UFRGS - UNIVERSIDADE FEDERAL DO RIO GRANDE DO SUL

INSTITUTO DE CIÊNCIAS BÁSICAS DA SAÚDE

DEPARTAMENTO DE BIOQUÍMICA PROF. TUISKON DICK

PROGRAMA DE PÓS-GRADUAÇÃO EM CIÊNCIAS BIOLÓGICAS: BIOQUÍMICA

Efeitos das glicotoxinas sobre o metabolismo energético do sistema nervoso central e o estado redox no sistema periférico de ratos: Possíveis efeitos prejudiciais do diabetes e benéficos do ácido graxo poli-insaturado Omega 3

Adriano Martimbianco de Assis

Tese apresentada ao Curso de Pós-Graduação em Ciências Biológicas - Bioquímica, da Universidade Federal do Rio Grande do Sul, com requisito para obtenção do título de Doutor em Ciências Biológicas-Bioquímica

Porto Alegre, Dezembro de 2011.

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“Quando você pensa que sabe todas as respostas, vem a vida e muda todas as perguntas.”

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RESUMO

Durante as últimas décadas a incidência de obesidade e diabetes tipo 2 vem aumentando assustadoramente, principalmente, em crianças. Recentes estudos sugerem que a hiperglicemia crônica, situação comum em diabéticos sem controle sobre a glicemia, altera mecanismos intracelulares, incluindo aumento na via dos polióis, ativação da proteína quinase C, aumento da via das hexosaminas e aumento na formação de produtos finais da glicação avançada (AGEs). Complicações vasculares como a nefropatia e a neuropatia são comumente encontradas em pacientes diabéticos, assim como falha na função cardíaca e hepática. Os AGEs estão relacionados com o aumento de: estresse oxidativo, dano ao ADN, citocinas pró-inflamatórias e resistência à insulina. Nos últimos anos muitas drogas têm sido testadas para prevenção ou diminuição dos efeitos das glicotoxinas, principalmente no diabetes, entretanto poucas obtiveram sucesso. Na presente tese, demonstramos que as glicotoxinas endógenas e exógenas afetam o metabolismo de alguns aminoácidos e o metabolismo energético do sistema nervoso central de maneira a prejudicar sua homeostase. Podemos concluir, também, que os Ω3-PUFAs demonstraram melhorar o estado redox no fígado e no rim de ratos diabéticos alimentados com uma dieta hiperlipídica aquecida. Estes resultados podem sugerir que os ácidos graxos poli-insaturados Omega 3 (Ω3-PUFAs) possam ser adjuvante no tratamento de algumas doenças como diabetes mellitus e resistência à insulina.

ABSTRACT

During the last decades the incidence of obesity and type 2 diabetes has increased alarmingly, especially in children. Some studies suggest that chronic hyperglycemia, a situation common in diabetics with no control over blood glucose levels, alters intracellular mechanisms, including increased polyol pathway, activation of protein kinase C, increased of Hexosamines pathway and increased formation of advanced glycation end products (AGEs). Vascular complications such as nephropathy and neuropathy are commonly found in diabetic patients, as well as cardiac and liver failure. AGEs are related to the increase: oxidative stress, DNA damage, proinflammatory cytokines and insulin resistance. In recent years several drugs have been tested for preventing or mitigating the effects of glycotoxins, especially in diabetes, but few have succeeded. In this thesis, we demonstrate that endogenous and exogenous glycotoxins affect metabolism of some amino acids and energy metabolism of the central nervous system in a manner detrimental homeostasis. We can also conclude that Ω3-PUFAs shown to improve the redox state in liver and kidney of diabetic rats fed a heated fat diet. These results may suggest that polyunsaturated fatty acids Omega 3 (Ω3-PUFAs) can be used as adjuvant in the treatment of some diseases such as diabetes mellitus and insulin resistance.

Lista de Abreviaturas

ADN: Ácido Desoxirribonucléico

AGEs: Produtos Finais de Glicação Avançada (do inglês *Advanced Glycation end Products*)

CAT: Catalase

CML: Carboximetil-lisina

DAG: Diacilglicerol

DM: Diabetes Mellitus

EAO: Espécies Ativas de Oxigênio

GPx: Glutationa Peroxidase

IL-6: Interleucina 6

IL-8: Interleucina 8

IKK: I Kapa B Kinase

IRS-1: Substrato do Receptor de Insulina 1

JNK: Jun Kinase

LC-PUFA: Ácidos Graxos poli-insaturados de cadeia longa

MCP1: Proteína Quimiotática de Monócitos 1

NF-kB: Fator Nuclear kB

Ω3-PUFAs: Ácidos Graxos Poliinsaturados Omega 3

PARP: Poli (ADP ribose) polimerase

PKC: Proteína Cinase C

RAGE: Receptor para AGE

RI: Resistência à Insulina

SOD: Superóxido Dismutase

TNF-α: Fator Alfa de Necrose Tumoral

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1. Introdução

1.1. Resistência à Insulina

A resistência a insulina é definida como uma resposta inadequada dos tecidos em que a insulina atua. Tecidos como o músculo esquelético, fígado e tecido adiposo, podem ter os efeitos fisiológicos da insulina circulante prejudicados. As principais características da resistência à insulina nesses tecidos são a diminuição da captação de glicose estimulada pela insulina no músculo esquelético, prejuízo na inibição, por efeitos da insulina, de produção de glicose no fígado e diminuição na habilidade da insulina de inibir a lipólise no tecido adiposo (Schenk et al., 2009).

O processo pelo qual se desenvolve a resistência a insulina é complexo e ainda não está totalmente estabelecido. A obesidade e dietas ricas em gordura podem ativar vias intracelulares como a IKK/NF-κB e a via da JNK em adipócitos, hepatócitos e outros tipos celulares. Ligantes de TNF-α e IL-1, receptores Toll ou de AGEs, quando acionados aumentam a formação de espécies reativas de oxigênio, ativação de diversas vias da proteína cinase C (PKC), estresse de reticulo endoplasmático e via das ceramidas. Esse processo resulta em ativação da IκB cinase (IKKβ) e conseqüente ativação do fator nuclear κB (NF-κB), que aumenta a expressão de diversos marcadores inflamatórios que resultam na resistência à insulina. A obesidade também promove a ativação da via da JNK, que promove a fosforilação do receptor de insulina em serina, que regula negativamente sua atuação (Shoelson et al., 2006).

Estudos em humanos obesos e resistentes à insulina mostram claramente a relação entre ativação crônica de vias de sinalização inflamatórias e decréscimo da sensibilidade à insulina. Por exemplo, elevados

níveis de Fator de necrose tumoral – alfa (TNF-α), Interleucina 6 (IL-6) e Interleucina 8 (IL-8) foram encontrados em pacientes diabéticos e com resistência à insulina (Roytblat et al., 2000; Sartipy e Loskutoff, 2003).

A resistência à insulina aumenta a produção hepática de glicose que determina uma maior produção de insulina pelas células β-das ilhotas de Langerhans, num primeiro momento há hipertrofia e hiperplasia das células β. Com o aumento da lipotoxicidade, glicotoxicidade e do estresse oxidativo nas células β há uma morte acentuada das mesmas (Unger e Orci, 2000).

A resistência à insulina, associada ao diabetes mellitus, parece ser geneticamente determinada, mas sua ocorrência é claramente afetada por fatores ambientais e processos secundários a diabetes mellitus. A resistência à insulina é um dos primeiros fatores etiológicos na patogênese da doença micro vascular na diabetes mellitus e sua implicação em complicações macro vasculares posteriores.

1.2. Diabetes Mellitus

O diabetes mellitus (DM) corresponde a um grupo de alterações metabólicas caracterizadas principalmente pela hiperglicemia, resultado de defeitos na ação ou secreção de insulina, ou em ambos.

O Diabetes Mellitus do tipo I (DM1), também conhecido como Insulino-dependente, inclui todas as formas que são causadas por destruição primária auto-imune das células β do pâncreas ou algum outro tipo de defeito primário da função deste tipo celular, que leve a uma secreção de insulina insuficiente para os tecidos periféricos alvo. As primeiras alterações dessa patologia são a cetoacidose (concentração aumentada de corpos cetônicos) e a glicemia elevada, com valores acima de 125 mg/dL em jejum (ADA, 2009). Vários

sintomas podem estar associados como a sede, poliúria, polidipsia e perda de peso (Alberti et al., 1998).

Já o Diabetes Mellitus do tipo II (DM2), chamado de diabetes não insulino-dependente, é resultante da resistência dos tecidos periféricos alvo à ação da insulina, combinada a uma secreção inadequada de tal hormônio. Essa resistência geralmente está associada com a obesidade, como demonstrado em um estudo envolvendo adultos com média de idade de 62.5 anos, com DM2, no qual foi relatado que 68% dos pacientes tinham sobrepeso, e 32% obesidade. Esses valores também estavam associados a níveis inadequados de pressão arterial, perfil lipídico e controle glicêmico (Daousi et al., 2005).

.Em 2008, segundo a Organização Mundial de Saúde (OMS) estima-se que cerca de 250 milhões de pessoas sejam diabéticas, o que significa 7,9% da população adulta. Segundo projeções da OMS, a população de indivíduos diabéticos a nível mundial vai aumentar até 2025 em mais de 50%, representando cerca de 380 milhões de pessoas. Pacientes com diabetes tipo I ou II têm uma propensão de cerca de 40% para complicações diabéticas microvasculares, mesmo tendo um controle de glicemia aceitável (UK prospective diabetes study group, 1998; Narayan et al., 2003). A maior causa de mortalidade em pessoas com diabetes mellitus tipo II são as doenças cardiovasculares (Kannel e McGee, 1979).

No Brasil, entre 1999 e 2001, foram gastos com internações de indivíduos com DM cerca de 243,9 milhões de reais. Existe a especulação de que se as medidas mínimas de prevenção e controle da doença fossem aplicadas corretamente, poderia haver uma redução de 50% nesse valor

(Gellar and Nansei, 2009) de quinze a vinte e quatro anos de idade (Artal et al., 2010). Com os altos índices dessa doença por todo o país, é natural que haja um grande custo na manutenção da melhor qualidade de vida possível desses pacientes.

A morbidade causada pelo DM é tradicionalmente classificada em doenças micro e macrovasculares (Huebschmann et al., 2006). Embora as complicações macrovasculares tenham recebido maior atenção, as complicações microvasculares são características no diabetes e a hiperglicemia contribui para o seu desenvolvimento. Vários mecanismos relativos à hiperglicemia crônica são, hipoteticamente, intermediários de complicações micro e macrovasculares, que incluem a via do poliol, mediada pela aldolase redutase, a via das hexosaminas, a ativação da proteína cinase C, a geração de estresse oxidativo, ativação da poli (ADP ribose) polimerase (PARP) e o acúmulo de produtos avançados de glicação (Brownlee, 2001; Beckman et al., 2002).

As complicações crônicas do DM compreendem a nefropatia, com possível evolução para falência renal, retinopatia, com possibilidade de cegueira, cardiopatia, com possibilidade de falha cardíaca, neuropatia, com possibilidade de neurodegeneração e artropatias, com risco de úlceras nos pés, amputações, entre outras. Pessoas com diabetes apresentam elevado risco de doença vascular aterosclerótica, como doenças coronarianas, arterial periférica e vascular cerebral (Beckman et al., 2002).

Grande parte do dano tecidual e morte celular associada com a hiperglicemia crônica no diabetes são mediadas por radicais livres. Na hiperglicemia de pacientes diabéticos, o estresse oxidativo gerado é devido,

primeiramente, ao excesso de produção de radicais livres e, em segundo lugar, ao aumento da oxidação de substratos (açucares, gorduras não saturadas, proteínas glicadas), aumento da auto-oxidação da glicose e a um decréscimo de antioxidantes (Brownlee, 2004).

A ligação entre estresse oxidativo e os Produtos Avançados de Glicação (*do inglês Advanced Glycation End Products* – AGEs) pode, em parte, ser explicada pela relação entre hiperglicemia e disfunção endotelial e dano tecidual. Estudos demonstraram que a interação entre AGEs e seu receptor (RAGE) induz a ativação de estresse oxidativo, estimula a produção e liberação de citocinas, amplificando seu dano tecidual (Wautier et al., 1994).

1.3. Produtos Avançados de Glicação (AGEs)

Os Produtos Avançados de Glicação (AGEs) são um grupo heterogêneo de moléculas, produzidas por glicação e oxidação não enzimática *in vivo*. A glicação é a principal causa espontânea de dano a proteínas celulares e extracelulares em sistemas fisiológicos, acometendo cerca de 0,1 a 0,2 % de resíduos de arginina e de lisina (Thornalley, 1999a; Thornalley et al., 2003).

Na figura 1, podemos ver a glicação protéica (conhecida como Reação de Maillard) ocorrendo entre açucares redutores e grupos amino livres das proteínas, via adição nucleofílica, formando bases de Schiff. Essas bases são rearranjadas para uma forma mais estável e essencialmente irreversível, chamada de produtos de Amadori. Durante esta reorganização, grupos intermediários carbonila são acumulados. Estes compostos são conhecidos como α-dicarbonilas ou oxoaldeídos, incluindo a 3-deoxiglicosona e o metilglioxal (Baynes e Thorpe, 1999). As α-dicarbonilas têm a habilidade de

reagir com grupos amino, sulfidrila e guanidina em proteínas (Lo et al., 1994; Frye et al., 1998). As α-dicarbonilas podem também reagir com grupos lisina e arginina de proteínas, formando compostos estáveis como os aductos N-ε-(carboximetil) lisina (Basta et al., 2008).

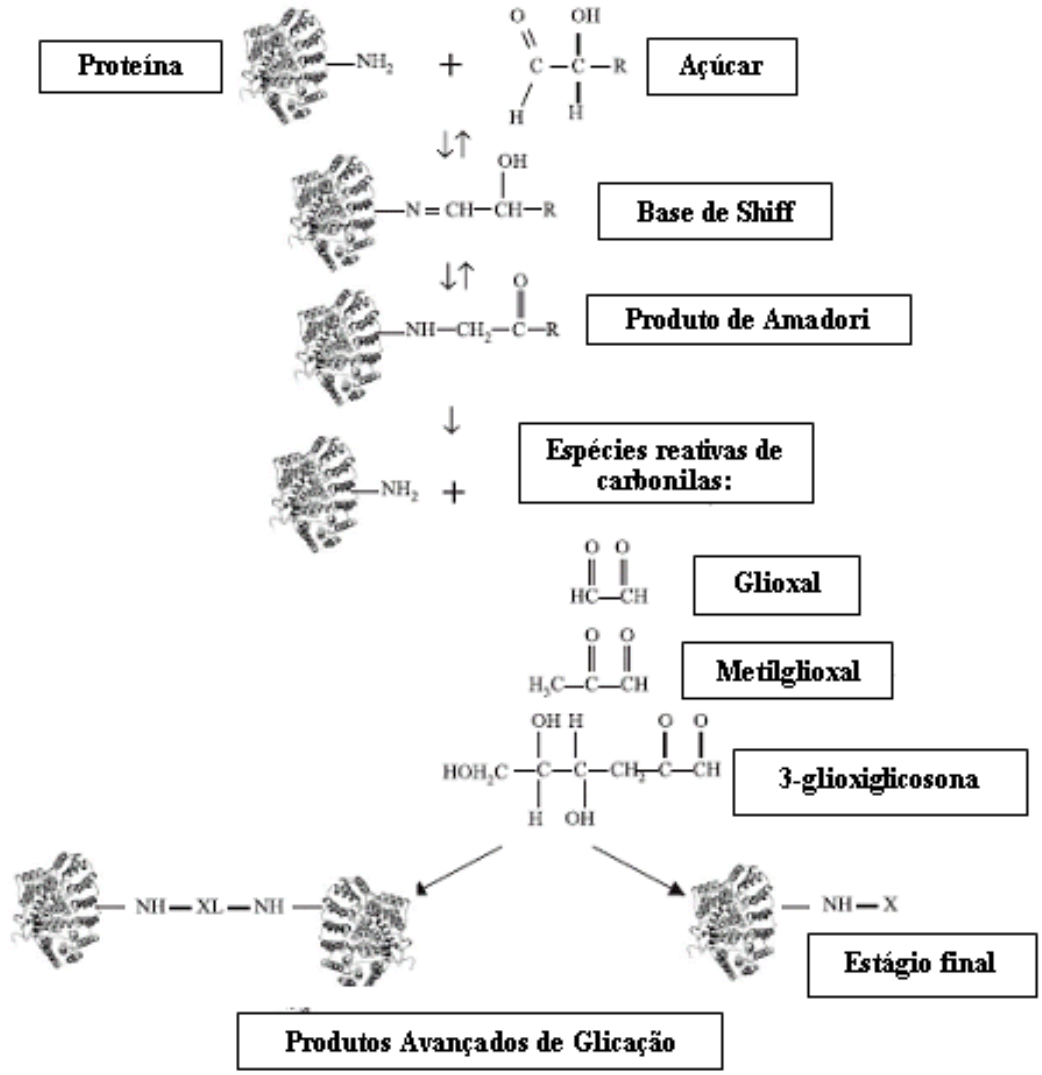

Figura 1. Etapas da Reação de Maillard.

Devido à heterogeneidade de estruturas de AGEs, pode ocorrer formação de ligações cruzadas. As proteínas afetadas por este processo geralmente são estáveis e de longa vida como o colágeno. A formação de ligações cruzadas induzida por ligação de AGEs aumenta a rigidez da matriz

protéica, impedindo seu funcionamento, bem como aumenta a resistência à remoção por processos proteolíticos, afetando o processo de reposição tecidual. Esses processos permitem o avançar do envelhecimento e aceleram o diabetes (Paul e Bailey, 1999).

Atualmente, são conhecidos diferentes tipos de AGEs, sendo classificados de acordo com a sua origem. Takeuchi et al., (2004), reconheceram seis classes distintas de AGEs: os derivados de glicose (AGE-1), os derivados de outros carboidratos, como os de gliceraldeído (AGE-2), os de alfa-dicarbonila, como os glicoaldeídos (AGE-3), metilglioxal (AGE-4), glioxal (AGE-5), 3-deoxiglicosona (AGE-6).

Glioxal, metilglioxal e 3-deoxiglicosona são formados na glicação de proteínas por glicose, entretanto, glioxal é também formado na peroxidação de lipídios, metilglioxal pela fragmentação de trioses fosfato e pelo catabolismo da acetona e de treonina, e a 3-deoxiglicosona a partir de glicose-3-fosfato. Em alguns casos, a glicação está envolvida não somente com a glicose, mas também com compostos formados pela glicação por outros compostos já formados por glicoxidação. A partir da glicose, a via não oxidativa pode gerar pirralina; na via oxidativa pode gerar pentosidina e N-6-carboximetilisina (Niwa, 1999).

Os AGEs são importantes na etiopatogenia do diabetes tipo II porque modificam proteínas intracelulares, de matriz celular e proteínas circulantes (Brownlee, 2004). Os AGEs são os principais responsáveis pela retinopatia, nefropatia e neuropatia diabética, já que se formam intracelularmente e extracelularmente (Ahmed, 2005), são provenientes de alimentos (Vlassara e

Uribarri, 2004), cigarro (Cerami et al., 1997) e são prejudiciais independentemente da hiperglicemia.

Estes produtos de glicação são formados em excesso, principalmente durante o envelhecimento e o diabetes mellitus (DM).No caso do DM, uma das conseqüências prejudiciais da hiperglicemia crônica e do constante estresse oxidativo, causados por um controle glicêmico inadequado, é a formação acelerada dos AGEs *in vivo*, via reação de Maillard (Lapolla et al., 2005). Esta hiperglicemia crônica pode resultar em um significante acúmulo de AGEs em certas proteínas de vida longa como as do cristalino. O cristalino está sujeito a uma progressiva modificação por AGEs, ocorrendo um escurecimento lenticular e acúmulo de ligações cruzadas (Monnier et al., 1981). O resultado dessa opacificação é a formação de cataratas, processo associado com diabetes e envelhecimento.

Os AGEs acumulam-se em proteínas da matriz extracelular, como no processo fisiológico de envelhecimento (Frye et al., 1998); entretanto, isso ocorre mais rapidamente em indivíduos com diabetes mellitus do que em indivíduos não diabéticos (Schleicher et al., 1997). Também foi demonstrado que há uma grande correlação entre a importância dos depósitos de AGEs e a severidade das complicações diabéticas (Brownlee et al., 2001).

Como já foi citado anteriormente, a formação e o acúmulo de AGEs são características em tecidos de indivíduos mais idosos (envelhecimento) e em pacientes com diabetes mellitus, sendo que esses produtos estão implicados com a patogênese das complicações micro e macrovasculares no diabetes (Brownlee, 2001). A disfunção microvascular, incluindo o espessamento da membrana basal, aumento da permeabilidade vascular estado pré-trombótico,

e redução de fluxo sanguíneo, é uma característica onipresente na doença microvascular da retina, rins e nervos periféricos (Singh et al., 2001).

O aumento de AGEs séricos está associado com o aumento da doença arterial coronariana em indivíduos com diabetes tipo II (Kilhovd et al., 1999). Os AGEs podem estar relacionados com a aterosclerose de várias maneiras, incluindo aumento da disfunção endotelial, aumento de LDL vascular, aumento da desestabilização de placas ateroscleróticas, proliferação neoíntima (membrana interna) (Zhou et al., 2003) e inibição da recuperação vascular após injúria.

A inflamação e disfunção endotelial fornecem condições favoráveis para a progressão gradual de ateroma em indivíduos diabéticos (Basta et al., 2004b). Os mecanismos ateroscleróticos mediados por AGEs incluem a óxido nítrico sintase induzível (iNOS) (Bucala et al., 1993), resistência à regeneração vascular do colágeno em crosslinks, redução da remoção do LDL e pela redução do reconhecimento do receptor de LDL pelo LDL modificado por AGEs (Bucala et al. 1994; Brownlee et al., 1991).

Os AGEs têm sido identificados também, a partir de fontes exógenas como tabaco e em certos alimentos, principalmente aqueles que sofrem aquecimento. (O'brien e Morrissey, 1989). Estes AGEs exógenos são os principais causadores destes efeitos deletérios ao organismo, podendo ser mais deletérios do que os AGEs endógenos (Huebschmann et al., 2006).

1.3.1. Fontes endógenas

Pessoas diabéticas têm níveis mais elevados de AGEs do que as não diabéticas porque tanto a hiperglicemia como o estresse oxidativo contribuem para esse acúmulo. Estudos mostram níveis de AGEs 20 a 30% mais

elevados em pessoas com diabetes sem complicações e níveis de 40 a 100% mais altos em pessoas com diabetes tipo II com complicações por doença arterial coronariana ou microalbuminúria (Sharp et al., 2003).

Indivíduos com doença renal em estágio final apresentam aumento significativo de AGEs circulantes comparados com indivíduos saudáveis (cinco a dez vezes) (Raj et al., 2000). O transplante renal normalizou os níveis de AGEs em indivíduos com doença renal terminal (Makita et al., 1991). A literatura indica que o turnover de AGEs é dinâmico e que os AGEs endógenos são determinados pela produção de AGEs através da hiperglicemia crônica e estresse oxidativo.

1.3.2. Fontes exógenas

A formação de AGEs geralmente é endógena, porém, estes podem ser derivados de fontes exógenas como tabaco e alimentos (Vlassara et al., 2002; Cerami et al., 1997).

Os AGEs derivados da dieta são similares aos AGEs endógenos no que diz respeito a suas propriedades pró-oxidantes e inflamatórias (Cai et al.,2002; Vlassara e Uribarri, 2004). Aminolipídios provenientes de dietas hiperlipídicas (4-hidroxinonenal, N-carboximetilisina (CML) e seus análogos) são também alvos de peroxidação (Bucala et al., 1993; Fu et al., 1996). Assim, a ingestão de produtos de glicação e lipoxidação pode acelerar a geração de radicais livres induzindo ao estresse oxidativo via carbonilação (Miyata et al., 2003). A auto-oxidação da glicose também é acompanhada pela geração de espécies ativas de oxigênio, tal como o radical superóxido (Wolff e Dean, 1987).

Em humanos, diabéticos ou não, uma única refeição com alto teor de AGEs leva a uma aumento significativo de AGEs séricos, comparado a uma refeição com baixo nível de AGEs. Cerca de 10% dos AGEs são absorvidos, e 2/3 do total absorvido é retido (Koschinsky et al., 1997). O epitélio intestinal absorve derivados primários (Produtos de Amadori), assim como AGEs intermediários e tardios (Finot, 2005). Mono, di ou tripeptídeos modificados por AGEs podem ser prontamente transportados através da parede intestinal levando consigo um ou mais produtos de glicação. A presença, na maioria dos alimentos, de dois produtos derivados da formação dos AGEs (Metilglioxal e N-Carboximetilisina) permitiu estudos que confirmam seu papel tóxico em sistemas de múltiplas finalidades (Cai et al., 2002; Lin et al., 2002; Peppa et al 2003a; Vlassara e Uribarri, 2004).

Goldberg et al., 2004, em seu trabalho quantificaram para fins comparativos o conteúdo de 250 alimentos comumente utilizados pelos humanos, alguns deles estão listados na Tabela 1. A cocção a altas temperaturas (exemplo: assado, grelhado, fritura e cozimento) aumenta significativamente os índices dos produtos finais de glicação avançada (Goldberg et al., 2004), enquanto o cozimento dos alimentos a temperaturas mais baixas, por menor tempo e com maior conteúdo de água (fervura ou vapor) permitem um menor aumento destes produtos. (Goldberg et al., 2004; Uribarri et al., 2005).

Tabela 1. Conteúdo de AGEs em alimentos preparados por métodos de cozimento padrão. (* AGEs foram quantificados através de um anticorpo para N-Carboximetilisina). Adaptado de Goldberg et al., 2004.

Alimentos	AGE* (kU/g ou /ml de alimento)
Gorduras	
Amêndoas, assadas	66,5 kU/g
Óleo de Oliva	120 kU/ml
Manteiga	265 kU/g
Maionese	94 kU/g
Proteínas	
Peito de frango, grelhado x 15 min	58 kU/g
Peito de frango, frito x 15 min	61 kU/g
Carne, fervida x 60 min	22 kU/g
Carne, grelhada x 15 min	60 kU/g
Atum, assado	6 kU/g
Atum, grelhado	51 kU/g
Queijo americano	87 kU/g
Queijo	56 kU/g
Ovo frito	27 kU/g
Gema de ovo fervida	12 kU/g
Tofu cru	8 kU/g
Tofu grelhado	41 kU/g
Carboidratos	
Pão integral de trigo	0.54 kU/g
Panqueca caseira	10 kU/g
Leite de vaca integral	0.05 kU/mL
Leite humano integral	0.05 kU/mL
Emfamil (fórmula infantil)	4.86 kU/mL
Maçã	0.13 kU/g
Banana	0.01 kU/g
Cenouras	0.1 kU/g
Feijões verdes	0.18 kU/g

1.4. Receptores para Produtos Avançados de Glicação

A importância em compreender a relação dos AGEs com seus receptores está no fato de que estes desempenham papéis negativos e positivos nas ações e destinos dos AGEs (Huebschmann et al., 2006). No seu papel positivo, alguns receptores normalmente ajudam na retirada dos AGEs da circulação e podem auxiliar a atenuar os efeitos pró-oxidantes dos AGEs. Em contrapartida, o receptor para AGE (RAGE) e outros receptores parecem

ativar uma resposta estressante, desecandeando inflamação e disfunção celular.

As principais moléculas AGE-ligantes descritas incluem o complexo AGE-receptor específico, composto por R1, R2 e R3 (Li et al., 1996), receptor para AGE (RAGE) e receptores como o CD-36 (Ohgami et al., 2002) e SCR-II (Araki et al., 1995).

Entre os receptores de AGEs que aumentam sua remoção (pretensa ação benéfica), incluem-se AGE-R1 e lisozima (Li et al., 1996; Lu et al., 2004). O AGE-R1 é ativo em ligação e degradação de AGE-ligante específico. A baixa expressão de AGE-R1 em rins de camundongos diabéticos não obesos é associada a altos níveis de AGEs nos tecidos e com doença renal. Da mesma forma, células mononucleares circulatórias humanas de indivíduos diabéticos com complicações diabéticas severas apresentaram baixa expressão de AGE-R1 e alto nível de AGEs séricos (He et al., 2001). AGE-R1 pode exercer uma ação protetora contra a ativação celular promovida por AGEs e RAGE.

RAGE é um membro multiligante da superfamília das imunoglobulinas de superfície celular, que foi primeiramente descrito como um receptor para aductos modificados por glicosilação não enzimática entre proteínas e lipídios, que ocorre principalmente no diabetes mellitus (Neeper et al., 1992). RAGE é expresso em uma grande quantidade de tecidos, mas principalmente no coração, pulmões e músculo esquelético. Este receptor é uma proteína de aproximadamente 45 kDa, originalmente isolada do endotélio pulmonar bovino (Schmidt et al., 1992). Este receptor consiste de 403 aminoácidos em humanos, ratos e camundongos. A região extracelular deste receptor consiste

em um domínio imunoglobulina tipo V, seguido por dois domínios imunoglobulina tipo C, estabilizadas por duas pontes dissulfeto entre resíduos de cisteína (Dattilo et al., 2007).

A via de sinalização do RAGE pode ser inicializada por diversos ligantes, como AGEs, proteínas S100, anfoterina, proteína beta amilóide, entre outros (Schmidt et al., 2001; Chavaskis et al., 2003). O sistema de receptores AGEs podem ser regulados por fatores relacionados ao diabetes mellitus, assim como glicose, insulina, AGEs e espécies ativas de oxigênio (Shanmugam et al., 2003; Müller et al., 2004).

1.5. Glioxal e Metilglioxal

O glioxal e o metilglioxal são espécies reativas de carbonilas com potente ação de glicação. Eles são formados pela degradação de proteínas glicadas, intermediários glicolíticos e peroxidação lipídica e reagem com proteínas para formar os AGEs (Thornalley et al., 2005).

Os efeitos tóxicos do glioxal e do metilglioxal são fatores que podem contribuir para a etiopatogenia da doença cardiovascular, do diabetes mellitus, da doença de Parkinson e da doença de Alzheimer (O'brien et al., 2005; Shangary et al., 2003). As concentrações de glioxal e metilglioxal estão aumentadas em pacientes com diabetes mellitus e uremia. Estas dicarbonilas são em torno de 2000 vezes mais reativas que a glicose em reações de glicação (Zeng et al, 2006).

O glioxal é um dialdeído altamente reativo que se origina endogenamente da autoxidação da glicose e ascorbato, da oxidação do DNA e da peroxidação lipídica (Shangari et al., 2003, Wells-Knecht et al., 1995). A peroxidação lipídica é conhecida por ser uma cadeia de reações

iniciada por lipídeos oxidados por radicais livres (Halliwell et al., 2000). Na presença de ERO os lipídios reagem com o oxigênio para produzir radicais alquila e peroxila que se propagam por uma cadeia de radicais livres e formam hidroperóxidos como produtos primários (Droge et al., 2002). Os radicais alcoxil e peroxil podem causar danos às membranas protéicas das células levando a modificação da estrutura e diminuição da fluidez da membrana celular resultando na alteração das suas propriedades fisiológicas. A destruição da membrana celular causa a perda da função das organelas podendo levar à morte celular (Droge et al., 2002). A peroxidação lipídica resulta em uma mistura complexa de hidroperóxidos e produtos secundários de oxidação, incluindo peróxidos cíclicos.

O metilglioxal é formado a partir do gliceraldeído-3-P e da dihidroxiacetona-P, do catabolismo da glicina e treonina e do metabolismo da acetona (Kalapos et al., 1999). No jejum 37% do acetoacetato é transformado em acetona, na cetoacidose diabética esta porcentagem atinge 50% (Owen et al., 1982; Reichard et al., 1986). Tanto o metilglioxal quanto o glioxal são agentes alcoilantes bifuncionais que reagem não enzimaticamente com grupos amino e sulfidril de biomoléculas, formando AGEs (Thornalley et al., 2001). Produtos de ligação cruzadas são formadas quando os propagadores (compostos como o glioxal, metilglioxal e 3-deoxiglicosona provenientes da degradação dos AGEs) reagem com a lisina; enquanto derivados do imidazol são formados com resíduos da arginina (Westwood et al., 1997). Glioxais reagem também com os grupos amino de DNA/RNA e lipídios (Roberts et al., 2003).

O acúmulo do glioxal, metilglioxal e de outros α-oxaldeídos nas células levam a modificação do DNA, dando espaço a mutagênese e

apoptose e a modificação de proteínas podendo levar a degradação de proteínas, inibição de enzimas e, ainda, respostas imunes mediadas por citocinas. O acúmulo celular de α-oxaldeídos é esperado em contribuir para a toxicidade associada com a oxidação do GSH, na toxicidade química, e em patologias crônicas, como a diabetes mellitus, onde a formação do glioxal e do metilglioxal esta aumentada. Sendo assim, o aumento da glicação por α-oxaldeídos é vista como uma conseqüência do estresse oxidativo. (Abordo et al, 1999).

O glioxal e o metilglioxal constituem uma parte significante dos compostos carbonilas liberados pelos veículos automotores (Grosjean, 1984).

1.6. Sistema Glioxalase

O glioxal e o metilglioxal são detoxicificados endogenamente pelo sistema da glioxalase, que é presente no citosol de todas as células de mamíferos (Abordo et al., 1999). O sistema das redutases é menos importante na detoxificação dos glioxais, este sistema inclui a aldeído redutase e a carbonil redutase. O sistema da glioxalase converte o glioxal a glicolato e o metilglioxal a D-lactato na presença de glutationa (GSH). Este sistema é dependente de duas enzimas (a glioxalase I e glioxalase II) e ainda da glutationa (Shangari et al., 2004). Os efeitos citotóxicos do glioxal e do metilglioxal nas células e tecidos ocorrem na maioria das vezes através da indução de espécies ativas de oxigênio (EAO) (Di Loreto et al., 2004) Sob condições de estresse oxidativo, como na situação de hiperglicemia, onde citocinas estão sendo liberadas, os níveis de GSH diminuem e isto prejudica

a detoxificação do glioxal e metilglioxal através do sistema da glioxalase (Shangari et al., 2005).

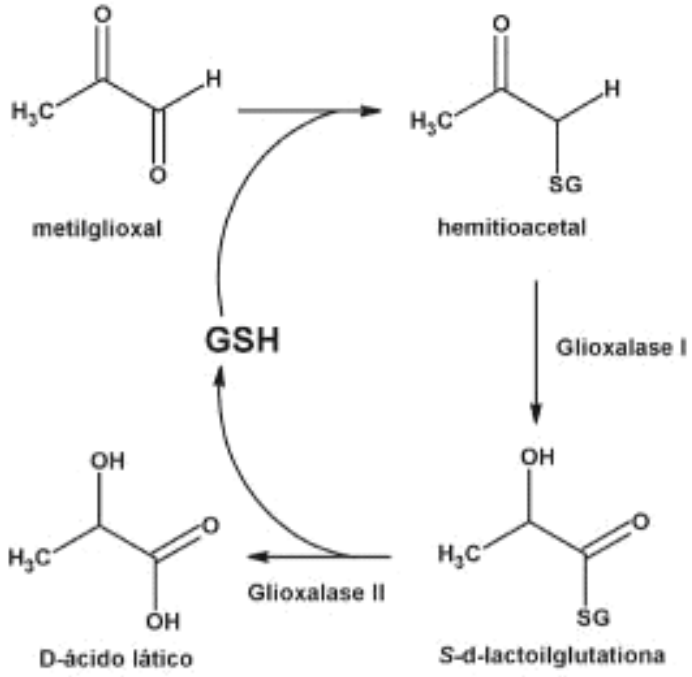

Figura 2. Sistema das Glioxalases.

1.7. Metabolismo do Sistema Nervoso Central

O diabetes mellitus (DM) é uma doença complexa, envolvendo múltiplos órgãos, que estão implicados em uma grande variedade de comunicação cruzada. Além do pâncreas endócrino, muitos orgãos desempenham um papel no metabolismo da glicose e na desregulação metabólica, incluindo o fígado, músculos, tecido adiposo, intestino e rim. Inúmeros artigos têm sido publicados descrevendo os respectivos papéis dos órgãos mais importantes na fisiopatologia da DM tipo II (De Fronzo, 2009) e as complicações associadas com DM que podem ocorrer em diversos órgãos periféricos (Melendez-Ramirez, 2010). No entanto, o cérebro [sistema nervoso central (SNC)] é muitas vezes esquecido nessa literatura. No entanto, o cérebro pode ser considerado o maestro da orquestra de todos os órgãos

envolvidos na regulação da glicose e do metabolismo energético (Figura 3).

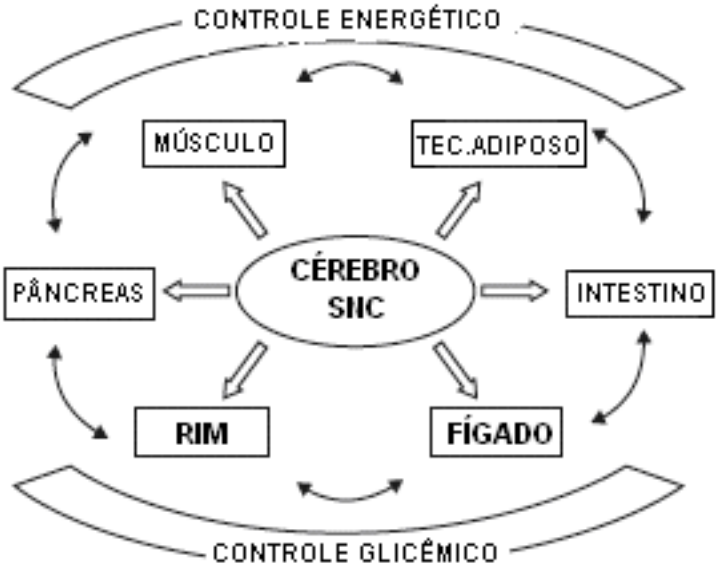

Figura 3. Regulação da glicose e do metabolismo energético.

A alta prevalência de complicações no SNC decorrentes do DM é um problema que está ganhando mais aceitação e atenção. Não só complicações agudas bem conhecidas (por exemplo, coma, acidente vascular cerebral), mas também doenças crônicas (por exemplo, encefalopatias demência) foram recentemente descritas detalhadamente (Sima, 2010; Stiles e Seaquist, 2010).

Evidências recentes sugerem alterações morfológicas, eletrofisiológicas e cognitivas associadas à hiperglicemia crônica. Muitas das alterações observadas no SNC de pacientes diabéticos e modelos animais de diabetes lembram mudanças observadas no envelhecimento normal. Os pontos centrais em comum entre o diabetes e as mudanças do SNC relacionadas a idade levaram à teoria do envelhecimento cerebral avançado em pacientes diabéticos (Wrighten et al., 2009).

O cérebro representa apenas 2% do peso corporal total, mas que recebe 15% do fluxo total de sangue fornecido pelo sistema cardiovascular, além disso, consome pelo menos 25% de toda a glicose circulante e 20% do

oxigênio disponível no corpo em condições de repouso (Magistretti et al., 1999).

Lactato é conhecido como o produto final da glicólise sob condições anaeróbicas (ou condições insuficientes de oxigenação). Este é o caso dos músculos que produzem grandes quantidades de lactato a partir da glicose durante episódios de alta intensidade de atividade física. No cérebro, apesar do fato de que o lactato foi por muito tempo considerado um desperdício metabólico e potencialmente um composto tóxico, ele é hoje reconhecido não só como um substrato energético valioso para os neurônios, mas possivelmente até mesmo como fonte preferencial de energia em certas circunstâncias (Pellerin, 2003; Wyss et al., 2011; Draouli e Fuon, 2011).

O lactato, assim como os corpos cetônicos são membros da família de compostos conhecidos como ácidos monocarboxílicos. Uma vez que são moléculas hidrofílicas, eles não podem cruzar membranas celulares por difusão simples. Assim, transportadores específicos são necessários para estes compostos serem transportados por diferentes tipos celulares. Uma família de prótons ligados a transportadores foi identificada nos últimos anos e são conhecidos coletivamente como os transportadores de ácidos monocarboxílicos ou MCTs (Pierre e Pellerin, 2005). Os astrócitos expressam MCT1, mas também exibem MCT4 (Pellerin et al., 2005). Ambos os transportadores estão mais provavelmente envolvidos na liberação de lactato pelos astrócitos. No entanto, estes dois transportadores diferem em sua afinidade para o lactato, o MCT1 exibe um menor K_m do que o MCT4 para este substrato (3.5 vs 34.7 mM, respectivamente). Em contraste, na grande maioria dos neurônios foi encontrado um transportador alta afinidade o MCT2 (K_m = 0,7 mM) (Pierre et al., 2002).

1.8. Estresse Oxidativo

Como revisado extensamente por Dröge (2002), durante o processo de produção de ATP pela cadeia respiratória, ocorrido na matriz mitocondrial, o oxigênio tem a função de receber elétrons para formar água e dar prosseguimento ao processo de fosforilação oxidativa. No entanto, durante esse processo, pode ocorrer a formação de Espécies Ativas de Oxigênio (EAO). Entre eles, o mais comumente encontrado é o Radical Superóxido (O_2^-). Essas moléculas possuem um elétron desemparelhado na sua última camada de valência, o que faz com que se tornem altamente reativas, pois buscam a estabilidade através retirada de um elétron de outras moléculas. Entretanto esse processo pode causar dano a diversos tecidos e componentes celulares. Os metabólitos gerados a partir do O_2^-,como o peróxido de hidrogênio (H_2O_2), não são radicais livres, pois não possuem um elétron desemparelhado, mas são altamente reativos, sendo então conhecidos como Espécies Ativas de Oxigênio (EAO). Ainda existem outras fontes de espécies reativas, não necessariamente provenientes do O_2, e são conhecidas de um modo geral como Radicais Livres (RL).

Os RL são produzidos em diversas situações do nosso metabolismo, e são altamente reativos, podendo proporcionar dano ao acido desoxirribonucléico (ADN), a proteínas e a lipídeos de membranas plasmáticas (Aladag et al., 2009).

Liu (2008), em sua revisão, estabelece que as células possuem métodos para se defender e conter os malefícios gerados a partir das EAOs. Entre elas podemos agrupar as defesas enzimáticas das enzimas superóxido desmutase (SOD), catalase (CAT) e glutationa peroxidase (GPx), e as defesas não enzimáticas das vitaminas A, C, E, flavonóides, ácido úrico, entre outros.

Um organismo encontra-se em uma situação de estresse oxidativo quando há um desequilíbrio entre os sistemas antioxidantes e pró-oxidantes, sendo que os últimos estão de forma aumentada.

A enzima SOD é capaz de converter imediatamente o O_2^- a H_2O_2, que é detoxificado a água tanto pela CAT quanto pela GPx na mitocôndria. Outra enzima importante para a manutenção do estado redox é a glutationa redutase que regenera glutationa que foi usada como doador de hidrogênio pela GPx durante a eliminação do H_2O_2 (Johansen et al., 2005). Na figura 4 está ilustrada cada uma das reações enzimáticas.

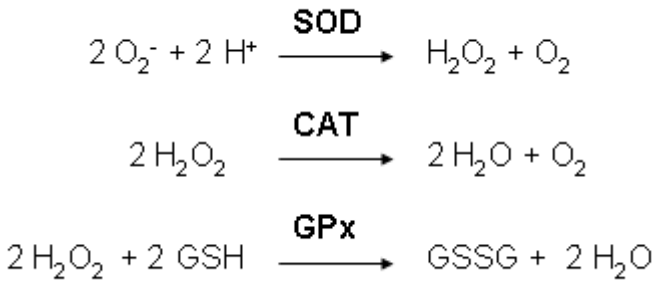

Figura 4. Reações promovidas pelas enzimas antioxidantes (Superóxido dismutase – SOD; Catalase – CAT e Glutationa peroxidase – GPx).

Na Patologia do diabetes mellitus, o estresse oxidativo participa ativamente da maioria dos eventos deletérios ao paciente. As EAOs estão presentes de forma a incentivar a via dos polióis, são resultado da produção intracelular de produtos de glicação avançada e frutos da ativação da PKC (Brownlee, 2005).

Como intensamente revisado por Maiese (2007), tanto o processo de dano celular no DM1, quanto o processo de resistência a insulina no DM2, estão ligados a presença de estresse oxidativo celular. Um aumento de EAOs devido a elevações nos níveis da glicose circulante também é uma das causas que leva a esse quadro de estresse oxidativo. Isso esta associado a uma

diminuição na sensibilidade a insulina e destruição das células produtoras de insulina no pâncreas.

Em um trabalho em que sessenta e três pacientes com diabetes mellitus do tipo 2 fizeram parte, foram avaliados padrões de atividade enzimática de SOD e GPx, para comparar com um grupo controle de trinta e sete indivíduos não diabéticas. O resultado é que as enzimas antioxidantes se encontram em maior atividade no grupo diabético, provando assim que esses pacientes se encontram em um estado de desbalanço redox (Liu et al., 2008).

Já em um estudo com pacientes com DM1, em que fizeram parte trinta e cinco crianças, sendo vinte com saúde normal e quinze com DM1, foi feito um perfil do estado redox desses indivíduos, sendo avaliados parâmetros de lipoperoxidação, oxidação de proteínas e defesas antioxidantes enzimáticas e não-enzimáticas. A lipoperoxidação foi encontrada aumentada no estado diabético. Todos os parâmetros de defesas antioxidantes não-enzimáticas e enzimáticas foram encontrados diminuídos no grupo diabético (Ramakrishna e Jailkhani, 2007).

O estresse oxidativo também está ligado a uma série de doenças que são as principais causas de mortes na atualidade. Entre elas podemos citar as doenças cardiovasculares, o derrame cerebral, doenças neurodegenerativas como o Alzheimer e Parkinson, e o câncer (Rahman, 2007).

1.9. Ácidos Graxos Poli-insaturados

Os ácidos graxos poli-insaturados de cadeia longa (LC-PUFA), chamados de ácido eicosapentaenóico (EPA; 20:5 n-3) e ácido docosaexaenóico (DHA; 22:6 n-3), que são abundantemente encontrados em peixes marinhos, tem ação hipolipidêmica, reduzem eventos cardíacos e

regridem a progressão da aterosclerose (Ruxton et al., 2004). Pesquisas recentes onde os Ω3-PUFAs foram incluídos na dieta de diabéticos demonstraram grandes efeitos sobre o perfil lipídico e os parâmetros corporais dos pacientes (Nettleton e Katz, 2005). Muitos estudos em humanos obesos observaram que após a suplementação com Ω3-PUFAs houve uma grande redução da adiposidade. Já em pacientes diabéticos com o mesmo tipo de suplementação não houve diferença em relação à glicemia. Em roedores alimentados com uma dieta hiperlipídica os Ω3-PUFAs foram eficazes em prevenir a obesidade e melhoraram a tolerância à glicose (Neschen et al., 2007).

Muitos dos efeitos benéficos dos Ω3-PUFAs são amplamente atribuídos ao Receptor Ativador da Proliferação de Peroxisomos (PPAR) (Sanderson et al., 2008).

Atualmente, a recomendação do comitê nutricional da Associação Americana do Coração (AHA) especifica que pacientes sem doença coronariana documentada devem ingerir óleo de peixe duas vezes por semana. Já pacientes com doença coronariana documentada devem ingerir cerca de 1g de EPA:DHA diariamente, de preferência a partir de óleo de peixe, contudo, suplementos podem ser usados, e indivíduos com hipertrigliceridemia devem ingerir de 2 a 4g de EPA:DHA diariamente em cápsulas (Thorsdottir et al., 2007).

Neste sentido, embora estudos com a suplementação destes ácidos graxos em longo prazo sejam necessários, os Ω3-PUFAs da dieta ou suplementados devem ser considerados como parte de uma estratégia efetiva no tratamento de doenças como diabetes, obesidade e síndrome metabólica.

2. Objetivos

2.1. Objetivo Geral

Tendo em vista a grande prevalência do diabetes mellitus na população mundial que a tornou uma das principais doenças da atualidade, gerando grande impacto na sociedade, economia e na saúde coletiva; da ampla utilização de alimentos aquecidos a altas temperaturas pela sociedade; do fácil acesso a alimentos ricos em gorduras; e de poucas estratégias eficazes no combate desta doença; o objetivo geral desta tese foi responder a seguinte questão central: quais são os efeitos das glicotoxinas (endógenas e exógenas) sobre o metabolismo energético do sistema nervoso central e o estado redox do sistema periférico de ratos diabéticos e não diabéticos, e ainda se podem ser alterados através da dieta com os ácidos graxos poli-insaturados ômega 3?

Para responder a esta questão procuramos avaliar os parâmetros abaixo que se caracterizaram como nossos objetivos específicos.

2.2. Objetivos específicos

1. Verificar os efeitos *in vitro* do glioxal e metilglioxal (400 μM) sobre o metabolismo dos aminoácidos glicina (0,2 mM), leucina (0,2 mM), alanina (0,2 mM), glutamina (2 mM), glutamato (0,2 mM) e glucose (5 mM), lactato (10 mM), acetato (1 mM) em córtex cerebral de ratos de 10 dias de idade pós-natal e de ratos adultos (3 meses de idade pós-natal).

2. Estudar os efeitos *in vivo* de uma dieta rica em produtos avançados de glicação e do acréscimo do ácido graxo poliinsaturado ômega 3 nesta dieta sobre a oxidação de glicose, lactato, betahidroxibutirato, captação de 2-

deoxiglicose e transportadores de ácido monocarboxílico em hipocampo de ratos adultos diabéticos e não diabéticos.

3. Detectar os efeitos *in vivo* de uma dieta rica em produtos avançados de glicação e do acréscimo do ácido graxo poliinsaturado ômega 3 nesta dieta sobre o estado redox, conteúdo de lipídios e metabolismo do glicogênio em fígado de ratos adultos diabéticos e não diabéticos.

4. Determinar os efeitos *in vivo* de uma dieta rica em produtos avançados de glicação e do acréscimo do ácido graxo poliinsaturado ômega 3 nesta dieta sobre o estado redox e os produtos avançados de glicação em rim de ratos adultos diabéticos e não-diabéticos.

3. Resultados

Os resultados desta tese serão apresentados na forma de 2 artigos publicados e na forma de 2 manuscritos a serem submetidos à publicação.

3.1. Artigo publicado na revista Brain Research (2009).

Effects of glyoxal or methylglyoxal on the metabolism of amino acids, lactate, glucose and acetate in the cerebral cortex of young and adult rats.

DOI: 10.1016/j.brainres.2009.12.008

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BRAIN RESEARCH

Research Report

Effects of glyoxal or methylglyoxal on the metabolism of amino acids, lactate, glucose and acetate in the cerebral cortex of young and adult rats

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ABSTRACT

The in vitro effects of glyoxal and methylglyoxal on the metabolism of glycine, alanine, leucine, glutamate, glutamine, glucose, lactate and acetate were evaluated in cortico-cerebral slices from young (10-day-old) or adult (3-month-old) rats. In a first set of experiments with cortico-cerebral slices from young animals, the compounds glyoxal or methylglyoxal at 400 μM, increased the oxidation of alanine, leucine and glycine to CO_2 and decreased the protein synthesis from these amino acids. Lipid synthesis from alanine, leucine and glycine was not changed in the cortico-cerebral slices from young rats after glyoxals exposure. Moreover, glutamine oxidation to CO_2 decreased by glyoxals exposure, but glutamate oxidation was not affected. In a second set of experiments with brain slices from adult animals, glycine metabolism (oxidation to CO_2, conversion to lipids or incorporation into proteins) was not changed by glyoxals exposure. In addition, the oxidation rates of glucose, lactate, acetate, glutamine and glutamate to CO_2 were also not modified. Taken together, these results indicate that glyoxal disrupts the energetic metabolism of the rat cerebral cortex in vitro. However, only young animals were susceptible to such events, suggesting that the immature cerebral cortex is less capable of dealing with glyoxal than the mature one.

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1. Introduction

Cardiovascular disease is one of the main causes of mortality among diabetic patients (Hobbs, 2006). High levels of advanced glycation end products (AGEs) induce significant oxidative stress and play an important role in the pathogenesis of diabetic cardiovascular disease (Brownlee, 2001).

Although it is well known that AGEs are a product of hyperglycemia, these compounds are also derived from specific diet types. In fact, foods with high lipid and protein contents result in the highest AGE levels. This may be caused by high levels of free radicals released in the course of various lipoxidation reactions, which catalyze the formation of AGEs and advanced lipoxidation end products (ALEs) from amine-containing lipids during cooking of fats and meats. Glycoxidation and lipoxidation are promoted by heat, the absence of moisture, and the presence of metals (Goldberg et al., 2005).

The pathological effects of AGEs are related to their ability to modify the chemical and biological properties of native molecules by forming cross-linked species and their ability to bind to several cellular receptors (Schmidt et al., 1994; Vlassara, 2001). Glucose and other reducing sugars can react with the amino group of proteins, nucleic acids and lipids, forming a relatively unstable Schiff base (almidine) that is soon enolized to a more stable form in an Amadori rearrangement to create a ketoamine known as an "Amadori compound". In the subsequent second half reaction, the Amadori compound is subjected to complex reactions such as dehydration, rearrangement, and condensation to produce carbonyl compounds such as glyoxal and methylglyoxal, which are much more reactive than the parent sugars (Lapolla et al., 2005). The main source of methylglyoxal in physiological systems comes to degradation of triosephosphates (Lapolla et al., 2005).

Glyoxal and methylglyoxal appear to play important roles in the pathogenesis of angiopathies in diabetes, aging and neurodegenerative processes (Goldin et al., 2006; Ramasamy et al., 2005). These aldehydes are capable of inducing cellular damage and protein glycation, leading to the formation of AGEs (Thornalley, 2005), which, in turn, may also contribute to cytotoxicity and lead to proinflammatory responses. Cellular concentration of methylglyoxal and glyoxal is 1–2 μM and median toxic concentrations are 300 μM–1 mM (Dobler et al., 2006). Although the central nervous system is a target of these compounds (Di Loreto et al., 2008), there are no data in the literature concerning the effects of glyoxal and methylglyoxal on energetic metabolism in this tissue. In the present study, the in vitro effects of glyoxal and methylglyoxal on the metabolism of the amino acids glycine, alanine, leucine, glutamine and glutamate, as well as on the metabolism of the energetic fuels glucose, lactate and acetate, were evaluated in cortico-cerebral slices from young (10-day-old) and adult (3-month-old) rats.

2. Results

2.1. Experiments with cortico-cerebral slices derived from young rats

Considering the critical role of amino acid oxidation in the energetic homeostasis of the CNS (Schelp and Burini, 1995), we first investigated the effects of 400 μM glyoxal or methylglyoxal on the rates of alanine, leucine and glycine oxidation to CO_2 in cortico-cerebral slices derived from young (10-day-old) rats (Fig. 1). Glyoxal significantly increased (around 40%) the oxidation of these three amino acids to CO_2. Methylglyoxal significantly increased (around 30%) alanine oxidation to CO_2.

Fig. 2 depicts the effects of 400 μM glyoxal or methylglyoxal on the rate of alanine, leucine and glycine conversion to lipids in cortico-cerebral slices derived from young (10-day-old) rats. No significant effects were observed. With respect to the incorporation of amino acids into proteins (Fig. 3), 400 μM glyoxal significantly decreased the synthesis of proteins from alanine, leucine and glycine (around 30%, 25% and 65%, respectively). However, methylglyoxal did not show significant effects (Fig. 3).

2.2. Experiments with cortico-cerebral slices derived from adult rats

Taking into account that the developing CNS is more susceptible to pro-oxidative damage, when compared to the adult one (Costa et al., 2004), we also performed experiments using cortico-cerebral slices derived from adult (3-month-old) rats in order to compare with experiments using young animals. Fig. 4 shows the effects of 400 μM glyoxal or methylglyoxal on the rate of glycine metabolism in cortico-cerebral slices derived from adult rats. Neither glyoxal nor methylglyoxal affected the conversion of glycine to CO_2, lipids or proteins. Moreover, glyoxal did not affect the oxidation rates of glucose, lactate or acetate to CO_2 in the cortico-cerebral slices derived from adult rats (Fig. 5). Due to the crucial role of glutamine in the maintenance of the metabolic and neurotransmission in glutamatergic neurons, we also performed a comparative study on the effects of 400 μM glyoxal or methylglyoxal on the rate of glutamine oxidation to CO_2 in the cortico-cerebral slices derived from both young and adult rats (Fig. 6). Both glyoxal and methylglyoxal (at 400 μM)

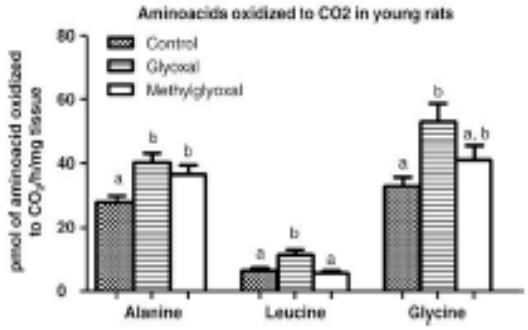

Fig. 1 – Effects of glyoxal or methylglyoxal on the oxidation of amino acids to CO_2 in cortico-cerebral slices from young rats. For incubation details, see Experimental procedures. Amino acid oxidation is expressed as pmol of amino acid oxidized to CO_2/h/mg tissue and represented as mean± SEM (n=6 per group). Different letters indicate statistical differences between groups in each amino acid oxidation assay, as evaluated by one-way analysis of variance followed by the Duncan multiple-range test (assuming $p<0.05$).

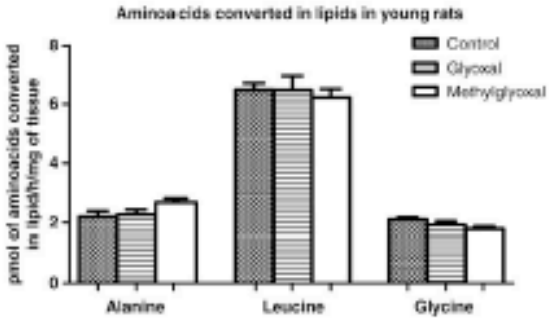

Fig. 2 – Effects of glyoxal or methylglyoxal on the conversion of amino acids to lipids in cortico-cerebral slices from young rats. For incubation details, see Experimental procedures. Data are expressed as pmol of amino acid converted into lipids/h/mg of tissue and represented as mean ± SEM (n=6 per group). No statistical differences were found between groups in each amino acid assay of lipid incorporation, as evaluated by one-way analysis of variance followed by the Duncan multiple-range test (assuming $p<0.05$).

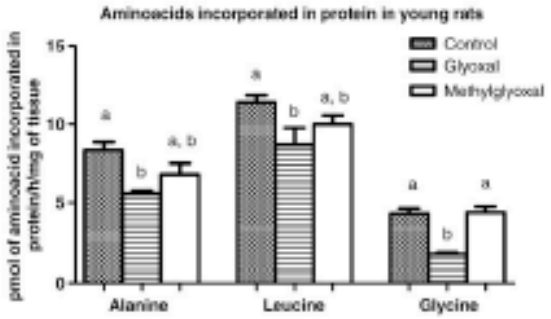

Fig. 3 – Effects of glyoxal or methylglyoxal on the incorporation of amino acids to proteins in cortico-cerebral slices from young rats. For incubation details, see Experimental procedures. Data are expressed as pmol of amino acid incorporated into proteins/h/mg of tissue and represented as mean ± SEM (n=6 per group). Different letters indicate significant differences ($p<0.05$) by one-way analysis of variance followed by the Duncan multiple-range test. Different letters indicate statistical differences between groups in each amino acid assay of protein incorporation, as evaluated by one-way analysis of variance followed by the Duncan multiple-range test (assuming $p<0.05$).

significantly decreased (20–25%) glutamine oxidation to CO_2 in the cortico-cerebral slices derived from young animals. However, no such effect was observed in the cortical slices from adult animals. Neither glyoxal nor methylglyoxal modified the oxidation of glutamate to CO_2 in the cortico-cerebral slices from young and adult animal (Fig. 7).

3. Discussion

The most important finding of this study was that glyoxal, which has been reported to contribute to the formation of advanced glycation end products (AGEs) (Thornalley, 2003), increased the rate of CO_2 production from the amino acids alanine, leucine and glycine in cortico-cerebral slices derived from young rats. Conversely, the incorporation of these amino acids into proteins was decreased after glyoxal exposure. Although it has been reported that glyoxals are able to affect the energetic metabolism, which can lead to decreased ATP levels and mitochondrial dysfunction (de Arriba et al., 2007), there are no data in the literature on the effects of glyoxals (methylglyoxal or glyoxal) on the metabolism of amino acids in cerebral tissues.

Although the exact intracellular concentration of glyoxals in neurons is not yet known, methylglyoxal concentrations of up to 300 μM have been measured in cultured CHO cells, most of it bound to cysteine residues and glutathione (Chaplen et al., 1998). In addition, the concentration of these aldehydes can increase when the concentrations of their precursors (triosephosphates) are elevated, such as in triosephosphate deficiency, hyperglycemia and impaired glucose utilization (Ahmed et al., 2003). Therefore, the glyoxal concentration (400 μM) used in our study appears to be relevant from a physiological point of view.

The metabolism of amino acids is extremely important to the homeostasis of the central nervous system (CNS). In addition to their crucial roles as precursors of neurotransmitters and proteins, all of the amino acids can be oxidized to CO_2 and H_2O, contributing to the energetic homeostasis of the CNS (Schelp and Burini, 1995). The increased oxidation of alanine, glycine and leucine observed in the cortico-cerebral slices exposed to glyoxal was an unexpected event observed in our experimental protocol. de Arriba et al. (2007) showed that methylglyoxal induced a decrease in mitochondrial membrane potential and intracellular ATP levels in cultured

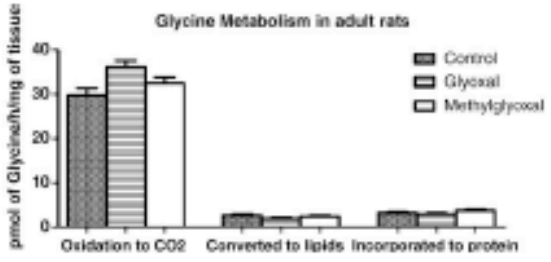

Fig. 4 – Effects of glyoxal or methylglyoxal on the metabolism of glycine in cortico-cerebral slices from adult rats. For incubation details, see Experimental procedures. Glycine oxidation is expressed as pmol of amino acid oxidized to CO_2/h/mg tissue. Glycine conversion to lipids and incorporation into proteins are expressed as pmol of glycine converted into lipids/h/mg of tissue and pmol of amino acid incorporated into proteins/h/mg of tissue, respectively. Data are represented as mean ± SEM (n=6 per group). No statistical differences in oxidation or incorporation were found between groups in each glycine assay, as evaluated by one-way analysis of variance followed by the Duncan multiple-range test (assuming $p<0.05$).

SH-SY5Y neuroblastoma cells, suggesting that the oxidative (mitochondrial) metabolism is decreased after exposure to glyoxals. However, our results showed increased CO_2 production from alanine, glycine and leucine. This effect might represent a compensatory response to the inhibition of glyceraldehyde-3-phosphate dehydrogenase by glyoxals (Morgan et al., 2002), which is responsible for decreased oxidation of glucose, the main substrate for neurons.

Although the rates of alanine, leucine and glycine oxidation to CO_2 were increased in the cortico-cerebral slices from young animals after glyoxal exposure, the rate of glutamine oxidation was decreased. The latter effect was observed after exposures to both glyoxal and methylglyoxal. This indicates that the effects of glyoxals on the oxidative profile of the amino acids in the cortico-cerebral slices depend on the specific amino acid and do not represent a general phenomenon. Glutamine oxidation is of particular importance to neurons. In fact, glutamine can first be generated from glutamate in astrocytes in a reaction catalyzed by the astrocyte-specific enzyme, glutamine synthetase (GS) (Martinez-Hernandez et al., 1977). Astrocyte-derived glutamine is used by neurons for the re-synthesis of glutamate. This glutamate–glutamine pathway constitutes a major portion of the glutamate pool in the brain (Berl et al., 1962). In neurons, glutamate can be transported into synaptic vesicles, but can also be oxidized to CO_2. The lower oxidation rates of glutamine observed in the cortico-cerebral slices exposed to both glyoxals could be related to either decreased glutamine uptake or decreased glutaminase activity. Our results indicate that glutamate oxidation to CO_2 was not changed by glyoxal or methylglyoxal exposure in young and adult rats; possibly, decreased glutaminase activity reduces glutamine oxidation in young rats. It is important to mention that glutamate and glutamine entrance into the CNS is insignificant (Yudkoff et al., 2008) and that the endogenous synthesis of these amino acids is dependent upon pyruvate carboxylase, an enzyme specifically found in astrocytes. Moreover, more than 1/3 of nitrogen in the glutamate pool comes from leucine (Yudkoff et al., 1996) and, therefore, an increment of leucine oxidation induced by glyoxal could change the metabolic relationships of the glutamate–glutamine–leucine cycle.

It is important to mention that the effects of glyoxals were more pronounced in the cortico-cerebral slices derived from young rats. In contrast, these events were not observed in the slices derived from adult animals. These results suggest that the developing brain is more susceptible to and less capable of

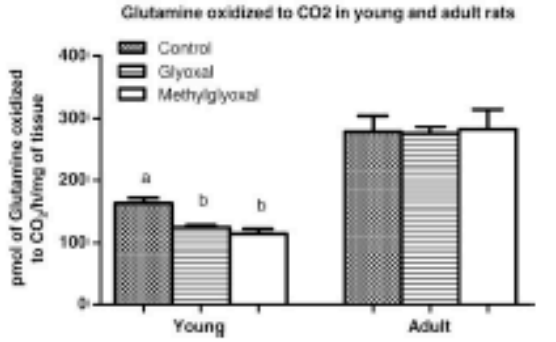

Fig. 6 – Effects of glyoxal or methylglyoxal on the oxidation of glutamine to CO_2 in cortico-cerebral slices from young and adult rats. For incubation details, see Experimental procedures. Glutamine oxidation is expressed as pmol of glutamine oxidized to CO_2/h/mg tissue and represented as mean ± SEM ($n = 6$ per group). Different letters indicate statistical differences between groups for the glutamine oxidation assay in young animals, as evaluated by one-way analysis of variance followed by the Duncan multiple-range test (assuming $p < 0.05$). No differences were found in adult animals.

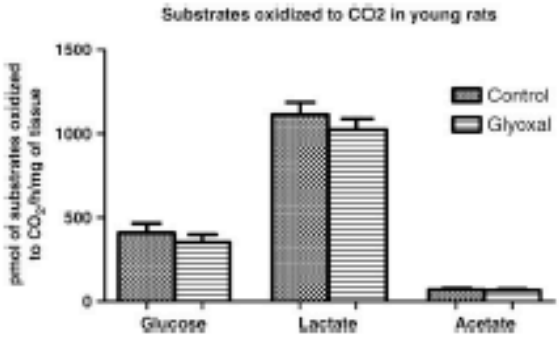

Fig. 5 – Effects of glyoxal on the oxidation of glucose, lactate and acetate in cortico-cerebral slices from adult rats. For incubation details, see Experimental procedures. Rates of oxidation are expressed as pmol of substrate oxidized to CO_2/h/mg tissue and represented as mean ± SEM ($n = 6$ per group). No statistical differences in substrate oxidation were found between groups in each assay, as evaluated by one-way analysis of variance followed by the Duncan multiple-range test (assuming $p < 0.05$).

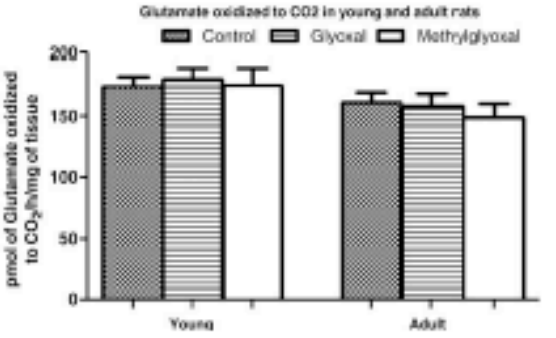

Fig. 7 – Effects of glyoxal or methylglyoxal on the oxidation of glutamate to CO_2 in cortico-cerebral slices from young and adult rats. For incubation details, see Experimental procedures. Glutamate oxidation is expressed as pmol of glutamine oxidized to CO_2/h/mg tissue and represented as mean ± SEM ($n = 6$ per group). No statistical differences were found in glutamate oxidation to CO_2 between groups in young and adult rats, as evaluated by one-way analysis of variance, followed by the Duncan multiple-range test (assuming $p < 0.05$).

dealing with glyoxals. From a mechanistic point of view, it is known that glyoxals can interact with and deplete intracellular glutathione (Di Loreto et al., 2008). The fact that glyoxals only had significant effects in the cortico-cerebral slices from young animals might be related to the lower levels of glutathione in the cerebral cortex of young animals. In agreement with this idea, we have previously showed that weanling pups present low glutathione levels in the cerebral cortex and that the levels of this important intracellular antioxidant increase during the early post-natal development (Stringari et al., 2008). The lower glutathione levels in the cerebral cortex of young animals might allow for the interaction of glyoxals with the sulfhydryl groups of proteins involved in energetic metabolism and antioxidant protection (Morgan et al., 2002). Since glycine is supplied by astrocytes to neurons in the form of the dipeptide cysteinyl-glycine, which contributes to the maintenance of intracellular glutathione homeostasis (Dringen et al., 1999), the increased glycine oxidation to CO_2 after glyoxal exposure could also contribute to decreased glutathione levels in the cortico-cerebral slices of young rats. In fact, the only route for glycine catabolism in the CNS involves the glycine cleavage system, which is specifically found in astrocytes (Sato et al., 1991; Bommaccanti et al., 1996). Therefore, it is possible to conceive that glyoxal caused an increase in the activity of the glycine cleavage system. However, it may be noted that energy metabolisms of glucose, lactate and acetate were preserved.

Altogether, these results indicate that the glyoxals studied here (mainly glyoxal) can alter the energetic metabolism of amino acids in the rat cerebral cortex under *in vitro* conditions. Although it is well known that glyoxal and methylglyoxal play important roles in the pathogenesis of neurodegenerative processes (Ramasamy et al., 2005), the relationship between the present data with the development of neuropathological conditions represents an unsolved question. Moreover, it is important to emphasize that only young animals were susceptible to these effects, suggesting that the immature cerebral cortex is less able to deal with glyoxals compared to the mature tissue. Taking into account that the developing CNS is dependent on amino acids for neurotransmitters and protein synthesis to a higher extent than the mature one, additional studies examining the neurotoxic effects of such aldehydes on the developing brain appear to be important.

4. Experimental procedures

4.1. 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 [U-^{14}C]glycine (100 mCi/mmol), L[U-^{14}C]leucine (323 mCi/mmol), L[U-^{14}C]alanine (150 mCi/mmol), D[U-^{14}C] glucose (250–360 mCi/mmol), L[U-^{14}C]lactate (56–186 mCi/mmol), and [U-^{14}C]acetate (45–60 mCi/mmol) were from Perkin-Elmer (Boston, MA, USA). L[U-^{14}C]glutamine (250 mCi/mmol) was from Amersham International (Buckinghamshire, UK).

4.2. Animals

Wistar rats (10 days (male or female) or 3 months old (male)) were obtained from our own breeding colony at the Department of Biochemistry (Universidade Federal do Rio Grande do Sul). They were maintained at 21–23°C, on a 12:12-h light/dark cycle, with free access to water and commercial laboratory diet (NUVILAB, Porto Alegre, Brazil).

4.3. Tissue preparation

Six rats were killed by decapitation in each experiment. The complete cerebral cortex was dissected, weighed, and cut into 0.3-mm slices using a McIlwain tissue chopper. The protocol used in this research followed the guidelines of the Committee on Care and Use of Experimental Animal Resources, School of Veterinary Medicine and Animal Science of the University of São Paulo, Brazil, and was approved by the Ethics Committee (Comitê de Ética no Uso de Animais—UFRGS).

4.4. Incubations

For the measurement of lipid or protein synthesis and CO_2 production, brain-cortical slices (between 60 and 70 mg) were incubated in 1.0 mL Krebs Ringer bicarbonate (KRb) buffer pH 7.4, containing either (i) 5.0 mM D-glucose + 0.2 mM L-alanine + 0.2 μCi L-[U-^{14}C] alanine; (ii) 5.0 mM D-glucose + 0.2 mM L-leucine + 0.2 μCi L-[U-^{14}C] leucine; (iii) 5.0 mM D-glucose + 0.2 mM glycine + 0.2 μCi [U-^{14}C] glycine; (iv) 5.0 mM D-glucose + 0.2 mM L-leucine + 0.2 μCi L-[U-^{14}C] leucine; (v) 2.0 mM glutamine + 0.2 μCi L-[U-^{14}C]glutamine; (vi) 10.0 mM L-lactate + 0.2 μCi L-[U-^{14}C]lactate; (vii) 5.0 mM glucose + 0.2 μCi D-[U-^{14}C]glucose; (viii) 1.0 mM acetate + 0.2 μCi [U-^{14}C]acetate; (ix) 0.2 μM L-glutamate + 0.1 μCi L-[U-^{14}C] glutamate. Before incubation, the reaction medium was gassed 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 3 filter paper were hung from the stoppers. Slices were incubated at 35°C for 1 h in a Dubnoff metabolic shaker (60 cycles/min) according to the method of Dunlop et al. (1975) and Ferreira et al. (2007). Glyoxal or methylglyoxal at final concentration of 400 μM was added in the incubation media. Preliminary results indicated that a 1-h pre-incubation (without radio-labeled substrates) followed by 1-h incubation with these glyoxals was not different from a single 1-h incubation in the presence of these glyoxals (data not shown). 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 flask contents 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 radioactivity was measured. The resulting precipitate was dissolved in

concentrated formic acid and radioactivity was measured. This radioactivity represents protein synthesis from amino acids. All the results were expressed with respect 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.

4.5. Statistical analyses

Data were analyzed by one-way analysis of variance, followed by the Duncan multiple-range test. Differences were considered significant when $p<0.05$.

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Effect of high fat thermolyzed diet on monocarboxylate transporters and energetic metabolism in the hippocampus of diabetic rats and the benefits of the Ω3 polyunsaturated fatty acids

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Abstract

Diabetes mellitus (DM), a state of chronic hyperglycemia, is a common disease and one of the leading causes of morbidity and mortality in developed countries. The aim of this study was to evaluate the effect of high fat thermolyzed diet on monocarboxylate transporters and energetic metabolism in the hippocampus of diabetic rats and the benefits of the Ω3 polyunsaturated fatty acids. Diabetes was induced by an intraperitoneal (i.p.) injection of alloxan (150 mg kg^{-1} b.w.). Rats were divided into six groups: (1) the control group (C, n=10), which received standard laboratory rat chow; (2) the control diabetic group (D, n=10), which received an injection of alloxan and the same diet as group 1; (3) the high fat thermolyzed diet group (HFTD, n=10), which received a diet enriched with fats that had been heated for 60 min at 180 °C to which a vitamin mixture was added after heating; (4) the high fat thermolyzed diet diabetic group (D+HFTD, n=10), which received an injection of alloxan and the same diet as group 3; (5) the high fat thermolyzed diet + Ω3 polyunsaturated fatty acid group (HFTD+Ω3), which received a diet enriched with fats that had been heated for 60 min at 180 °C to which a vitamin mixture and an omega 3 fatty acid supplement was added after heating; and (6) the high thermolyzed fat diet + Ω3-polyunsaturated fatty acid diabetic group (D+HFTD+Ω3, n=10), which received an injection of alloxan and the same diet as group 5. After one month of diet the rats were sacrificed by decapitation and the hippocampi quickly removed and processed for further analysis. In the glucose oxidation to CO_2 we didn't observe statistical differences. However, we note that the HFTD diet and diabetes increases the β-hydroxybutyrate oxidation to CO_2, and we have a decrease in the lactate oxidation only in D+HFTD group. In western blot, we observed an increase in the MCT1 and MCT2 immunocontent only in the D+HFTD group. A high fat thermolyzed diet decreased lactate oxidation

and increased β-hydroxybutyrate oxidation, MCT1 and 2 immunocontent in diabetic rats (D+HFTD) in the hippocampus. We note a relationship between oxidation this substrates and his transporter. The omega 3 was effective in normalizing the monocarboxylic acids metabolism.

Keywords: Advanced glycation end products; energetic metabolism; diabetes; monocarboxylate transporter; Ω3-polyunsaturated fatty acids; high fat diet.

Introduction

A major problem in public health today is the increasing incidence of age-related chronic diseases, most prominently obesity, diabetes, and cardiovascular and renal disorders. Some nutritional habits can lead to metabolic alterations that certainly have a critical influence not only on health in general but more specifically on brain function. This notion is supported by recent observations implying that the typical diet of Western countries nowadays, rich in sugar and saturated fat, and the associated metabolic consequences for the organism may contribute to cognitive decline in ageing and accelerate the course of dementia in Alzheimer's disease (Kalmijn, 2000).

Glucose is classically considered the main energy substrate of the brain. In recent years, however, evidence has been provided for the notion that the brain can make use of alternative energy substrates under specific circumstances. For example, fatty acids provided by the diet readily cross the blood–brain barrier (BBB) and can contribute significantly to fulfil brain energy needs (Ebert *et al.* 2003). In addition to their direct utilization by the brain, fatty acids are converted in the liver to ketone bodies, i.e. *β*-hydroxybutyrate and acetoacetate, which can be also subsequently oxidized by brain cells. Under normal circumstances, the concentration of ketone bodies in the circulation is low and their utilization by the adult brain is considered of little physiological significance. But it can be reactivated during prolonged ketosis, diabetes and fasting (Cremer, 1982;Hawkins *et al.* 1986). Ketone bodies may thus become an important source of energy for the brain, depending of their blood concentration (Morris, 2005). In addition to its formation from glucose within the brain parenchyma, notably by astrocytes (Pellerin, 2003), it was shown that elevation of blood lactate, as occurs during moderate exercise, is not only

sufficient to promote its use by the brain (Dalsgaard *et al.* 2004) but it also reduces in parallel cerebral glucose consumption (Smith *et al.* 2003).

Monocarboxylate transporters (MCTs) form a large family of proton-linked carriers that have the ability to transport lactate, pyruvate and the ketone bodies (Halestrap & Meredith, 2004; Pierre & Pellerin, 2005). In the central nervous system, three MCTs have been identified and their distribution determined at the cellular level. MCT1 is mostly expressed by endothelial cells and astrocytic processes in rodents (Pellerin *et al.* 1998; Pierre *et al.* 2000; Baud *et al.* 2003) and in humans (Froberg *et al.* 2001; Chiry *et al.* 2006). MCT2 is the major neuronal transporter in the rodent brain (Pierre *et al.* 2002) while MCT4 is exclusively expressed in astrocytes (Pellerin *et al.* 2005).

A common feature of brain circuit components, such as dendrites, synaptic membranes, and other membranes of the nervous system, is that they are richly endowed in docosahexaenoic acid (DHA), the main member of the omega-3 essential fatty acid family. DHA is avidly retained and concentrated in the nervous system and known to play a role in neuroprotection, memory, and vision (Bazan et al., 2011).

De Assis et al. 2009, showed that normal rats fed with a high thermolyzed fat diet for 12 months had significant increases in hippocampus DNA damage compared to those fed a normal high fat diet. In this study, we investigated the effect of high fat thermolyzed diet on monocarboxylate transporters and energetic metabolism (glucose, lactate and β-hydroxybutyrate) in hippocampus of diabetic rats and the benefits of the Ω3 polyunsaturated fatty acids

Methods

Chemicals

Sodium hydroxide was purchased from J.T. Baker Chemical Company, Phillisburg, NJ, USA. L[U-^{14}C]lactate (56-186 mCi/mmol) and Optiphase Hi-Safe 3 were purchased from Perkin-Elmer (Boston, MA, USA). Alloxan monohydrate and sodium L-lactate were obtained from Sigma-Aldrich, St. Louis, MO, USA. Electrophoresis/immunoblotting reagents were from Bio-Rad Laboratories (Hercules, CA, USA). D-[U-^{14}C]-Glucose (297 mCi/mmol) and L-[U-^{14}C]-Lactate (152 mCi/mmol) was purchased from Amersham International (Little Chalfont, Bucks, UK). [1-^{14}C]-3-Hydroxybutyric acid, sodium salt (50 mCi/mmol) was purchased from American Radiolabeled Chemicals, Inc. (Saint Louis, MO, USA). We used anti-MCT1 and anti-MCT2 antibody characterized in Pierre *et al.* 2000.

Animals and Treatment

Treatments and diets

Diabetes mellitus was induced by an intraperitoneal (i.p.) injection of alloxan (150 mg kg^{-1} b.w.), and non-diabetic animals received a saline solution (NaCl 0.9%), also via an i.p. injection. After 10 days, blood samples were obtained from rats after a 12h fasting to determine the plasma glucose concentration. Only animals that presented with 15–30 mmol/l glucose concentration were included in the study.

Sixty adult Wistar rats (male, 60 days old) from the Central Animal House of the Department of Biochemistry were maintained under a standard dark–light cycle (lights on between 7:00 a.m. and 7:00 p.m.) at room temperature (22±2 °C). All experiments were conducted in accordance with

the Guiding Principles in the Use of Animals in Toxicology, adopted by the Society of Toxicology in July 1989, and all experiments were approved by our ethics committee for animal use at the Federal University of Rio Grande do Sul. Rats were divided into six groups, to see more details Figure 1: (1) the control group (C, n=10), which received standard laboratory rat chow; (2) the control diabetic group (D, n=10), which received an injection of alloxan and the same diet as group 1; (3) the high fat thermolyzed diet group (HFTD, n=10), which received a diet enriched with fats that had been heated for 60 min at 180 °C to which a vitamin mixture was added after heating; (4) the high fat thermolyzed diet diabetic group (D+HFTD, n=10), which received an injection of alloxan and the same diet as group 3; (5) the high fat thermolyzed diet + Ω3 polyunsaturated fatty acid group (HFTD+Ω3), which received a diet enriched with fats that had been heated for 60 min at 180 °C to which a vitamin mixture and an omega 3 fatty acid supplement was added after heating; and (6) the high thermolyzed fat diet + Ω3-polyunsaturated fatty acid diabetic group (D+HFTD+Ω3, n=10), which received an injection of alloxan and the same diet as group 5. Diet thermolyzation was based on the study by Sandu et al., 2005, which indicated a high AGE content (around 1 unit/μg) in a high fat diet after thermolyzation. The salt and vitamin compositions used were the same as those described by Horwitz et al., 1980. During the treatment period of four weeks, the animals had free access to food and water, to see more details about diet Table 1.

Tissue Preparation

Rats were killed by decapitation. Blood was collected immediately after decapitation into heparinized tubes. The whole blood was centrifuged at 2500 x

g for 10 min to yield the plasma fraction, which was used for subsequent biochemical analyses.

The brains of the rats were removed in no more than 1 min. These were placed on a Petri plate dish with filter paper and over ice. The brains were washed with Krebs Ringer bicarbonate buffer solution, pH 7.4, at 3°C. Hippocampus was dissected and: (i) weighted and cut into slices to lactate oxidation to CO_2 or (ii) homogenized in a buffer of 0.32 M sucrose containing Hepes 1mM, MgCl2 1mM, NaHCO3 1mM, phenyl-methyl-sulphonyl fluoride 0.1mM, pH 7.4, in the presence of a complete set of protease inhibitors (Complete, Roche, Switzerland).

Blood Biochemical Parameters

Plasma glucose, lactate (Labtest, MG, Brazil) and β-Hydroxybutyrate (Cayman Chemical Company, MI, USA) concentrations were measured using commercial kits. Reactions were performed using the Labmax apparatus.

Substrate Oxidation to CO_2

For the measurement of lactate, glucose and B-Hydroxybutyrate oxidation to CO_2, hippocampus was dissected, weighed, and cut into 0.3-mm slices using a McIlwain tissue chopper. This was incubated in closed bottles in a medium containing Krebs Ringer bicarbonate buffer (pH 7.4) with 10 mM sodium L-lactate and 0.2 µCi L[U-^{14}C]lactate (56-186 mCi/mmol). Incubations were carried out in flasks after contents were gassed with a 95% O_2:5% CO_2 mixture for 1 min. Flasks were subsequently sealed with rubber caps. The slices of tissue were incubated at 37°C for 1 h in a metabolic shaker (60 cycles/min), according to the method of Dunlop et al., 1975. Incubation was stopped by adding 0.20 ml TCA 50% through the rubber cap. Subsequently, 0.1 ml of 2 M

sodium hydroxide was injected into central wells. The flasks were shaken further for 30 min at 37°C to trap CO_2. Next, the contents of the central well were transferred to vials and assayed for CO_2 radioactivity in a liquid-scintillation counter. All results were expressed considering the initial specific activity of the incubation medium. The CO_2 production rate was constant during 30, 60 and 90 min of incubation.

Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and immunoblot

Proteins (20 µg) were separated by SDS-PAGE on 10% (w/v) acrylamide and 0.275% (w/v) bisacrylamide gels and were then electrotransferred onto nitrocellulose membranes. The membranes were then incubated in Tris-buffered saline Tween-20 [TBS-T; 20 mM Tris–HCl, pH 7.5, 137 mM NaCl, 0.05% (v/v) Tween 20] containing 1% (w/v) non-fat milk powder for 1 h at room temperature. Subsequently, the membranes were incubated for 12 h with the appropriate primary antibody (MCT1, 1:800; MCT2, 1:600 (Pierre et al., 2000) and β-Actin, 1:10000 (Sigma Aldrich)). After washing in TBS-T, blots were incubated with horseradish peroxidase-linked anti-immunoglobulin G (IgG) antibodies for 1.5 h at room temperature. Chemiluminescent bands were detected, and densitometric analysis was performed using Image-J® software.

Statistical Analyses

Biochemical data are expressed as the mean ± S.D. All analyses were performed using the Statistical Package for the Social Sciences (SPSS 16.0) software. Likewise, the differences between all groups were analyzed using

one-way ANOVA and Tukey's post hoc test. *P*-values were considered significant when $P < 0.05$.

Results

Table 2 shows the biochemical parameters in our experimental groups. We observed a significant difference ($P<0.05$) between the plasma glucose of diabetic and non-diabetic rat, thus demonstrating that our model was effective in inducing diabetes mellitus. However, diabetic rats fed a HFTD or HFTD+Ω3 diet showed a decrease in their glucose concentration. This effect was attributed to a lower amount of carbohydrate in these diets as compared to the control diet. However, in adding polyunsaturated fatty acids in the diet, we noticed a significant increase ($P<0.05$) in plasma lactate in these groups. We also evaluated the concentration of β-hydroxybutyrate in plasma and we can observe that diet and diabetes increased significantly ($P<0.05$) the amount of ketone bodies, however omega 3 was effective to prevent this increase just in non-diabetic rats (HFTD+ Ω3).

In the Figure 2, we can observe the effect of different diets on β-hydroxybutyrate oxidation to $^{14}CO_2$ (A) and lactate oxidation to $^{14}CO_2$ (B). A high fat thermolyzed diet increased β-hydroxybutyrate oxidation significantly ($P<0.05$) in both diabetic and non-diabetic rats (HFTD and D+HFTD), when we added omega 3 supplements, we can observe the same increase (HFTD+ Ω3 and (D+HFTD+ Ω3). Already, in the Figure 2B we can observe a significant ($P<0.05$) decrease in lactate oxidation caused by the group D+HFTD in relation to other groups.

In Figure 3, we show the results of the analysis of glucose metabolism. We did not find a significant difference in both levels the oxidation (Figure 3A) and glucose uptake (Figure 3B) among all groups.

Figure 4 shows the immunocontent of MCT1 (Figure 4A) and MCT2 (Figure 4B). All diabetic groups and groups supplemented with a high fat diet, showed significant ($P<0.05$) increases in the immunocontent of MCT1 when compared to the non-diabetic control group (Figure 4A). This increased was higher in the D+HFTD in relation to the others groups. In the Figure 4B, only the diabetic rats that received the high fat thermolyzed diet (D+HFTD) showed a significant increase ($P<0.05$) in MCT2 immunocontent when compared to the non-diabetic control group. However, supplementation of their diet with omega 3 in diabetic group (D+HFTD+Ω3) reduced these values to below normal levels (that of the control).

Discussion

The high prevalence of CNS complications resulting from DM is a problem that is gaining more acceptance and attention. Recent evidence suggests morphological, electrophysiological and cognitive changes associated with chronic hyperglycaemia. Many of the CNS changes observed in diabetic patients and animal models of diabetes are reminiscent of the changes seen in normal aging. The central commonalities between diabetes-induced and age-related CNS changes have led to the theory of advanced brain aging in diabetic patients (Wrighten et al., 2009). Furthermore, the patterns of volumetric and neurocognitive deficits in diabetic populations have been shown to be rather similar to those reported in populations of individuals with major depressive disorders (McIntyre et al., 2010). It is not known, however, whether these observations may partly explain the known connection between DM and increased risk of depression (Nouwen et al., 2010).

In our study, we evaluated effect of high fat thermolyzed diet on monocarboxylate transporters and energetic metabolism in the hippocampus of diabetic rats and the benefits of the Ω3-polyunsaturated fatty acids. The most prominent results we obtained in this work was that high fat thermolyzed diet decreased lactate oxidation (Figure 2B) and increased β-hydroxybutyrate oxidation (Figure 2 A), MCT1 (Figure 4A) and MCT2 (Figure 4B) immunocontent in diabetic rats (D+HFTD) in the hippocampus. We note a relationship between oxidation this substrates and his transporter. The omega 3 was effective in normalizing the monocarboxylic acids metabolism. However, we don't know if this effect of Ω3-PUFAs is a reflection of its peripheral action or if it actually action in the CNS.

In adulthood, cerebral MCT1 expression was shown to be up-regulated

after 3 weeks of a ketogenic diet (Leino et al. 2001). Pierre et al., 2007, shows in your study to describe gradual and extensive changes in the expression of all three MCT isoforms found in the central nervous system in adult animals exposed to a high fat diet, as well as elevated expression of the same MCT isoforms in brains of genetically obese or diabetic mice.

It is widely accepted that central nervous system (CNS) neurons rely on glucose to maintain metabolism, but several studies have shown that lactate, a product of the glycolytic pathway, acts as an energy source for rat brain neurons and astroglia in vitro (Vicario et al., 1991). Lactate can support synaptic function in the hippocampal slice (Schurr et al., 1988) and appears to be necessary for recovery of this preparation from hypoxia (Schurr et al., 1997). Glia release lactate under conditions of hypoxia or hypoglycemia, (Walz and Mukerji, 1988) and lactate release has also been demonstrated in conscious rats in response to a variety of metabolic insults (Korf, 1996).

Altogether, these results indicate that high fat thermolyzed diet and diabetes can affect energetic metabolism, maily lactate metabolism and monocarboxylate transporters in astrocyte (MCT1) and neuron (MCT2). Moreover, the Ω3-PUFAs seem to normalize the changes promoted by diet and diabetes, but we can not say exactly whether this effect is central or peripheral. Further studies are necessary for a better understanding of these molecular mechanisms.

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Legends

Figure 1. Experimental model. Groups are diabetic and non-diabetic rats fed a control diet (C), high fat thermolyzed diet (HFTD) and high fat thermolyzed diet + Ω3 (HFTD+Ω3).

Figure 2. β-Hydroxybutyrate oxidation to $^{14}CO_2$ (A) and Lactate oxidation to $^{14}CO_2$ (B) in hippocampus of diabetic and non-diabetic rats fed a control diet (C), high fat thermolyzed diet (HFTD) and high fat thermolyzed diet + Ω3 (HFTD+Ω3). β-Hydroxybutyrate and lactate oxidation is expressed as pmol of substrate oxidized to CO2/h/mg tissue and represented as the mean ± SD (n=10 per group). Different asterisks on the same row indicate a significant difference ($P< 0.05$) all groups were analyzed using one-way ANOVA and Tukey's post hoc test.

Figure 3. Glucose oxidation to $^{14}CO_2$ (A) and Uptake of 2-deoxy-D-glucose (B) in hippocampus of diabetic and non-diabetic rats fed a control diet (C), high fat thermolyzed diet (HFTD) and high fat thermolyzed diet + Ω3 (HFTD+Ω3). Uptake of 2-deoxy-D-glucose is expressed as pmol of uptake of 2-deoxy-D-glucose/30min/mg tissue. Glucose oxidation is expressed as pmol of glucose oxidized to CO2/h/mg tissue. All results are presented as the mean ± SD (n=10 per group). Different asterisks on the same row indicate a significant difference ($P< 0.05$) all groups were analyzed using one-way ANOVA and Tukey's post hoc test.

Figure 4. Representative and quantitative Western blot analysis of the hippocampus MCT1 (A) and MCT2(B) immunocontent from diabetic and non-diabetic rats fed a control diet (C), high fat thermolyzed diet (HFTD) and high fat thermolyzed diet + Ω3 (HTFD+Ω3). The graphic represents the means ± SD (n=3 per group). Different asterisks indicate a significant difference ($P< 0.05$) all groups were analyzed using one-way ANOVA and Tukey's post hoc test.

Table 1. The composition of control (C), high fat thermolyzed (HFTD) and high fat thermolyzed + Ω3 (HFTD+Ω3) experimental diets that were fed to diabetic and non-diabetic rats.

Composition	Control Diet (%)	High Fat Thermolyzed Diet (%)	High Fat Thermolyzed Diet + Ω3 (%)
Commercial bran	-	20.5	20.5
Soy Protein Isolate [a]	27.0	15.9	15.9
Corn Starch	55,5	-	-
Sucrose	5.0	10.0	10.0
Vitamin mix [b]	1.0	1.0	1.0
Mineral salt mix[c]	4.0	2.0	2.0
DL-Methionin [d]	0.3	0.3	0.3
DL-Lysine [e]	0.3	0.3	0.3
Soy Oil	5.0	1.0	1.0
Lard	-	49.0	46.0
Ω 3 Fatty Acid [f]	-	-	3.0

[a] Soy Protein Isolate, purity 97% (from Solae, Esteio, Brazil).

[b] Vitamin mixture: mg/100g of diet (from Roche, SãoPaulo, Brazil): vitamin A (retinyl acetate),4; vitamin D (cholecalciferol), 0.5; vitamin E (DL-α-tocopheryl acetate), 10; menadione, 0.5; choline, 200; PABA, 10; inositol,10; niacine (nicotinic acid),4; pantothenicacid (calcium D- pantothenate), 4;riboflavin, 0.8;thiamin (thiamine hydrochloride), 0.5; pyridoxine (pyridoxine hydrochloride),0.5; folic acid, 0.2; biotin [D-(+)- biotin], 0.04; vitamin B12, 0.003.

[c] Mineral salt mixture: mg/100 g of diet (from Roche, SãoPaulo, Brazil): NaCl, 557; KI,3.2 ;KH_2PO_4, 1556; $MgSO_4$, 229; $CaCO_3$, 1526; $FeSO_4–7H_2O$, 108; $MnSO_4–H_2O$, 16; $ZnSO_4–7H_2O$, 2.2; $CuSO_4–5H_2O$, 1.9; $CoCl–6H_2O$, 0.09.

[d] D-L-Methionin (from Merk, Rio de Janeiro, Brazil).

[e] DL-Lysine (from Merk, Rio de Janeiro, Brazil).

[f] Fish oil: Docosahexaenoic Acid (DHA) 5 : 1 Eicosapentaenoic Acid (EPA) (Naturalis SA, Brazil.)

Note: Salt and vitamin composition are according to Horwitz.

Table 2. Biochemical parameters in diabetic and non-diabetic rats fed a control diet (C), high fat thermolyzed diet (HFTD) and high fat thermolyzed diet + Ω3 (HFTD+Ω3). Glucose and lactate levels are expressed as nmol/L. β-Hydroxybutyrate concentrations are expressed in mM. All parameters represented as the mean ± SD (n=10 per group). Different asterisks on the same row indicate a significant difference (P< 0.05) all groups were analyzed using one-way ANOVA and Tukey's post hoc test.

Biochemical Parameters	C	D	HFTD	D+HFTD	HFTD+ω3	D+FTD+ω3
Glucose (mmol/L)	5.2 ± 0.4	22.2 ± 2.8 *	5.2 ± 0.5	20.2 ± 2.0 *	5.07 ± 1.3	21.9 ± 2.6 *
Lactate (mmol/L)	3.84 ± 0.93	3.96 ± 0.75	4.65 ± 0.68	4.44 ± 0.69	5.55 ± 0.49 *	5.51 ± 0.99 *
β-Hydroxybutyrate (mM)	0.42 ± 0.19	2.03 ± 0.37 *	1,45 ± 0.42*	3.66 ± 1.46*	0.45 ± 0.022	3.24 ± 1,17*

Figure 1.

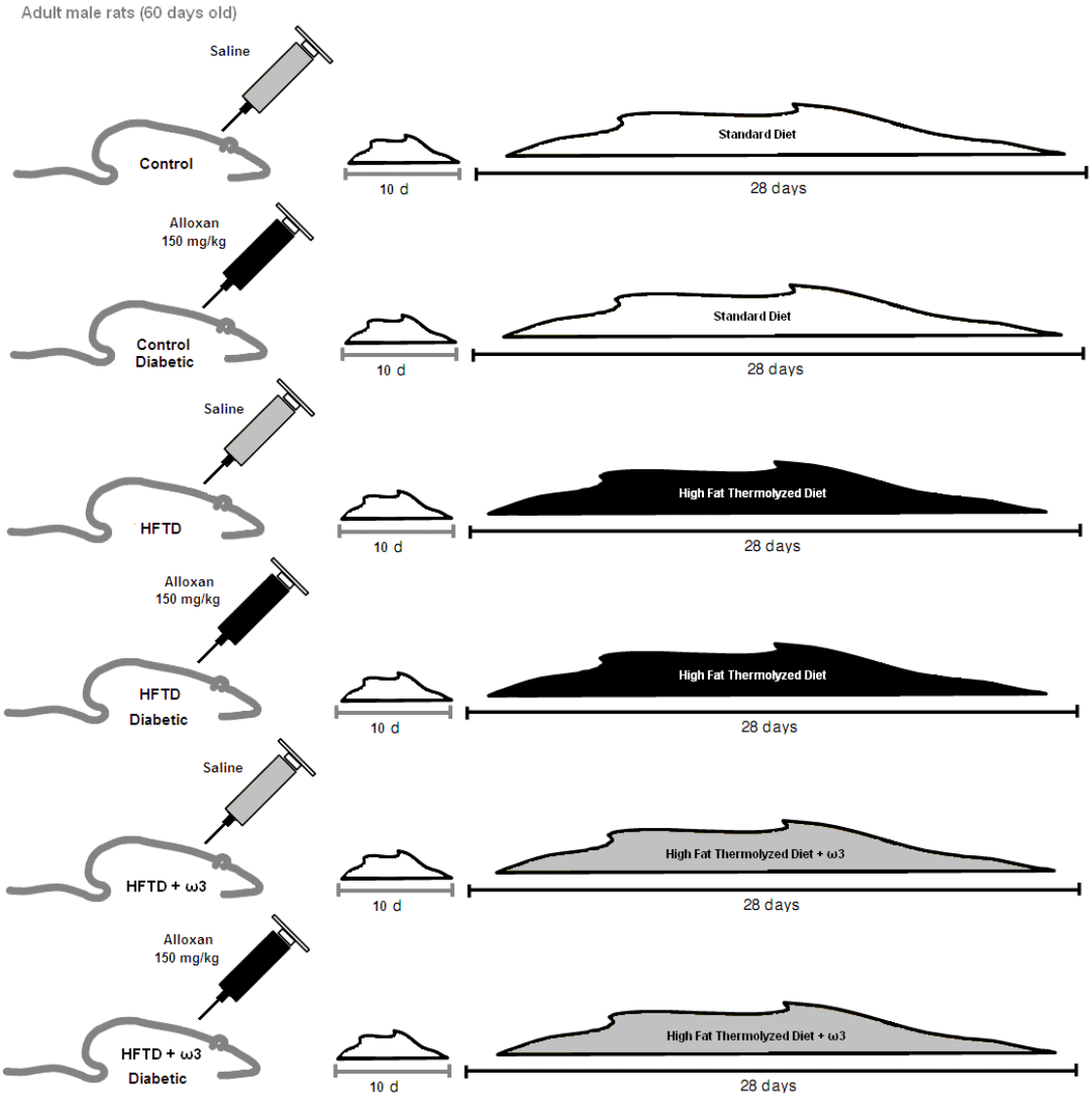
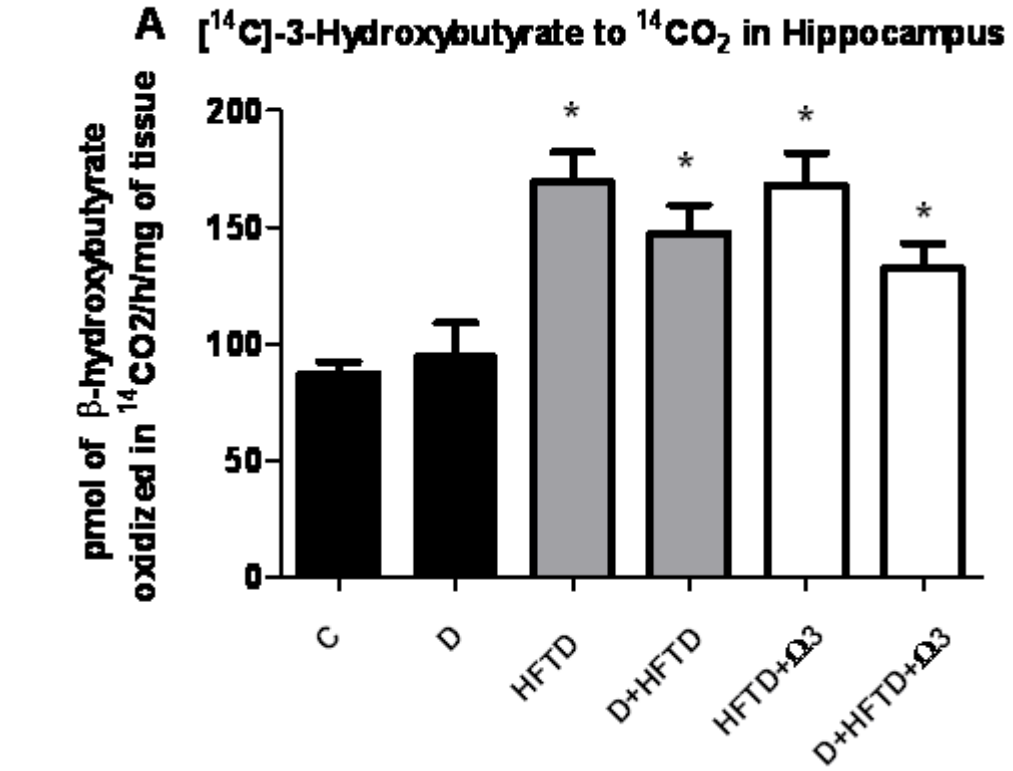

Adult male rats (60 days old)
Saline
Control
10 d
Standard Diet
28 days
Alloxan
150 mg/kg
Control
Diabetic
10 d
Standard Diet
28 days
Saline
HFTD
10 d
High Fat Thermolyzed Diet
28 days
Alloxan
150 mg/kg
HFTD
Diabetic
10 d
High Fat Thermolyzed Diet
28 days
Saline
HFTD + ω3
10 d
High Fat Thermolyzed Diet + ω3
28 days
Alloxan
150 mg/kg
HFTD + ω3
Diabetic
10 d
High Fat Thermolyzed Diet + ω3
28 days

Figure 2


A
[14C]-3-Hydroxybutyrate to 14CO2 in Hippocampus
pmol of β-hydroxybutyrate oxidized in 14CO2/h/mg of tissue
200
150
100
50
0
*
*
*
*
C
D
HFTD
D+HFTD
HFTD+Ω3
D+HFTD+Ω3

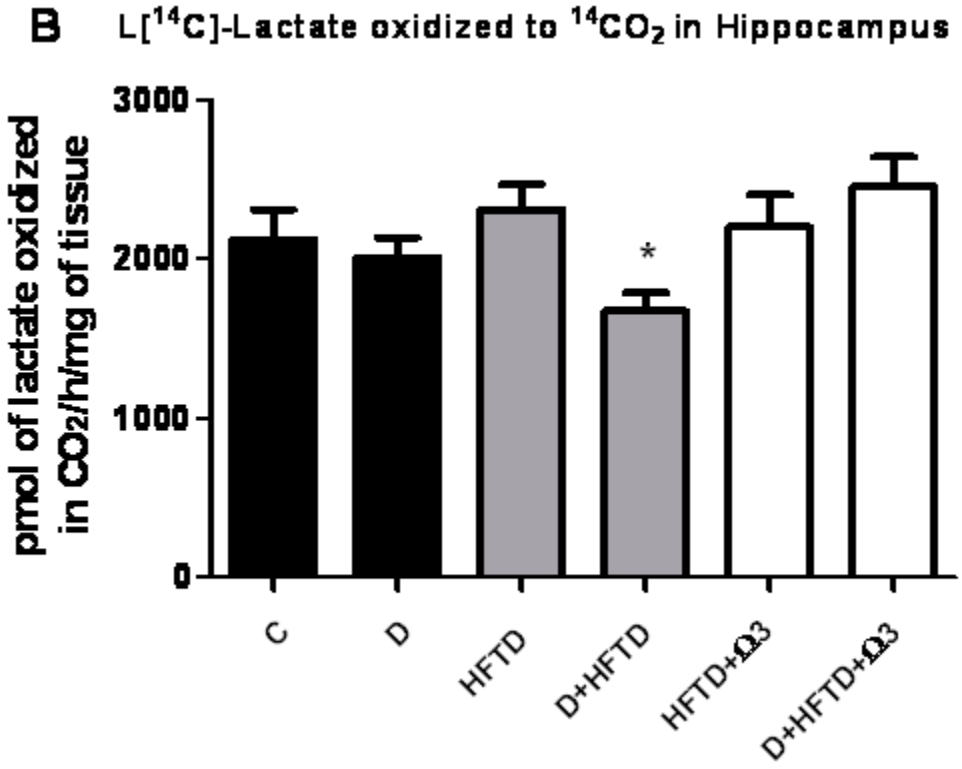

B
L[14C]-Lactate oxidized to 14CO2 in Hippocampus
pmol of lactate oxidized in CO2/h/mg of tissue
3000
2000
1000
0
*
C
D
HFTD
D+HFTD
HFTD+Ω3
D+HFTD+Ω3

Figure 3

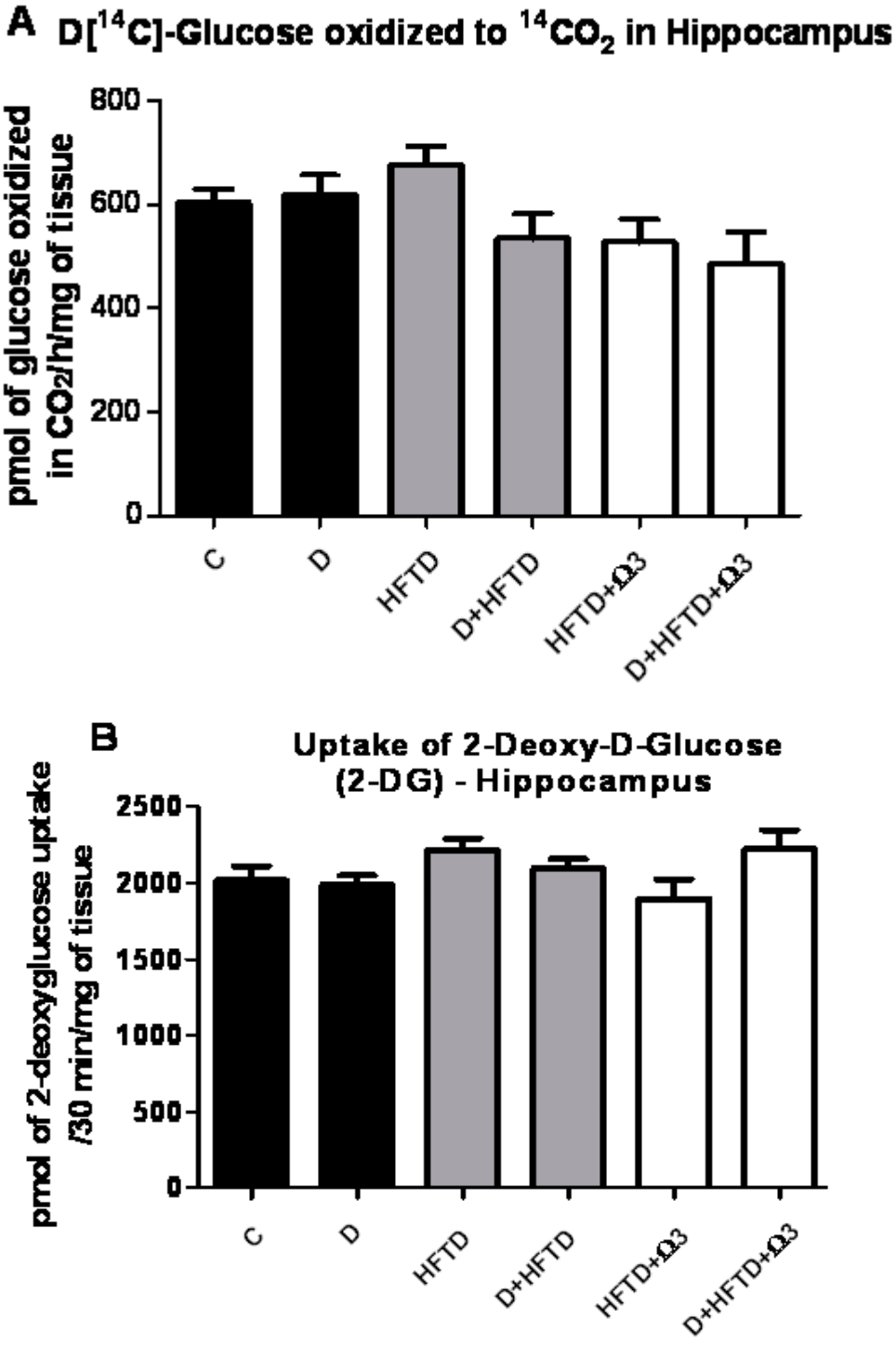
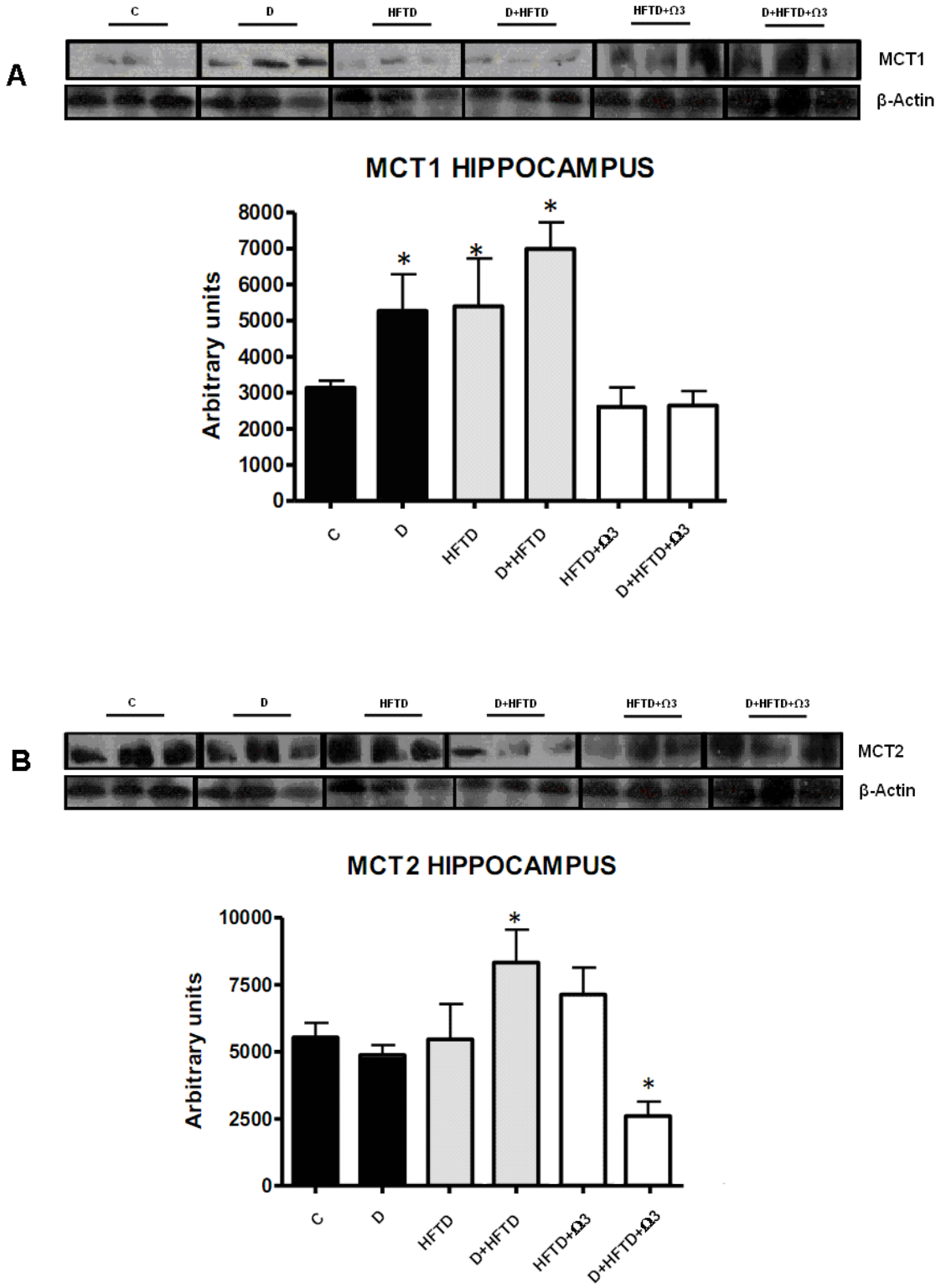

A
D[14C]-Glucose oxidized to 14CO2 in Hippocampus
pmol of glucose oxidized in CO2/h/mg of tissue
800
600
400
200
0
C
D
HFTD
D+HFTD
HFTD+Ω3
D+HFTD+Ω3
B
Uptake of 2-Deoxy-D-Glucose (2-DG) - Hippocampus
pmol of 2-deoxyglucose uptake /30 min/mg of tissue
2500
2000
1500
1000
500
0
C
D
HFTD
D+HFTD
HFTD+Ω3
D+HFTD+Ω3

Figure 4.


A
C
D
HFTD
D+HFTD
HFTD+Ω3
D+HFTD+Ω3
MCT1
β-Actin
MCT1 HIPPOCAMPUS
Arbitrary units
8000
7000
6000
5000
4000
3000
2000
1000
0
*
B
MCT2
β-Actin
MCT2 HIPPOCAMPUS
10000
7500
5000
2500

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Ω3-Polyunsaturated fatty acids prevent lipoperoxidation, modulate antioxidant enzymes, and reduce lipid content but do not alter glycogen metabolism in the livers of diabetic rats fed on a high fat thermolyzed diet

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Abstract Ω3-Polyunsaturated fatty acids (Ω3-PUFAs) are known to act as hypolipidaemics, but the literature is unclear about the effects that Ω3-PUFAs have on oxidative stress in obese and diabetic patients. In this study, our aim was to investigate the effects of Ω3-PUFAs on oxidative stress, including antioxidant enzyme activity and hepatic lipid and glycogen metabolism in the livers of diabetic and non-diabetic rats fed on a high fat thermolyzed diet. Rats were divided into six groups: (1) the control group (C), (2) the control diabetic group (D), (3) the high fat thermolyzed diet group (HFTD), which were fed a diet that was enriched in fat that was heated for 60 min at 180°C, (4) the high fat thermolyzed diet diabetic group (D + HFTD), (5) the high fat thermolyzed diet + Ω3 polyunsaturated fatty acid group (HFTD + Ω3), and (6) the high fat thermolyzed diet + Ω3 polyunsaturated fatty acid diabetic group (D + HFTD + Ω3). The most important finding of this study was that Ω3-PUFAs are able to reduce triglycerides, non-esterified fatty acid, lipoperoxidation levels, advanced glycation end products, SOD/CAT enzymatic ratio, and CAT immunocontent and increase SOD2 levels in the livers of diabetic rats fed with a HFTD. However, Ω3-PUFAs did not alter the observed levels of protein damage, blood glucose, or glycogen metabolism in the liver. These findings suggest that Ω3-PUFAs may represent an important auxiliary adjuvant in combating some diseases like diabetes mellitus, insulin resistance, and non-alcoholic fatty liver disease.

Keywords Advanced glycation end products · Glycogen metabolism · Diabetes · Oxidative stress · Ω3-Polyunsaturated fatty acids · High fat diet

Marcos L. S. Perry—*In memoriam*.

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Introduction

Diabetes mellitus affects people worldwide and poses major public health and socioeconomic challenges. Hyperglycemic states are supposed to induce the overproduction of reactive oxygen species (ROS) through various mechanisms including the increased non-enzymatic formation of advanced glycation end products (AGEs), increased polyol pathway flux, and increased O_2-production through the activation of mitochondrial and protein kinase C (PKC)-dependent NAD(P)H oxidases in many tissues [1].

Recent studies suggest that oxidative stress increases with diabetes mellitus. This may be due to a decreased antioxidant potential, increased free-radical production, alterations in enzymatic pathways related to the redox state (such as the polyol and glyoxalase pathways), and changes in the compartmentalization of metal ions. Together, such

factors promote increases in oxidative stress levels in diabetic patients [2, 3].

Reactive derivatives from non-enzymatic glucose–protein condensation reactions as well as lipids and nucleic acids exposed to reducing sugars, such as glucose or glyceraldehyde, form a heterogeneous group of irreversible adducts called AGEs [4]. The formation and accumulation of AGEs in various tissues, which is known to progress during a normal aging process, is extremely accelerated in patients with diabetes mellitus [5, 6]. One important component of AGE accumulation with aging appears to be the sustained exposure to dietary AGEs (dAGEs), which contributes to the overloading of anti-AGE receptors and depletion of anti-oxidant reserves [7]. There is accumulating evidence that an interaction between AGEs and receptor for advanced glycation end products (RAGEs) elicits oxidative stress generation and subsequently modulates gene expression in various types of cells including hepatocytes and hepatic stellate cells [8, 9].

The liver is not only a target organ for circulating AGEs but also an important site for their clearance and catabolism. Indeed, the liver can sequester a number of circulating senescent macromolecules such as AGEs [10]. It has long been known that glucose is stored in the liver in the form of liver glycogen. Early studies on glycogen metabolism in type 1 diabetic patients using liver biopsies revealed conflicting results, reporting either increased or decreased liver glycogen concentrations [11].

Epidemiological studies also reported a lower incidence of type 2 diabetes mellitus in individuals who consumed Ω3-polyunsaturated fatty acids (Ω3-PUFAs), while intake of total saturated and/or monounsaturated fats was associated with an increased risk of type 2 diabetes in glucose-intolerant individuals [12]. The beneficial effects of Ω3-PUFAs consumption on liver disease were mainly attributed to their effects in reducing triglyceride levels, increasing high density lipoprotein cholesterol, and improving endothelial function through anti-inflammatory mechanisms and reduced platelet aggregation [13]. Many studies show that it is very important to maintain a ratio at Ω6 5:1 Ω3 allowing a greater conversion of alpha-linolenic acid to docosahexaenoic acid [14]. However, this recommendation is more common for a normal situation (not pathological).

Our group has previously demonstrated the deleterious effects of both endogenous and exogenous AGEs [15, 16]. de Assis et al. [17] showed that normal rats fed with a high thermolyzed fat diet for 12 months had significant increases in blood DNA damage and liver lipoperoxidation compared to those fed a normal high fat diet. Thus, the aim of this study was to investigate the effects of Ω3-PUFAs on oxidative stress, antioxidant enzyme activity, and hepatic lipid and glycogen metabolism in the livers of diabetic and non-diabetic rats fed with a high fat thermolyzed diet.

Materials and methods

Animals and chemicals

Sixty adult Wistar rats (male, 60 days old) from the Central Animal House of the Department of Biochemistry were maintained under a standard dark–light cycle (lights on between 7:00 a.m. and 7:00 p.m.) at room temperature (22 ± 2°C). All experiments were conducted in accordance with the Guiding Principles in the Use of Animals in Toxicology, adopted by the Society of Toxicology in July 1989, and all experiments were approved by our ethics committee for animal use at the Federal University of Rio Grande do Sul. D-[U-^{14}C]-Glucose (297 mCi/mmol) was purchased from Amersham International (Little Chalfont, Bucks, UK). Alloxan monohydrate and all other chemicals were obtained from Sigma-Aldrich (St. Louis, MO, USA).

Treatments and diets

Diabetes mellitus was induced by an intraperitoneal (i.p.) injection of alloxan (150 mg kg^{-1} b.w), and non-diabetic animals received a saline solution (NaCl 0.9%), also via an i.p. injection. After 10 days, blood samples were obtained from rats in fasting state to determine the plasma glucose concentration. Only animals that presented with 15–30 mmol/l glucose concentration were included in the study.

Rats were divided into six groups: (1) the control group (C, $n = 10$), which received standard laboratory rat chow; (2) the control diabetic group (D, $n = 10$), which received an injection of alloxan and the same diet as group 1; (3) the high fat thermolyzed diet group (HFTD, $n = 10$), which received a diet enriched with fats that had been heated for 60 min at 180°C to which a vitamin mixture was added after heating; (4) the high fat thermolyzed diet diabetic group (D + HFTD, $n = 10$), which received an injection of alloxan and the same diet as group 3; (5) the high fat thermolyzed diet + Ω3 polyunsaturated fatty acid group (HFTD + Ω3), which received a diet enriched with fats that had been heated for 60 min at 180°C to which a vitamin mixture and an Ω3 fatty acid supplement was added after heating; and (6) the high thermolyzed fat diet + Ω3-PUFA diabetic group (D + HFTD + Ω3, $n = 10$), which received an injection of alloxan and the same diet as group 5. Diet thermolyzation was based on the study by Sandu et al. [18], which indicated a high AGE content (around 1 unit/μg) in a high fat diet after thermolyzation. The salt and vitamin compositions used were the

same as those described by Horwitz [19]. During the treatment period of 4 weeks, the animals had free access to food and water.

Tissue preparation

Rats were killed by decapitation, and the blood was collected immediately in heparinized tubes. Retroperitoneal and epididymal fat tissues were dissected and weighted. The whole blood sample was then centrifuged at 2500×*g* for 10 min to yield the plasma fraction, which was used for subsequent biochemical analyses. The liver was dissected and was then either weighed and cut into 0.3-mm slices using a McIlwain tissue chopper for glycogen synthesis or weighed and stored at −80°C for future analyses of oxidative stress, western blotting, glycogen, triglyceride, and cholesterol concentrations.

Blood biochemical parameters

Plasma glucose, triacylglycerol (TAG), total cholesterol, and HDL cholesterol levels were measured using commercial kits (Labtest, MG, Brazil). Reactions were performed using the Labmax apparatus (Labtest, MG, Brazil). The plasma non-esterified fatty acid (NEFA) activity was measured using a commercial kit (Roche Diagnostics, Germany). To analyze the amount of AGEs, we checked the concentration of Carboxymethyllysine in serum by ELISA anti-CML antibodies (R&D Systems, Minneapolis, MN, USA).

Oxidative stress and antioxidant enzyme activity

Measurement of protein carbonyl

The oxidative damage to proteins was measured by quantifying the number of carbonyl groups by reaction with 2,4-dinitrophenylhydrazine (DNPH). Proteins were precipitated by the addition of 20% trichloroacetic acid (TCA) and were resuspended in 10 mM DNPH, and the absorbance of the resulting solution at 370 nm was recorded [20]. Results are expressed as nmol carbonyl/mg protein.

Thiobarbituric acid reactive species (TBARS)

In order to assess the extent of lipoperoxidation, we detected TBARS formation through a heated and acidic reaction. This is widely adopted as a method for the measurement of lipid redox states, as previously described [21]. The samples were mixed with 0.6 ml of 10% TCA and 0.5 ml of 0.67% thiobarbituric acid and were then heated in a boiling water bath for 25 min. The level of TBARS was determined by measuring the absorbance of each sample at 532 nm. The concentration of TBARS in the samples was then determined from a calibration curve using 1,1,3,3-tetramethoxypropane (which had been subjected to the same treatment as the supernatants) as a standard. Results are expressed as nanomoles of TBARS per milligram of protein.

Measurement of non-protein thiol content

An aliquot of the supernatant from homogenates precipitated with 20% TCA was diluted in 0.1% SDS and 0.01 M 5,5′-dithionitrobis-2-nitrobenzoic acid (DTNB) in ethanol was added. An intense yellow color developed, and the absorbance at 412 nm was recorded after 20 min [22]. Simultaneously, the absorbance of a blank sample, which was identical to the analyzed samples except for the absence of DTNB, was also recorded. The non-protein thiol content of each sample was calculated, after subtraction of the blank absorbance, using the following equation and the molar extinction coefficient of 13,600 M^{-1} cm^{-1}: [(final absorbance/13,600)V]/1000/Q, where "*V*" is the volume (in ml) of the sample used in the assay and "*Q*" is the amount of protein (in mg) in the sample used to perform the assay.

Antioxidant enzyme activity

Catalase (CAT) activity was measured as previously described [23]. The rate of decrease in absorbance at 240 nm was measured as a function of H_2O_2 degradation by CAT. Superoxide dismutase (SOD) activity was assessed by quantifying the inhibition of superoxide-dependent adrenaline autooxidation by recording the absorbance of the samples at 480 nm [24].

Glycogen metabolism

Hepatic glycogen synthesis

Levels of hepatic glycogen synthesis were measured as follows. The livers of rats were dissected and cut into 300 µm slices using a McIlwain tissue chopper (100–120 mg). The liver slices were incubated in a beaker with media containing Krebs–Ringer bicarbonate buffer (pH 7.4), 5 mM glucose, and 0.2 µCi D[U-^{14}C]glucose. Incubations were performed at room temperature in media that had previously been treated with a 95% O_2:5% CO_2 mixture for 1 h. Liver slices (between 100 and 120 mg) were then incubated at 37°C for 1 h in a metabolic shaker (60 cycles/min) according to the method of Dolnikoff et al. [25]. The incubations were stopped by placing the bottles in ice, and subsequently, 1 ml of 60% KOH was added to each beaker. After 15 min in a boiling water bath, 3 ml of

96% ethanol was added to the tubes to precipitate the glycogen. After precipitation, glycogen was suspended in 0.2 ml of water, and scintillation liquid (Opti-Phase Hi-Safe3 from PerkinElmer-USA) was added. The samples were then assessed in a scintillation liquid counter.

Hepatic glycogen concentration

The hepatic glycogen concentration was measured using the Krisman method [26].

Hepatic lipids

In order to measure the hepatic lipid content, frozen liver samples were thawed on ice and homogenized in deionized water. Extraction and isolation of lipids to yield dried lipid extracts were performed using the technique described by Folch et al. [27]. The hepatic cholesterol and triglycerides content of the lipid extracts were then assayed enzymatically by colorimetry.

Western blotting analysis

Proteins (20 µg) were separated by SDS–PAGE on 10% (w/v) acrylamide and 0.275% (w/v) bisacrylamide gels and were then electrotransferred onto nitrocellulose membranes. The membranes were then incubated in Tris-buffered saline Tween-20 [TBS-T; 20 mM Tris–HCl, pH 7.5, 137 mM NaCl, 0.05% (v/v) Tween 20] containing 1% (w/v) non-fat milk powder for 1 h at room temperature. Subsequently, the membranes were incubated for 12 h with the appropriate primary antibody (CAT, 1:6000; Superoxide dismutase, 1:2000 and β-Actin, 1:10000). After washing in TBS-T, blots were incubated with horseradish peroxidase-linked anti-immunoglobulin G (IgG) antibodies for 1.5 h at room temperature. Chemiluminescent bands were detected, and densitometric analysis was performed using Image-J® software.

Statistical analyses

Biochemical data are expressed as the mean ± SD. All the analyses were performed using the Statistical Package for the Social Sciences (SPSS 16.0) software. Likewise, the differences between all groups were analyzed using ANOVA and Tukey's post-hoc test. P values were considered significant when $P < 0.05$.

Results

In Table 1, non-diabetic groups we had a significant ($P < 0.05$) increase in body weight and adipose tissue in rats treated with high fat thermolyzed diets (HFTD and HFTD + Ω3), this result was expected for us although treatment with the diet is only 1 month, for composition experimental diets details, see Table 2. Already in the diabetic groups, we had a reduction in body weight of all rats, though animals that received the high fat thermolyzed diets (HFTD and HFTD + Ω3) showed a smaller reduction in body weight and a significant increased in adipose tissue in relation to the diabetic control group (D) Table 1.

Table 3 shows the biochemical parameters in our experimental groups. We observed a significant difference ($P < 0.05$) between the plasma glucose of diabetic and non-diabetic rats (an increase of about 400%), thus demonstrating that our model was effective in inducing diabetes mellitus. However, diabetic rats fed a HFTD or HFTD + Ω3 diet showed a decrease in their glucose concentration. This effect was attributed to a lower amount of carbohydrate in these diets as compared to the control diet. We found no significant difference in the plasma cholesterol and HDL of the groups tested, although the groups that received only HFTD (both diabetic and non-diabetic) appeared to show higher levels of cholesterol. Diabetes mellitus and HFTD did not affect the level of plasma NEFAs but promoted an increase in plasma triglycerides. However, in adding polyunsaturated fatty acids in the diet, we noticed a significant reduction ($P < 0.05$) in plasma triglycerides and NEFAs in these groups. We also evaluated the concentration of AGEs in serum by measuring Carboxymethyllysine (CML) and we can observe that diet (HFTD and HFTD + Ω3) increased significantly ($P < 0.05$) the amount of CML in non-diabetics rats. All diabetic rats had an increase of CML, however, supplementation with Ω3-PUFAs having partially prevented this increase.

In Table 4, we can observe the effect of different diets on hepatic lipids and hepatic glycogen metabolism. A high fat thermolyzed diet (HFTD) significantly increased ($P < 0.05$) hepatic triglycerides content in diabetic and non-diabetic rats. However, supplementation of their diet with Ω3 (HFTD + Ω3) reduced these values to normal levels (that of the control) in the non-diabetic group. A HFTD increased the levels of hepatic cholesterol, although this increase only proved to be significant ($P < 0.05$) in diabetic rats. In contrast to triglyceride content, supplementation with Ω3 did not affect the levels of hepatic cholesterol. In addition, we noted increased levels of hepatic cholesterol in both diabetic groups compared to the non-diabetic groups, although the values were not statistically significant. We did not find a significant difference in the levels of hepatic glycogen metabolism in the experimental groups studied. However, diabetes mellitus has a tendency to decrease hepatic glycogen concentration.

We show the results of the analysis of oxidative stress and antioxidant enzyme activity in Table 5. A high fat

Table 1 Body parameters in diabetic and non-diabetic rats fed a control diet (C), high fat thermolyzed diet (HFTD), and high fat thermolyzed diet + Ω3 (HFTD + Ω3)

Body parameters	C	D	HFTD + Ω3	D + HFTD + Ω3	HFTD	D + HFTD
Initial body weight (g)	282 ± 18.5	278 ± 14.3	285 ± 25.1	284 ± 22.5	275 ± 12.8	281 ± 17.4
Final body weight (g)	314 ± 23.2	226 ± 32.7*	331 ± 48.9	252 ± 39.3*	335 ± 22.4	242 ± 48.7*
Body weight gain (g)	32 ± 7.2	−52 ± 12.6**	46 ± 9.3*	−32 ± 5.9**	60 ± 11.8*	−39 ± 9.5**
Retroperitoneal adipose tissue (g)	2.1 ± 0.1	0.2 ± 0.0*	6.3 ± 1.9**	2.8 ± 0.6	8.6 ± 3.1**	3.2 ± 0.8
Epididymal adipose tissue (g)	1.2 ± 0.09	0.2 ± 0.0*	2.4 ± 0.4**	1.0 ± 0.2	3.5 ± 0.7**	1.2 ± 0.3*

Body weight and adipose tissue weight are expressed as grams (g), all parameters represented as the mean ± SD ($n = 10$ per group). *Different asterisks* on the same row indicate a significant difference ($P < 0.05$) and all groups were analyzed using ANOVA and Tukey's post-hoc test

Table 2 The composition of control (C), high fat thermolyzed (HFTD), and high fat thermolyzed + Ω3 (HFTD + Ω3) experimental diets that were fed to diabetic and non-diabetic rats

Composition	Control diet (%)	High fat thermolyzed diet (%)	High fat thermolyzed diet + Ω3 (%)
Commercial bran	–	20.5	20.5
Soy protein isolate[a]	27.0	15.9	15.9
Corn starch	55.5	–	–
Sucrose	5.0	10.0	10.0
Vitamin mix[b]	1.0	1.0	1.0
Mineral salt mix[c]	4.0	2.0	2.0
DL-Methionin[d]	0.3	0.3	0.3
DL-Lysine[e]	0.3	0.3	0.3
Soy oil	5.0	1.0	1.0
Lard	–	49.0	46.0
Ω3 fatty acid[f]	–	–	3.0

Salt and vitamin composition are according to Horwitz

[a] Soy protein isolate, purity 97% (from Solae, Esteio, Brazil)

[b] Vitamin mixture: mg/100 g of diet (from Roche, SãoPaulo, Brazil): vitamin A (retinyl acetate), 4; vitamin D (cholecalciferol), 0.5; vitamin E (DL-α-tocopheryl acetate), 10; menadione, 0.5; choline, 200; PABA, 10; inositol, 10; niacine (nicotinic acid), 4; pantothenicacid (calcium D-pantothenate), 4; riboflavin, 0.8; thiamin (thiamine hydrochloride), 0.5; pyridoxine (pyridoxine hydrochloride), 0.5; folic acid, 0.2; biotin [D-(+)-biotin], 0.04; vitamin B12, 0.003

[c] Mineral salt mixture: mg/100 g of diet (from Roche, SãoPaulo, Brazil): NaCl, 557; KI, 3.2; KH_2PO_4, 1556; $MgSO_4$, 229; $CaCO_3$, 1526; $FeSO_4$–$7H_2O$, 108; $MnSO_4$–H_2O, 16; $ZnSO_4$–$7H_2O$, 2.2; $CuSO_4$–$5H_2O$, 1.9; CoCl–$6H_2O$, 0.09

[d] DL-Methionin (from Merk, Rio de Janeiro, Brazil)

[e] DL-Lysine (from Merk, Rio de Janeiro, Brazil)

[f] Fish oil: docosahexaenoic acid (DHA) 5 : 1 eicosapentaenoic acid (EPA) (Naturalis SA, Brazil.)

thermolyzed diet increased SOD activity significantly ($P < 0.05$) in both diabetic and non-diabetic rats (HFTD and D + HFTD). When we added Ω3 supplements, the SOD activity was reduced in non-diabetic rats (HFTD + Ω3), and this reduction in activity was even more evident ($P < 0.05$) in diabetic rats (D + HFTD + Ω3). CAT activity in non-diabetic groups increased only in the HFTD group ($P < 0.05$). Diabetes caused an increase in CAT activity in all diabetic groups (D, D + HFTD + Ω3 and D + HFTD) relative to the non-diabetic rats. We did not find any difference in the SOD/CAT ratio in non-diabetic groups. However, in analyzing the SOD/CAT ratio in the diabetic groups, for this ratio, we verified an increase in the D and D + HFTD groups and a significant ($P < 0.05$) decrease in the D + HFTD + Ω3 group. The groups that received supplementation with polyunsaturated fatty acids (HFTD + Ω3 and D + HFTD + Ω) showed significantly ($P < 0.05$) lower levels of lipoperoxidation compared to the other groups. All groups fed high fat thermolyzed diets (HFTD, D + HFTD/D, HFTD + Ω3 and D + HFTD + Ω3) showed a significant ($P < 0.05$) increase in protein carbonyl levels relative to the control groups (C and D). We did not observe any differences in the non-protein thiol content between groups.

A drastic reduction ($P < 0.05$) in the hepatic immunocontent of CAT was observed in groups for which diets were supplemented with polyunsaturated fatty acids (Ω3-PUFAs) relative to groups that did not receive

Table 3 Biochemical parameters in diabetic and non-diabetic rats fed a control diet (C), high fat thermolyzed diet (HFTD), and high fat thermolyzed diet + Ω3 (HFTD + Ω3)

	C	D	HFTD + Ω3	D + HFTD + Ω3	HFTD	D + HFTD
Glucose (mmol/l)	5.4 ± 0.5	27.5 ± 6.8*	5.8 ± 1.2	20.2 ± 4.0*	5.6 ± 0.4	19.9 ± 4.2*
Cholesterol (mg/dl)	56.9 ± 12.8	76.1 ± 16.4	59.0 ± 16.3	66.2 ± 14.3	77.1 ± 25.9	71.5 ± 10.4
Triglycerides (mg/dl)	73.4 ± 23.2	147.3 ± 60.4*	67.1 ± 18.6	104.7 ± 42.7*	140.9 ± 67.0*	262.7 ± 78.5**
HDL (mg/dl)	23.5 ± 5.3	26.0 ± 4.8	24.7 ± 5.6	29.5 ± 6.4	25.5 ± 5.7	25.6 ± 2.5
Non-esterified fatty acids (NEFA) (μM)	566.1 ± 60.5	583.0 ± 45.1	378.7 ± 60.7*	427.7 ± 143.5*	523.6 ± 126.5	616.4 ± 227.6
Carboxymethyllysine (% Control of CML)	100 ± 4.1	163.6 ± 13.9**	112.2 ± 6.3*	124.3 ± 16.4*	114.7 ± 5.5*	151.8 ± 11.3**

Cholesterol, high density lipoprotein (HDL), and triglyceride levels are expressed as mg/dl. Free fatty acid concentrations are expressed in μM/ml, the glucose concentration is expressed as mmol/l, and carboxymethyllysine are expressed by % of a control group. All the parameters represented as the mean ± SD ($n = 10$ per group). *Different asterisks* on the same row indicate a significant difference ($P < 0.05$) and all groups were analyzed using ANOVA and Tukey's post-hoc test

Table 4 Hepatic lipids and glycogen metabolism in diabetic and non-diabetic rats fed a control diet (C), high fat thermolyzed diet (HFTD), and high fat thermolyzed diet + Ω3 (HFTD + Ω3)

	C	D	HFTD + Ω3	D + HFTD + Ω3	HFTD	D + HFTD
Hepatic triglycerides (mg/%)	2.29 ± 0.34	2.02 ± 0.51	2.90 ± 1.41	4.31 ± 0.94*	3.86 ± 1.03**	4.70 ± 1.17**
Hepatic cholesterol (mg/%)	1.03 ± 0.09	1.10 ± 0.15	1.26 ± 0.13	1.30 ± 0.09*	1.24 ± 0.16	1.31 ± 0.12*
Hepatic glycogen concentration (mg/%)	5.5 ± 0.8	4.8 ± 1.6	4.2 ± 0.4	3.1 ± 1.1	4.2 ± 0.4	3.6 ± 1.2
Hepatic glycogen synthesis (pmol/mg^{-1} tissue h^{-1})	75.0 ± 10.1	74.5 ± 13.0	82.1 ± 6.4	82.4 ± 13.8	80.0 ± 5.6	82.7 ± 8.9

Glycogen synthesis from D-[U-^{14}C]-glucose is expressed in pmol of glucose incorporated into glycogen/100 mg tissue/h. Concentrations of hepatic glycogen, triglycerides, and cholesterol are expressed in mg/100 mg of tissue and are represented as the mean ± SD ($n = 10$ per group). *Different asterisks* on the same row indicate a significant difference ($P < 0.05$) and all groups were analyzed using ANOVA and Tukey's post-hoc test

Table 5 Oxidative stress and antioxidant enzymes in the livers of diabetic and non-diabetic rats fed a control diet (C), high fat thermolyzed diet (HFTD), and high fat thermolyzed diet + Ω3 (HFTD + Ω3)

	C	D	HFTD + Ω3	D + HFTD + Ω3	HFTD	D + HFTD
Superoxide dismutase (U/mg protein)	73.1 ± 6.5	87.4 ± 18.1	92.6 ± 27.1	57.8 ± 7.9 *	124.4 ± 19.2**	113.1 ± 18.7**
Catalase (U/mg protein)	39.5 ± 5.1	50.1 ± 11.2	43.1 ± 4.4	61.3 ± 10.1*	71.1 ± 3.8*	64.2 ± 10.6*
SOD/CAT ratio (arbitrary units)	1.85 ± 0.1	2.20 ± 0.6	1.92 ± 0.5	0.95 ± 0.1*	1.75 ± 0.2	2.12 ± 0.4
Lipoperoxidation (nmol TBARS/mg protein)	0.055 ± 0.01	0.075 ± 0.03	0.033 ± 0.01*	0.049 ± 0.01*	0.056 ± 0.01	0.074 ± 0.01
Protein carbonyl (nmol/mg protein)	0.59 ± 0.29	0.53 ± 0.15	0.81 ± 0.11*	0.81 ± 0.16*	0.85 ± 0.13*	0.75 ± 0.14*
Non-protein sulfhydryl groups (nmol/mg protein)	0.030 ± 0.008	0.033 ± 0.006	0.029 ± 0.007	0.026 ± 0.005	0.041 ± 0.007	0.037 ± 0.008

SOD and CAT activities are expressed in U/mg protein. The SOD/CAT ratio is expressed in arbitrary units. Lipoperoxidation, protein carbonyl and non-protein thiol contents are expressed in nmol/mg protein. All results are presented as the mean ± SD ($n = 10$ per group). *Different asterisks* on the same row indicate a significant difference ($P < 0.05$) and all groups were analyzed using ANOVA and Tukey's post-hoc test

Ω3-PUFA supplements (Fig. 1a). All diabetic groups and groups supplemented with a high fat diet, including groups that received Ω3-PUFAs supplements, showed significant ($P < 0.05$) increases in the hepatic immunocontent of SOD2 when compared to the non-diabetic control group (Fig. 1b).

Discussion

In this study, we evaluated the effect of a high fat thermolyzed diet on the liver of diabetic rats and the possible beneficial effect of Ω3-PUFAs. The most important finding of this study was that Ω3-PUFAs are able to reduce

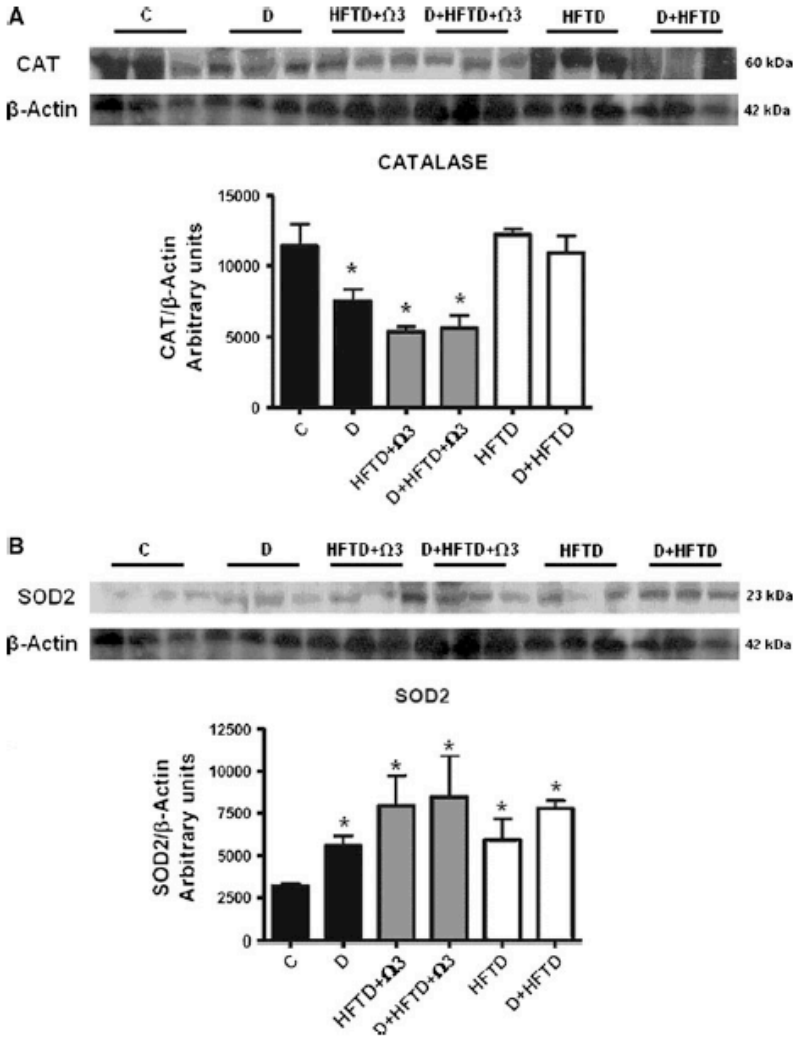

Fig. 1 Representative and quantitative western blot analysis of the liver CAT (**a**) and SOD2 (**b**) immunocontent from diabetic and non-diabetic rats fed a control diet (C), high fat thermolyzed diet (HFTD), and high fat thermolyzed diet + Ω3 (HTFD + Ω3). The graphic represents the means ± SD ($n = 3$ per group). *Different asterisks* indicate a significant difference ($P < 0.05$) and all groups were analyzed using ANOVA and Tukey's post-hoc test

triglycerides (in both plasma and the liver), NEFAs, lipoperoxidation and modulate antioxidant enzyme activity and immunocontent in the livers of diabetic rats supplemented with a high fat thermolyzed diet. However, these Ω3-PUFAs did not affect the observed levels of protein damage, blood glucose, or glycogen metabolism in the liver.

The most common cause of obesity in man is a chronic intake of a hypercaloric diet rich in (saturated) fats. Obesity can lead to an increase in hepatic import and synthesis of triglycerides and a decrease in hepatic triglyceride export and oxidation. This results in the accumulation of triglycerides in hepatocyte, which promotes an increase in oxidative stress and cytokines proinflammation [28, 29]. It is known that fish oil diminishes the effect of lifestyle-related diseases like diabetes mellitus and hyperlipidemia [30]. The triglyceride-lowering effects of Ω3-PUFAs have been thoroughly researched [31, 32], and studies have consistently demonstrated that supplementation with 2–4 g/d of Ω3-PUFAs reduces circulating triglycerides up to 34% in hypertriglyceridemic patients [33]. In a recent study, Sato et al. [34] demonstrated that the anti-obesity effect of Ω3-PUFAs in diet-induced obesity is associated with the suppression of hepatic lipogenesis and steatosis. Corroborating these authors, our results showed that both diabetes mellitus and a high fat thermolyzed diet for 1 month increases the levels of triglycerides in both plasma and the liver and that Ω3-PUFAs have been effective in decreasing these values (Tables 3, 4). These results further emphasize the beneficial effect of Ω3-PUFAs in both the protection from and the reversal of hyperlipidemia. Although levels of

NEFAs did not change in diabetic or high fat thermolyzed groups, we observed a beneficial effect of Ω3-PUFAs (Table 3). These beneficial effects are largely mediated by peroxisome proliferator-activated receptors (PPARs). PPAR-α and PPAR-δ (-β) represent the main targets [35]; however, recent studies have shown the importance of the inhibition of SREBP-1c [36]. The hypolipidaemic and anti-obesity effects of Ω3-PUFAs probably depends on the in situ suppression of lipogenesis and on an increase in fatty acid oxidation in several tissues [37, 38]. This metabolic switch might reduce accumulation of toxic fatty acid derivatives while protecting insulin signaling in the liver [39].

Although we did not observe significant differences in the metabolism of glycogen, our results suggest a decrease in the hepatic glycogen content of diabetic rats. This reduction is more drastic in the diabetic groups that received a high fat thermolyzed diet (Table 4). An in vitro study showed that AGEs decreased tyrosine phosphorylation of IRS-1 and subsequently reduced glycogen synthesis in insulin-exposed Hep3B cells and the association of the p85 subunit of phosphatidylinositol 3-kinase with IRS-1 [40]. These results may justify our finding that a high fat diet enriched in AGEs (HFTD) reduced observed glycogen concentrations. Insulin promotes storage of glucose in the form of glycogen, and as expected in this study, alloxan-induced diabetes mellitus led to decreased glycogen content in the liver. Human studies have also reported lower glycogen content in the livers of type 2 diabetics [41, 42]. This phenomenon is already well described in the literature [43], but little is known about the impact of hepatic glycogen content on the regulation of glucose metabolism in the liver from in vivo models. In a recent study, the author suggests that the beneficial effects of Ω3-PUFAs on glucose metabolism are most strongly related to their interaction with PPARs. On the other hand, SREBP-1c has been shown to play a more important role in lipid metabolism in obese mice [44]. Our results demonstrate that lipid metabolism is more susceptible to the beneficial effects of supplementation with Ω3-PUFAs over a 4-week period than glucose metabolism. However, studies with longer periods of supplementation appear to show that Ω3-PUFAs have more beneficial effects on glucose metabolism [45].

Obesity and diabetes mellitus represent chronic inflammatory states that lead to increases in ROS, AGEs, and several immunomodulatory factors that can contribute to non-alcoholic fatty liver disease (NAFLD) and liver injury [46]. Many earlier studies have used Ω3-PUFAs to prevent oxidative stress that results from NAFLD, a high fat diet or diabetes mellitus. However, results in the literature are contradictory. Some studies suggested that the intake of a diet high in fish oil results in high Ω3-PUFA content in membrane lipids and enhances lipid peroxidation in the organs, blood, and urine of experimental animals and humans [47, 48]. Some others, however, suggest that low doses of fish oils do not significantly affect lipid peroxidation in vivo [49, 50]. In our study, we have shown that Ω3-PUFAs were effective in reducing and preventing increased lipid peroxidation that is promoted by diet and diabetes mellitus (Table 5). We believe that this beneficial result is observed as a consequence of the intermediate size of the dose used in this study (3 g Ω3-PUFAs/100 g diet) as compared to data from the literature and the fact that we used this dose over a short period of time (4 weeks). We can also correlate the decrease in NEFAs (Table 3) with the reduction of lipid peroxidation in rats supplemented with Ω3-PUFAs (Table 5) because it is already known that NEFAs induce a stress-signaling pathway in the liver. In our study, we identified an increase in protein carbonyl levels in rats fed with a high fat thermolyzed diet (Table 5), including rats whose diets were supplemented with Ω3-PUFAs. These findings corroborate the study of Matsuzawa-Nagata et al. [51] who reported that protein carbonyl levels were increased by 35% in the livers of mice fed a high fat diet, compared to control mice, after 6 weeks. In our study, we showed that diabetes mellitus, a high fat thermolyzed diet and Ω3-PUFAs could modulate enzyme activity We observed an increase in SOD activity in HFTD and D + HFTD, suggesting that O_2^- production is increased during treatment with this diets, however, SOD activity of HFTD + Ω3 did not change and D + HFTD + Ω3 decreased suggesting an attenuation in the increase of SOD activity caused by HFTD. The immunoblot of SOD2 increased in this groups making a regulation in the substrate level and decreasing the enzymatic activity. These results suggesting that O_2^- production is decreased which corroborating the result of lesser lipoperoxidation by HFTD + Ω3 (Table 5). Bauer et al. [52] demonstrated that an increase in NEFA and impaired adiponectin bioactivity both contribute to reduced levels of SOD2 protein in monocytes of type 2 diabetes patients. Indeed the study of Bauer et al. was conducted with subjects with DM2 and our model (alloxan) is DM1, however, once installed the pathology of diabetes mellitus, the pro-inflammatory and pro-oxidant response are very similar in both types (DM1 and DM2). [53, 54]. The study of Flachs et al. [55] observed that feeding mice with a high fat diet enriched with EPA/DHA concentrate (6% EPA, 51% DHA) for 5 weeks leads to elevated systemic concentrations of adiponectin and suggested that this increase could explain, to some extent, the anti-diabetic properties of these Ω3-PUFAs.

In this study, supplementation with Ω3-PUFAs decreased levels of lipoperoxidation and hepatic lipids and increased the SOD2 immunocontent. A combination of prior evidence from the literature and the results presented

here lead us to propose that Ω3-PUFAs reduce the amount of NEFAs and increase the concentration of adiponectin. This, in turn, leads to increases in the levels of SOD2, which subsequently prevents HFTD-mediated increases in lipoperoxidation and oxidative stress. Our findings confirm the beneficial effects of Ω3-PUFAs on lipid metabolism and oxidative stress in the liver, which may represent an important adjuvant to therapies currently used in the treatment of some diseases like diabetes mellitus, insulin resistance, and non-alcoholic fatty liver disease.

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Ω3-Polyunsaturated fatty acids prevent the development of renal disease in diabetic rats fed a high fat thermolyzed diet by inhibiting the formation of reactive oxygen species

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Abstract

Recent studies suggest that oxidative stress increases with diabetes mellitus. This may be due to a decreased antioxidant potential, increased free-radical production, alterations in enzymatic pathways related to the redox state (such as the polyol and glyoxalase pathways), and changes in the compartmentalization of metal ions. In this study, we verified a possible beneficial effect of to prevent the development of renal disease in diabetic rats fed a high fat thermolyzed diet. Rats were divided into six groups: (1) the control group (C), (2) the control diabetic group (D), (3) the high fat thermolyzed diet group (HFTD), which were fed a diet that was enriched in fat that was heated for 60 min at 180 °C, (4) the high fat thermolyzed diet diabetic group (D+HFTD), (5) the high fat thermolyzed die t+ Ω3 polyunsaturated fatty acid group (HFTD+Ω3), (6) the high fat thermolyzed diet + Ω3 polyunsaturated fatty acid diabetic group (D+HFTD+Ω3). Our findings suggest that Ω3-PUFAs reduce reactive species of oxygen, modulate antioxidant defense but not alter antiglycation defense. Further studies are necessary for a better understanding of the molecular mechanisms involved in Ω3-PUFAs prevent oxidative damage.

Keywords: Advanced Glycation End Products; Oxidative Stress; Ω3-Polyunsaturated Fatty Acids; Renal disease; Kidney.

1. Introduction

Recent studies showed a major problem in public health is the increasing incidence of age-related chronic diseases, most prominently obesity, diabetes, and renal disorders. These have in common elevated oxidant stress (OS) linked to a inflammatory state (1,2).

Advanced glycation end products (AGEs) are a heterogeneous group of compounds with significant prooxidant and proinflammatory actions (3,4). They form in the body under physiologic conditions produced by the reaction of reducing sugars and reactive aldehydes with the free amino group of proteins, lipids, and nucleic acids (5). Their rate of endogenous formation is markedly enhanced in diabetes mellitus (4). They also form externally during the heat processing of food (6).

Diet is a major source of AGEs and other oxidants that are generated during exposure of food to heat (7). The most important determinant of the levels of AGEs in the food is the method of cooking, especially the cooking temperature (7). Of course, AGE levels also depend on the content of proteins, lipids, and carbohydrates. Thus, meals cooked at high temperatures and under dry conditions have the highest AGE content, especially if there is a high fat content. We found a close association between the content of AGEs in the diet and serum AGE levels, OS, and inflammatory mediators across a spectrum of healthy, diabetic, and renal-failure subjects (7,8,9).

AGEs are modified in the body by enzymatic degradation, innate defenses, and receptor-dependent uptake and degradation.The major degradative enzymes involved are the glyoxalase I and II system. The glyoxalase system, consisting of glyoxalase I, glyoxalase II and using a catalytic amount of reduced glutathione, converts dicarbonyl compounds (-

oxoaldehydes) to their corresponding hydroxy acids such as methylylglyoxal to d-lactate (10). Since the reaction of the dicarbonyl compounds (or their thiol esters) with glyoxalase I is the rate-limiting step, glyoxalase I activity indirectly determines the rate of AGE formation, and therefore might be of particular interest in aging and age-related diseases.

Eicosapentaenoic acid (EPA) is one of the Ω3 polyunsaturated fatty acids (Ω3-PUFAs) which are contained in fish oil. It was shown that EPA has many effects such as anti-thrombotic, hypolipidaemic, anti-atherogenic, anti-inflammatory and anti-mitogenic actions. Moreover, recent studies have shown that dietary supplementation with Ω3-PUFAs retards disease progression in human and experimental renal diseases. There is also a report that EPA improved albuminuria in type 2 diabetic patients (11). However, the role of EPA in the progression of diabetic nephropathy is not fully understood.

It is postulated that one of the effects of Ω3-PUFAs on diabetic nephropathy might be increased antioxidant enzymes and decrease reactive oxygen species. In this study, we verified a possible beneficial effect of Ω3-PUFAs to prevent the development of renal disease in diabetic rats fed a high fat thermolyzed diet.

2. Material and Methods

2.1. Animals and chemicals

Sixty adult Wistar rats (male, 60 days old) from the Central Animal House of the Department of Biochemistry were maintained under a standard dark–light cycle (lights on between 7:00 a.m. and 7:00 p.m.) at room temperature (22±2 °C). All experiments were conducted in accordance with the Guiding Principles in the Use of Animals in Toxicology, adopted by the Society of Toxicology in July 1989, and all experiments were approved by our ethics committee for animal use at the Federal University of Rio Grande do Sul. Alloxan monohydrate and all other chemicals were obtained from Sigma-Aldrich (St. Louis, MO, USA). Anti-catalase antibody, anti-superoxide dismutase 2 antibody and anti-β-actin antibody were obtained from Calbiochem (Gibbstown, USA), Abcam (Cambridge, MA, USA) and Sigma-Aldrich (St. Louis, MO, USA), respectively.

2.2. Treatments and diets

Diabetes mellitus was induced by an intraperitoneal (i.p.) injection of alloxan (150 mg kg^{-1} b.w.), and non-diabetic animals received a saline solution (NaCl 0.9%), also via an i.p. injection. After 10 days, blood samples were obtained from rats in fasting state to determine the plasma glucose concentration. Only animals that presented with 15–30 mmol/l glucose concentration were included in the study.

Rats were divided into six groups: (1) the control group (C, n=10), which received standard laboratory rat chow; (2) the control diabetic group (D, n=10), which received an injection of alloxan and the same diet as group 1; (3) the high fat thermolyzed diet group (HFTD, n=10), which received a diet enriched

with fats that had been heated for 60 min at 180 °C to which a vitamin mixture was added after heating; (4) the high fat thermolyzed diet diabetic group (D+HFTD, n=10), which received an injection of alloxan and the same diet as group 3; (5) the high fat thermolyzed diet + Ω3 polyunsaturated fatty acid group (HFTD+Ω3), which received a diet enriched with fats that had been heated for 60 min at 180 °C to which a vitamin mixture and an omega 3 fatty acid supplement was added after heating; and (6) the high thermolyzed fat diet + Ω3-polyunsaturated fatty acid diabetic group (D+HFTD+Ω3, n=10), which received an injection of alloxan and the same diet as group 5. Diet thermolyzation was based on the study by Sandu et al. (12), which indicated a high AGE content (around 1 unit/μg) in a high fat diet after thermolyzation. The salt and vitamin compositions used were the same as those described by Horwitz et al., (13). During the treatment period of four weeks, the animals had free access to food and water.

2.3. Tissue Preparation

Rats were killed by decapitation, and the blood was collected immediately in heparinized tubes. The whole blood sample was then centrifuged at 2500 x *g* for 10 min to yield the plasma fraction, which was used for subsequent biochemical analyses. The kidney was dissected stored at - 80 °C for future analyses of oxidative stress and western blotting.

2.4. Insulin Tolerance Test

An insulin tolerance test was performed after 26 days of diet treatment. Insulin (1 U insulin/kg) was injected intraperitoneally after 6 h of starvation, and blood was collected by a small puncture on the tail immediately prior to the injection,

as well as 30, 60, and 120 min afterward. Each time, glucose was measured by a glucometer (AccuChek Active, Roche Diagnostics, USA).

2.5. Oxidative stress and antioxidant enzyme activity

2.5.1. Measurement of ROS

To assess the ROS formation, 2′,7′-dichlorofluorescin (DCFH-DA, Sigma) was used as a probe (14). Within the cell, esterases cleave the acetate groups on DCFH-DA, thus trapping the reduced probe (DCFH) intracellularly. ROS in the cells leads to the oxidation of DCFH, yielding the fluorescent product DCF. An aliquot of the sample was incubated with DCFH-DA (100 μM) at 37°C for 30 min, chilling the reaction mixture in ice terminated the reaction. The formation of the oxidized fluorescent derivative (DCF) was monitored at excitation and emission wavelengths of 488 and 525 nm, respectively, using a fluorescence spectrophotometer. The free radical content was quantified using a DCF standard curve and results were expressed as nanomoles of DCF formed per milligram of protein.

2.5.2. Measurement of protein carbonyl

The oxidative damage to proteins was measured by quantifying the number of carbonyl groups by reaction with 2, 4-dinitrophenylhydrazine (DNPH). Proteins were precipitated by the addition of 20% trichloroacetic acid (TCA) and were resuspended in 10mM DNPH, and the absorbance of the resulting solution at 370 nm was recorded (15). Results are expressed as nmol carbonyl/mg protein.

2.5.3. Thiobarbituric acid reactive species (TBARS)

In order to assess the extent of lipoperoxidation, we detected thiobarbituric acid reactive species (TBARS) formation through a heated and acidic reaction. This is widely adopted as a method for the measurement of lipid redox states, as previously described (16). The samples were mixed with 0.6 mL of 10% TCA and 0.5 mL of 0.67% thiobarbituric acid and were then heated in a boiling water bath for 25 min. The level of TBARS was determined by measuring the absorbance of each sample at 532 nm. The concentration of TBARS in the samples was then determined from a calibration curve using 1,1,3,3-tetramethoxypropane (which had been subjected to the same treatment as the supernatants) as a standard. Results are expressed as nanomoles of TBARS per milligram of protein.

2.5.4. Measurement of non-protein thiol content

An aliquot of the supernatant from homogenates precipitated with 20% TCA was diluted in 0.1% SDS and 0.01 M 5,5′-dithionitrobis-2-nitrobenzoic acid (DTNB) in ethanol was added. An intense yellow color developed, and the absorbance at 412 nm was recorded after 20 min (17). Simultaneously, the absorbance of a blank sample, which was identical to the analyzed samples except for the absence of DTNB, was also recorded. The non-protein thiol content of each sample was calculated, after subtraction of the blank absorbance, by using the following equation and the molar extinction coefficient of 13,600 M^{-1} cm^{-1}: {[(final absorbance/13,600)V]/1000}/Q, where “V” is the volume (in mL) of the sample used in the assay and “Q” is the amount of protein (in mg) in the sample used to perform the assay.

2.5.5. Catalase assay

Catalase (CAT) activity was measured as previously described (18). The rate of decrease in absorbance at 240 nm was measured as a function of H_2O_2 degradation by catalase.

2.5.6. Superoxide dismutase assay

Superoxide dismutase (SOD) activity was assessed by quantifying the inhibition of superoxide-dependent adrenaline autooxidation by recording the absorbance of the samples at 480 nm (19).

2.5.7. Glutathione peroxidase assay

GSH-Px activity was measured according to the method described by Wendel, (20) using tert-butyl hydroperoxide as substrate. Nicotinamide adenine dinucleotide phosphate (NADPH) disappearance was monitoredspectrophotometrically at 340 nm in a medium containing 2 mM glutathione, 0.15 U/ml glutathione reductase, 0.4 mM azide, 0.5 mM tert-butyl hydroperoxide and 0.1 mM NADPH. One GSH-Px unit is defined as 1 µmol of NADPH consumed per minute and the specific activity is represented as units per milligram of protein.

2.6. Measurement of glyoxalase I activity

Glyoxalase I activity was assayed according to Mannervik et al. (21). The assay was carried out in 96-well microplates using a microplate spectrophotometer (UV Star - Greiner). The 200 µl/well reaction mixture contained 50 mM sodium-phosphate buffer pH 7.2, 2 mM methylglyoxal (MG) and 1 mM glutathione (GSH; pre-incubated for 30 min at room temp). To the buffer, 10–20 µg protein from the sample was added per well. The formation of S-(D)-lactoylglutathione was monitored at 240 nm for 15 min at 25ºC. A unit of glyoxalase I activity is defined as the amount of enzyme that catalyzes the

formation of 1 μmol of S-(D)-lactoylglutathione per minute. Specific activity is expressed in units per milligram of protein.

2.7. Western blotting analysis

Proteins (20 μg) were separated by SDS-PAGE on 10% (w/v) acrylamide and 0.275% (w/v) bisacrylamide gels and were then electrotransferred onto nitrocellulose membranes. The membranes were then incubated in Tris-buffered saline Tween-20 [TBS-T; 20 mM Tris–HCl, pH 7.5, 137 mM NaCl, 0.05% (v/v) Tween 20] containing 1% (w/v) non-fat milk powder for 1 h at room temperature. Subsequently, the membranes were incubated for 12 h with the appropriate primary antibody (Catalase, 1:6000; Superoxide dismutase, 1:2000, β-Actin, 1:10000). After washing in TBS-T, blots were incubated with horseradish peroxidase-linked anti-immunoglobulin G (IgG) antibodies for 1.5 h at room temperature. Chemiluminescent bands were detected, and densitometric analysis was performed using Image-J® software.

2.8. Statistical Analyses

Biochemical data are expressed as the mean ± S.D. All analyses were performed using the Statistical Package for the Social Sciences (SPSS 16.0) software. Likewise, the differences between all groups were analyzed using one-way ANOVA and Tukey's post hoc test. *P*-values were considered significant when $p < 0.05$.

3. Results

Figure 1 shows the Insulin Tolerance Test in diabetic groups (A), non-diabetic groups (B) and area under a curve (C). We observed a significant difference ($P<0.05$) between the diabetic control group (D) and diabetic groups supplemented with high fat diet (D+HFTD and D+HFTD+Ω3). However, diabetic rats fed a HFTD or HFTD+Ω3 diet showed a decrease in their glucose concentration. This effect was attributed to a lower amount of carbohydrate in these diets as compared to the control diet. In both graphs we can notice a trend in the Ω3-PUFAs improve insulin sensitivity.

In Figure 2, we tried to assess the effect of diabetes, high fat thermolyzed diet and Ω3-PUFAs on the lipoperoxidation (A), ROS (B), and damage to protein (C, D). We did not find a significant difference in the levels of lipoperoxidation (Figure 2A) and thiol content (Figure 2D) in the experimental groups studied. However, Ω3-PUFAs were significantly ($P<0.05$) effective in preventing the increase of reactive species (Figure 2B) in both groups (HFTD+Ω3and D+HFTD+Ω3). In addition, we noted significant ($P<0.05$) increased levels of protein carbonyl in all diabetic groups compared to the non-diabetic groups, although Ω3-PUFAs had no effect on this parameter.

Figure 3 shows the effect of our experimental model over the antioxidant enzymes activity, Superoxide dismutase (A), Catalase (B) Glutathione peroxidase (C) and ratio of them - SOD / CAT + GPx (D). We observe a significant ($P<0.05$) decreased of SOD (Figure 3A) and CAT (Figure 3B) activity in all diabetic groups compared to the non-diabetic groups. Although, diabetes and HFTD decreased ($P<0.05$) GPx activity, when added Ω3-PUFAs in the diet we can observe an increased ($P<0.05$) in GPx activity just in diabetic

group (D+HFTD+ Ω3). In addition, in the SOD / CAT + GPx ratio we noted an increased in this ratio by high fat thermolyzed diet and a normalizing these values by Ω3-PUFAs.

Figure 4 shows the immunocontent of Catalase (A) and Superoxide dismutase 2 (B). We observed a significant ($P<0.05$) decreased in kidney immunocontent of CAT in diabetic (D) and D+HFTD+Ω3. Althogh, only the high fat thermolyzed diet is able to increased immunocontent of SOD2 in diabetic and non-diabetic groups (HFTD and D+HFTD).

In the Figure 5, we verified the glyoxalase I activity, one of the anti-glycation defenses. Only diabetic group (D) increased significant ($P<0.05$) glyoxalase I activity in relation other experimental groups.

4. Discussion

In this study, we evaluated a possible beneficial effect of Ω3-PUFAs to prevent the development of renal disease in diabetic rats fed a high fat thermolyzed diet. The most important finding of this study was that Ω3-PUFAs are able to modulate antioxidant enzymes and prevent lipoperoxidation, reducing reactive species of oxygen in kidney of diabetic rats supplemented with high fat thermolyzed diet. However, these Ω3-PUFAs did not affect the observed levels of glyoxalase I in kidney and insulin sensivity.

We cannot deny a possibility that the correction of metabolic abnormalities by Ω3-PUFAs may contribute to the improvement of diabetic nephropathy in the present study. However, Shimizu et al. (22) reported that EPA administration improved urinary ACR without affecting blood pressure levels, glycaemic control and lipid metabolism (22). In your study, Donadio et al., (23) showed that dietary supplementation with Ω3-PUFAs retards disease progression in non-diabetic renal diseases including IgA nephropathy. Therefore, besides the effects of EPA on ITT or hypertriglyceridemia, we assume that EPA has a direct renal effect on diabetic nephropathy.

Hydrogen peroxide (H_2O_2) is an important contributor of the oxidative damage which is related with SOD activity. When the hydrogen peroxide concentration is relatively low within the tissue, this disintegration is made mainly by the GPx enzyme because of the low Km value of GPx with respect to CAT (24). However, when its concentration is high enough, both CAT and GPx enzymes are responsible for the detoxification process (25). According to our results (Figure 3), the decrease observed in SOD, CAT and GPx activity a indicated transcriptional repression in diabetic kidney tissues which we have also observed previously in diabetic liver tissues (26). Corroborating with our

results that diabetes reduce enzymes antioxidant activy, It has been demonstrated that AGEs decreases SOD activity *in vivo* and *in vitro* (27). Superoxide dismutase catalyzes the dismutation of radical superoxide anion radical into oxygen and hydrogen peroxide. A decrease in CAT activity might be due to a direct inhibition of SOD by AGEs, as superoxide directly inhibits catalase (28).

In conclusion, our findings suggest that Ω3-PUFAs reduce reactive oxygen species, modulate antioxidant defense, maily a gluthatione peroxidase activity, but not alter antiglycation defense. Further studies are necessary for a better understanding of the molecular mechanisms involved in Ω3-PUFAs prevent oxidative damage via inhibtion of reactive oxygen species.

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Legends

Figure 1. Insulin Tolerance Test in the kidney of diabetic (A) and non-diabetic (B) rats fed a control diet (C), high fat thermolyzed diet (HFTD) and high fat thermolyzed diet + Ω3 (HFTD+Ω3). Blood glucose level is expressed in mg/dL. All results are presented as the mean ± SD (n=6 per group). Different asterisks on the same row indicate a significant difference ($P< 0.05$) all groups were analyzed using one-way ANOVA and Tukey's post hoc test.

Figure 2. Oxidative stress in the kidney of diabetic and non-diabetic rats fed a control diet (C), high fat thermolyzed diet (HFTD) and high fat thermolyzed diet + Ω3 (HFTD+Ω3). Lipoperoxidation (A), DCFH (B), protein carbonyl (C) and non-protein thiol contents (D) are expressed in nmol/mg protein. All results are presented as the mean ± SD (n=10 per group). Different asterisks on the same row indicate a significant difference ($P< 0.05$) all groups were analyzed using one-way ANOVA and Tukey's post hoc test.

Figure 3. Antioxidant enzymes activity in the kidney of diabetic and non-diabetic rats fed a control diet (C), high fat thermolyzed diet (HFTD) and high fat thermolyzed diet + Ω3 (HFTD+Ω3). SOD (A), CAT (B) and GPx (C) activities are expressed in U/mg protein. The SOD/CAT+GPx (D) ratio is expressed in arbitrary units. All results are presented as the mean ± SD (n=10 per group). Different asterisks on the same row indicate a significant difference ($P< 0.05$) all groups were analyzed using one-way ANOVA and Tukey's post hoc test.

Figure 4. Representative and quantitative Western blot analysis of the kidney CAT (A) and SOD2 (B) immunocontent from diabetic and non-diabetic rats fed a control diet (C), high fat thermolyzed diet (HFTD) and high fat thermolyzed diet + Ω3 (HTFD+Ω3). The graphic represents the means ± SD (n=3 per group). Different asterisks indicate a significant difference ($P< 0.05$) all groups were analyzed using one-way ANOVA and Tukey's post hoc test.

Figure 5. Glyoxalase I activity in kidney of diabetic and non-diabetic rats fed a control diet (C), high fat thermolyzed diet (HFTD) and high fat thermolyzed diet + Ω3 (HFTD+Ω3). Glyoxalase I was expressed in U/mg protein. All results are presented as the mean ± SD (n=10 per group). Different asterisks on the same row indicate a significant difference ($P< 0.05$) all groups were analyzed using one-way ANOVA and Tukey's post hoc test.

Figure 1.

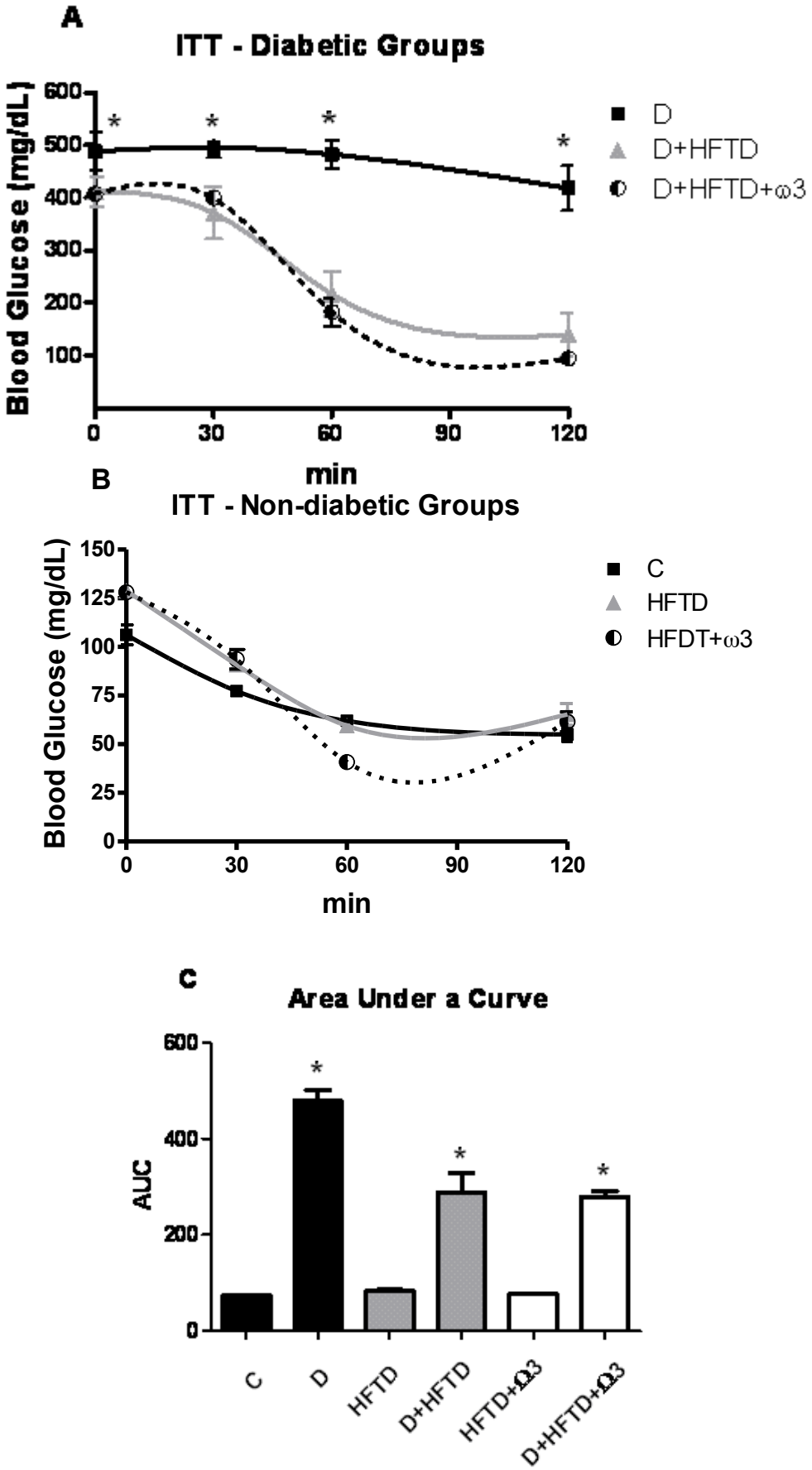

A
ITT - Diabetic Groups
Blood Glucose (mg/dL)
600
500
400
300
200
100
0
30
60
90
120
min
D
D+HFTD
D+HFTD+ω3
B
ITT - Non-diabetic Groups
Blood Glucose (mg/dL)
150
125
100
75
50
25
0
0
30
60
90
120
min
C
HFTD
HFDT+ω3
C
Area Under a Curve
AUC
600
400
200
0
C
D
HFTD
D+HFTD
HFTD+Ω3
D+HFTD+Ω3

Figure 2.

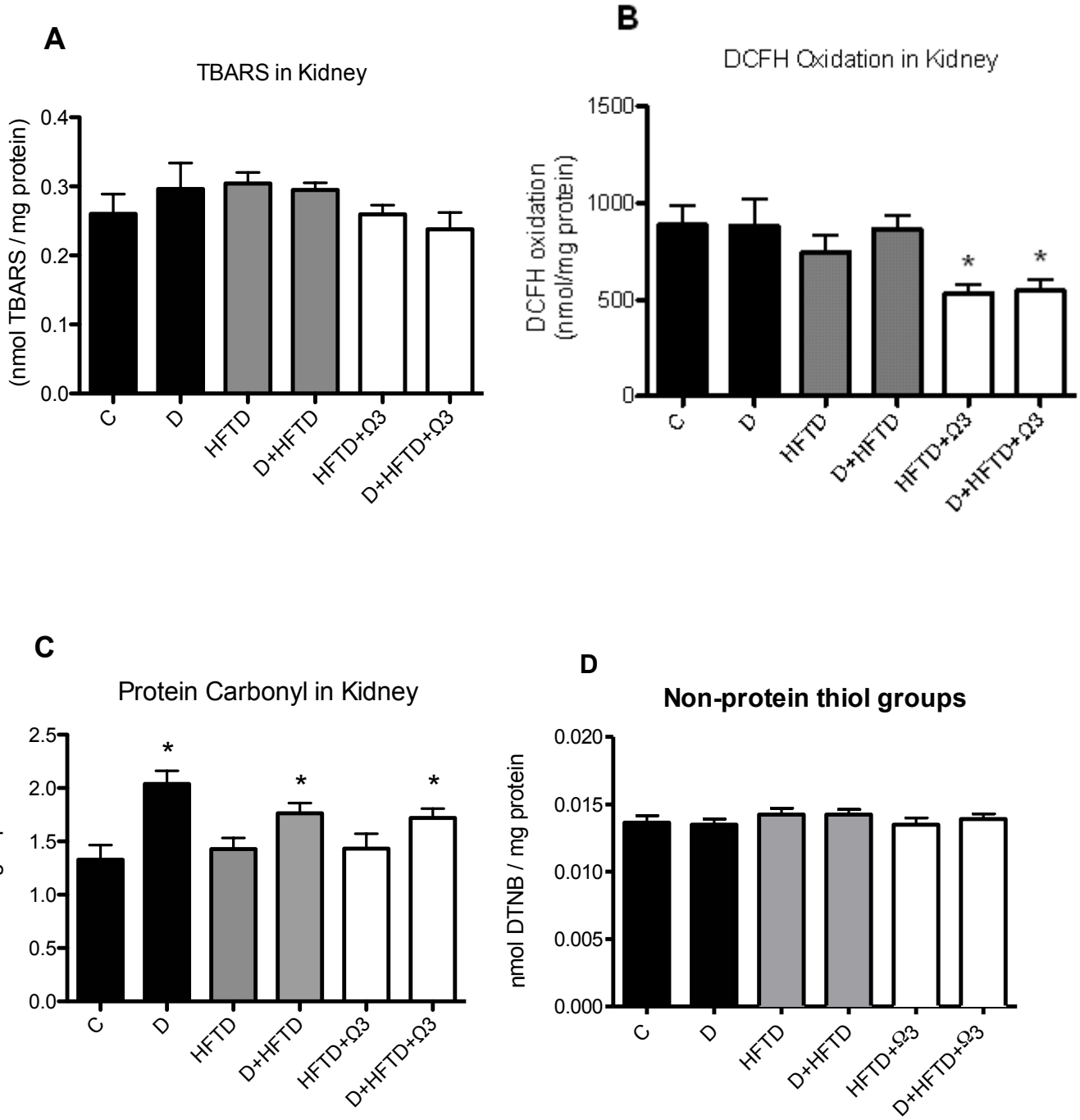

A
TBARS in Kidney
(nmol TBARS / mg protein)
0.4
0.3
0.2
0.1
0.0
C
D
HFTD
D+HFTD
HFTD+Ω3
D+HFTD+Ω3
B
DCFH Oxidation in Kidney
DCFH oxidation (nmol/mg protein)
1500
1000
500
0
*
*
C
D
HFTD
D+HFTD
HFTD+Ω3
D+HFTD+Ω3
C
Protein Carbonyl in Kidney
mmol / mg of protein
2.5
2.0
1.5
1.0
0.5
0.0
*
*
*
C
D
HFTD
D+HFTD
HFTD+Ω3
D+HFTD+Ω3
D
Non-protein thiol groups
nmol DTNB / mg protein
0.020
0.015
0.010
0.005
0.000
C
D
HFTD
D+HFTD
HFTD+Ω3
D+HFTD+Ω3

Figure 3.

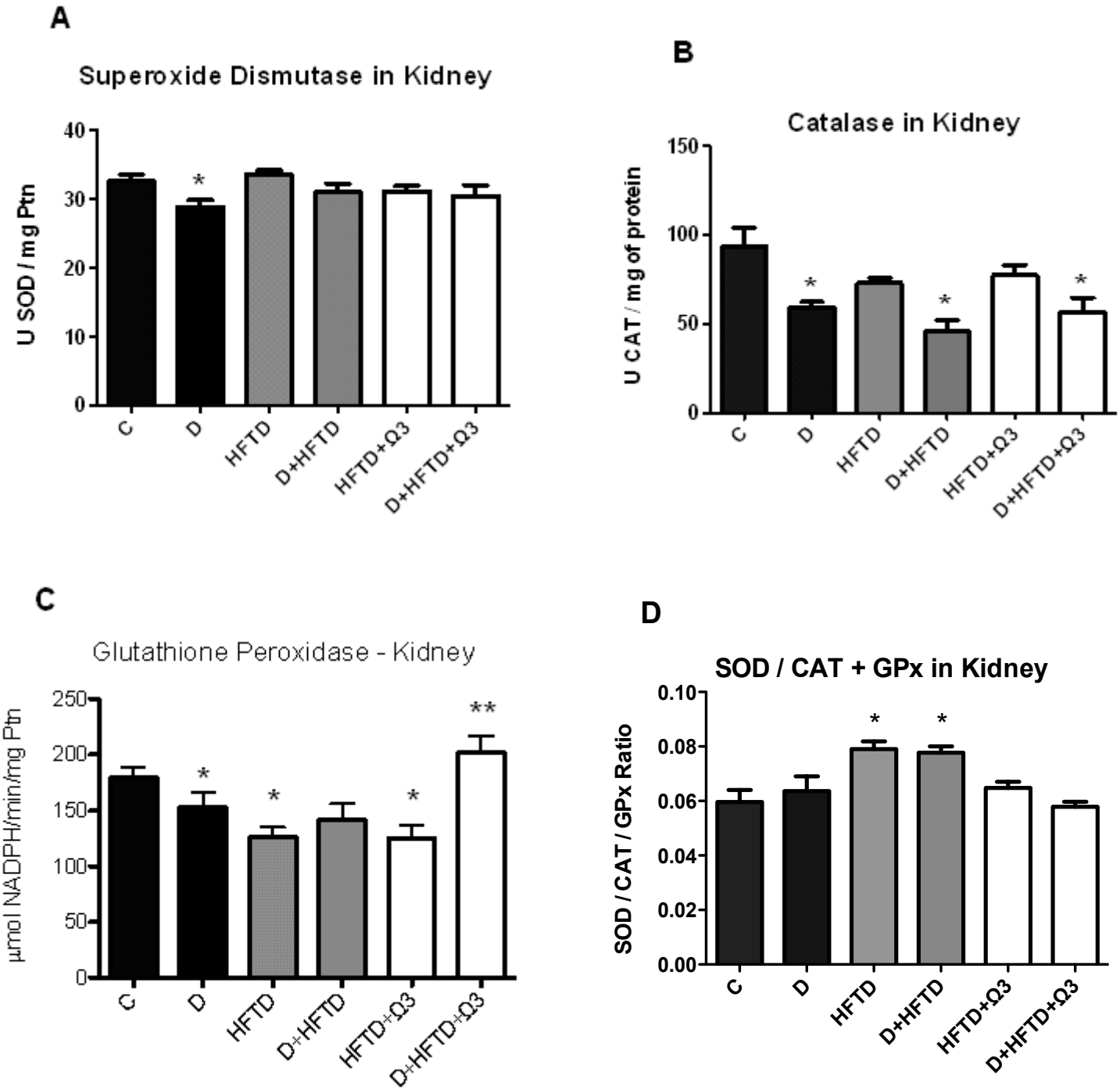

A
Superoxide Dismutase in Kidney
U SOD / mg Ptn
40
30
20
10
0
*
C
D
HFTD
D+HFTD
HFTD+Ω3
D+HFTD+Ω3
B
Catalase in Kidney
U CAT / mg of protein
150
100
50
0
*
*
*
C
D
HFTD
D+HFTD
HFTD+Ω3
D+HFTD+Ω3
C
Glutathione Peroxidase - Kidney
µmol NADPH/min/mg Ptn
250
200
150
100
50
0
*
*
*
**
C
D
HFTD
D+HFTD
HFTD+Ω3
D+HFTD+Ω3
D
SOD / CAT + GPx in Kidney
SOD / CAT / GPx Ratio
0.10
0.08
0.06
0.04
0.02
0.00
*
*
C
D
HFTD
D+HFTD
HFTD+Ω3
D+HFTD+Ω3

Figure 4.

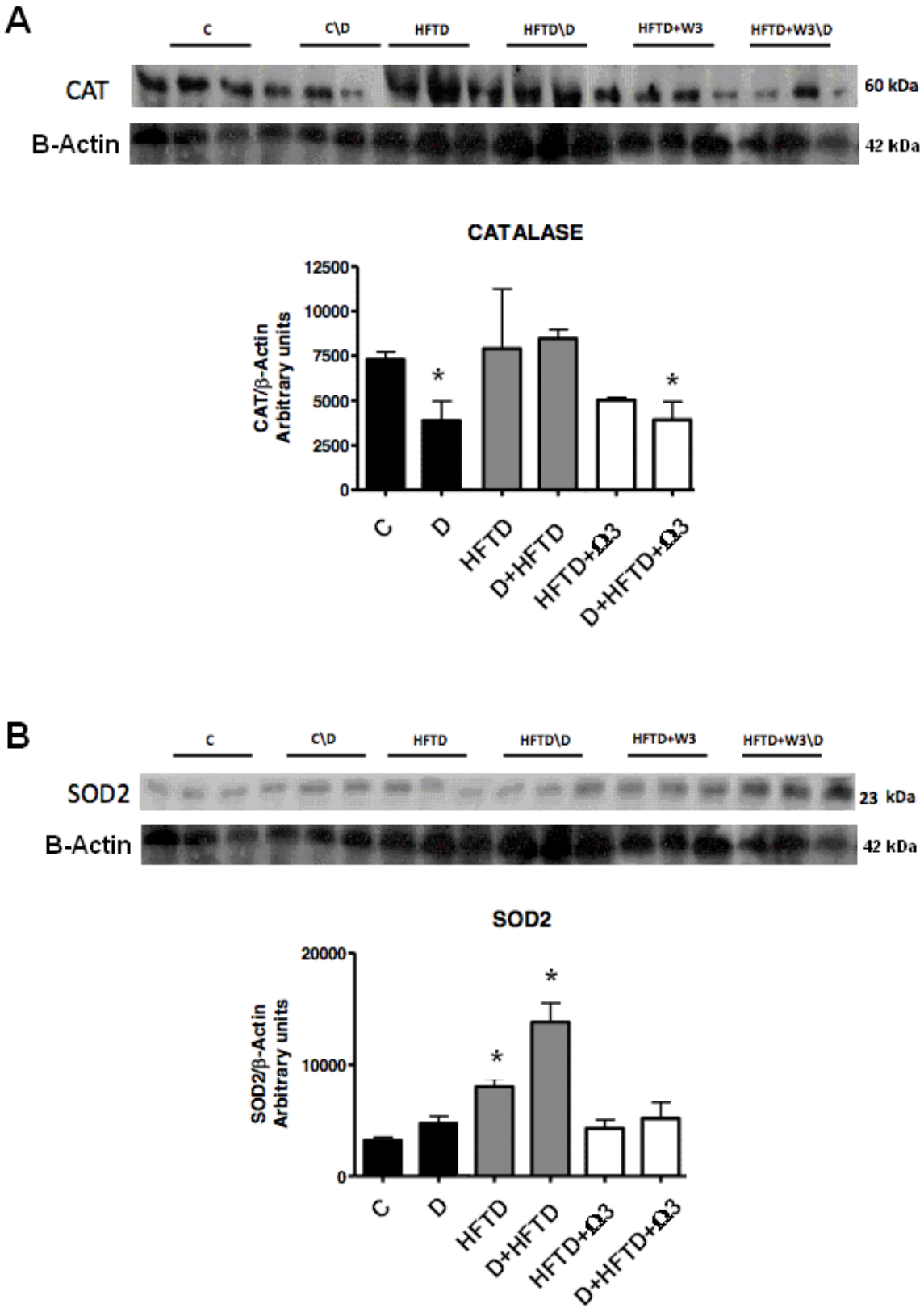
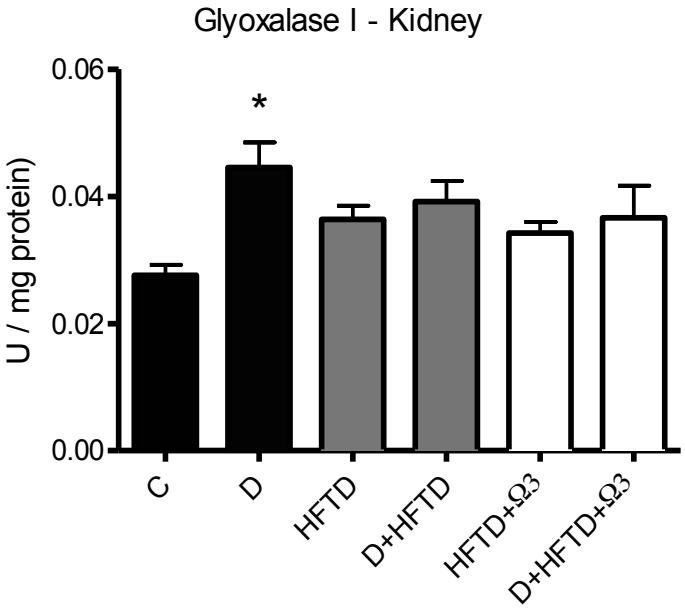

A
C
C\D
HFTD
HFTD\D
HFTD+W3
HFTD+W3\D
CAT
60 kDa
B-Actin
42 kDa
CATALASE
CAT/β-Actin Arbitrary units
12500
10000
7500
5000
2500
0
C
D
HFTD
D+HFTD
HFTD+Ω3
D+HFTD+Ω3
B
SOD2
23 kDa
B-Actin
42 kDa
SOD2
SOD2/β-Actin Arbitrary units
20000
10000
0

Figura 5.


Glyoxalase I - Kidney
U / mg protein)
0.06
0.04
0.02
0.00
*
C
D
HFTD
D+HFTD
HFTD+Ω3
D+HFTD+Ω3

4. Discussão

Um possível mecanismo de dano ao tecido induzido por hiperglicemia persistente na diabetes mellitus é a formação de produtos avançados de glicação (AGEs). Endogenamente, açúcares reduzidos reagem não enzimaticamente com grupos amino de proteínas, lipídeos e ácidos nucléicos através de uma série de reações que formam bases de Shiff seguida da formação de produtos de Amadori para a produção de AGEs. O glioxal e o metilglioxal são espécies reativas de carbonilas com ação de glicação, formados pela degradação de proteínas glicadas, intermediários glicolíticos e peroxidação lipídica e reagem com proteínas para formar os AGEs. A preparação de alimentos a elevadas temperaturas (130°C) é a principal fonte exógena dos AGEs. Este processo é dependente do tempo e da temperatura em que os alimentos são aquecidos, bem como da composição dos mesmos (Shangari e O'brien, 2004).

Baseado nestes conceitos, este trabalho buscou avaliar os efeitos causados pelas glicotoxinas no sistema nervoso central e periférico de ratos em dois principais modelos:

- Estudo *in vitro*: para verificar o efeito dos glioxais endógenos diretamente no tecido alvo (SNC). (Item 3.1)
- Estudo *in vivo*: Diabetes + Dieta Hiperlipídica Aquecida - para verificar a combinação de uma fonte exógena com uma fonte endógena de AGEs e seus efeitos em tecidos alvo do diabetes. (Itens 3.2, 3.3 e 3.4).

4.1. Estudo *in vitro*

4.1.1. Efeitos do glioxal e metilglioxal sobre o metabolismo de

aminoácidos, lactato, glicose e acetato no cortex cerebral de ratos adultos e ratos jovens.

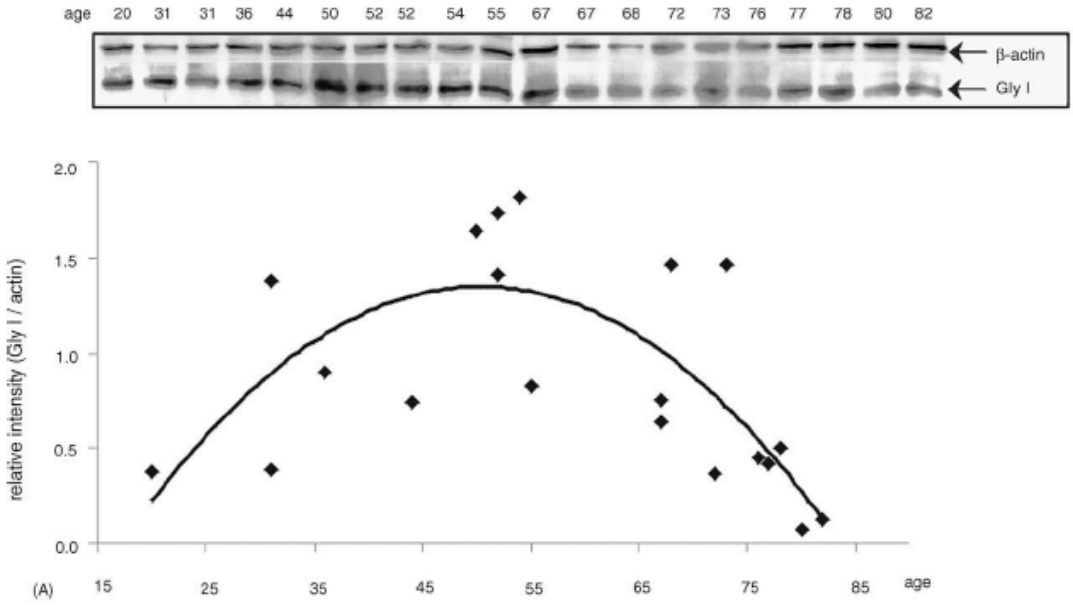
No item 3.1 desta tese nós verificamos os efeitos do glioxal e metilglioxal sobre o metabolismo de alguns aminoácidos, lactato, glicose e acetato em fatias de córtex cerebral de ratos de 10 dias de idade pós-natal e de ratos adultos (3 meses de idade pós-natal). Embora a concentração intracelular exata de glioxal em neurônios ainda é desconhecida, concentracões de metiglioxal até 300 μM foram medidos em cultura de células CHO, a maioria deles ligado a resíduos de cisteína e glutationa (Chaplen et al., 1998). Além disso, a concentracão destes aldeidos pode aumentar quando as concentracões de seus precursors está aumentada, como na hiperglicemia (Ahmed et al., 2005). Contudo, a concentração de glioxal (400uM) utilizada em nosso estudo parece ser relevante a partir de um ponto de vista fisiológico.

No item 3.1, tivemos como achado mais importante que o glioxal, que tem sido relatado a contribuir para a formação de produtos de glicação avançada (AGEs) (Thornalley, 2005), aumentou a taxa de produção de CO_2 a partir dos aminoácidos alanina, leucina e glicina em fatias córtico-cerebrais derivados de ratos jovens. O fato de que os glioxais só tiveram efeitos significativos em córtex dos animais jovens pode estar relacionado com níveis mais baixos de glutationa. Corroborando com esta idéia, Stringari et al., 2008, demonstrou que filhotes após a fase de desmame apresentam níveis baixos de glutationa no cortex cerebral. Por outro lado, a incorporação desses amino ácidos em proteínas foi diminuída após a exposição ao glioxal. Embora tenha sido relatado que os glioxais são capazes de afetar o metabolismo energético, o que pode levar à diminuição dos níveis de ATP e a disfunção mitocondrial

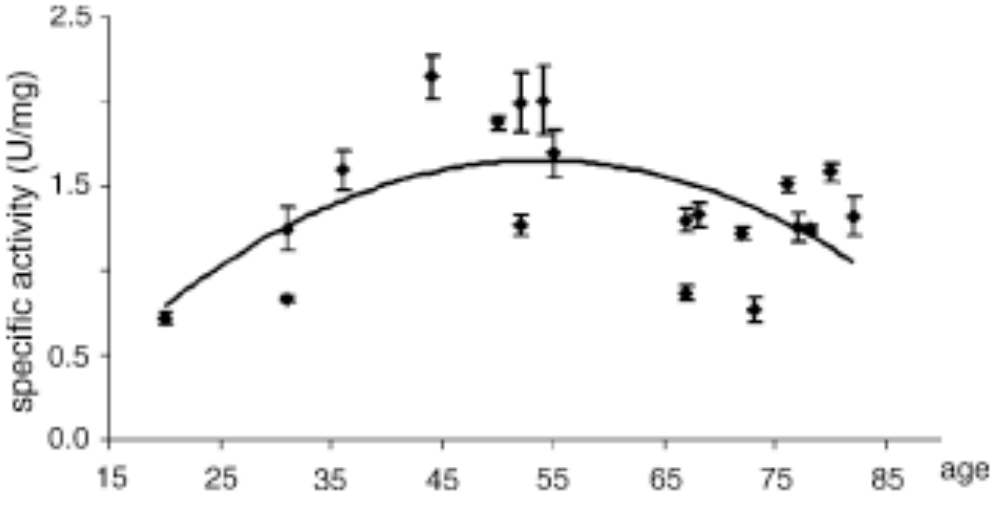
(De Arriba et al., 2007), não existem dados na literatura sobre os efeitos dos glioxais (metilglioxal ou glioxal) sobre o metabolismo de aminoácidos em tecidos cerebral.

Outro fato de extrema relevância que pode justificar os efeitos tóxicos dos glioxais somente em ratos jovens é que a enzima glioxalase I parece ter sua atividade e expressão de forma idade-dependente, ou seja, tendo seu intervalo de atividade e expressão dos 18 aos 80 anos de idade (Kuhla et al., 2004). Como podemos avaliar a Figura 5 na página seguinte, seu pico de ação parece ser por volta dos 55 anos de idade.

A



B



C

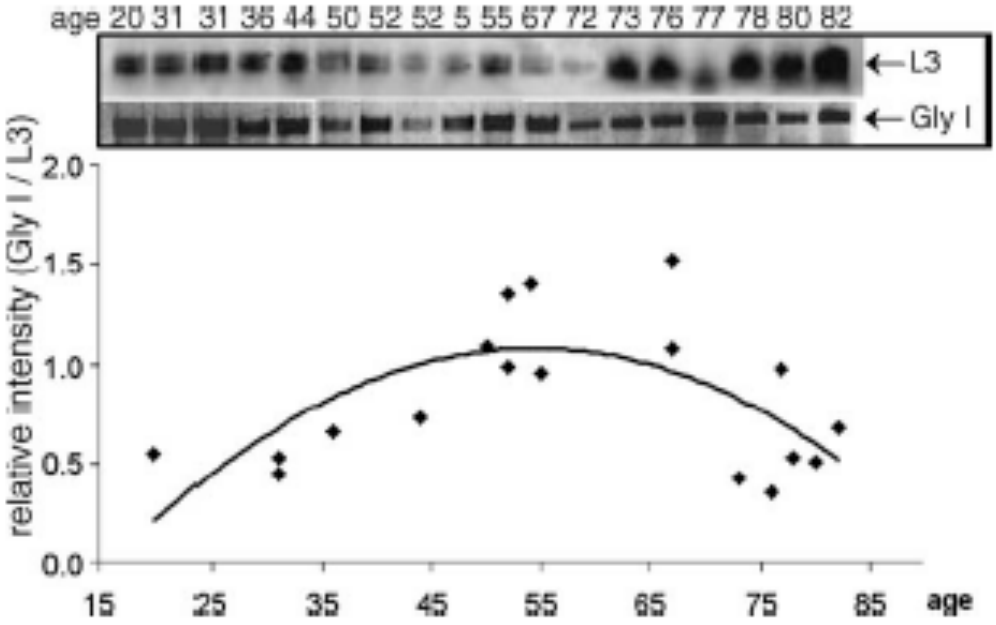


Figura 5. Análise por western blotting do imunoconteúdo da Glioxalase I (Figura 5A), atividade enzimática da Glioxalase I (Figura 5B) e níveis de RNA da Glioxalase I determinados por RT-PCR (Figura 5C) em amostras de cérebros humanos de diferentes idades (área de brodmann 22) (Kuhla et al., 2004).

Estes dados da Figura 5 em conjunto com os resultados encontrados no item 3.1., onde os glioxais (principalmente o glioxal) podem alterar o metabolismo energético de aminoácidos em córtex cerebral de ratos sob condições *in vitro*, sugerem que organismos jovens, animais ou humanos, são mais suscetíveis ao ataque de glioxais devido a menor ação da glioxalase I e menor nível de glutationa no sistema nervoso central.

Observação: Alguns dos resultados do item 3.1. foram utilizados em uma dissertação de mestrado (Schmidt, 2009). Contudo, novos experimentos foram feitos e os resultados antigos refeitos afim de confeccionar o artigo para publicação em uma revista internacional indexada. Ainda, o autor da presente

tese é considerado primeiro autor no artigo do item 3.1.

4.2. Estudo *in vivo*:

4.2.1. Efeitos da dieta hiperlipídica aquecida sobre o metabolismo energético do sistema nervoso central em ratos diabéticos

No item 3.2 buscamos investigar os efeitos de uma dieta hiperlipídica aquecida (rica em AGEs) nos transportadores de ácidos monocarboxílicos e metabolismo energético (lactato, glicose e β-hidroxibutirato) no hipocampo de ratos diabéticos e os possíveis benefícios da suplementação com o ácido graxo poliinsaturado omega 3. Neste manuscrito nós escolhemos trabalhar somente com o hipocampo, pois Pierre et al., 2007, em seu trabalho demonstraram que esta estrutura é mais suscetível a alterações promovidas por dieta hiperlipídica.

Há pouca informação disponível sobre a regulação da expressão dos transportadores de ácidos monocarboxílicos (MCTs) no sistema nervoso central, especialmente em relação aos diferentes estados metabólicos. Os MCTs têm sido implicados na transferência intercelular de lactato em diferentes órgãos, incluindo o cérebro (Brooks, 1986; Pellerin e Magistretti, 1994). Além disso, eles estão envolvidos na importação e na utilização de substratos energéticos, tais como o lactato e corpos cetônicos em situações metabólicas particulares.

A exposição a uma dieta hiperlipídica aquecida estabelece um novo ambiente metabólico para o cérebro conduzindo a maioria das prováveis adaptações metabólicas específicas. Em ratos adultos, a expressão de MCT1 mostrou-se aumentada após três semanas de alimentação com dieta cetogênica (Leino et al., 2001). A resistência a ação da insulina é bem descrita

em tecidos, como músculo esquelético e o fígado, mas foi recentemente proposta também afetando o sistema nervoso central (Isganaitis e Lustig, 2005). As consequências metabólicas específicas da resistências à insulina no cérebro ainda não foram completamente exploradas, contudo a superexpressão dos transportadores de ácidos monocarboxílicos pelos astrócitos (MCT1) e neurônios (MCT2), como observado no item 3.2 poderia ser uma resposta para superar a resistência à insulina cerebral e seu impacto sobre a utilização dos substratos energéticos pelo sistema nervoso central. Por outro lado, Mori et al., 2004, em seu estudo com humanos obesos demonstrou que uma refeição diária rica em Ω3-PUFAs em um programa de redução de peso foi capaz de reduzir os níveis de leptina e insulina, assim como a pressão arterial. Deste modo, podemos supor que as alterações dos Ω3-PUFAs sobre a ação da insulina esta diretamente relacionada com os efeitos destes ácidos graxos no metabolismo energético e os transportadores de ácidos monocarboxílicos.

Neste item, podemos observar que a dieta hiperlipídica aquecida foi capaz de diminuir a oxidação de lactato a CO_2 e aumetar a oxidação de β-hidroxibutirato a CO_2, ainda esta mesma dieta aumentou o imunoconteúdo de MCT1 e MCT2 em hipocampo de ratos diabéticos. De acordo com este item, notamos uma relação entre a oxidação destes substratos e seus transportadores. Embora os Ω3-PUFAs foram eficazes em normalizar o metabolismo energético no hipocampo de ratos diabéticos, devido à falta de trabalhos anteriores na literatura não sabemos se este efeito é um reflexo de sua ação periférica ou se realmente ele tem uma ação central.

4.2.2. Efeitos da dieta hiperlipídica aquecida sobre o estresse oxidativo, conteúdo lipídico e metabolismo do glicogênio em fígado de ratos diabéticos e a possível ação protetora do ácido graxo poliinsaturado Omega 3 (Ω3-PUFAs)

No item 3.3. tivemos como objetivo principal investigar os efeitos dos Ω3-PUFAs sobre o estresse oxidativo, incluindo a atividade das enzimas antioxidantes, lipídios hepáticos e do metabolismo do glicogênio no fígado de ratos diabéticos e não diabéticos alimentados com uma dieta hiperlipídica aquecida.

Neste, a suplementação de 4 semanas com Ω3-PUFAs parece ter mais efeitos sobre o metabolismo lipídico do que o metabolismo glicídico. Nós observamos que o diabetes e a dieta hiperlipídica aquecida reduzem as concentrações de glicogênio hepático, contudo os Ω3-PUFAs parecem não alterar este metabolismo.

Por outro lado, os efeitos positivos dos Ω3-PUFAs na redução de triglicerídeos e colesterol hepáticos demonstrados no item 3.3 têm sido relatados em diversos estudos utilizando modelos animais (Aguilera et al., 2004) e humanos (Oh, 2005). Os Ω3-PUFAs podem inibir a atividade da enzima acil-CoA diacilglicerol aciltransferase que é responsável pela síntese de triglicerídeos (Rustan et al., 1988). Ratos alimentados com Ω3-PUFAs demonstraram níveis séricos de colesterol total mais baixos do que o grupo controle, devido ao aumento da excreção biliar (Balasubramaniam et al., 1985). Estes efeitos hipolipidêmicos e anti-obesidade dos Ω3-PUFAs dependem provavelmente da supressão *in situ* da lipogênese e o aumento na oxidação de ácidos graxos em vários tecidos (Flachs et al., 2005). Estes

efeitos metabólicos podem reduzir o acúmulo de susbtâncias tóxicas derivadas dos ácidos graxos, com o diacilglicerol, protegendo a sinalização da insulina no fígado (Neschen et al., 2007).

Neste item 3.3, a suplementação com Ω3-PUFAs promoveu a diminuição dos níveis de lipídios e lipoperoxidação hepática, contudo aumentou o imunoconteúdo de SOD2. Uma combinação com evidências anteriores na literatura e os resultados apresentados neste capítulo nos levam a propor que os Ω3-PUFAs reduzem a quantidade de ácidos graxos não-estereificados e aumentam a adiponectina plasmática (Bauer et al., 2011). Esta por sua vez aumenta os níveis de SOD2, que por sua vez previne a lipoperoxidação induzida por dieta hipelipídica aquecida.

No item 3.3 confirmamos os efeitos benéficos dos Ω3-PUFAs sobre o metabolismo lipídico e estresse oxidativo no tecido hepático, o que talvez possa representar um importante adjuvante nas terapias atualmente utilizadas no tratamento de algumas doenças como diabetes mellitus e resistência à insulina.

4.2.3. Efeitos da dieta hiperlipídica aquecida sobre o estresse oxidativo e os produtos avançados de glicação em rim de ratos diabéticos e a possível ação protetora do ácido graxo poliinsaturado Omega 3 (Ω3-PUFAs)

No item 3.4 tivemos como objetivo principal investigar um possível efeito benéfico dos Ω3-PUFAs em prevenir o desenvolvimento da doença renal em ratos diabéticos alimentados com uma dieta hiperlipídica aquecida.

A diabetes mellitus é uma das principais causas de doença renal crônica. Cerca de 20-30% dos doentes com diabetes mellitus tipo I e tipo II

desenvolvem nefropatia (Rossing et al., 1994). A probabilidade da progressão para nefropatia é maior na diabetes mellitus tipo 1 (DM1), mas a grande maioria dos doentes com doença renal crônica são portadores de diabetes mellitus tipo 2 (DM2), devido à sua maior prevalência. Contudo, no item 4.2.3 nossos achados sugerem que os Ω3-PUFAs podem reduzir as espécies ativas de oxigênio, modular as defesas antioxidantes enzimáticas, principalmente a glutationa peroxidase, entretanto parecem não afetar os sistemas antiglicação.

Corroborando com nossos resultados, em um estudo semelhante ao nosso, foram administradas dietas ricas em diferentes ácidos graxos poli-insaturados (Ω3-PUFAs ou Ω6-PUFAs) em ratos diabéticos por um longo período (30 semanas), contudo, somente a dieta rica em Ω3-PUFAs foi eficaz em prevenir o desenvolvimento da doença renal promovida pelo diabetes mellitus. Os animais que receberam a dieta rica em Ω3-PUFAs não demonstraram aumento da excreção de albumina na urina, glomeruloesclerose, fibrose tubulointersticial, hipertensão e inflamação características da doença renal promovida pelo diabetes mellitus (Garman et al., 2009).

A inflamação é um importante fator patogênico ligado ao desenvolvimento da doença renal no diabetes mellitus (Ziyadeh, 2004). Zhao et al., 2007, em seu estudo demonstrou que o diabetes mellitus é capaz de aumentar a densidade de macrófagos ativados e a expressão proteica de IL-6 e MCP-1 no córtex renal, compatível com o aumento da inflamação tecidual. Neste mesmo trabalho, o tratamento com Ω3-PUFAs reverteu estes parâmetros demonstrando os efeitos anti-inflamatórios deste composto sobre o diabetes mellitus. Já esta bem descrito na literatura que a inflamação está intimamente ligada ao estresse oxidativo. De acordo com a literatura e baseado em nossos

resultados acreditamos que os efeitos anti-inflamatórios dos Ω3-PUFAs estão relacionados com os dados mostrados no item 3.4. Em resumo, a ação anti-inflamatória dos Ω3-PUFAs estaria diminuindo a ativação de vias inflamatórias como a via do NFkB, a ativação destas vias leva ao aumento de espécias ativas de oxigênio promovendo o estresse oxidativo tecidual, entretanto uma diminuição destas vias (Zhao et al., 2007) levaria á uma diminuição nas espécies ativas de oxigênio, resultado encontrado por nós no item 3.4, representando um menor dano tecidual e prevenindo assim a progressão da doença renal no diabetes mellitus.

Todavia, mais estudos são necessários para melhor entendimento do mecanimso de ação dos Ω3-PUFAs prevenindo danos oxidativos via inibição das espécies ativas de oxigênio.

5. Conclusões

Os resultados que foram apresentados na presente tese, sugerem que as glicotoxinas endógenas e exógenas afetam o metabolismo de alguns aminoácidos (glicina, leucina, alanina, glutamina e glutamato) e o metabolismo energético do sistema nervoso central de maneira a prejudicar sua homeostase. Além disso, observamos que o diabetes mellitus aliado a glicotoxinas exógenas alteram principalmente o metabolismo do lactato em hipocampo de ratos.

Podemos concluir também que os Ω3-PUFAs demonstraram melhorar o estado redox no fígado e no rim de ratos diabéticos alimentados com uma dieta hiperlipídica aquecida. Estes resultados são muito promissores uma vez que muitas das complicações do diabetes mellitus tem como causa principal o aumento de espécies ativas de oxigênio e nitrogênio.

Com relação aos nossos objetivos específicos concluímos que:

- Nosso estudo *in vitro,* demonstrou que o córtex cerebral de ratos jovens é mais suscetível a ação dos glioxais sobre o metabolismo de aminoácidos, e que o glioxal parece ter um efeito mais nocivo do que o metilglioxal neste modelo.
- Quando avaliamos os efeitos das glicotoxinas no sistema nervoso central através da dieta, concluímos que certos estados metabólicos como o diabetes e a alimentação com uma dieta hiperlipídica aquecida em conjunto podem diminuir a utilização de certos substratos, assim como aumentar seus transportadores, acarretando em conseqüências metabólicas negativas. Ainda, os Ω3-PUFAs de alguma forma ainda não bem compreendida parecem ter um efeito benéfico neste modelo.

- Em relação ao fígado, a suplementação com Ω3-PUFAs foi eficaz em reduzir os parâmetros de estresse oxidativo estudados por nós, assim como o metabolismo lipídico em ratos diabéticos alimentados com uma dieta hiperlipídica aquecida. Reforçando o papel benéfico dos Ω3-PUFAs descritos na literatura de forma controvérsa.

- No rim, a suplementação com Ω3-PUFAs demonstrou prevenir o aumento do estresse oxidativo causado pela ingesta de uma dieta hiperlipídica aquecida e o diabetes mellitus, contudo não foi eficaz em modificar o sistema anti-glicação da glioxalase. Sabemos que a nefropatia diabética é uma das doenças que mais acomete pacientes com diabetes mellitus, nossos resultados demonstram uma ação positiva dos Ω3-PUFAs perante esta condição, esta ação pode estar relacionada com o efeito antiinflamatório já descrito dos ácidos graxos poli-insaturados.

6. Perspectivas

- Verificar o efeito *in vitro* de substâncias antioxidantes sobre o metabolismo energéticos em nosso modelo de incubação com glioxal e metilglioxal em fatias de córtex cerebral.

- Verificar o efeito *in vitro* de substâncias antioxidantes sobre estado redox em nosso modelo de incubação com glioxal e metilglioxal em fatias de córtex cerebral.

- Estudar o sistema anti-glicação das glioxalases em nosso modelo de incubação com glioxal e metilglioxal em fatias de córtex cerebral.

- Avaliar parâmetros moleculares para verificar o efeito do diabetes e da dieta hiperlipídica aquecida no metabolismo do lactato.

- Estudar histológicamente o rim e o fígado dos animais diabéticos alimentados com dieta hiperlipídica aquecida e suplementados com Ω3-PUFAs.

- Estudar o metabolismo do glicogênio em diferentes órgãos dos animais diabéticos.

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8. Anexos

8.1. Instruções para o autor – Revista Hippocampus

Author Guidelines

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Papers reporting original research will be the major substance of the journal, but occasional short Commentaries will also be published. The Commentaries will be of three types: Historical Reviews of individual careers or areas of research; Updating Reviews that briefly summarize the state of knowledge in a particular subject area; and Speculative Reviews , in which new perspectives or hypotheses are outlined. The Speculative Reviews may take the form of Point-Counterpoint presentations by two or more authors with differing viewpoints on a topic area. The publication of a Commentary in Hippocampus will normally follow an invitation to the author(s) from the Editor. However, anyone interested in contributing a Commentary or suggesting a topic for one is invited to contact the Editor. The Editor also wishes to encourage neurobiologists to contribute to the journal by writing short letters, which will be considered for publication in a separate Letters to the Editor section. This section will provide a medium for communication and discussion, not only of points that arise from papers published in Hippocampus , but also of topics of general interest to the readership of the journal. The Editor reserves the right to invite replies or comments to such letters at his discretion.

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