músculo, atividade da SOD, catalase e resíduos sulfidril no pâncreas, fígado e rim de ratos adultos submetidos ao tratamento intraperitoneal agudo com SFN pós-indução de diabetes.

Parte II

Capítulo 1

Chronic sulforaphane oral treatment accentuates blood glucose impairment and may affect GLUT3 expression in cerebral cortex and hypothalamus of rats fed with a highly palatable diet

(*submetido ao periódico Nutrition Research*)

Chronic sulforaphane oral treatment accentuates blood glucose impairment and may affect GLUT3 expression in cerebral cortex and hypothalamus of rats fed with a highly palatable diet

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List of abbreviations

SFN; sulforaphane

SC; standard chow

HP; highly palatable

GTT; glucose tolerance test

AUC; area under the curve

HDL; high density lipoprotein

TAG; triacylglycerol

ALT; alanine aminotransferase

AST; aspartate aminotransferase

SDS; sodium dodecyl sulfate

SDS-PAGE; sodium dodecyl sulfate polyacrylamide gel electrophoresis

GLUT1; glucose transporter 1

GLUT3; glucose transporter 3

ANOVA; analysis of variance

CNS; central nervous system

BBB; blood-brain barrier

RNA; ribonucleic acid

GLUT4- glucose transporter 4

Abstract

Obesity and insulin resistance are the key factors underlying the etiology of major health problems like hypertension, diabetes and stroke. These important health issues lead researches to investigate new approaches to prevent and treat obesity and insulin resistance. Good candidates are the phytochemical compounds that have being extensively studied in the field. Therefore, the aim of this study was to test whether sulforaphane (SFN, 1mg/kg, 4 months treatment), a potent antioxidant enzymes inducer present in cruciferous vegetables, had some beneficial effects on obesity and insulin resistance induced by a highly palatable (HP) diet in male Wistar rats. Glucose tolerance, serum and hepatic lipids levels, lipid profile, ALT, AST, urea and creatinine, GLUT1 and GLUT3 levels in cerebral cortex, hippocampus and hypothalamus were analyzed. Glucose tolerance was lower in the HP diet groups, especially in the HP group treated with SFN. Except for the liver triacylglycerols, no differences were found in serum lipids, hepatic and kidney markers of the HP diet groups. Although expression of GLUT1 was similar between groups for all three brain structures analyzed, expression of GLUT3 in cortex and hypothalamus had a tendency to be lower in the HP diet group treated with SFN. In conclusion, SFN at the specific dose was able to accentuate glucose intolerance and may affect GLUT3 expression in the cerebral cortex and hypothalamus.

Key words: sulforaphane, insulin resistance, highly palatable diet, glucose transporter type 1, glucose transporter type 3.

1. Introduction

Obesity and insulin resistance are key factors in the etiology of major diseases like hypertension, diabetes, cardiovascular disease, stroke and even some types of cancer [1,2,3]. The link between excessive body weight and insulin dysfunction in the above mentioned disease is the increase of body fat that leads to an increased release of free fatty acids into the blood, which accumulates in other tissues [4,5]. This set of alterations culminates in hyperglycemia and increased oxidative stress resulting in health problems [6]. It is important to remember that one of the main causes of obesity and insulin resistance in modern world is the elevated consumption of food with high content of sugar and fat [7].

Results from our group demonstrated that animals fed with a highly palatable diet (enriched with sucrose) for four months, used as a model to induce obesity, had increased oxidation of proteins in cerebral frontal cortex and anxiety -like behavior, elevated body fat, and high levels of glucose and hepatic triacylglycerols[8]. Although the mechanisms behind these changes are unknown, it is clear that alterations in glucose metabolism are implicated.

So, studies with phytochemicals compounds that can prevent or attenuate metabolic derangements have being extensively developed with several positive results [9, 10, 11]. It has been recently demonstrated that sulforaphane (SFN), a potent antioxidant that acts as a phase 2 enzymes inducer present in cruciferous vegetables like broccoli and cauliflower [12], prevent streptozotocin induced-diabetes [13]. Therefore, the aim of this work was to study the effect of oral sulforaphane administration, at concentration similar to that obtained by the ingestion of 150g of fresh broccoli [14], on obesity and insulin resistance induced by a highly palatable(HP) diet. To address this question we analyzed peripheral and central parameters of the glucose metabolism in a dietary model to induce obesity with highly palatable diet.

2. Methods and materials

2.1. Reagents

R, S -Sulforaphane was purchased from LKT Laboratories (St. Paul, MN). Diagnostic kits were obtained from Labtest® (Lagoa Santa, MG, Brazil).

2.2. Animals and diets

Twenty one 60-days old male Wistar rats were obtained from the Central Animal House of our Department and were maintained under a standard dark-light cycle (lights on between 7:00 a.m. and 7:00 p.m.), at a room temperature of 22 $\pm$ 2°C. Animal care followed the official governmental guidelines in compliance with the Federation of Brazilian Societies for Experimental Biology and was approved by the Ethics Committee of the Federal University of Rio Grande do Sul, Brazil.

Rats were divided into three groups: (1) the control group (SC, n=7), which received standard laboratory rat chow (50 % carbohydrate, from starch, 22 % protein and 4 % fat); (2) the highly palatable diet group (HP, n=7), which received a enriched sucrose diet (65 % carbohydrates being 34 % in condensed milk, 23% from starch and 8 % from sucrose), 25 % protein, 10 % fat derived from soybean oil) (3) the highly palatable diet group which received the same enriched sucrose diet + sulforaphane (HP+SFN, n=7). All animals had free access to food and water during the four months of treatment.

2.3. Oral sulforaphane treatment

All animals were gavaged daily, in the early morning with either deionized water (SC and HP groups) or sulforaphane (SFN) 1mg/kg diluted in deionized water (HP+SFN). The SFN solution was prepared weekly, adjusted to animals' body weight, and kept under refrigeration.

2.4. Glucose tolerance test (GTT)

A glucose tolerance test was performed one week before the animals were sacrificed. A 50% glucose solution was injected into the animals (i.p, 2 mg/g) after 6h of fasting [15]. Blood sample

was taken by a small tail puncture immediately before and 30, 60, and 120 minutes after the injection. At each time, glucose was measured with a glucosimeter (AccuChek Active, Roche Diagnostics®, USA). The glucose levels were also evaluated by the analysis of the area under de curve (AUC).

2.5. Blood and Tissue preparation

At the end of four months all groups were anesthetized by sodium tiopenthal (40 mg/Kg), blood samples were taken by cardiac puncture and immediately centrifuged at 5000x g for 10 minutes to obtain serum and evaluate biochemical parameters. Liver, retroperitoneal and epididymal fat pads were dissected and weighted. Cerebral cortex, hippocampus and hypothalamus were dissected and a sample of each was obtained to perform posterior western blotting analysis.

2.6. Biochemical parameters

Serum levels of total cholesterol, high density lipoprotein (HDL) cholesterol, triacylglycerols (TAG), urea, creatinine and enzymatic activities of alanine aminotransferase (ALT) and aspartate aminotransferase (AST) were measured with commercial kits (Labtest®- Lagoa Santa, MG, Brazil). Liver triacylglycerol (TAG) and cholesterol were measured using a 100 mg liver sample, which was homogenized in a 1:20 saline solution (0.9%). The assay was performed with commercial kits (Roche Diagnostics, Indianapolis, USA) using an aliquot of this mixture. Liver cholesterol was determined by the method of Bergmeyer [16].

2.7. Western blotting analysis

Cerebral cortices, hippocampi and hypothalamus were dissected out and immediately homogenized in a 25 mM HEPES solution (pH 7.4) with 0.1% SDS and protease inhibitor cocktail (Sigma, USA). Samples (20 μg protein/well) were placed in a 10% SDS–PAGE mini-gel and transferred to a nitrocellulose membrane using a Trans-Blot system (Bio-Rad, São Paulo/SP, Brazil). Membranes were processed as follow: (1)blocking with 5% bovine serum albumin (Sigma, USA) for 1 h; (2) incubation with primary antibody overnight: 1:200 rabbit anti-GLUT1 and rabbit

anti-GLUT3 (Santa Cruz Biotechnology); (3) incubation with horseradish peroxidase-conjugated secondary antibody for rabbit 1:2000 (GE Healthcare UK Limited) overnight and (4) chemioluminescence (ECL, Amersham Pharmacia Biotech, São Paulo/SP, Brazil) was detected using X-ray films (Kodak X-Omat, Rochester, NY, USA). The same steps were repeated to incubate the membranes with anti-β-actin primary antibody overnight 1:3000, followed by incubation with horseradish peroxidase-conjugated secondary antibody for mouse 1:2000. The films were scanned and bands intensities were analyzed using Image J software (developed at the US National Institutes of Health and available on the web site (http://rsb.info.nih.gov/nih-image/). In order to determine the adequate amount of protein to be assayed, different protein concentrations were carried out in the same gel for each antibody tested.

2.8. Statistical analysis

Data were analyzed with SPSS 17.0 software. Glucose tolerance test were analyzed by Repeated Measures analyses of variance (ANOVA) and post-hoc Bonferroni's test. Parametric variables were tested by One-Way ANOVA and post-hoc Duncan's test and non-parametric variables were tested by Kruskall Wallis's test followed by Dunns' post-hoc. Results are expressed as mean ± standard deviation or median (min-max).

3. Results

The animals body weight did not change after four months of diet and treatment. However the adipose tissue, represented by retroperitoneal and epididymal fat pads, was two fold higher in HP and HP+SFN groups than the SC group (Table 1, $P>0.05$ and $P<0.05$, respectively). Although the liver TAG, a marker of insulin resistance, was elevated in HP and HP+SFN groups (Table 1, $P<0.05$), the liver weight, liver cholesterol, serum TAG, total cholesterol and HDL cholesterol were similar for all groups (Table 2, $P>0.05$).

In order to evaluate the chronic effects of sulforaphane in the liver and kidney function ALT, AST, urea and creatinine were measured and the levels of theses markers in the blood were similar for all groups, suggesting absence of hepato- and nephrotoxicity effect by the chronic use of SFN (Table 2, $P>0.05$).

On the other hand, glucose intolerance induced by HP diet was accentuated in HP+SFN group when compared with SC and HP group. Fasting glycemia at time 0 was equal in all groups ($P>0.05$) (Figure 1A). However, after glucose overload, the glycemic levels in HP and HP+SFN groups were higher than SC group at 30 minutes, and the levels for the HP+SFN was higher than HP group ($P<0.05$). This difference between the two HP groups disappear at 60 minutes of test, remaining both, HP+SFN and HP more intolerant to glucose than the SC group ($P<0.05$). At the end of the test the glycemic levels were similar for all groups, with a slightly increased for both HP groups compared with SC group ($P>0.05$). The difference in glycemic levels during the test can be appreciated by the area under the curve (AUC), with statistical differences only for SC and HP groups (Figure 1B, $P<0.05$).

We also measured the expression of glucose transporters in the cerebral cortex, hipoccampus and hypothalamus. In cerebral cortex we found no difference in GLUT1 expression (Figure 2A, $P>0.05$). However, the expression of GLUT3 tended to be lower in the HP+SFN (Figure 2B,

P=0.05) when compared with HP e SC groups. Also, we were able to observe a tendency of decrease GLUT3 levels with the increased blood glucose levels for all groups (negative correlation between GLUT3 levels and AUC; r=-0.4, p=0.05, data not shown). This data suggests a possible influence of glycemic levels on GLUT3 expression in the cortex. No correlation was observed between GLUT1 expression in the cerebral cortex and AUC values. Like the GLUT1 and GLUT3 expression in the hippocampus (Figure 3A and B, P>0.05), the GLUT1 expression in hypothalamus was similar in all groups (Figure 4A, P>0.05). However, there was a tendency to decrease the expression of hypothalamic GLUT3 in HP+SFN (Figure 4B, P=0.06) when compared with HP e SC groups. Similar to what we observed in the cerebral cortex, here was a tendency of decrease GLUT3 levels in this structure with the increased blood glucose levels for all groups (negative correlation between GLUT3 levels and AUC; r=-0.5, p=0.06, data not shown). Again, no correlation was found between hypothalamic GLUT1 levels and AUC values.

4. Discussion

This was the first study that demonstrated the effects of chronic oral administration of SFN in a model of obesity and insulin resistance induced by HP diet. Surprisingly we found an increased glycemia in HP diet + SFN treated animals, since the dose of SFN used (1mg/kg) was based on the amount of SFN present in 150g of fresh broccoli [14], a portion commonly ingested in a human meal.

As demonstrated by previous studies from our group [17,18] HP diet induced obesity and insulin resistance, based on increased levels of adipose tissue, glucose intolerance and hepatic triacylglycerols when compared with SC, even without changes on serum lipid profile. SFN had no effects in preventing or ameliorating any alteration promoted by the HP diet, and did not produce a toxic effect in the liver or kidney functionality, suggested by the unaltered levels of ALT, AST, urea and creatinine. On the other hand, the increased glycemic levels in GTT at 30 minutes in HP diet +SFN-treated animals indicated higher glucose intolerance than HP group. This hypothesis is reinforced by the tendency of decreased GLUT3 expression in the cortex and hypothalamus in HP diet + SFN -treated animals, what was not observed in animals receiving only HP diet.

GLUT1 and GLUT3 are glucose transporters present in central nervous system (CNS). While GLUT1 is involved in glucose crossing through the endothelial cells of the blood-brain barrier (BBB), GLUT3 transports glucose into the neural cells [19]. Nevertheless, whether hyperglycemia increases or decreases GLUT-1 and GLUT-3 expression in the brain is still on debate. Zhang and colleagues [20] observed an increase of GLUT1 and GLUT3 mRNA levels in diabetic rats with or without brain ischemia and reperfusion. Likewise, Peeyush et al, Reagan et al and Sherin et al [21-23] described an increased GLUT3 gene expression in brainstem, hippocampus and cortex of diabetic rats who received different types of treatments. On the other hand, it has been described decrease in GLUT1 expression in hypothalamus [24], thalamus, cerebellum and

hippocampus, mediated by high glucose levels [25]. Similar to our findings, Hou et al [26] demonstrated decreased GLUT1 and GLUT3 expression in the brain of diabetic rats, with transporters levels being negatively correlated to blood glucose. However, it is important to mention that all studies cited above measured mostly GLUT1 and GLUT3 mRNA that may not always be translated into protein content. Different from glucose transporter 4 (GLUT4), GLUT1 and GLUT3 are not regulated by insulin but rather by blood glucose levels. It seems that the hyperglycemia down regulates brain glucose transporters in an attempt to protect the brain against damage caused by an influx of glucose into the cell. The critical point is when blood glucose levels reduces and cells fails in correct this down regulation, resulting in a relative insufficiency of glucose in brain, favoring neurological damage [26].

In this study we did not investigate the mechanism underlying the hyperglycemia in presence of SFN, and even not being one of our goals, it still is a limitation of our data. Different from our results, Song et al [13] demonstrated that SFN in lower dose (40μg/kg, ip) and in a very short period of treatment (3 days), was able to prevent streptozotocin-diabetes by decreasing reactive oxygen species production and suppressing NF-κB pathway in pancreas, preserving β-cells and insulin production. It is important to emphasize that the SFN used in our protocol was an isolated compound, absorbed directly, without intestinal enzymatic mechanisms, which is a different process compared to SFN contained in cruciferous vegetables. It is known that vegetables can have antinutrional factors that affect absorption of some nutrients. That could explain why isolated SFN had a hyperglycaemic effect different from the oral intake of fresh broccoli. Regardless, it is important to remember that the benefit or toxicity of antioxidants usually is dose-dependent, what is well-explained by the concept of hormesis, defined as a biphasic dose-response of cells or organisms to an exogenous or endogeneous factor (chemical agents, dietary ingestion, oxidative stress, etc.) in which the factor induces stimulatory or beneficial effects at low doses and inhibitory or adverse effects at high doses [27].

In conclusion, SFN at the specific dose was able to accentuate glucose intolerance and tend to decrease the GLUT3 expression in the cerebral cortex and hypothalamus without impairment of any other parameters evaluated. Further studies are necessary to understand the mechanism underlying these alterations, testing different SFN doses that might be harmless and at the same time preventing or improving the alterations caused by obesity and insulin resistance.

Acknowledgments

This work is dedicated to life and memory of Marcos Luiz Santos Perry, beloved friend, mentor and Professor of Biochemistry.

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Legends of tables and figures.

Table 1. Body composition and lipid content in the liver of SC, HP and HP+SFN groups (*n=7*). Data are express as mean ± standard deviation. Statistical analysis performed with One Way Anova followed by Duncan's post-hoc test. **[a,b]** = Different letters mean P< 0.05 among groups.

Table 2. Serum biochemical parameters of SC, HP and HP+SFN groups (*n=7*). Data are express as mean ± standard deviation or median (min-max). Statistical analysis performed with One Way Anova followed by Duncan's post-hoc test or Kruskall-Wallis test. **[a]**= Same letter mean no differences among groups.

Figure 1. (A) Glucose Tolerance Test (GTT) and (B) Area Under de Curve (AUC) of all groups (*n=7*). For GTT, blood glucose was measured before (0) and 30, 60 and 120 minutes after glucose injection (2mg/g body weight). Data are expressed as mean± standard deviation of groups. Statistical analysis performed with Repeated Measures Anova followed by Bonferroni's test for GTT and One Way Anova followed by Duncan's post-hoc test for AUC. **[a,b,c]** = Different letters mean P< 0.05 among groups.

Figure 2. Western blotting analysis of glucose transporters (A) GLUT-1 and (B) GLUT-3 in cerebral cortices of SC (white bars), HP (black bars) and HP+SFN groups (filled bars) (*n=4*). Data are expressed as mean ± standard deviation (*n*=4 animals/group. At the top of the figure are representative images of the immunocontent of transporters. β-Actin was used as a protein loading control. Statistical analysis performed with One Way Anova followed by Duncan's post-hoc test.

Figure 3. Western blotting analysis of glucose transporters (A) GLUT-1 and (B) GLUT-3 in hippocampi of SC (white bars), HP (black bars) and HP+SFN groups (filled bars) (*n=4*). Data are expressed as mean ± standard deviation (*n*=4 animals/group). At the top of the figure are representative images of the immunocontent of transporters. β-Actin was used as a protein loading control. Statistical analysis performed with One Way Anova followed by Duncan's post-hoc test.

Figure 4. Western blotting analysis of glucose transporters (A) GLUT-1 and (B) GLUT-3 in hypothalamus of CT (white bars), HP (black bars) and HP+SFN groups (filled bars) (*n=4*). Data are expressed as mean ± standard deviation (*n*=4 animals/group). . At the top of the figure are representative images of the immunocontent of transporters. β-Actin was used as a protein loading control. Statistical analysis performed with One Way Anova followed by Duncan's post-hoc test.

Table 1. Body composition and lipid content in liver.

	SC (n=7)	HP (n=7)	HP+SF (n=7)
Final body weight (g)	328 ± 17 [a]	343 ± 26 [a]	339 ± 15 [a]
Adipose Tissue (RP+EP) (g)	8 ± 3 [a]	16 ± 4 [b]	15 ± 3 [b]
Liver weight (g)	10 ± 1 [a]	10 ± 1 [a]	9 ± 1 [a]
Liver TAG (mg%)	0.9 ± 0.3 [a]	1.5 ± 0.3 [b]	1.4 ± 0.4 [b]
Liver Cholesterol (mg%)	1.7 ± 0.4 [a]	1.7 ± 0.4 [a]	1.4 ± 0.2 [a]

Table 2. Serum biochemical parameters.

	SC (n=7)	HP (n=7)	HP+SF (n=7)
Serum TAG (mg/dL^{-1})	92 (43-222) [a]	99 (52-176) [a]	115 (34-269) [a]
Total Cholesterol(mg/dL^{-1})	62 ± 6 [a]	64 ± 8 [a]	57 ± 11 [a]
HDL Cholesterol(mg/dL^{-1})	34 ± 2 [a]	38 ± 1 [a]	38 ± 1 [a]
ALT(IU/L)	3 (2-5) [a]	3 (2-6) [a]	4 (2-6) [a]
AST(IU/L)	8 (4-13) [a]	8 (4-17) [a]	13 (4-27) [a]
Urea (mg/dL^{-1})	54 ± 8 [a]	46 ± 9 [a]	49 ± 8 [a]
Creatinine (mg/dL^{-1})	0.4 ± 0.1 [a]	0.4 ± 0.1 [a]	0.4 ± 0.1 [a]

Figure 1

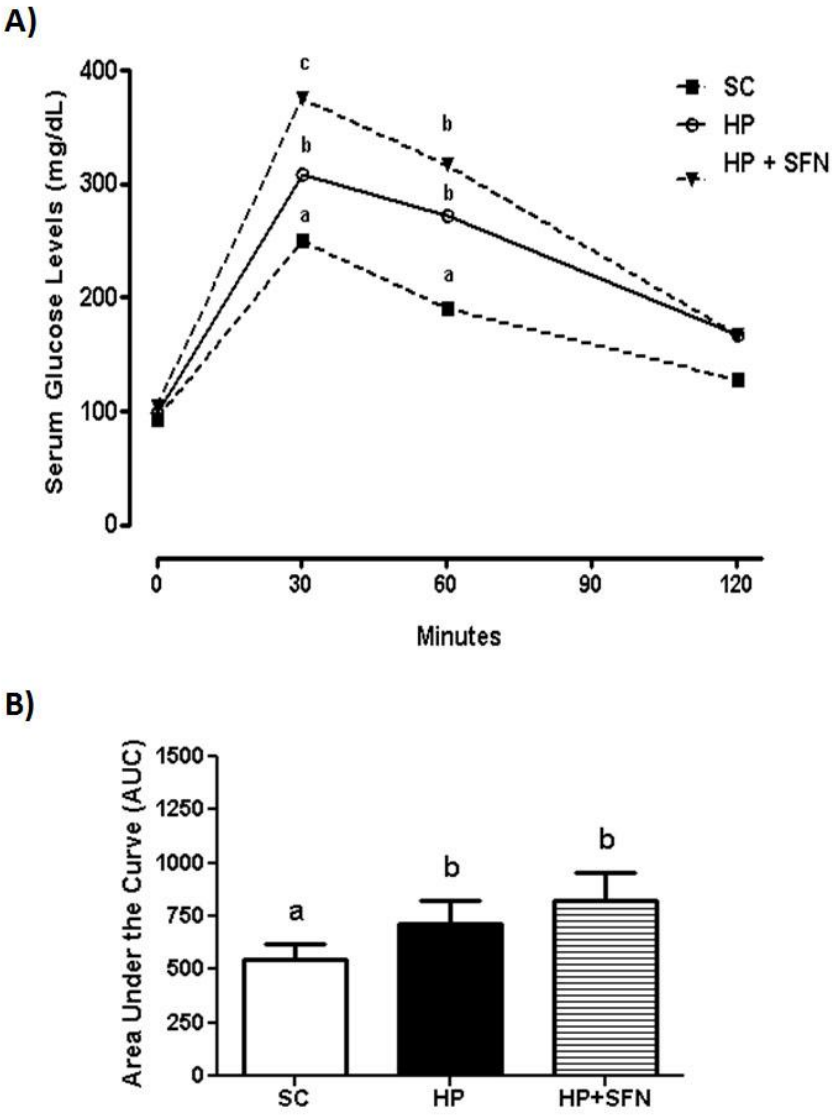

A)
Serum Glucose Levels (mg/dL)
400
300
200
100
0
0
30
60
90
120
Minutes
SC
HP
HP + SFN
a
b
c
B)
Area Under the Curve (AUC)
1500
1250
1000
750
500
250
0
SC
HP
HP+SFN
a
b
b

Figure 2

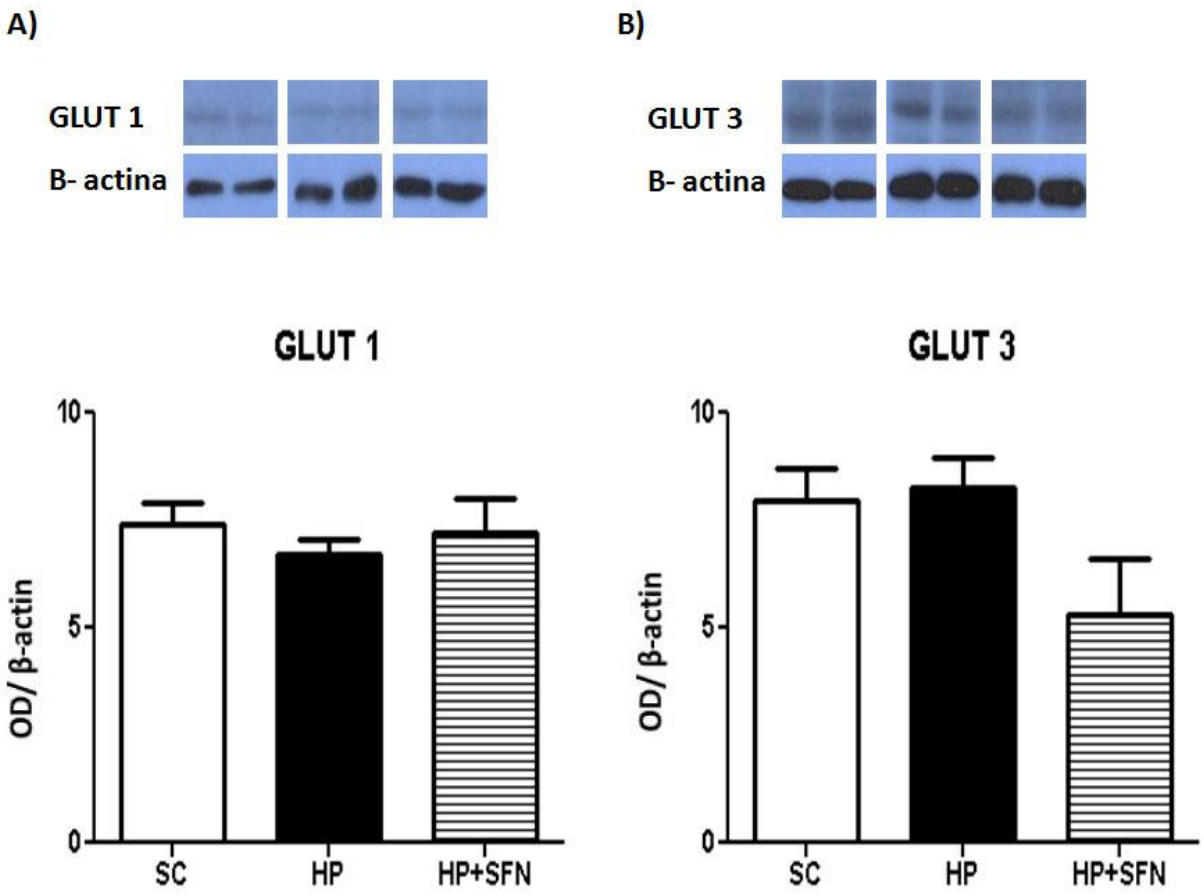

A)
GLUT 1
B- actina
B)
GLUT 3
B- actina
GLUT 1
OD/ β-actin
10
5
0
SC
HP
HP+SFN
GLUT 3
OD/ β-actin
10
5
0
SC
HP
HP+SFN

Figure 3

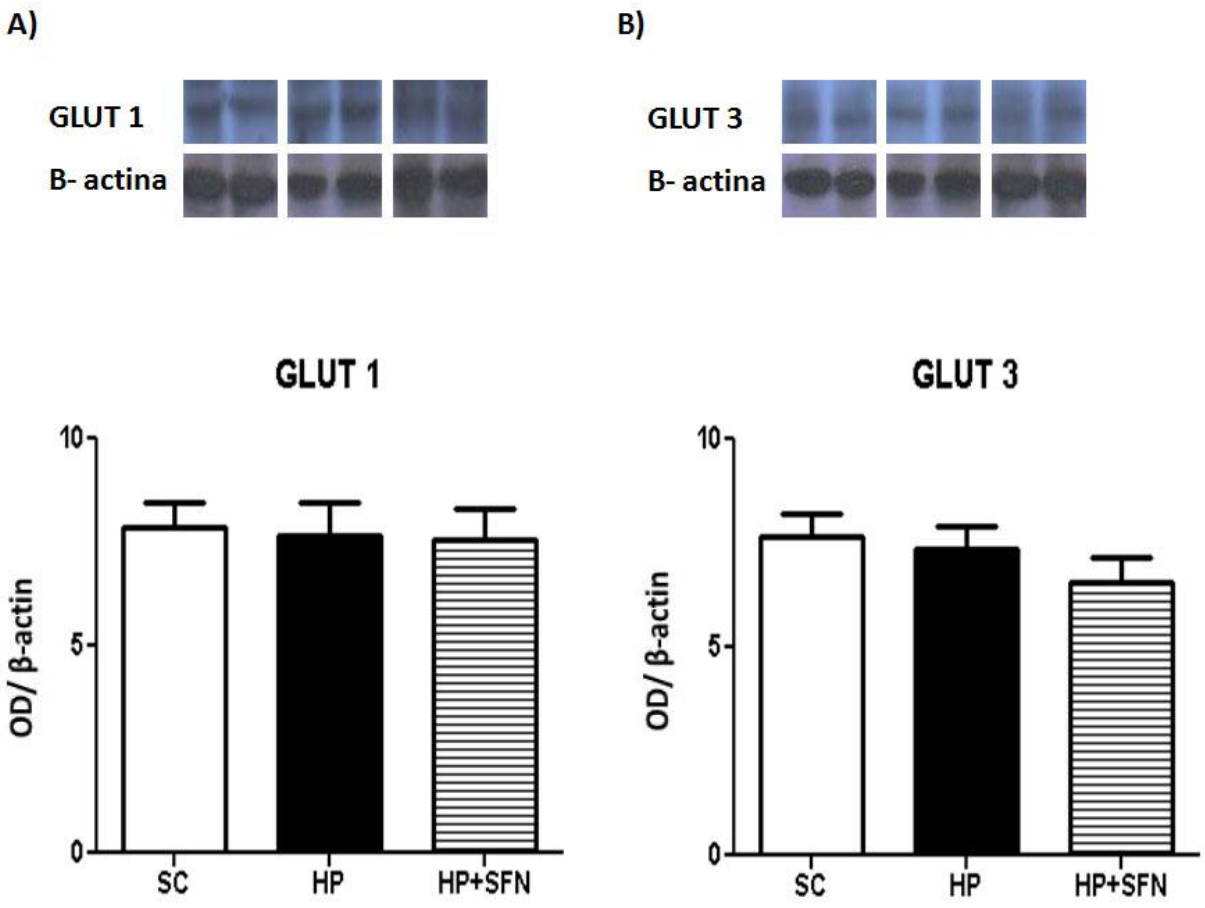
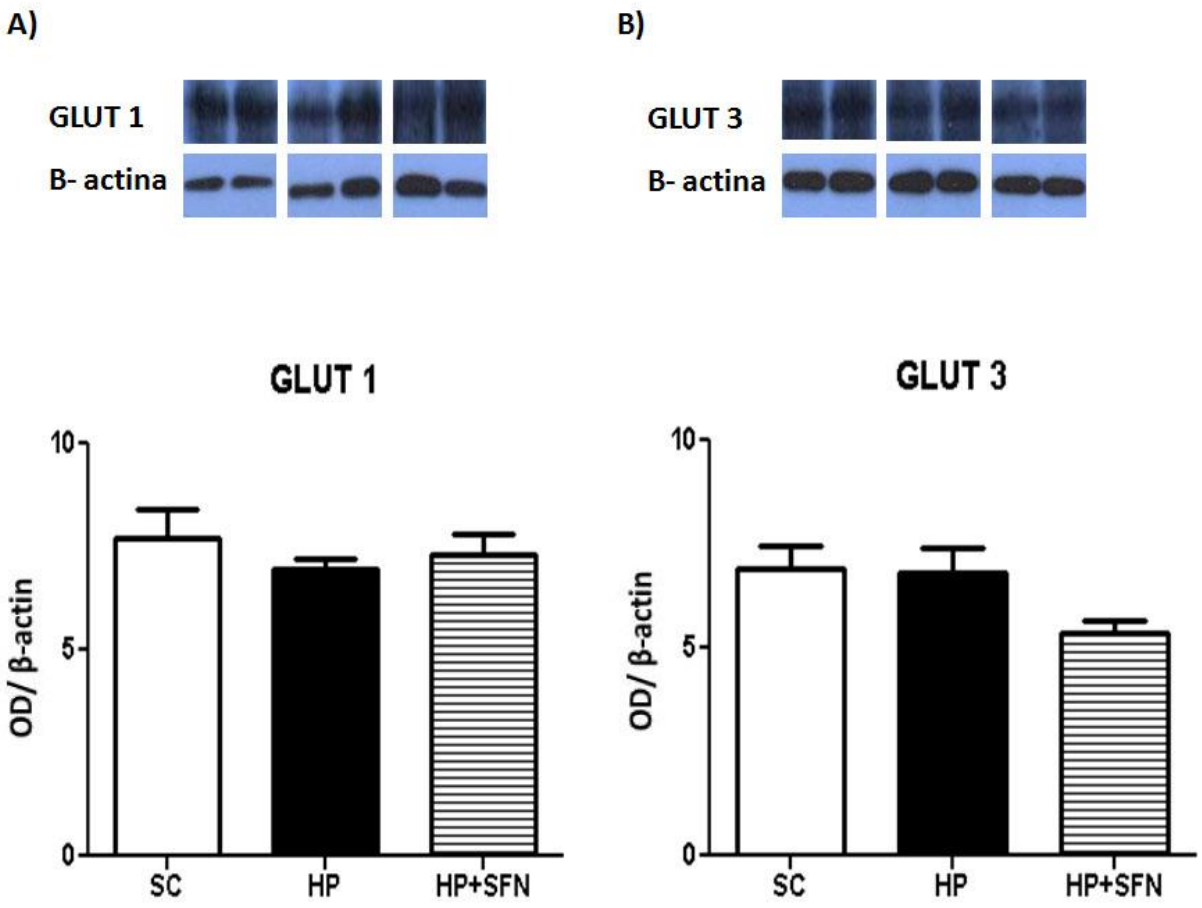

A)
GLUT 1
B- actina
B)
GLUT 3
B- actina
GLUT 1
OD/ β-actin
10
5
0
SC
HP
HP+SFN
GLUT 3
OD/ β-actin
10
5
0
SC
HP
HP+SFN

Figure 4


A)
GLUT 1
B- actina
B)
GLUT 3
B- actina
GLUT 1
OD/ β-actin
10
5
0
SC
HP
HP+SFN
GLUT 3
OD/ β-actin
10
5
0
SC
HP
HP+SFN

Capítulo 2

Characterization of sulforaphane effects in neurochemical parameters of young and adult rats cerebral cortex.

(*submetido ao periódico Neurochemical Research*)

Characterization of sulforaphane effects in neurochemical parameters of young and adult rats cerebral cortex.

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Abstract

Sulforaphane (SFN) is a phytochemical compound, with potent antioxidant properties, that recently demonstrated neurological benefits against brain injury. As most of those studies use pathological models to evaluate SFN actions, our goal was to characterize in vitro effects of SFN different doses (0, 0.25, 0.5, 1, 2, 5 and 10μM) under physiological conditions, using slices of cerebral cortex from young and adult rats. Cell viability, metabolic parameters, intracellular reactive oxygen species (ROS) production, Na^+-K^+-ATPase and glutamine Syntethase (GS) activities were evaluated. No alterations in cell viability could be observed, but SFN 0.25 and 0.5μM increased CO_2 production and protein synthesis, while 5 and 10μM decreased the same parameters in cerebral cortex of young rats, but not in adults. A positive dose dependent correlation was found between Na^+-K^+-ATPase activity and SFN concentration, mainly in adult cerebral cortex and free radical production was slightly elevated at this age too, when treated with SFN high dose. The reduction of protein synthesis in young brain cortices was followed by decreased activity of GS, which was not observed in adults. We conclude that SFN may increase or decrease CO_2 production only in young rat cerebral cortex, probably by excessive Na+,K+-ATPase activity and decline of protein synthesis and activity of GS, promoted by SFN high dose, may favor cell derangements, which can be accentuated by ROS production.

Key words: sulforaphane; energy metabolism; cell viability; Na^+-K^+-ATPase; glutamine synthetase; reactive oxygen species.

1. Introduction

Non-nutrient molecules derived from plant secondary metabolism, called phytochemicals, have been target in a large number of epidemiological, *in* vivo and *in* vitro studies. Phytochemicals present in the human diet includes several groups, such as the most popular sulfur compounds, terpenoids and polyphenols [1].

Sulforaphane (SFN) is a sulfur compound, an isothiocynate derived from the hydrolysis of the glucosinolate glucoraphanin by the enzyme myrosinase, which is present in human intestine and in vegetables [2,3], and can be found in cruciferous of the genus *Brassica* including cauliflower, broccoli, cabbage, radish, and Brussels sprouts [4,5]. Although these compound has some direct antioxidant effects that have yet to be elucidated [6], studies suggested that the main actions of SFN are mediated by improvement of antioxidant defenses through the nuclear factor E2-related factor (Nrf2)- antioxidant response element (ARE) pathway, like Heme oxygenase-1, NAD(P)H: quinone oxidoreductase, glutathione-S-transferase, gamma-glutamyl cysteine ligase, and glutathione reductase promoting gene expression [7].

Besides its well known anticarcinogenic properties [8,9,10] recently neurological benefits of SNF in brain injury has been investigated and several studies have been performed in order to evaluate the SFN protective effect in experimental models of focal cerebral ischemia, brain inflammation, intracerebral hemorrhage, brain ischemia and reperfusion [11,12,13,14], showing promising perspectives about neuroprotection and therapeutical actions at some concentrations of the compound. However some of those works also demonstrated that according to SFN concentration it could be observed loss of protective effect against injury or even toxicity [15,16,17].

As most of those studies use pathological models to evaluate the SFN actions against brain damage and almost no data can be found about SFN effects in brain normal conditions the aim of the present study was to characterize this phytochemical effects, using different doses, on cellular

viability, metabolic parameters and free radicals production of slices from cerebral cortex of young and adult animals under physiological conditions, considering age effects involved in central nervous system maturity.

2. Material and methods

2.1. Chemicals

Chloroform, formic acid and methanol were obtained from Merck SA, Porto Alegre, Brazil. Hyamine hydroxide was purchased from J.T. Baker Chemical Company, Phillisburg, NJ, USA, and, l[U-^{14}C]leucine (323 mCi/mmol), were from Perkin-Elmer (Boston, MA, USA). All other reagents were purchased from Sigma Chemical CO (St. Louis, MO).

2.2. Animals

Male Wistar rats (ten day old and sixty day old) were obtained from the Central Animal House of the Biochemistry Department, Federal University of Rio Grande do Sul, Brazil. They were kept under a standard dark-light cycle (lights on between 7:00 a.m. and 7:00 p.m.) at a room temperature of 22 $\pm$ 2°C. Animal care followed the official governmental guidelines according to the Federation of Brazilian Societies for Experimental Biology and it was approved by the Ethics Committee of the Federal University of Rio Grande do Sul, Brazil.

2.3. Slice preparation and incubation system

Animals were killed by decapitation, brains were rapidly removed, cortices were dissected over ice and transverse sections (400 μm) were prepared using a McIlwain tissue chopper. After that the slices were incubated in 1.0 mL Dulbecco buffer, pH 7.2, containing 5.0 mM D-glucose + 0.2 mM L-leucine+ 0.2 μCi L-[U-^{14}C] leucine (Sigma Chemical Co., St. Louis, MO, USA) and 0, 0.25, 0.5, 1, 2, 5 or 10 μM of R,S-Sulforaphane (LKT Laboratories, St. Paul, MN) during 90min and viability tests, CO2 production, lipid and protein synthesis were performed.

2.3.1. Lactate and lactate dehydrogenase (LDH) assay

LDH is a cytosolic enzyme that converts pyruvate to lactate and vice-versa and alterations in membrane permeability promote release of lactate and LDH from the inside of the cell to the medium of incubation. Membrane damage was determined by measuring lactate and lactate dehydrogenase released into the medium using commercial kits (Labtest, Minas Gerais, Brazil).

2.3.2. MTT Colorimetric assay

Slice viability assay was performed by the colorimetric [3(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide] (MTT, Sigma) evaluating the activity of dehydrogenases that reduces this substrate to formazan, mostly in mitochondria. After the standard incubation period (1h30min), slices were incubated with 0.5 mg/ml of MTT at 37°C for 45 min. The formazan product generated during the incubation was solubilized in dimethyl sulfoxide and measured at 560 and 630 nm. Only viable slices are able to reduce MTT. Results were expressed as a percentage of control group (SFN 0μM).

2.3.3. CO_2 production, lipid and protein synthesis

Before incubation for 90min, the reaction medium was oxigenated with a 95% O_2:5% CO_2 mixture for 1 min. Flasks were sealed with rubber caps and parafilm. Glass center wells containing a folded 60 mm/4 mm piece of Whatman 3filter paper were hung from the stoppers. Slices were incubated at 35°C for 1h in a Dubnoff metabolic shaker (60 cycles/min) according to the method of Dunlop et al.[18] and Ferreira et al.[19]. Incubations were stopped by adding 0.25 mL 50% TCA through the rubber cap. Then 0.20 mL of 1 M sodium hydroxide was injected into the central wells. The flasks were shaken for an additional 30 min at 35°C to trap CO_2. Afterwards, the contents of the central well were transferred to vials and assayed for CO_2 radioactivity in a liquid scintillation counter. The incubated slices were homogenized and transferred to tubes. After centrifugation, the precipitate was washed three times with 10% TCA, and lipids were extracted with

chloroform/methanol (2:1). The chloroform/methanol phase was evaporated in vials and the radioactivity was measured. The resulting precipitate was dissolved in concentrated formic acid and the radioactivity was measured, representing protein synthesis from L-leucine. All results were expressed in relation to the initial specific activity of the incubation medium. The CO_2 production rate and the lipid and protein synthesis were constant over 30, 60 and 90 min of incubation.

2.4. Na^+,K^+-ATPase activity assay

Na^+,K^+-ATPase is a plasmatic membrane enzyme related to cell energetic metabolism. Dissected cortices were homogenized in 10 volumes (1:10, w/v) of 0.32M sucrose solution containing 5.0 mM HEPES and 1.0 mM EDTA, pH 7.4 (Medium buffer). The homogenate was centrifuged at 1000 g for 10 minutes and the supernatant was used for the assay. The reaction mixture for Na^+,K^+-ATPase activity assay contained 5.0 mM $MgCl_2$, 80.0 mM NaCl, 20.0 mM KCl and 40.0 mM Tris–HCl, pH 7.4, with samples standardized at 1mg/ml protein, where 0.5μM or 5μM SFN was dissolved, and the incubation time was also 90min. The reaction was initiated by ATP addition. Controls were carried out under the same conditions with and without the addition of 1.0 mM ouabain. Na^+,K^+-ATPase activity was calculated by the difference between the two assays, as described by Wyse et al. [20]. Released inorganic phosphate (Pi) was measured by the method of Chan et al. [21]. Specific enzyme activity was expressed as nmol Pi released per min per mg of protein. All samples were run in duplicates.

2.5. Glutamine Syntethase activity assay

The enzymatic assay was performed, as previously described [22] in order to complement the results of leucine metabolism, involved in glutamate synthesis. Briefly, homogenate (0.1 mL) was added to 0.1 mL of reaction mixture containing (in mM): 10 MgCl2; 50 L-glutamate; 100 imidazole–HCl buffer (pH 7.4); 10 2-mercaptoethanol; 50 hydroxylamine–HCl; 150 ATP and incubated for 15 min at 37 °C. The reaction was stopped by the addition of 0.4 mL of a solution containing (in mM): 370 ferric chloride; 670 HCl; 200 trichloroacetic acid. After centrifugation, the

supernatant's absorbance was measured at 530 nm and compared to the absorbance generated by standard quantities of -glutamylhydroxamate, treated with ferric chloride reagent.

2.6. DCFH oxidation

Intracellular ROS production was detected using the nonfluorescent cell permeating compound, 2'-7'-dichlorofluorescein diacetate (DCFH-DA). DCFH-DA is hydrolyzed by intracellular esterases to dichlorofluorescin (DCFH), which is trapped within the cell. This nonfluorescent molecule is then oxidized to fluorescent dichlorofluorescin (DCF) by the action of cellular oxidants. Slices were treated with DCFH-DA (10μM) for 30 min at 37C. Following DCFH-DA exposure, the slices were scraped into PBS with 0.2% Triton X-100. The fluorescence was measured in a plate reader (Spectra Max GEMINI XPS, Molecular Devices, USA) with excitation at 485 nm and emission at 520 nm [23].

2.7. Protein determination

Protein concentration was measured by the method of Bradford [24], using bovine serum albumin as standard.

2.8. Statistical Analysis

Data were analyzed with SPSS 16.0 software by One-Way ANOVA and post-hoc Tukey's test, followed by Pearson's correlation for some parameters. All results were obtained by three independent experiments and are expressed as mean ± standard deviation. In order to simplify data interpretation we compared all SFN concentrations only to the control group (SFN 0 μM).

3. Results

Starting the characterization of SFN effects by cellular viability tests we found that both lactate and LDH release from cerebral cortex of young animals were not different in any tested concentration, even when lactate decreased after exposed to SFN 2, 5 and 10μM (Figure 1A and 1C, $P>0.05$). Also no difference was observed in formazan production by reduced MTT (Figure 1B,

P>0.05). The same results were observed in cerebral cortex of adult animals, where lactate liberation was slightly higher in the control group, however, the reduced MTT and LDH release also did not differ statically (Figure 2A, B and C, respectively, P>0.05). These set of results indicate that all tested concentrations were innocuous to cellular viability.

The next step of this characterization was the measurement of cellular energy and synthesis parameters. In young animals the lower doses of SFN, 0.25 and 0.5 μM, were able to increase CO_2 production (7.6±3.3 and 6.9±2.0 pmol L-leucine oxidized, respectively vs. 4.4±2.7, P<0.05, Figure 3A) and protein synthesis (13.8±3.3 and 12.4±2.6 pmol L-leucine incorporated into protein, respectively vs. 6.5±2.2, P<0.05, Figure 3B) when compared to the control group. On the other hand, the highest doses, 5 and 10μM, decreased these two parameters, when compared to the same control group (2.2±0.6 and 2.3±0.9 pmol L-leucine oxidized, respectively vs. 4.4±2.7, P<0.05, Figure 3A and 3.2±1.1 and 3.7±1.7 pmol L-leucine incorporated into protein, respectively vs. 6.5±2.2, P<0.05, Figure 3B). Lipid synthesis slightly increased with the lower doses, but with no statistical significance when compared with other tested concentrations (P>0.05, Figure 3C). In adult animals we did not observe any alteration in the same parameters, although fluctuation of values are present mostly in CO_2 production, but with no statistical significance (P>0.05, Figure 4A). Protein and lipid synthesis also did not differ between groups (P>0.05, Figure 4B and 4C).

Since energy production was altered in some SFN tested concentrations we decide to evaluate Na^+,K^+-ATPase activity, a plasmatic membrane bound enzyme that, beyond be involved in cell ionic exchanges, is an important component of cell energetic metabolism. To test the activity we used SFN in two different doses, low (0.5μM) and high (5μM), to be compared with the control group (SFN 0μM). We observed that Na^+,K^+-ATPase activity was increased in cerebral cortex of young rats two fold higher with 5μM of SFN when compared to 0 and 0.5μM (219±27 nmol Pi/min/protein mg vs. 111±19 and 113±22 nmol Pi/ min/protein mg, respectively, P<0.01, Table 1).

No differences was observed among the 0.5μM and the control group, and there was a positive correlation between the SFN dose and enzyme activation (r=0.9, P<0.01, Table 1). Different from young, in the adult animals, Na^+,K^+-ATPase activity was increased in both concentration of SFN, 0.5μM and 5μM, when compared to control (171±10 and 236±47, respectively, vs. 90±10 nmol Pi/ min/protein mg, P<0.01, Table 1). Again, a positive correlation between SFN dose and enzymatic activation was observed (r=0.8, P<0.01, Table 1), even in a different age.

Alterations in Na^+,K^+-ATPase are frequently related to increased free radicals (ROS) production and for this reason we quantify the oxidation of DCFH to DCF in the same SFN concentrations used to test Na^+,K^+-ATPase activity. Compared to control group (SFN 0μM), which was interpreted as 100%, SFN 0.5μM slightly decreased the production of ROS (97%) while SFN 5μM increased a little this same parameter (114%) in the cortices of young animals, although these differences were not significant (Figure 5A, P>0.05). In adult brain cortices SFN 0.5μM increased ROS production (107%) and 5μM demonstrate the same increased as observed in young animals (114%) compared to SFN 0μM, but again without statistical significance (Figure 5B, P>0.05).

Besides changes in energy production some concentrations of SFN also influenced protein synthesis and the metabolism of aminoacids in brain is also linked to neurotransmitters synthesis and that is why we evaluate glutamine synthetase (GS) activity. We could observe that there is a tendency of decrease in GS activity between SFN 0μM and SFN 5μM (P=0.06, Table 2) in young animals while in adult animals no differences could be observed between groups (P>0.05, Table 2).

4. Discussion

Several studies evaluated SFN action in the central nervous system (CNS) and found only beneficial effects, independent of tissue, cell, experimental protocol and concentration used [11,14,25, 26]. However, in this characterization study, we observed some different effects in

cerebral cortex of young and adult animals and although the tested concentrations of SFN did not alter cellular viability it did produced modifications in some basic functions of brain cortex, Na^+,K^+-ATPase and GS activity.

The incubation system showed that in adult brain cortices no differences were found in CO2 production, lipid and protein synthesis. However lower doses of SFN (0.25 and 0.5μM) were able to stimulate energy production and protein synthesis from leucine in cerebral cortex of young animals, while the opposite effect was observed with SFN 5 and 10 μM, without changes in lipids synthesis. It has been demonstrated that metabolism of amino acids is extremely important to the homeostasis of the CNS, especially leucine that crosses the blood brain barrier most swiftly than any other amino acid, and constitutes the major donor of NH_2 groups (at least 25-30%) to glutamate synthesis [27,28]. Therefore, the "leucine-glutamate cycle" is essential for continuous formation of glutamate, the main excitatory neurotransmitter in the CNS [29]. The decreased of protein synthesis from leucine in young animals cortices treated with SFN 5 and 10 μM may indicate that this aminoacid could have being used in other pathways, like glutamate synthesis. In this sense we evaluated the activity of GS, that converts glutamate to glutamine, and we observed that SFN 5μM decrease the enzymatic activity at this same age, even though only a statistical trend was found between groups (P=0.06). If more leucine is being used to provide glutamate and GS converts less glutamate to glutamine is expected that more glutamate accumulates in the cell, promoting toxic and pro-oxidant effects, once the neurotoxicity of excessive glutamate is well known [30,31,32]. Complementing this hypothesis we point to SFN 0.25 and 0.5μM effects on increased protein synthesis that, in parallel, have no differences in GS activity when compared to control group. Corroborating the findings of CO2 production, lipid and protein synthesis in adult brain cortices, GS activity also did not differ between groups in both SFN tested concentrations.

In addition to the essential roles as neurotransmitters and proteins precursors, all of the amino acids can be oxidized to CO_2 and H_2O, contributing to the energetic homeostasis of the CNS [33]. The lower rate of CO_2 production found in brain cortices incubated with SFN 5 and 10μM may indicate deficit in aerobic glycolysis, which could result either from inhibition of one or more glycolytic enzymes, or from an inhibition of the Krebs cycle and/or the respiratory chain [34].

Another possibility to explain the lower rate of CO_2 production in young animals is the alteration of some enzymes outside glycolytic pathway but also related to energy utilization, like Na^+,K^+-ATPase, which is an essential component of plasma membrane involved in energy metabolism by consumption of large amount of ATP [35,36]. Na+,K+-ATPase activity had an increased activity when incubated with SFN 5μM and also a positive dose dependent correlation in both young and adult animals. When incubated with SFN 0. 5μM only in adults we could observe the same increase, but apparently this effect of SFN 0.5 and 5μM in Na+,K+-ATPase in adult cerebral cortex has no impact in the other evaluated parameters, unlike young animals. These alterations may be attributed to the immaturity of the CNS in young animals making it more susceptible to any type of derangements and goes in favor to the findings of a previous study from our group, which compared energetic metabolism in young and adult cerebral cortex, and observed alterations in amino acid oxidation against oxidative insult only in young animals, suggesting that the immature cerebral cortex is less able to deal with insults compared to the mature tissue [37]. Moreover, to our knowledge there are no studies evaluating the SFN effects in CO2 production, protein and lipid synthesis.

Studies evaluating the effect of other phytochemicals on the Na+,K+-ATPase activity usually shows a reversal of enzymatic activity, which is decreased in pathological conditions. A common explanation could be the protective effect of these compounds against free radicals associated to the plasma membrane fatty acids support, protection and preservation [38,39,40]. Based on this we evaluated ROS production using the same SFN concentrations used in Na+,K+-

ATPase activity and, even without statistical significance, ROS production was higher when submitted to SFN 5μM in both ages and when submitted to SFN 0.5μM in adults. These were the same pattern of change found in Na+,K+-ATPase activity and, although we have no other mechanism which profoundly explain this, it is important to highlight that the benefit or toxicity of phytochemicals, and the type of gene expression promoted by antioxidant compounds frequently are dose dependent.

In summary, our data suggests that SFN can alter the aerobic energy production and protein synthesis only in young rat cerebral cortex, according to concentration used, without modifying lipid synthesis and also without loss of cellular viability. The changes in energy production could be related to the excessive Na+,K+-ATPase activity and the decreased protein synthesis could be negatively affecting the tissue since GS activity is impaired.

Conflict of interest

The authors declare that they have no conflict of interest.

Acknowledgments

This work is dedicated to life and memory of Marcos Luiz Santos Perry, beloved friend, mentor and Professor of Biochemistry. Also, we are very grateful for excellent contributions and support of Marcelo Ganzella and Gisele Hansel.

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Legend of figures and table

Figure 1. Cellular viability tests in cerebral cortices of young animals (n=9): Lactate (A), MTT (B) and Lactate Dehydrogenase (LDH) assay (C). Data are expressed as mean± standard deviation. Statistical analysis performed with One Way Anova followed by Tukey's test.

Figure 2. Cellular viability tests in cerebral cortices of adult animals (n=9): Lactate (A), MTT (B) and Lactate Dehydrogenase (LDH) assay (C). Data are expressed as mean± standard deviation. Statistical analysis performed with One Way Anova followed by Tukey's test.

Figure 3. Incubation of young animals cerebral cortices with L-leucine (n=6): oxidation to CO_2 (A), protein (B) and lipid (C) synthesis. Data are expressed as mean± standard deviation of pmol L-leucine oxidized or incorporated (for details see Material and Methods section). Statistical analysis performed with One Way Anova followed by Tukey's test. ***** = $P<0.05$ compared to control group (0 μM SFN).

Figure 4. Incubation of adult animals cerebral cortices with L-leucine (n=6): oxidation to CO_2 (A), protein (B) and lipid (C) synthesis. Data are expressed as mean± standard deviation of pmol L-leucine oxidized or incorporated (for details see Material and Methods section). Statistical analysis performed with One Way Anova followed by Tukey's test.

Figure 5. DCFH oxidation in young (A) and adult (B) cerebral cortices (n=6). Data are expressed as percent of control group (0 μM SFN, 100%). Statistical analysis performed with One Way Anova followed by Tukey's test.

Table 1. Na^+, K^+ ATPase activity in young and adult cerebral cortices (n=6). Data are expressed as mean± standard deviation. Statistical analysis performed with One Way Anova followed by Tukey's test and Pearson's Correlation. Different letters means $P<0.05$ among groups.

Table 2. Glutamine Synthetase activity in young and adult cerebral cortices (n=6). Data are expressed as mean± standard deviation. Statistical analysis performed with One Way Anova followed by Tukey's test. # indicates $P=0.06$ (trend) compared to control group (0 μM SFN).

Figure 1

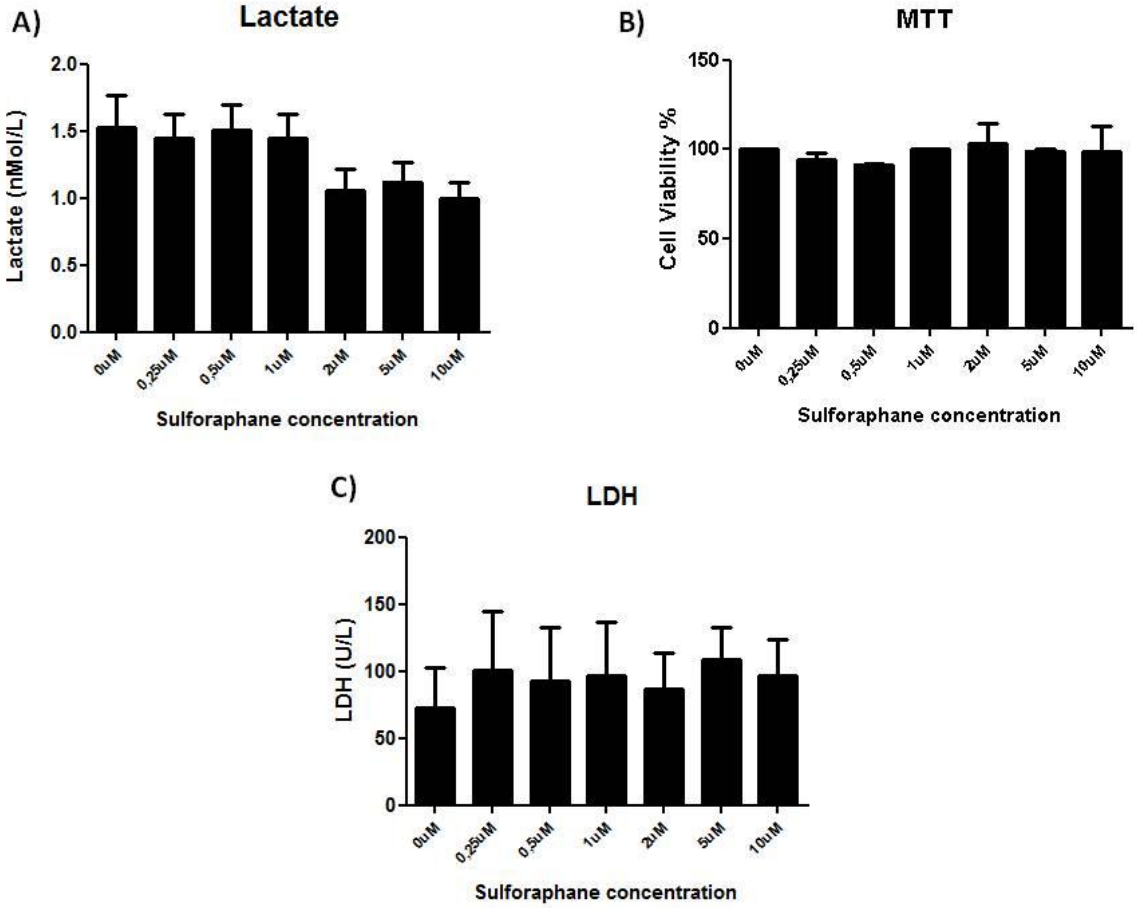
A)
Lactate
Lactate (nMol/L)
2.0
1.5
1.0
0.5
0.0
0uM
0,25uM
0,5uM
1uM
2uM
5uM
10uM
Sulforaphane concentration
B)
MTT
Cell Viability %
150
100
50
0
0uM
0,25uM
0,5uM
1uM
2uM
5uM
10uM
Sulforaphane concentration
C)
LDH
LDH (U/L)
200
150
100
50
0
0uM
0,25uM
0,5uM
1uM
2uM
5uM
10uM
Sulforaphane concentration

Figure 2

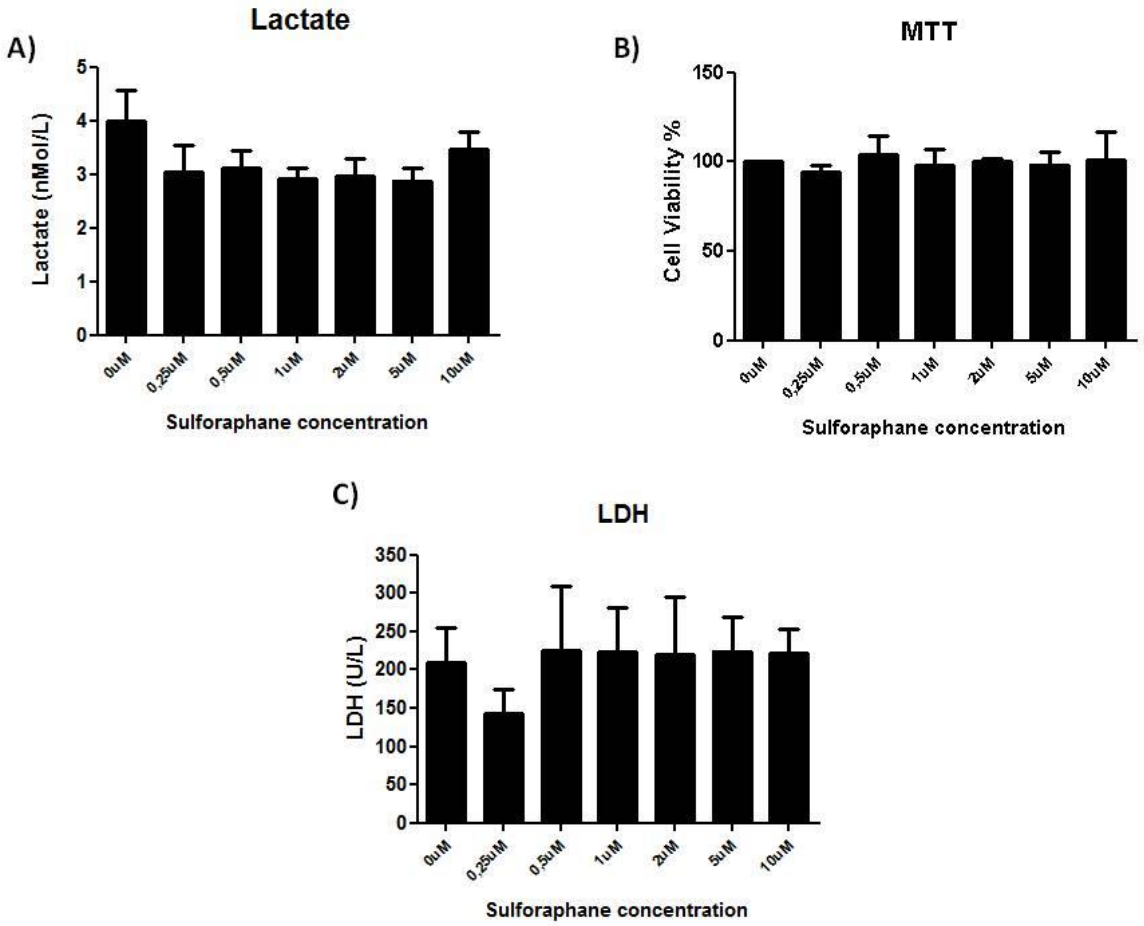
A)
Lactate
Lactate (nMol/L)
0
1
2
3
4
5
0uM
0,25uM
0,5uM
1uM
2uM
5uM
10uM
Sulforaphane concentration
B)
MTT
Cell Viability %
0
50
100
150
0uM
0,25uM
0,5uM
1uM
2uM
5uM
10uM
Sulforaphane concentration
C)
LDH
LDH (U/L)
0
50
100
150
200
250
300
350
0uM
0,25uM
0,5uM
1uM
2uM
5uM
10uM
Sulforaphane concentration

Figure 3

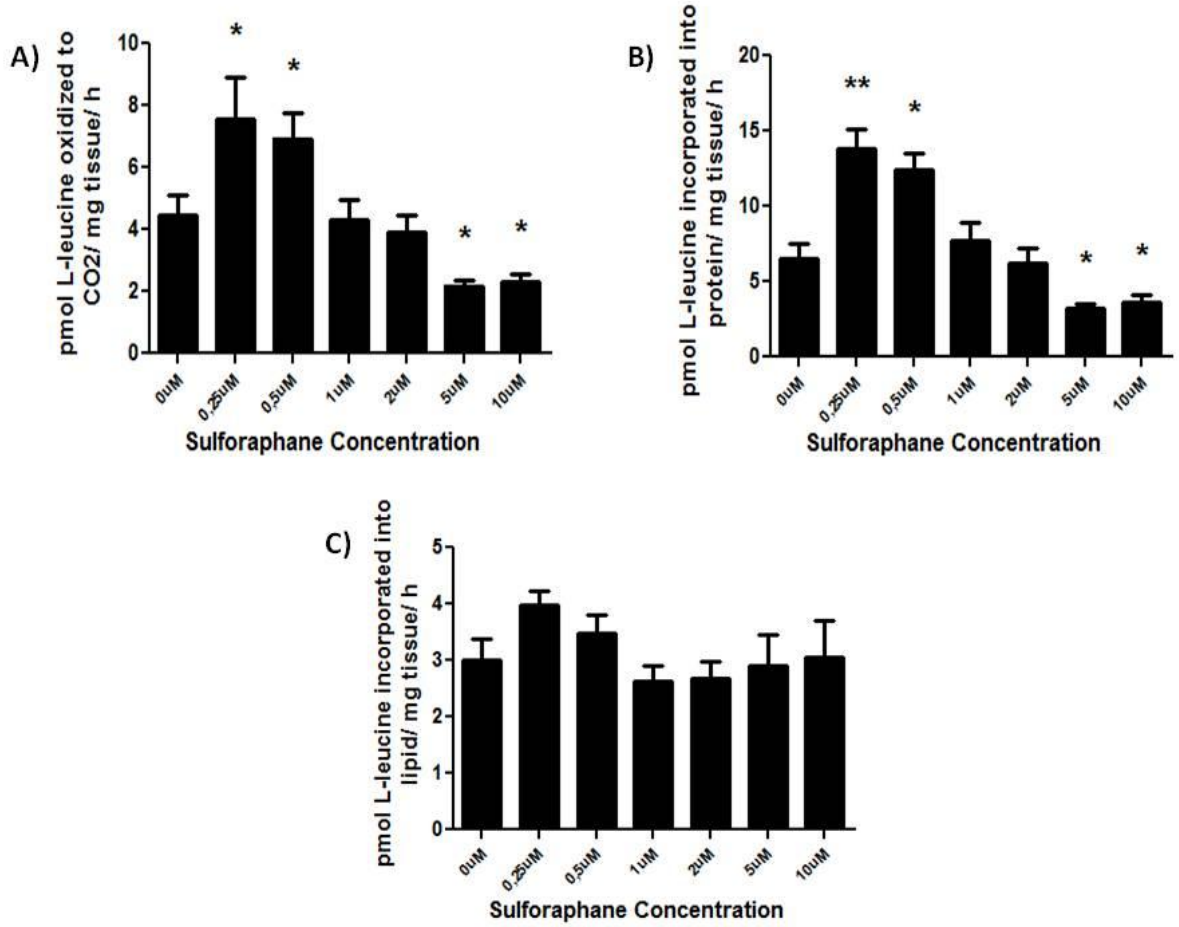
A)
pmol L-leucine oxidized to CO2/ mg tissue/ h
0
2
4
6
8
10
*
*
*
*
0uM
0,25uM
0,5uM
1uM
2uM
5uM
10uM
Sulforaphane Concentration
B)
pmol L-leucine incorporated into protein/ mg tissue/ h
0
5
10
15
20
**
*
*
*
0uM
0,25uM
0,5uM
1uM
2uM
5uM
10uM
Sulforaphane Concentration
C)
pmol L-leucine incorporated into lipid/ mg tissue/ h
0
1
2
3
4
5
0uM
0,25uM
0,5uM
1uM
2uM
5uM
10uM
Sulforaphane Concentration

Figure 4

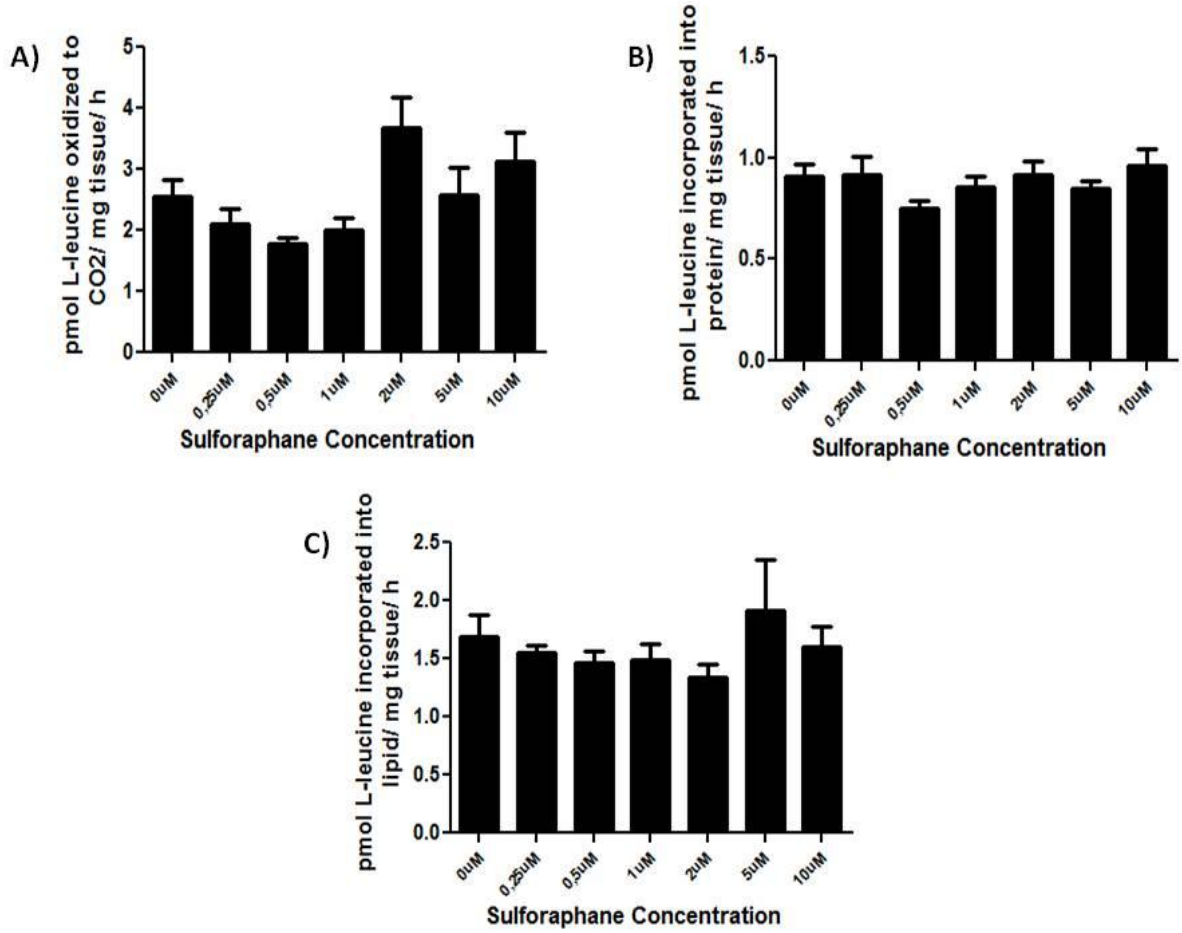
A)
pmol L-leucine oxidized to CO2/ mg tissue/ h
0
1
2
3
4
5
0uM
0,25uM
0,5uM
1uM
2uM
5uM
10uM
Sulforaphane Concentration
B)
pmol L-leucine incorporated into protein/ mg tissue/ h
0.0
0.5
1.0
1.5
0uM
0,25uM
0,5uM
1uM
2uM
5uM
10uM
Sulforaphane Concentration
C)
pmol L-leucine incorporated into lipid/ mg tissue/ h
0.0
0.5
1.0
1.5
2.0
2.5
0uM
0,25uM
0,5uM
1uM
2uM
5uM
10uM
Sulforaphane Concentration

Figure 5

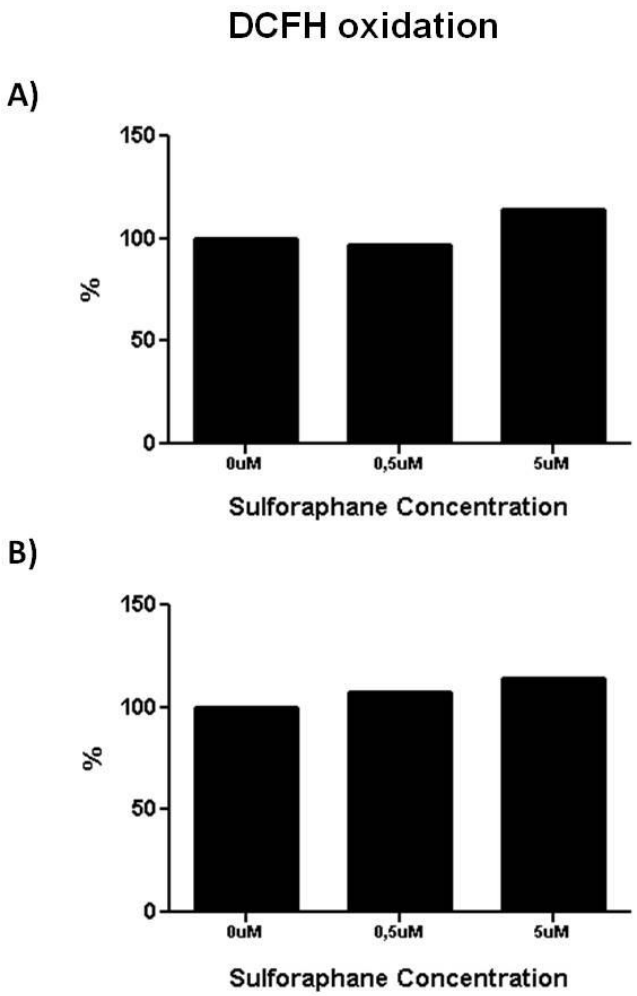
DCFH oxidation
A)
150
100
50
0
%
0uM
0,5uM
5uM
Sulforaphane Concentration
B)
150
100
50
0
%
0uM
0,5uM
5uM
Sulforaphane Concentration

Table 1. Na^+, K^+ ATPase activity in young and adult brain cortices.

SFN concentrations	0 μM	0,5 μM	5μM	Correlation
Young animals (nmol Pi/ min/protein mg)	111±19	113±22	219±27*	r=0.9, p<0.01
Adult animals (nmol Pi/ min/ protein mg)	90±10	171±10*	236±47*	r=0.8, p<0.01

Table 2. Glutamine synthetase activity in young and adult brain cortices

SFN concentrations	**0 μM**	**0,5 μM**	**5μM**
Young animals (μmol/h/mg protein)	0.21±0.02	0.18±0.02	0.16±0.03[#]
Adult animals (μmol/h/mg protein)	0.73±0.03	0.72±0.05	0.70±0.05

Capítulo 3

Metabolic effects of sulforaphane oral treatment in streptozotocin-diabetic rats.

(*aceito para publicação no periódico Journal of Medicinal Food*)

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ORIGINAL ARTICLE

Metabolic Effects of Sulforaphane Oral Treatment in Streptozotocin-Diabetic Rats

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ABSTRACT Diabetes has reached epidemic levels in the whole world, and the use of bioactive compounds that may have the capacity to prevent and treat diabetes is of great interest. Sulforaphane (SFN) is a compound which is found in cruciferous vegetables and that acts as both a potent antioxidant and regulator of gene expression. The aim of this study was to evaluate the effect of SFN in diabetes induced by streptozotocin (STZ). Male Wistar rats were gavaged with water or 0.1, 0.25, or 0.5 mg/kg of SFN before an injection of STZ (80 mg/kg). Animals treated with SFN showed fasting glycemia, insulin sensitivity, and hepatic glycogen concentrations, similar to the control group (nondiabetic), and different from the diabetic group. Diabetic animals also presented elevated levels of serum triacylglycerols (TAG), urea, and creatinine, and all SFN doses were able to reverse these alterations. However, the same doses of SFN accentuated alterations in total cholesterol, alanine, and aspartate aminotransferase levels, and had no effect on hepatic TAG, HDL cholesterol, and uptake of 2-deoxy glucose in adipose tissue and soleum muscle. Based on the effects inferred by the present data, SFN presented some positive effects against diabetes induction, although the impairment of hepatic function and cholesterol levels were aggravated after treatment with the compound.

KEY WORDS: ***diabetes mellitus*** • ***streptozotocin*** • ***sulforaphane***

INTRODUCTION

DIABETES MELLITUS (DM) is a set of alterations that culminates in hyperglycemia, and the progress of these alterations is directly related to pancreatic β-cell loss of function.[1] Epidemiological studies have shown that the incidence of DM patients is rising at a pandemic level worldwide, and it is estimated that by the year 2025, more than 300 million people will be inflicted.[2] As a consequence, hyperglycemia promotes impairment of the liver, kidneys, eyes, vascular system, peripheral nerves, and even brain functions.[3–5] Abnormalities in lipid metabolism are also a very common feature of diabetes, generating an appropriate environment for atherosclerosis, most of the time due to elevated levels of cholesterol, triacylglycerols (TAG), and lower levels of HDL cholesterol presented by diabetic individuals.[6–8]

In the face of this scenario, there is an increased scientific interest about bioactive compounds that may have the capacity to prevent either the occurrence of diabetes or the disease progression.[9–11] Sulforaphane (SFN) is a glycoside sulphide belonging to the isothiocyanates group that is found in cruciferous vegetables, mostly broccoli and cauliflower. Isothiocyanates are a class of anticarcinogenic phase II enzyme inducers, responsible by detoxification processes,[12,13] that act as activators of transcription factor NF-E2–related factor-2 (Nrf2) which regulates gene expression through the promoter antioxidant response element. Nrf2 regulates the transcription of protective and metabolic enzymes such as glutathione reductase and NADPH-quinone oxireductase.[14] Although most of the evidence of SFN benefits is related to the prevention and inhibition of the carcinogenesis process,[15–17] recent data show other functions related to this compound, such as antiinflammatory, antiapoptotic, cardiac, and neuroprotective properties, besides preserved pancreatic function in streptozotocin (STZ)-diabetic rats.[13,18,19]

Based on these data, the aim of this study was to evaluate the effect of SFN oral administration in the clinical parameters of diabetes management in a rat experimental model of this disease.

MATERIALS AND METHODS

Reagents

R, S -SFN was purchased from LKT Laboratories (St. Paul, MN). STZ and glycogen was obtained from Sigma Chemical Co. (St. Louis, MO). 2-[U-14C]-Deoxy-D-Glucose (specific activity 308 mCi/mmol) was obtained from

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Amersham International (Buckinghamshire, UK). Optiphase Hi-Safe 3 was purchased from Perkin-Elmer (Boston, MA). All other chemicals used were obtained from Merck S.A., Porto Alegre, Brazil. Diagnostic kits were obtained from Labtest® (Lagoa Santa, MG, Brazil).

Animals and treatments (type 1 diabetes induction)

Sixty-day-old male Wistar rats were obtained from the Central Animal House of the UFRGS Biochemistry Department, the Federal University of Rio Grande do Sul, Brazil. They were maintained under a standard dark-light cycle (lights on between 7:00 a.m. and 7:00 p.m.) at a room temperature of 22°C ± 2°C and received a standard commercial diet and water *ad libitum*. Animal care followed the official governmental guidelines in compliance with the Federation of Brazilian Societies for Experimental Biology and was approved by the ethics committee of the Federal University of Rio Grande do Sul, Brazil.

To determine the effects of SFN, the rats were randomly divided into five groups: a control (CT) group, an STZ group, and three STZ groups pretreated with different SFN concentrations. During 3 days before diabetes induction, the rats were daily gavaged either with 300 μL of deionized water (CT and STZ group) or with 0.1, 0.25, and 0.5 mg/kg of SFN diluted in deionized water (SFN0.1, SFN0.25, and SFN0.5 groups, respectively). On the 4th day, in order to induce diabetes, the rats were intraperitoneally injected either with saline (CT group) or with STZ 80 mg/kg of body weight dissolved in saline (STZ, SFN0.1, SFN0.25, and SFN0.5 groups) (Sigma, St. Louis, MO), after an overnight fasting. The diabetes induction was confirmed 48 h after the injection, and only animals with a blood glucose ≥250 mg/dL^{-1} were considered diabetic.[20] The duration of the study was set as 10 days to evaluate the effects of SFN pretreatment on the acute alterations of the disease.

Insulin responsiveness test

An insulin responsiveness test (IRT) was performed 9 days after diabetes induction and before the test rats were fasted for 6 h. Subsequently, they were intraperitoneally injected with an insulin solution (Humulin; Lilly France S.A., Fergesheim, France) at a dose of 1 U/kg body weight. Blood samples were obtained from the cut tip of the tail immediately before, as well as 30, 60, and 120 min after the injection. The glycemia was determined using a glucometer (Accu-Chekt Active; Roche Diagnostics, Mannheim, Germany).

Blood and tissue preparation

One day after the IRT animals had been killed by decapitation, blood samples were immediately collected and centrifuged at 5000 *g* for 10 min to obtain serum. Liver, retroperitoneal and epididymal adipose tissue, and soleus skeletal muscle were dissected, weighted, and samples of these tissues were separated to perform a posterior specific analysis.

Blood biochemical parameters

Serum levels of total cholesterol, HDL cholesterol, TAG, urea, creatinine, and enzymatic activities of alanine aminotransferase (ALT) and aspartate aminotransferase (AST) were measured with commercial kits (Labtest- Lagoa Santa, MG, Brazil).

Hepatic and muscular glycogen concentration

Strips of soleus muscle weighting about 70–80 mg and samples of liver weighting about 150 mg were used to determinate hepatic and muscular glycogen concentrations, which were measured by the Krisman Method.[21]

Uptake of 2-deoxy glucose in soleus skeletal muscle and adipose tissue

Strips of soleus muscle weighting about 70–80 mg and samples of retroperitoneal adipose tissue weighting 200 mg were incubated at 37°C with gentle agitation for 30 min in KRB at pH 7.4, which was earlier oxygenated (95% O_2:5% CO_2 mixture for 1.0 min) and contained 5 mM glucose and 0.1 μCi 2-[U-14C]-Deoxy-D-Glucose. The medium was sparged with 95% O_2:5% CO_2 throughout the incubation to prevent tissue hypoxia. After incubation, the reaction was stopped by placing the flasks on ice (4°C). The incubation system was removed, and the slices were washed thrice with 1.0 mL KRB. The slices were transferred to tubes and centrifuged at 2000 *g* for 10 min. The supernatant was removed, and 0.2 mL of a 2 M NaOH solution was added to the pellet. After the tissue homogenization, the scintillation liquid (Optiphase HiSafe®) was added, and the radioactivity incorporation was measured in a Wallac scintillation counter (Turku, Finland).

Statistical analysis

Data were analyzed with SPSS 17.0 software. An insulin tolerance test was analyzed by Repeated Measures analysis of variance (ANOVA) and *post-hoc* Bonferroni's test. Parametric variables were tested by one-way ANOVA and *post-hoc* Tukey's test, and nonparametric variables were tested by the Kruskall–Wallis test. Results are expressed as either mean ± standard deviation or median (min-max).

RESULTS

Body composition, glycemia, and insulin sensitivity

Table 1 shows the body composition and pre- and post-induction fasting glycemia of the CT, STZ, and SFN (SFN0.1, SFN0.25, and SFN0.5) groups. There was no difference in body weight among the groups before diabetes induction, and even after all the groups had presented an increment of it after 10 days, the final weight was lower in the STZ, SFN0.1, SFN0.25, and SFN0.5 groups compared with the CT group ($P < .01$). A slight difference was observed in the SFN0.25 and SFN0.5 groups, which showed a higher body weight than the STZ and SFN0.1 groups

TABLE 1. BODY COMPOSITION, PRE- AND POSTINDUCTION GLYCEMIA OF CONTROL, STREPTOZOTOCIN, AND SULFORAPHANE GROUPS ($N=8$)

	CT	*STZ*	*STZ+SFN 0.1*	*STZ+SFN 0.25*	*STZ+SFN 0.5*
Initial body weight (g)	238±17	230±28	213±21	218±35	233±33
Final body weight (g)	313±14[a]	233±32[b]	240±34[b]	267±35[c]	266±24[c]
Retroperitoneal adipose tissue (g)	3±1[a]	1 (0.1–2.0)[b]	5±2[a]	3 (1.8–6.0)[a]	3±1[a]
Epididymal adipose tissue (g)	4±1[a]	2±1[b]	5±1[a]	5±1[a]	5±1[a]
Liver weight (g)	14±1[a]	9±1[b]	11±1[b]	12±2[b,c]	12±1[b,c]
Preinduction glycemia (mg/dL^{-1})*	89±12	88±13	87±14	89±14	96±15
Postinduction glycemia (mg/dL^{-1})*	104±11[a]	415±63[b]	119±55[a]	110±30[a]	122±33[a]

Data are expressed as mean±standard deviation or median (min-max). Statistical analysis performed with one-way ANOVA followed by either Tukey's test or Kruskall–Wallis test.

*Fasting.

[a,b,c]Different letters mean $P<.05$ among groups.

CT, control; SFN, sulforaphane; STZ, streptozotocin; ANOVA, analysis of variance.

($P<.05$). A comparison of the initial and final body weight resulted in the gain being only significant in CT ($P<.05$), although a trend could be observed in SFN0.25 and SFN0.5 ($P=.07$) (data not shown). The other groups exhibited some negative values related to this parameter because of some animals that did not gain weight.

With regard to retroperitoneal and epididymal adipose tissue, CT and all STZ+SFN groups were different from the STZ group, which had epididymal fat twofold lower than the other groups ($P<.01$). Liver weight also was lower in the STZ group compared with the other groups ($P<.05$) and again, SFN0.25 and SFN0.5 presented a subtle difference from the STZ and SFN0.1 groups ($P<.05$). Preinduction glycemia was measured in order to test whether there was any difference in this parameter among the groups before the STZ injection or SFN treatment, which was not observed ($P>.05$). Postinduction fasting glycemia was used to detect the animal sensibility to the treatment and to confirm diabetes diagnosis, which could be observed in the glycemia threefold higher in the STZ group compared with the CT and all STZ+SFN groups ($P<.01$). A test between pre- and postinduction fasting glycemia in the same group was also performed, and although all of them had an increase of this parameter, only STZ presented an elevated glycemia that was statistically significant (data not shown) ($P<.01$).

In order to test insulin sensitivity, the animals were submitted to IRT and at time 0, before an insulin injection, the STZ group showed glucose levels that were almost 300% higher than the other groups (Fig. 1A, $P<.01$). The difference between the STZ×CT and STZ×STZ+SFN groups was sustained during 30, 60, and 120 min after the injection ($P<.01$ along the test), and there were neither differences in all glycemic measures between the CT and STZ+SFN-treated groups nor differences between the SFN doses, which can be confirmed by the area under the curve (Fig. 1B, $P>.05$).

Glycogen concentration and 2-Deoxy Glucose uptake

Glycogen concentration was measured to test this model of diabetes and the possible effect of SFN; once untreated, diabetic subjects usually present lower levels of this energetic substrate, especially in the liver.[22,23] According to this, hepatic glycogen was decreased in STZ compared with the CT, SFN0.1, SFN0.25, and SFN0.5 groups (Fig. 2A, $P<.05$), and no differences among the other groups were found (Fig. 2A, $P>.05$). On the other hand, no differences

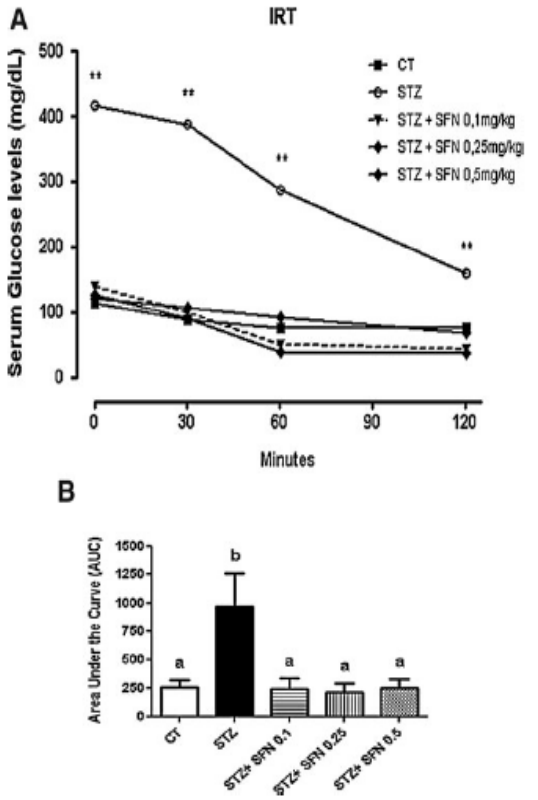

FIG. 1. Insulin Responsiveness Test (IRT) of groups ($n=8$). For IRT, blood glucose was measured before (0) and 30, 60, and 120 min after an insulin injection (1 UI/kg body weight). Data are expressed as mean±standard deviation of the groups. Statistical analysis was performed with Repeated Measures analysis of variance (ANOVA) followed by Bonferroni's test. $^{**}P<.01$ among groups. CT, control; SFN, sulforaphane; STZ, streptozotocin.

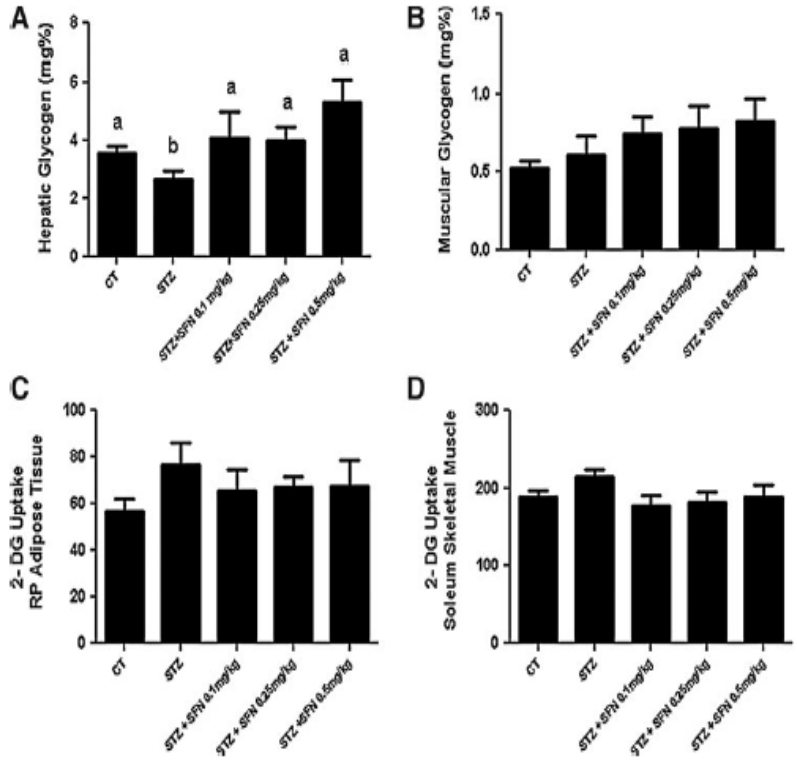

FIG. 2. Glycogen concentration and 2-Deoxy-Glucose (2-DG) uptake of the groups ($n=8$). **(A)** Hepatic glycogen concentration; **(B)** Muscular glycogen concentration; **(C)** Retroperitoneal (RP) Adipose Tissue 2-DG Uptake; **(D)** Soleus Muscle DG Uptake. Data are expressed as mean ± standard deviation. Statistical analysis was performed with one-way ANOVA, followed by Tukey's test. [a,b]Different letters mean $P<.05$ among the groups.

could be observed in muscular glycogen compared with the CT group (Fig. 2B, $P>.05$), although the concentration increased according to the SFN dose but without a statistically significant difference.

2-deoxy glucose uptake was verified to infer the adipose tissue and muscle ability after SFN treatment and diabetes induction once these tissues are insulin dependent to uptake glucose. Retroperitoneal adipose tissue did not show any statistical differences among the groups (Fig. 2C, $P>.05$), although the STZ group showed an increase in this parameter compared with the CT group. This same pattern occurred in 2-deoxy glucose uptake in the soleus skeletal muscle of the STZ group, which was slightly higher than the CT group, but was not different from all the STZ+SFN groups (Fig. 2D, $P>.05$, respectively) and again without a statistically significant difference between all of them.

Biochemical parameters

Considering the most common comorbidities of diabetes, we evaluated the lipid profile, hepatic, and kidney markers of the animals after the treatments. The STZ group presented elevated levels of total cholesterol, compared with the control, and SFN doses were not able to reverse this alteration, once the SFN0.1, SFN0.25, and SFN0.5 groups were different from CT (Table 2, $P<.05$). However, the opposite was observed in serum TAG levels, where 0.1, 0.25, and 0.5 mg/kg of SFN improved this parameter compared with the STZ group, being similar to CT (Table 2, $P<.05$ and $P>.05$ respectively); while HDL cholesterol did not vary among them. Although hepatic TAG showed no differences, ALT and AST activity was twofold higher in the STZ and all STZ+SFN groups compared with CT (Table 2, $P<.05$). With regard to kidney function, the urea levels of SFN0.1, SFN0.25, and SFN0.5 were lower than the CT and STZ groups, which did not differ (Table 2, $P<.05$ and $P>.05$, respectively), and creatinine levels were increased in only the STZ group compared with the others (Table 2, $P<.05$), with all the STZ+SFN groups being similar to the CT group.

DISCUSSION

Diabetes care focuses primarily on glucose control and lipid lowering, in order to avoid the disease progression and the major deleterious consequences of it, such as liver dysfunction, diabetic nephropathy, and cardiovascular disease. In this way, research conducted on bioactive compounds that could exert positive effects against those alterations is very relevant. Due to this, the aim of this study was about SFN effects on the main markers of diabetes clinical treatment. Moreover, to our knowledge, there are no studies that evaluate oral SFN treatment and its effects with regard to these same parameters.

In this diabetes model, the STZ+SFN-treated groups presented a positive effect in glycemia by the administration of this compound before the STZ injection, which is cytotoxic to β-cells and can be used to induce experimental diabetes in rodents.[24,25] Fasting glycemia and the glycemic curve from the IRT of SFN-treated groups proposes a preservation of pancreatic β-cells, once these two parameters are standard methods of determining glucose

TABLE 2. BIOCHEMICAL PARAMETERS ($N=8$)

	CT	*STZ*	*STZ+SFN 0.1*	*STZ+SFN 0.25*	*STZ+SFN 0.5*
Total cholesterol (mg/dL^{-1})	45±6[a]	67±10[b]	78±13[b]	109±33[b,c]	154±17[b,c]
HDL cholesterol (mg/dL^{-1})	30±3[a]	29±2[a]	29±4[a]	29±2[a]	29±3[a]
Serum TAG (mg/dL^{-1})	108±25[a]	187±58[b]	148±29[a]	115±36[a,c]	101±31[a,c]
Hepatic TAG (mg%)	1.2±0.4[a]	1.9±0.9[a]	1.9±0.3[a]	2.4±1[a]	1.9±0.5[a]
ALT (IU/L)	5±1[a]	13±2[b]	16±2[b]	13±2[b]	13±2[b]
AST (IU/L)	10±6[a]	18±3[b]	21±3[b]	23±4[b]	26±4[b]
Urea (mg/dL^{-1})	129±11[a]	105±19[a]	99±21[b]	82±21[b]	74±20[b]
Creatinine (mg/dL^{-1})	3.1±0.8[a]	5.3±1.7[b]	3.0±1.4[a]	2.3 (0.8–4.1)[a]	2.1 (1.3–3.6)[a]

Data are expressed as mean±standard deviation or median (min-max). Statistical analysis performed with one-way ANOVA or Kruskall–Wallis test.
[a,b,c]Different letters mean $P<.05$ between groups.
HDL, high-density lipoprotein; TAG, triacylglycerols; ALT, alanine aminotransferase; AST, aspartate aminotransferase.

intolerance and diabetes diagnosis.[26,27] To perform the glycemic curve, we choose not to perform a glucose tolerance test, because the STZ group already has fasting hyperglycemia without a glucose load. The SFN mechanism of action on glucose metabolism was demonstrated by Song *et al.*,[18] in a model of diabetes induced with STZ, which presented a decrease in reactive oxygen species production and an inhibition of the NF-κB pathway in the pancreas, thus preserving insulin secretion.

Weight loss is very common in decompensate diabetes, and decreased body weight in diabetic rats suggests the degradation of structural proteins that is possibly due to high levels of counter-regulatory hormones,[28,29] which also could explain the lower liver weight presented by the STZ and SFN groups, once not only structural proteins but also visceral proteins are susceptible to degradation.[30] With regard to this, SFN was not able to reverse these alterations in any group. Most of the times, protein degradation is followed by an increased lipolysis to sustain gluconeogenesis, decreasing adipose tissue, such as presented by the STZ group. However, all SFN doses succeeded to prevent the loss of retroperitoneal and epididymal adipose tissue, which were similar to the CT group.

To sustain hormonal catabolic effects, hepatic glycogen is an important key for the maintenance of blood glucose, and a decrease of it is an expected alteration that is related to the lack of insulin,[22,23] as can be observed in the STZ group. Lower levels of hepatic glycogen accentuate the degradation of body proteins, which are known to contribute as an energy source in gluconeogenesis according to the carbohydrates that are becoming unavailable.[31] This information corroborates to the final body weight and hepatic glycogen level presented by the STZ group. The three doses of SFN prevent this decrease, keeping the hepatic glycogen levels of these groups not only similar to the CT group, but also a little higher. In this way, it is known that some phytochemicals are capable of preventing the conversion of glucose-6-phosphate to glucose by the inhibition of glucose-6-phosphatase, preserving the glycogen concentration.[20,32,33] Surprisingly, no differences were found in muscular glycogen between the groups, as muscular glycogen usually decreases in animal models of diabetes using STZ,[34,35] and we assume that these levels do not decline in the STZ group due to the time of the disease (10 days), which is a limitation of our study, because it is shorter than most of the studies that quantify muscular glycogen in rodent models.[35,36] Regardless, the muscular glycogen content in the STZ+SFN groups slightly increased according to the increase in the SFN dose.

Another unexpected response was the absence of alterations in glucose uptake in the adipose tissue and soleus muscle in diabetic animals. Commonly, untreated diabetes impairs the ability of adipose tissue and muscle to uptake glucose properly due to the glucose transporter 4 present in them that enables them to be insulin dependent.[37,38] In this sense, a work by Cameron-Smith *et al.*[39] demonstrated that the *in vivo* muscular and adipose tissue uptake of 2-Deoxy-Glucose (2-DG) in STZ-diabetic rats can be higher than that in nondiabetic rats, proposing that this may occur in an attempt to compensate the alterations in insulin action.

Besides changes in glucose metabolism, other common features of diabetes are dyslipidemia, hepatic dysfunction, and nephropathy.[30,40,41] Unlike what was expected, SFN doses increased total cholesterol, ALT and AST enzymatic activity and decreased urea levels. The frequent elevation of transaminases levels is a sign of liver diseases,[41] and most of the time, hepatic dysfunction in diabetes occurs due to nonalcoholic fatty liver disease, mediated by the accumulation of TAG in the liver, which we could not observe. In the same way, the dose-dependent decrease of urea below CT levels could be considered indicative of the impairment of the urea cycle, as an accentuated decline of this metabolite could be related to hepatotoxicity.[42] We suggest that almost all these negative effects on total cholesterol, transaminases, and urea levels could be modulated by the association of STZ+SFN, as only STZ increases total cholesterol and ALT and AST levels besides decreasing urea levels, and the phytochemical may enhance this effect. Once our research group used another model of diabetes induction with a highly palatable diet for 4 months, concomitantly to SFN 1 mg/kg oral treatment daily, and this model exhibited no differences between the groups with regard to any of these same parameters (Souza *et al.*, unpublished data).

On the other hand, SFN was efficient to decrease serum TAG in a dose-dependent way, while HDL cholesterol, a protective molecule in the cardiovascular system, was apparently not affected by either STZ or SFN administration. All SFN doses demonstrated a protective effect against the toxicity of hyperglycemia in the kidney by lowering creatinine levels, which is very promising, because nephropathy is one of the most prevalent consequences of decompensate diabetes.[3,29] A recent work by Zheng *et al.*[43] showed that the molecular mechanism of SFN protection against diabetic nephropathy is through Nrf2 activation of antioxidant enzymatic defenses (NADPH-quinone oxireductase and γ-glutamylcysteine synthetase) and suggests that this same pathway could exert an insulin-independent action in the reduction of blood glucose.

In summary, our study presents some beneficial effects of SFN in the prevention of diabetes that is induced by STZ related to the maintenance of glycemic levels, hepatic glycogen, and kidney function which are similar to nondiabetic animals, besides the amelioration of serum TAG levels. The duration of diabetes in this study was not only a limitation in some parameters, such as muscular glycogen and 2-DG uptake in the adipose tissue and muscle, but it was also an attempt to understand the acute effects of diabetes on this model of prevention. The negative findings about total cholesterol, transaminases activity, and serum urea levels are possibly due to the enhancer effect of SFN on STZ derangements, once that in another model of diabetes induction without STZ and even with a higher dose of SFN, during more time of treatment, none of these alterations could be observed. More studies are required to fully explain the molecular mechanisms of SFN in peripheral tissues, although some positive effects could be inferred by the present data.

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AUTHOR DISCLOSURE STATEMENT

No competing financial interests exist.

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Capítulo 4

Sulforaphane ameliorates insulin responsiveness and lipid profile but do not increase enzymatic antioxidant response in diabetic rats.

(*artigo em construção*)

Sulforaphane ameliorates insulin responsiveness and lipid profile but do not increase enzymatic antioxidant response in diabetic rats.

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Abstract

Diabetes is one of the most prevalent chronic noncommunicable diseases and is characterized by hyperglicemia and increased oxidative stress. These two alterations are also responsible for main diabetic complications: cardiovascular disease, retinopathy, nephropathy and peripheric neuropathy. Diabetes progression is determinated by pancreatic β-cell failure, and recent studies shown that sulforaphane (SFN) is able to prevent this, preserving insulin production. Based on this, our goal was to test SFN effects on metabolic parameters related to diabetic complications and antioxidant defenses (SOD, CAT and SH groups) in pancreas, liver and kidney of non-diabetic and diabetic rats. Male Wistar rats were treated with water or SFN 0.5mg/kg i.p. during 21 days, after diabetes induction. Diabetic animals treated with SFN had levels of total cholesterol, Non-HDL cholesterol and TAG similar to non-diabetic and insulin responsiveness was higher than diabetic animals who did not receive the compound. No effect of SFN on SOD and CAT activity or SH groups was observed on pancreas, liver or kidney of treated animals. We conclude that SFN ameliorates some aspects involved in diabetes complications like lipid profile and insulin responsiveness, but not modulates antioxidant response exerted by SOD, CAT or SH groups on the structures evaluated.

Introduction

Modifications in life style and eating habits in the latter half of last century triggered changes on nutritional and epidemiological profile around the world, raising the prevalence of chronic noncommunicable diseases (CNCD). Such diseases include hypertension, cardiovascular disease, cancer and diabetes, among others (Truglio et al 2012).

Diabetes mellitus (DM) is one of the main CNCDs and is characterized by hyperglycemia resulting from decreased insulin production (type 1 DM) or lack of insulin action (type 2 DM) (ADA, 2008). Persistent hyperglycemia (untreated or decompensated) promotes oxidative stress, which is the basis of major diabetic complications, like atherosclerosis, neuropathy, nephropathy and retinopathy due to inability of these tissues to handle higher glucose levels inside the cells (Brownlee, 2005).

In both types of DM β-cell failure is the determinant of disease progression (DeFronzo, 2009). The loss of those cells is mainly mediated by inflammatory cytokines (IL-1, TNF-α), with activation of inflammatory pathways like NF-κB, which promotes increase of reactive species production (In't Veld, 2011). In this way, a recent work from Song and colleagues (2008) demonstrated that treatment with sulforaphane (SFN), a phytochemical compound, was able to prevent DM development by inhibition of NF-κB pathway and decrease oxidative stress.

SFN is a potent antioxidant which acts as inducer of phase II detoxification enzymes - NADPH quinoneoxidereductase (NQO1), hemeoxygenase-1 (HO-1) and γ-glutamylcysteine ligase (γ-GCL) - and also improve the activity of superoxide dismutase (SOD) and catalase (CAT) (Guerrero-Beltrán et al., 2012). Based on the described effects, the aim of this study was to test SFN treatment on metabolic parameters related to diabetic complications and antioxidant defenses of main organs affected by hyperglycemia in diabetic rats.

Materials and Methods

Reagents

R, S -Sulforaphane was purchased from LKT Laboratories (St. Paul, MN). Streptozotocin was obtained from Sigma Chemical Co. (St. Louis, MO). 2-[U-14C]-Deoxy-D-Glucose (specific activity 308 mCi/mmol) was obtained from Amersham International (Buckinghamshire, UK). Optiphase Hi-Safe 3 was purchased from Perkin-Elmer (Boston, MA, USA). All other chemicals used were obtained from Merck S.A., Porto Alegre, Brazil. Diagnostic kits were obtained from Labtest® (Lagoa Santa, MG, Brazil).

Animals and Treatments (type 1 diabetes induction and SFN administration)

60-day old male Wistar rats were obtained from the Central Animal House of UFRGS Biochemistry Department, Federal University of Rio Grande do Sul, Brazil. They were maintained under a standard dark-light cycle (lights on between 7:00 a.m. and 7:00 p.m.) at a room temperature of 22 $\pm$ 2°C and received a standard commercial diet and water *ad libitum*. Animal care followed the official governmental guidelines in compliance with the Federation of Brazilian Societies for Experimental Biology and was approved by the Ethics Committee of the Federal University of Rio Grande do Sul, Brazil.

To determine the effects of SFN on diabetes treatment, rats were randomly divided into 4 groups: a control non-diabetic group (CT, n=8), a sulforaphane non-diabetic group (SFN, n=8), a diabetic group (STZ, n=7) and a sulforaphane diabetic group (SS, n=8). Diabetes induction was carried out with a single intraperitoneal (i.p.) injection of streptozotocin (60 mg/kg) dissolved in citrate buffer pH 7.4 (diabetic groups) or just citrate buffer (non-diabetic groups), after an overnight fasting. The diabetes induction was confirmed 48 hours after the injection and only animals with blood glucose $\geq$ 250mg/dL^{-1} were consider diabetic (Fernandes et al., 2010). After that, CT and

STZ groups were treated daily with i.p. injection of deionized water and SFN and SS groups were treated with i.p. injection of SFN 0.5mg/kg, during 21 days.

Insulin Responsiveness test (IRT)

Insulin responsiveness test (IRT) was performed 20 days after diabetes induction and before the test rats were fasted for 6h. Subsequently, they were injected intraperitoneally with an insulin solution (Humulin; Lilly France S.A., Fergesheim, France) at a dose of 1U/kg body weight. Blood samples were obtained from the cut tip of the tail immediately before, 30, 60 and 120 min after the injection. The glycemia was determined using a glucometer (Accu-Chekt Active; Roche Diagnostics, Mannheim, Germany). The glucose levels were also evaluated by the analysis of the area under de curve (AUC).

Blood and Tissue preparation

One day after IRT animals were killed by decapitation and blood samples were immediately collected and centrifuged at 5000g for 10 minutes to obtain serum. Liver, kidney, pancreas and soleus skeletal muscle were dissected, weighted and samples of these tissues were separated to perform posterior specific analysis.

Blood biochemical parameters

Serum levels of total cholesterol, HDL cholesterol, triacylglycerols (TAG), urea, creatinine and enzymatic activities of alanine aminotransferase (ALT) and aspartate aminotransferase (AST) were measured with commercial kits (Labtest®- Lagoa Santa, MG, Brazil). Non-HDL cholesterol was calculated by subtracting the value of the total cholesterol and HDL cholesterol.

Uptake of 2-Deoxy Glucose in Soleus Skeletal Muscle

Strips of soleus muscle weighting about 70–80 mg were incubated at 37^{o}C with gentle agitation for 30 min in KRB at pH 7.4 that was previously oxygenated (95% O2:5% CO2 mixture for 1.0 min) and contained 5 mM glucose and 0.1 μCi 2-[U-14C]-Deoxy-D-Glucose. The medium was sparged with 95% O^2:5% CO^2 throughout the incubation to prevent tissue hypoxia. After

incubation, the reaction was stopped by putting the flasks on ice (4^{o}C). The incubation system was removed, and the slices were washed 3 times with 1.0 mL KRB. The slices were transferred to tubes and centrifuged at 2000g for 10 min. The supernatant was removed, and 0.2 mL of a 2M NaOH solution was added to the pellet. After the tissue homogenization, the scintillation liquid (Optiphase HiSafe®) was added, and the radioactivity incorporation was measured in a Wallac scintillation counter (Turku, Finland).

Estimation of antioxidant enzyme activities and sulfydril groups

Superoxide dismutase (EC 1.15.1.1) (SOD) activity was assessed by quantifying the inhibition of superoxide-dependent adrenaline autooxidation in a spectrophotometer at 480 nm, as previously described (Misra and Fridovich, 1972) and the results are expressed as units of SOD/mg of protein.

Catalase (EC 1.11.1.6) (CAT) activity was assayed by measuring the rate of decrease in H2O2 absorbance in a spectrophotometer at 240 nm, according to Aebi (1984), and the results are expressed as units of CAT/mg of protein. Bubble formation in oxygen generation by CAT activity was monitored and did not interfere with measurement of CAT activities in the linear range used to measure CAT activity.

Sulfhydryl groups (-SH) level were measured by according to Elman's method (1959), where samples were dilute in 10 mM of PBS and 10 mM boric acid, 0.2 mM EDTA (pH 8.5). Reduced thiol levels were determined by reacting samples with 5-thio-2-nitrobenzoic acid and measuring absorbance at 412 nm (ε412 nm = 27200 M^{-1} cm^{-1}). Results are expressed an nmol -SH/mg protein.

Statistical analysis

Data were analyzed with SPSS 17.0 software. Insulin tolerance test were analyzed by Repeated Measures ANOVA and post-hoc Bonferroni's test. Parametric variables were tested by

One-Way ANOVA and post-hoc Tukey's test and non-parametric variables were tested by Kruskall Wallis's test. Results are expressed as mean ± standard deviation or median (min-max).

Results

As demonstrated on Table 1, body and liver weight of the animals decrease in STZ and SS groups at the end of the treatment compared to CT and SFN ($P<0.05$) as opposed to kidney weight, which was higher in STZ and SS than CT and SFN groups ($P<0.05$).

Lipid profile of diabetic animal was improved by SFN treatment, since total cholesterol, Non-HDL cholesterol and TAG of SS group were lower than STZ and similar to CT and SFN, without differences in HDL-cholesterol between groups (Table 2, $P<0.05$ and $P>0.05$, respectively). About liver and kidney markers, ALT and AST levels did not differ (Table 2, $P>0.05$), but urea levels were elevated in STZ and SS groups, although creatinine was equal (Table 2, $P<0.05$ and $P>0.05$, respectively).

To test insulin sensitivity and evaluate hyperglycemia animals were submitted to IRT and at time 0, before insulin injection, STZ and SS groups showed glucose levels 400% higher than the other groups (Figure 1A, $P<0.01$). After 30 minutes of insulin injection, both STZ and SS remains with very close blood glucose levels and still elevated, but at 60 and 120 minutes SS group had an important decrease of glycemia (Figure 1A, $P<0.01$), which was not followed by STZ. CT and SFN groups remain equal during the whole test and with a glycemia much more lower than the other two. The insulin responsiveness between groups during the test can see on Area Under the Curve calculation (AUC), where STZ and SS were bigger than CT and SFN but still SS was lower than STZ (Figure 1B¸ $P<0.05$). To complement the data about insulin responsiveness, we decided to verify 2DG-glucose uptake by muscle, and we did not observe difference between groups, not even in those who are diabetic (Figure 2, $P>0.05$).

As oxidative stress is a feature of hyperglycemia, we evaluated SOD, CAT and SH groups on pancreas, liver and kidney of the treated animals. Pancreas of STZ group had lower SOD activity, which was not reversed by SFN treatment (Figure 3A, $P<0.05$). Regarding CAT, diabetic state elevated this enzyme activity on pancreas of both diabetic groups, yet without SFN effect (Figure 3B, $P<0.05$), and SH groups were similar between CT, SFN, STZ and SS (Figure 3C, $P>0,05$).

On liver, there was no difference in SOD activity and, opposite to pancreas, diabetic state decreased CAT activity in this organ (Figure 4A, $P>0.05$ and Figure 4B, $P<0,05$, respectively), which was not modified by SFN treatment. Once again, all groups were similar about SH content (Figure 4C, $P>0,05$).

On kidney, SOD activity was equal between groups (Figure 5A, $P>0.05$) and, surprisingly, CAT activity and SH groups were only elevated on SS group (Figure 5B and C, $P<0.05$), while all the other groups had close levels in both measures (Figure 5B and C, $P>0.05$).

Discussion

In the present study we demonstrated that SFN treatment can exert therapeutic effects on insulin responsiveness and lipid profile of diabetic animals even without increase antioxidant defenses on pancreas, which is the determinant organ of DM progression.

Loss of body weight is common in decompensate diabetes, since lack of insulin promotes increase of catabolic hormones and degradation of muscle proteins (Lager, 1991; Shamoon, 1992). The same explanation can be applied to decreased liver weight and the higher levels of urea, without changes in creatinine, once these three alterations were found only on STZ and SS, but not in non-diabetic groups. Another feature of diabetes is elevated kidney weight, also found in STZ and SS, due to glomerular hypertrophy that occurs by effect of decompensate glycemia, increasing glomerular filtration rate and glomerular pression. This increased pression damage the nephrons and

promotes adaptation of the remaining tissue, which works more and gets hypertrophied (Vallon and Thomson, 2012). Unfortunately SFN treatment was not able to ameliorate any of these parameters.

On the other hand, SS group had a better glycemia from the middle to the end of IRT compared to STZ, which proposes that SFN may exert some synergic insulin effect, since the changes on glycemia occur only after insulin injection. Considering that glycemic control is important to prevent diabetic complications and loss of β-cells (DeFronzo, 2009) we can infer that SFN may favor the preservation of pancreatic tissue. To complement results concerning to glycemia, we measure 2DG uptake by soleus muscle, but did not found any differences. A result similar to this was found in another work of our group (Souza et al., unpublished data) were 2DG uptake by soleus muscle of diabetic animals was not different from non diabetic and a hypothesis for that is maybe the tissue try to compensate alterations in insulin action that affects glucose transporter 4 (GLUT4) and normal glucose uptake overexpressing some components of insulin pathway (Cameron-Smith et al., 1997).

Besides ameliorating insulin responsiveness, SFN treatment also prevent dyslipidemia caused by DM keeping total cholesterol, Non-HDL cholesterol and TAG in lower levels. This is an important finding related to prevention of cardiovascular disease, which is the main cause of death in diabetic individuals (Vazzana et al., 2012).

Concerning absence of action on antioxidant defenses measured, the main action of SFN is to induce expression of phase II detoxification enzymes (NQO1, HO-1, γ-GCL), which we not evaluated, although some studies verify increase on SOD and CAT activity by effect of this compound (Guerrero-Beltrán et al., 2012). Lower antioxidant defenses and antioxidant capacity in diabetes is common, considering the pathophysiology of the disease that is based on increased oxidative stress by hyperglicemia (Styskal et al., 2012) and this is why decrease of SOD on pancreas and CAT on liver was not unexpected. The elevated activity of CAT and the SH groups on kidney only of SS group was an intriguing result that needs to be better investigated.

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Legends of tables and figures

Table 1. Body, liver and kidney weight of the CT, SFN, STZ and SS groups (*n=8*). Data are expressed as mean ± standard deviation. Statistical analysis performed with One Way Anova followed by Tukey's test. [a,b] = Different letters mean P< 0.05 among groups.

Table 2. Serum biochemical parameters (*n=8*). Data are expressed as mean ± standard deviation. Statistical analysis performed with One Way Anova followed by Tukey's test. [a,b] = Different letters mean P < 0.05 between groups. HDL = high density lipoprotein; TAG= triacylglycerol; ALT = alanine aminotransferase; AST= aspartate aminotransferase.

Figure 1. Insulin Responsiveness Test (IRT) of groups (*n=8*). For IRT, blood glucose was measured before (0) and 30, 60 and 120 minutes after insulin injection (1UI/kg body weight). Data are expressed as mean± standard deviation of groups. Statistical analysis performed with Repeated Measures Anova followed by Bonferroni's test. [a,b] = Different letters mean P< 0.05 among groups.

Figure 2. 2-Deoxy-Glucose (2-DG) Uptake on soleus muscle of groups (*n=8*). Data are expressed mean ± standard deviation. Statistical analysis performed with One Way Anova, followed by Tukey's test.

Figure 3. Estimation of antioxidant enzyme activities and sulfydril groups on pancreas of groups (*n=8*). A) SOD; B) CAT; C) SH groups. Data are expressed mean ± standard deviation. Statistical analysis performed with One Way Anova, followed by Tukey's test. [a,b] = Different letters mean P< 0.05 among groups. SOD = superóxido dismutase; CAT = catalase; SH = sulfydril.

Figure 4. Estimation of antioxidant enzyme activities and sulfydril groups on liver of groups (*n=8*). A) SOD; B) CAT; C) SH groups. Data are expressed mean ± standard deviation. Statistical analysis performed with One Way Anova, followed by Tukey's test. [a,b] = Different letters mean P< 0.05 among groups. SOD = superóxido dismutase; CAT = catalase; SH = sulfydril.

Figure 5. Estimation of antioxidant enzyme activities and sulfydril groups on kidney of groups (*n=8*). A) SOD; B) CAT; C) SH groups. Data are expressed mean ± standard deviation. Statistical analysis performed with One Way Anova, followed by Tukey's test. [a,b] = Different letters mean P< 0.05 among groups. SOD = superóxido dismutase; CAT = catalase; SH = sulfydril.

Table 1. Body, liver and kidney weight of the CT, SFN, STZ and SS groups.

	CT (n=8)	SFN (n=8)	STZ (n=7)	SS (n=8)
Initial body weight (g)	372±27	342±40	344±26	358±32
Final body weight (g)	407±16[a]	388±31 [a]	304±40[b]	314±56[b]
Liver weight (g)	13±1[a]	13±1[a]	11±1[b]	11±1[b]
Kidney weight (g)	1.3±0.1[a]	1.3±0.1[a]	1.5±0.1[b]	1.5±0.2[b]

Table 2. Serum biochemical parameters.

	CT	SFN	STZ	SS
Total Cholesterol (mg/dL^{-1})	60±4^{a}	57±9^{a}	95±20^{b}	74±10^{a}
HDL Cholesterol (mg/dL^{-1})	32±5	33±6	27±9	31±9
Non-HDL Cholesterol (mg/dL^{-1})	28±2^{a}	24±4^{a}	68±28^{b}	41±17^{a}
Serum TAG (mg/dL^{-1})	166±26^{a}	132±16^{a}	669±321^{b}	377±158^{a}
ALT (IU/L)	43±6	43±9	66±15	66±26
AST (IU/L)	198±47	192±50	189±57	200±53
Urea (mg/dL^{-1})	45±6^{a}	50±6^{a}	72±10^{b}	80±17^{b}
Creatinine (mg/dL^{-1})	0.32±0.03	0.31±0.02	0.30±0.03	0.31±0.04

Figure 1

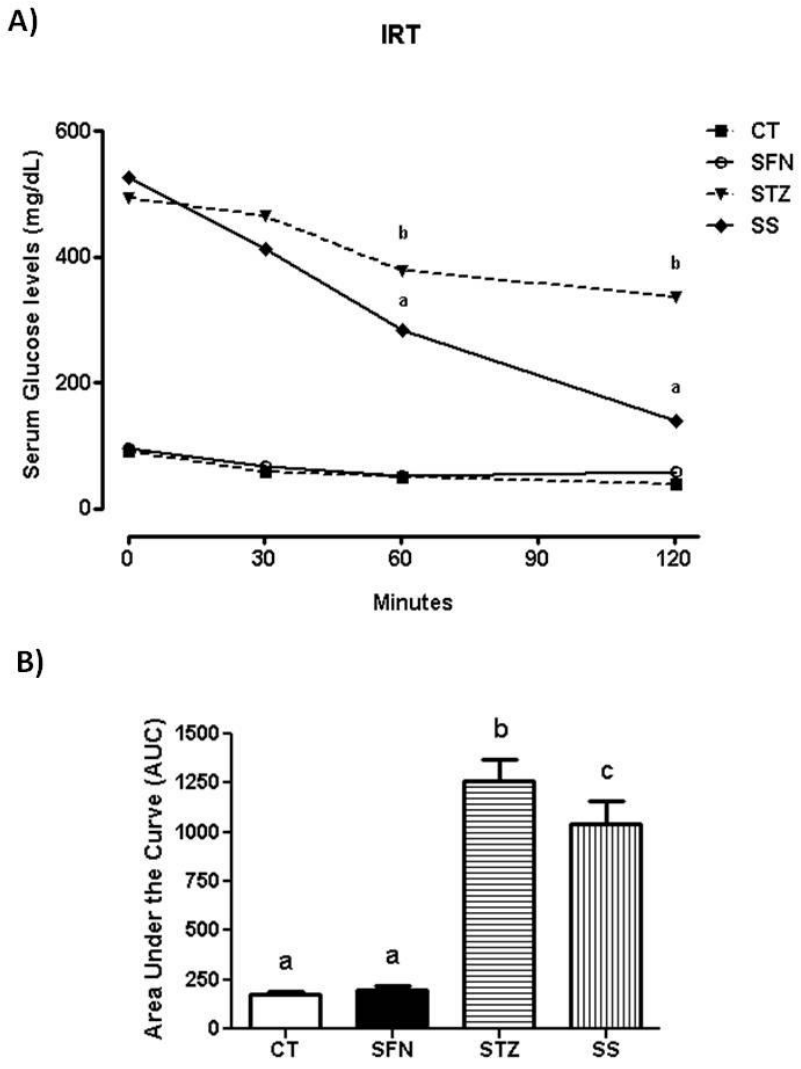
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Figure 2

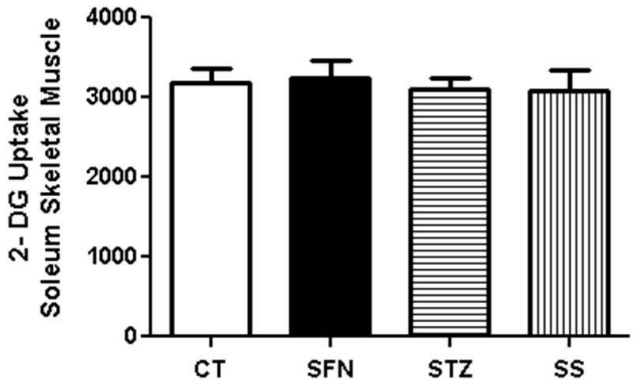
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Figure 3

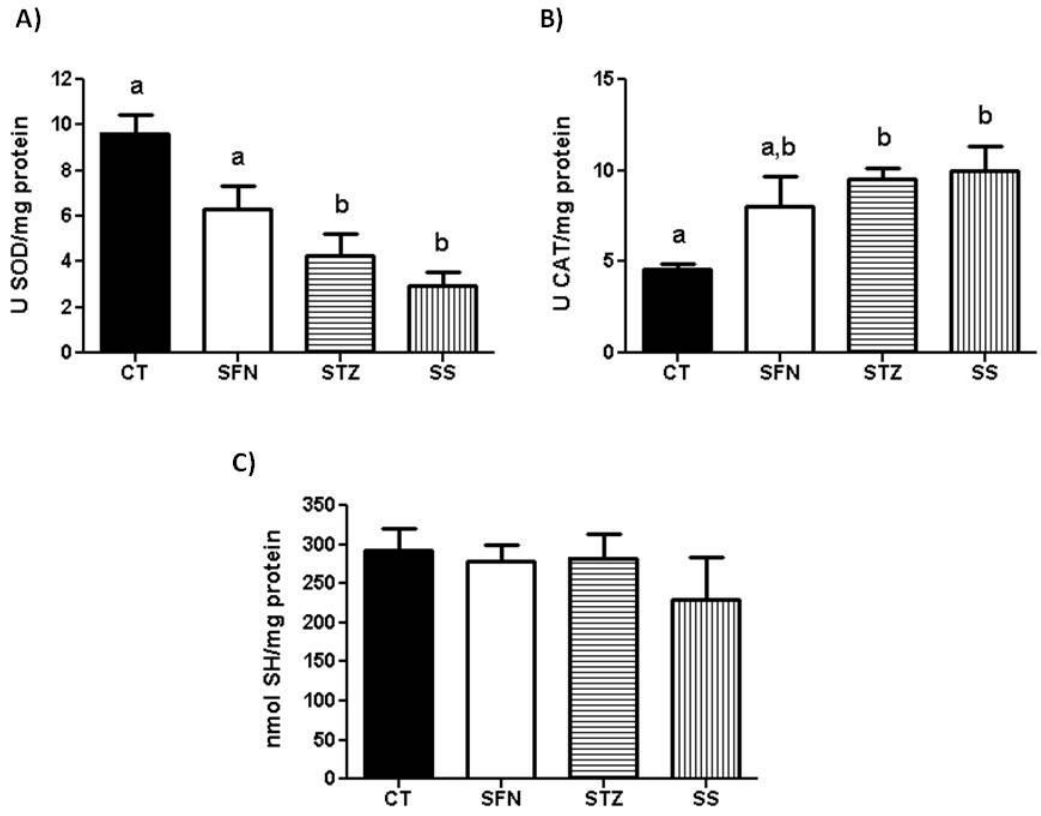
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Figure 4

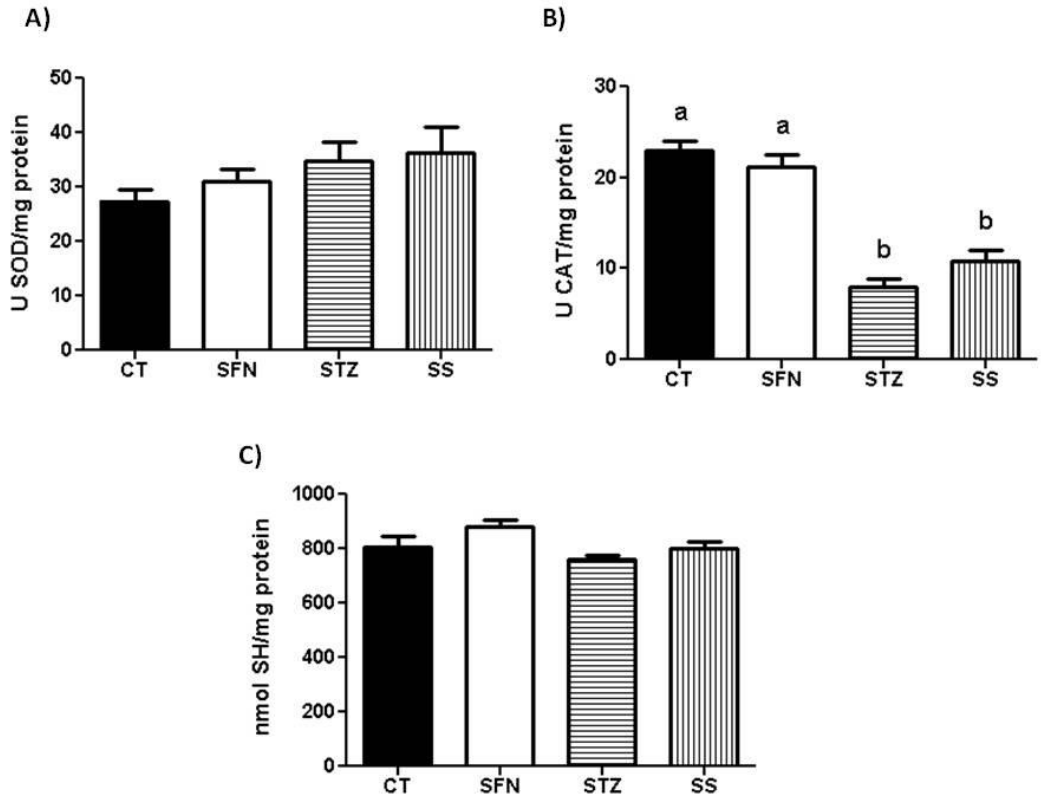
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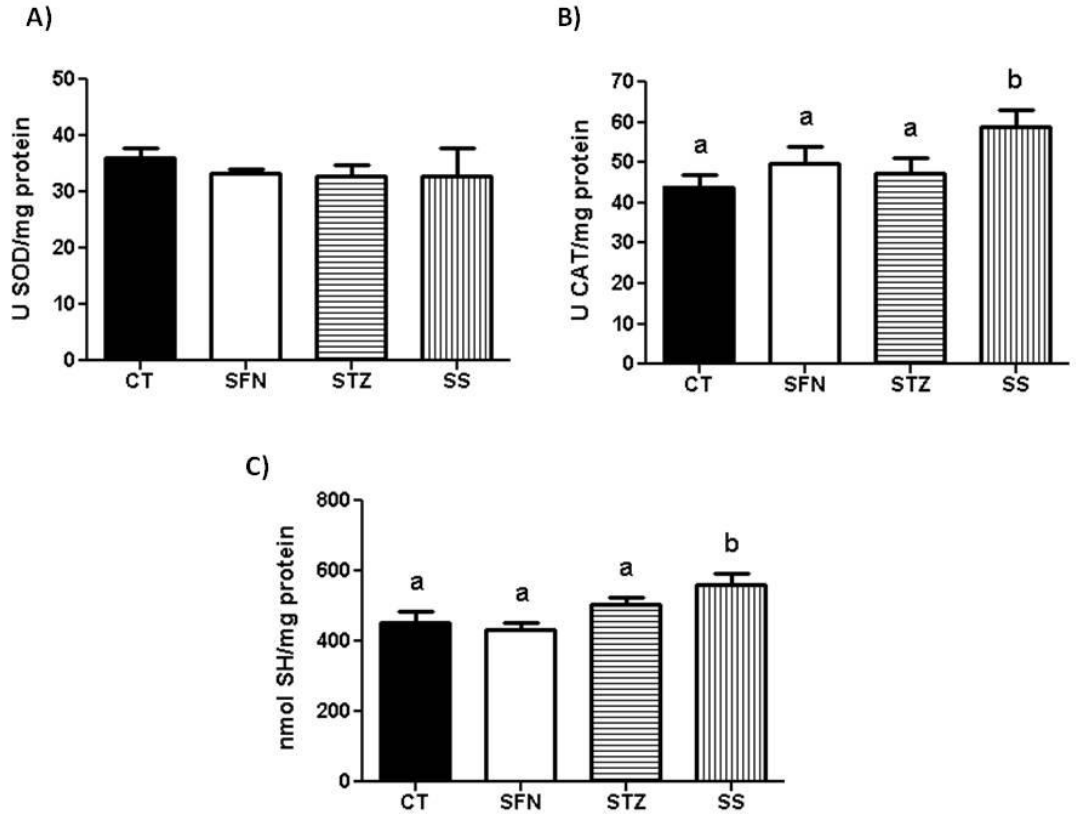
Figure 5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Parte III

DISCUSSÃO

O conjunto de dados obtidos sobre os efeitos do SFN na resistência à insulina, no diabetes induzido por estreptozotocina e no sistema nervoso central de ratos mostra que um mesmo composto pode ter um poder terapêutico ou deletério, de acordo com o quanto deste composto se usa. Isso remete à famosa frase do médico renascentista Paracelso (1493- 1541): "a diferença entre o remédio e o veneno está na dose".

Os dados epidemiológicos sobre os números do DM indicam o tamanho de sua magnitude em todo o mundo, assim como de suas comorbidades associadas e também dos altos custos que todas estas geram ao sistema de saúde (SBD, 2009). A busca incessante de novos compostos e moléculas que tratem, ou sejam coadjuvantes no tratamento do DM, é embasado nisto.

O SFN é um fitoquímico estudado desde o começo da década de 90, com consagrados efeitos anticarcinogênicos pelo seu alto poder antioxidante, mediado pela ativação do Nrf2 que age no núcleo celular aumentando a expressão de enzimas envolvidas principalmente em processos de detoxificação, especialmente de xenobióticos (Xue et al., 2008). Recentemente, este composto começou a ser avaliado em relação ao DM e à hiperglicemia, apresentando ainda poucos resultados (Song et al., 2008; Negi et al., 2011; Zheng et al., 2011). Por este motivo, esta molécula foi escolhida como objeto de estudo desta tese.

O efeito do tratamento oral crônico com SFN junto ao consumo da dieta hiperpalatável como acentuador da hiperglicemia foi inesperado, uma vez que a hipótese do trabalho era mensurar sua proteção contra as alterações glicêmicas desencadeadas pela dieta. A dose escolhida foi baseada na biodisponibilidade média de SFN em uma porção de 150g de brócolis (Hanlon et al.,2008), o que é facilmente consumido em uma refeição. Entretanto, é importante ressaltar que o composto utilizado é o isoticionato isolado, enquanto no alimento o temos na forma de glicosinolato (glicorafanina), que depende da ação da enzima mirosinase para ser hidrolizado e ficar

biodisponível. A atividade desta enzima, que está no próprio vegetal, é aumentada por ações como colher, picar e mastigar, sendo inativada ao entrar em contato com calor (cozer os alimentos) (Hanlon et al., 2008). A partir deste momento, degradação de glicorafanina em SFN e sua biodisponibilidade dependerão da ação da mirosinase intestinal, que dá continuidade ao processo digestivo (Lai et al., 2010). Tudo isso envolve uma série de passos que se tornam inexistentes quando é feita a administração do composto isolado. Além disso, os alimentos possuem fatores chamados "antinutricionais", os quais afetam a absorção de inúmeros nutrientes, acentuando novamente as diferenças entre as formas de ingestão.

Deste modo, é possível que os efeitos observados no tratamento oral crônico com SFN sejam relacionados à forma de administração do composto; entretanto, as alterações glicêmicas observadas também podem ser decorrentes da dose utilizada. Independente disto, o composto se mostrou não tóxico ao fígado e ao rim, ao menos não a ponto de alterar os níveis das principais provas de função hepática e renal, mesmo sendo ingerido diariamente por 4 meses.

A influência do SFN na expressão do transportador de glicose 3 (GLUT3) parece ser indireta, uma vez que este transportador é regulado pelos níveis glicêmicos (Hou et al., 2007). Entretanto, mediante este dado, optou-se por caracterizar *in vitro* os efeitos do SFN no córtex cerebral de ratos jovens e adultos, via curva de concentração. Embora este tenha sido um estudo *in vitro*, é importante dizer que o SFN passa a barreira hematoencefálica e age igualmente via Nrf2 no sistema nervoso central (SNC) (Jazwa et al., 2011).

Embora a curva de concentração deste composto não tenha alterado nenhum dos parâmetros de viabilidade celular, houve diferenças em funções basais do tecido, como produção de CO_2 e síntese proteica em animais jovens. As diferenças constaram de estimulação de ambas as funções quando o tecido foi exposto às concentrações mais baixas de SFN e diminuição das mesmas quando exposto às concentrações mais elevadas. Um padrão de resposta semelhante foi observado nos outros parâmetros complementares testados: atividade das enzimas Na^+-K^+-ATPase e glutamina

sintetase. As concentrações mais baixas de SFN não afetaram a atividade das enzimas, enquanto que as mais altas aumentaram a atividade da Na^+-K^+-ATPase, fazendo com que ela consumisse mais ATP, e diminuíram a atividade da glutamina sintetase, fazendo com que menos glutamato fosse convertido a glutamina. O maior consumo de ATP pela Na^+-K^+-ATPase pode ser uma explicação para menor produção de CO2, enquanto que a menor conversão de glutamato em glutamina acentua a gravidade de diminuição da síntese proteica, já que os aminoácidos que não estão sendo usados para este fim, ficam mais disponíveis para síntese de glutamato, que se acumula, além de já estar sendo menos removido da fenda sináptica. Considera-se ainda que estas alterações sejam passíveis de acontecer no cérebro de animais jovens e não dos adultos devido à imaturidade do SNC ainda em desenvolvimento de lidar com desajustes como estes (Schmidt et al., 2010).

Os resultados obtidos pela curva com diferentes concentrações de SFN associados aos resultados anteriores da glicemia e expressão de GLUT3 remetem ao conceito de hormese, definido como uma resposta bifásica de células e organismos à fatores exógenos ou endógenos (agentes químicos, ingestão dietética, estresse oxidativo), que é dose-dependente e na qual o fator indutor promove efeitos estimulatórios ou benéficos quando em pequenas concentrações ou inibitórios e adversos quando em elevadas concentrações (Speciale et al., 2011). Essa é uma ideia comum relacionada à antioxidantes, que sabidamente podem se tornar pró-oxidantes de acordo com a quantidade em que se encontram.

Considerando o conceito da hormese, buscou-se encontrar uma dose inócua de SFN testando três doses diferentes na prevenção do DM, administradas oralmente antes da indução que desta vez foi por meio da injeção de estreptozotocina (STZ), sendo a dose mais alta de SFN a metade da dose utilizada no modelo de indução de DM por dieta. Pudemos observar que todas elas conseguiram prevenir a indução, refletindo principalmente na glicemia de jejum e na curva glicêmica obtida pelo teste de sensibilidade à insulina, sendo estes dois parâmetros considerados padrão para diagnóstico de DM. Não houve diferença nas doses entre si, dentro dos parâmetros avaliados, que sugerissem

que alguma fosse melhor do que as outras. Os níveis glicêmicos obtidos propõem uma preservação das células β-pancreáticas e da secreção de insulina, sendo a perda destas células a maior determinante para progressão do DM, tanto do tipo 1 quanto do tipo 2 (DeFronzo, 2009). Fortalece essa proposição a preservação dos níveis de glicogênio hepático encontrado nos grupos pré-tratados com SFN, já que quando há déficit na produção de insulina os níveis desta reserva energética geralmente estão diminuídos (Mori et al., 2003; Fritsche et al., 2008). Contudo, intrigantemente os níveis de glicogênio muscular não foram menores nos animais diabéticos comparados aos demais, da mesma forma que a captação de glicose também não foi menor no músculo e no tecido adiposo dos mesmos, sendo estes tecidos dependentes de insulina para captação de 2-deoxi glicose por terem o transportador de glicose 4 (GLUT4). Neste sentido, Cameron-Smith e colaboradores (1997) demonstraram que a captação *in vivo* de 2-deoxi glicose pelo músculo e tecido adiposo de animais diabéticos (indução feita com estreptozotocina) pode ser maior do que em animais não diabéticos, em decorrência de mecanismos compensatórios da falta de ação da insulina nestes tecidos.

Outro efeito importante observado neste modelo de tratamento pré-indução foi a preservação da função renal, avaliada pelos níveis de uréia e creatinina, e a prevenção da trigliceridemia secundária ao DM, sendo estes dois aspectos muito relevantes considerando-se que a nefropatia diabética é uma das principais comorbidades do DM e a doença cardiovascular sua principal causa de morte (Zheng et al., 2011, Vazzana et al., 2012). Todavia, alguns efeitos deléterios foram observados, como aumento do colesterol total, das provas de função hepática e diminuição acentuada dos níveis de ureia – os quais reforçam a idéia de hepatoxicidade. Porém, acreditamos que estas alterações sejam decorrentes de alguma interação entre SFN e STZ, pois a exposição à esta droga, sem outros tratamentos, já altera estes parâmetros, os quais podem estar sendo potencializados pela associação de efeitos com o SFN. Corrobora com esta hipótese o fato de que no modelo utilizado com dieta hiperpalatável os animais foram submetidos à uma dose de SFN

muito maior e durante um período de tempo mais longo, onde não foram observadas nenhuma destas alterações.

Após constatarmos que as doses testadas no tratamento pré-indução foram efetivas na prevenção do DM, optou-se por ampliar o espectro de efeitos do SFN testando sua administração pós-indução de DM com STZ, escolhendo-se uma das doses já utilizadas. Embora ao final do tratamento não tenha sido encontrada diferença na glicemia de jejum entre os grupos diabéticos tratados ou não com SFN, uma maior responsividade à insulina pode ser observada no grupo diabético que recebeu o composto, propondo um efeito sinérgico com o hormônio, já que os níveis glicêmicos diminuíram somente após a administração do mesmo. Considerando que o controle glicêmico é o ponto mais importante do tratamento, pois evita a progressão e as complicações do DM, é de grande importância este achado. Na tentativa de encontrar justificativas para maior responsividade à insulina observada, avaliou-se a captação de 2- deoxi glicose pelo músculo e novamente não houve diferenças, tendo os animais diabéticos apresentados os mesmos níveis de captação do que os não-diabéticos.

Outro ponto importante foi a prevenção da dislipidemia secundária ao DM, tendo o SFN mantido os níveis de colesterol total, colesterol não-HDL e triglicerídeos semelhantes aos de animais não diabéticos. Novamente, menciona-se a relevância deste resultado considerando-se que a presença de DM adianta em 15 anos a idade para ocorrência de doença cardiovascular (Fox et al., 2004).

No intuito de mensurar a resposta antioxidante celular, foram avaliadas as atividades das enzimas SOD e catalase, bem como os grupamentos sulfidril em pâncreas, fígado e rins – órgãos muito afetados pela hiperglicemia – porém nenhum efeito do SFN sobre estes parâmetros pode ser observado.

A principal ação do SFN diz respeito a indução de enzimas de fase II (NQO1, HO-1, γ-GCL), as quais não foram avaliadas, logo não se pode dizer que o composto não exerceu nenhuma

ação antioxidante. Embora alguns trabalhos mostrem que o SFN pode estimular a atividade da SOD e da catalase, encontramos estas enzimas com menor atividade em determinados tecidos. Entretanto, defesas e capacidade antioxidante diminuídas são comuns em diabéticos¸ devido à fisiopatologia do DM, a qual tem por base o aumento do estresse oxidativo via hiperglicemia (Styskal et al., 2012) e por isso a diminuição da SOD no pâncreas e da catalase no fígado não foi um dado inesperado. Porém, a elevada atividade da catalase e dos grupos sulfidril exclusivamente no rim de animais diabéticos tratados com SF foi um resultado intrigante que precisa ser melhor investigado.

CONCLUSÃO

Baseado em nossos achados, concluímos que o SFN pode ter alguns efeitos preventivos/terapêuticos em relação ao DM, assim como sobre alguns aspectos enzimáticos e do metabolismo energético do sistema nervoso central. Contudo, estes efeitos são dependentes da concentração ou dose utilizada deste fitoquímico, o que se fundamenta pelo conceito de hormese.

É importante atentar para o fato de que os dados mostrados nos trabalhos desta tese são referentes à utilização do composto isolado, composto este que quando ingerido via alimento tem uma absorção e metabolização diferentes, podendo residir neste diferencial a forma mais segura de controle sobre a dose ingerida e a obtenção de somente efeitos benéficos à saúde.

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