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EFEITO PROTETOR DA N-ACETILCISTEÍNA SOBRE O ESTRESSE OXIDATIVO CAUSADO PELA ADMINISTRAÇÃO CRÔNICA DE ASPARTAME EM RATOS

TESE DE DOUTORADO

Isabela Andres Finamor

Santa Maria, RS, Brasil

2015

EFEITO PROTETOR DA N-ACETILCISTEÍNA SOBRE O ESTRESSE OXIDATIVO CAUSADO PELA ADMINISTRAÇÃO CRÔNICA DE ASPARTAME EM RATOS

Isabela Andres Finamor

Tese apresentada ao Curso de Doutorado do Programa de Pós-Graduação em Farmacologia, da Universidade Federal de Santa Maria (UFSM, RS), como requisito parcial para obtenção do grau de **Doutor em Farmacologia**.

Orientadora: Prof.ª Dr.ª Maria Amália Pavanato

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elaborada por
Isabela Andres Finamor

como requisito parcial para obtenção do grau de
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Aos meus familiares

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RESUMO

Tese de Doutorado
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EFEITO PROTETOR DA N-ACETILCISTEÍNA SOBRE O ESTRESSE OXIDATIVO CAUSADO PELA ADMINISTRAÇÃO CRÔNICA DE ASPARTAME EM RATOS

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Data e Local da Defesa: Santa Maria, 12 de março de 2015.

Esta tese avaliou os efeitos da N-acetilcisteína (150 mg/kg, intraperitoneal) frente às alterações bioquímicas e oxidativas ocasionadas pelo consumo oral diário de 40 mg/kg aspartame durante seis semanas por ratos. Para isto, foram realizados dois experimentos, onde, em ambos, os animais foram divididos em quatro grupos: Controle – receberam ambos os veículos, o do aspartame e o da N-acetilcisteína; NAC – receberam o veículo do aspartame, e a N-acetilcisteína; ASP – receberam o aspartame, e o veículo da N-acetilcisteína; ASP-NAC – receberam o aspartame e a N-acetilcisteína. O aspartame foi administrado durante seis semanas; enquanto que a N-acetilcisteína foi injetada apenas na quinta e sexta semana. Depois disto, os animais foram anestesiados, o sangue deles foi retirado, e o soro separado; e então, os ratos foram eutanasiados por exsanguinação. No primeiro experimento, o soro foi usado para a medida de glicose e dos biomarcadores de dano hepático e renal; o cérebro inteiro, o fígado e os rins foram removidos para análise dos parâmetros de estresse oxidativo. Conforme os resultados obtidos, de maneira geral, o aspartame ocasionou hiperglicemia e estresse oxidativo no cérebro inteiro e nos tecidos hepático e renal. O tratamento com N-acetilcisteína protegeu todos os tecidos estudados contra o dano oxidativo (peroxidação lipídica e carbonilação proteica), especialmente, por promover a síntese de glutationa reduzida (GSH), induzir as enzimas relacionadas ao metabolismo da mesma (glutationa peroxidase (GPx), glutationa redutase (GR) e glutationa S-transferase), e elevar os níveis de ácido ascórbico e o potencial reativo antioxidante total. No segundo experimento, o soro foi utilizado para a determinação de glicose e do perfil lipídico; o fígado foi retirado para a medida de glicose; e o cérebro inteiro foi separado nas seguintes estruturas: córtex cerebral, cerebelo, tronco encefálico, e hipotálamo; nas quais foi realizada a pesquisa de biomarcadores de estresse oxidativo. O consumo do aspartame causou uma produção elevada de glicose no fígado, hiperglicemia, hipertrigliceridemia e hipercolesterolemia; e, estresse oxidativo em todas as estruturas encefálicas. Embora o tratamento com a N-acetilcisteína não tenha reduzido a síntese de glicose hepática e nem a hiperglicemia, ela normalizou os níveis de triglicerídeos e de lipoproteína de alta densidade no soro. Este tratamento antioxidante também protegeu todas as estruturas encefálicas contra a peroxidação lipídica, aumentou os níveis de GSH e induziu as enzimas associadas à ela (GPx e GR), desencadeando diferentes respostas defensivas em cada estrutura encefálica. Portanto, os dados desta tese sugerem que a N-acetilcisteína atenua os danos oxidativos gerados pela ingestão deste edulcorante artificial. Estudos adicionais são necessários para elucidar as vias de sinalização envolvidas neste processo; bem como para determinar a quantidade dos metabólitos do aspartame (fenilalanina, aspartato e metanol) encontrada no plasma e cérebro dos ratos.

Palavras-chave: N-acetilcisteína. Aspartame. Estresse oxidativo. Glutationa.

ABSTRACT

Thesis of Doctoral Degree
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Federal University of Santa Maria

THE PROTECTIVE EFFECT OF N-ACETYLCYSTEINE ON THE OXIDATIVE STRESS CAUSED BY THE CHRONIC ADMINISTRATION OF ASPARTAME IN RATS

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Date and Place of Defense: March 12, 2015, Santa Maria.

This thesis evaluated the N-acetylcysteine effects (150 mg/kg, intraperitoneal) on the biochemical and oxidative changes caused by the daily oral consumption of aspartame at 40 mg/kg for six weeks by rats. For this purpose, were performed two experiments, in both the animals were divided in four groups: Control – received both aspartame and N-acetylcysteine vehicles; NAC – received aspartame vehicle, and N-acetylcysteine; ASP – received aspartame, and N-acetylcysteine vehicle; ASP-NAC – received both aspartame and N-acetylcysteine. The aspartame was administrated for six weeks; whereas the N-acetylcysteine was injected only in the fifth and sixth week. After it, the animals were anesthetized, their blood was removed, the serum was separated; and then, the rats were euthanized by exsanguination. In the first experiment, the serum was used for the measurement of glucose levels and also biomarkers of kidney and liver damage; the whole brain, liver and kidney were removed for the analysis of the oxidative stress parameters. As the results, in general, aspartame caused hyperglycemia and oxidative stress in the whole brain, and hepatic and renal tissues. N-acetylcysteine treatment protected all these tissues against the oxidative damage (lipid peroxidation and protein carbonylation), in especial, by promoting the reduced glutathione (GSH) synthesis, inducing the enzymes related to its metabolism (glutathione peroxidase (GPx), glutathione reductase (GR) and glutathione S-transferase), and elevating the ascorbic acid levels and total reactive antioxidant potential. In the second experiment, the serum was utilized for the determination of glucose content and lipid profile; the liver was removed for glucose measurement; and the whole brain was separated in the following structures: cerebral cortex, cerebellum, brainstem, and hypothalamus; in which was held the research of the oxidative stress biomarkers. The aspartame consumption caused an elevated glucose production in the liver, hyperglycemia, hypertriglyceridemia, hypercholesterolemia; and oxidative stress in all the brain regions. Although N-acetylcysteine treatment did not reduce neither the synthesis of liver glucose nor the hyperglycemia, it normalized the triglycerides and high-density lipoprotein cholesterol in the serum. This antioxidative treatment also protected all the brain regions against the lipid peroxidation, increased GSH levels and induced its associated enzymes (GPx and GR), triggering different defensive responses according each brain region. Therefore, the data of this thesis suggest that N-acetylcysteine attenuates the oxidative damage generated by the intake of this artificial sweetener. More studies were needed to elucidate the signaling pathways involved in this process; as well as to determine the aspartame metabolites levels (phenylalanine, aspartate and methanol) found in the rat plasma and brain.

Keywords: N-acetylcysteine. Aspartame. Oxidative stress. Glutathione.

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LISTA DE ABREVIATURAS E SIGLAS

ABAP	2,2'-azo-bis (2-amidinopropano)
ADI	Ingestão diária aceitável
ALP	Fosfatase alcalina
ALT	Alanina aminotransferase
ASP	Grupo aspartame
ASP-NAC	Grupo aspartame tratado com N-acetilcisteína
AST	Aspartato aminotransferase
CAT	Catalase
CDNB	1-cloro-2,4-dinitrobenzeno
CEUA	Comissão de Ética em Uso de Animais
CO_2	Dióxido de carbono
DHA	Ácido dehidroascórbico
DHR	Dehidroascorbato redutase
DNA	Ácido desoxirribonucleico
DNPH	2,4-dinitrofenilhidrazina
DTNB	5,5'-ditiobis-(2-ácido nitrobenzoico)
EAO	Espécies ativas de oxigênio
EDTA	Ácido etilenodiamino tetra-acético
FA	Fosfatase alcalina
Fe^{2+}	Íon ferroso
Fe^{3+}	Íon férrico
GPx	Glutationa peroxidase
GR	Glutationa redutase
GS-FDH	Formaldeído desidrogenase
GSH	Glutationa reduzida
GSSG	Glutationa oxidada
GST	Glutationa S-transferase
GSX	Glutationa S-conjugada
HCHO	Formaldeído
$HCOO^-$	Formato
H^+	Íon hidrogênio
H_2O	Água
H_2O_2	Peróxido de hidrogênio
H_2SO_4	Ácido sulfúrico
HCl	Ácido clorídrico
HDL	Lipoproteína de alta densidade
HRPO	Peroxidase de rabanate
IDA	Ingestão diária aceitável
i.p.	Intraperitoneal
KCl	Cloreto de potássio
LDL	Lipoproteína de baixa densidade
LOOH	Hidroperóxidos lipídicos
NaCl	Cloreto de sódio
NADPH	Nicotinamida adenina dinucleotídeo fosfato reduzido
$NADP^+$	Nicotinamida adenina dinucleotídeo fosfato
$NaHCO_3$	Bicarbonato de sódio
NaOH	Hidróxido de sódio

NPSH	Tiois não-proteicos
O_2	Oxigênio molecular
$O_2^{\bullet -}$	Radical ânion superóxido
$OH^{\bullet}$	Radical hidroxila
ROS	Espécies reativas de oxigênio
RX	Xenobióticos
SNC	Sistema Nervoso Central
SOD	Superóxido dismutase
TBARS	Substâncias reativas ao ácido tiobarbitúrico
TCA	Ácido tricloroacético
TRAP	Potencial antioxidante reativo total
v.o.	Via oral

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1 INTRODUÇÃO

Os aditivos alimentares são todos e quaisquer ingredientes adicionados intencionalmente aos alimentos sem o propósito de nutrir, com o objetivo de modificar suas características físicas, químicas, biológicas ou sensoriais, durante sua fabricação, processamento, preparação, tratamento, embalagem, acondicionamento, armazenagem, transporte ou manipulação. O próprio aditivo ou seus derivados, ao serem agregados, podem se converter em um componente de tal alimento. Esta definição não inclui os contaminantes ou substâncias nutritivas que sejam incorporadas aos alimentos para manter ou melhorar suas propriedades nutricionais. Os aditivos alimentares incluem os agentes de massa, antiespumantes, antiumectantes, antioxidantes, corantes, conservadores, espessantes, geleificantes, estabilizantes, aromatizantes, umectantes, reguladores de acidez, acidulantes, emulsificantes, melhoradores de farinha, realçadores de sabor, fermentos químicos, glaceantes, agentes de firmeza, sequestrantes, estabilizantes de cor, espumantes e edulcorantes (BRASIL, 1997).

Os edulcorantes são considerados substâncias diferentes dos açúcares que conferem sabor doce aos alimentos (BRASIL, 1997). Em 1988, foi determinado que os edulcorantes até então considerados fármacos, fossem registrados na Divisão Nacional de Alimentos e, assim, considerados alimentos (BRASIL, 1988). Este fato trouxe maior variedade e opções de produtos dietéticos aos portadores de *diabetes mellitus* (VIGGIANO, 2003). Atualmente, o uso de 15 aditivos edulcorantes em alimentos é permitido no Brasil, dentre esses estão os de origem natural: sorbitol, o manitol, a taumatina, os glicosídeos de esteviol, o xilitol, o eritrol, o maltitol, o isomaltitol e o lactitol, e os sintéticos: o acesulfame de potássio, o aspartame, o ciclamato, a sacarina, a sucralose e o neotame (BRASIL, 2008).

Esses edulcorantes também podem ser distinguidos de acordo com a quantidade de energia que são capazes de fornecer, sendo, portanto, classificados em nutritivos e não-nutritivos. Os edulcorantes nutritivos, como por exemplo, o sorbitol, o manitol, o xilitol, o eritrol, o maltitol, o isomaltitol e o lactitol, oferecem um sabor doce e uma fonte de energia. Os não-nutritivos, como, a taumatina, os glicosídeos de esteviol, o acesulfame de potássio, o aspartame, o ciclamato, a

sacarina, a sucralose e o neotame são doces sem fornecer energia ou quantidades insignificantes dela, podendo também ser denominados de “edulcorantes de alta intensidade”, pois são capazes de adoçar com a adição de um pequeno volume (AMERICAN DIETETIC ASSOCIATION, 2004).

Os edulcorantes são adicionados aos adoçantes dietéticos, os quais são definidos como adoçantes para dietas com restrição de sacarose, glicose (dextrose) ou frutose (BRASIL, 2008) e usados para atender às necessidades dos indivíduos sujeitos à restrição destes carboidratos, tais como os portadores de *diabetes mellitus*. Entretanto, eles são consumidos por todos aqueles que buscam o sabor doce sem a adição de energia, para auxiliar o controle do peso corporal e de doenças crônicas, como a obesidade, a hiperlipidemia, a cárie dental e certos distúrbios comportamentais, cujas taxas têm aumentado mundialmente (AMERICAN DIETETIC ASSOCIATION, 2004). Um estudo conduzido por Toledo e Ioshi (1995) demonstrou que, no Brasil, a sacarina (72%), o ciclamato (67%) e o aspartame (40%) estão entre os edulcorantes de alta intensidade mais consumidos por indivíduos em dieta de controle de peso (36%), portadores de *diabetes mellitus* (35%) e por aqueles que buscam perda de peso (23%), especialmente sob a forma de adoçantes de mesa e refrigerantes. Além disso, a mediana de ingestão de aspartame pelos usuários normais foi calculada em 2,9%, pelos diabéticos em 2,6% e em indivíduos em regime de controle de peso em 3,2% da ingestão diária aceitável (IDA) de 40mg/kg (TOLEDO E IOSHI, 1995).

O aspartame (L-aspartil-L-fenilalanina metil éster) teve seu sabor doce descoberto acidentalmente em 1965 por James Schlatter, um químico do Laboratório G. D. Searle, que estava desenvolvendo um novo tratamento para úlceras gástricas (MAZUR, 1984). Desde então, o aspartame foi o aditivo alimentar mais estudado antes de sua aprovação pela *Food and Drug Administration*, o órgão governamental norte-americano responsável pelo controle dos alimentos. Extensas pesquisas farmacológicas e toxicológicas foram realizadas em animais de laboratório, nas quais foram utilizadas doses muito maiores de aspartame do que os indivíduos poderiam possivelmente consumir. Baseado nos resultados destas avaliações, uma IDA de 50 mg/kg de peso corporal foi estabelecida para o aspartame nos Estados Unidos, enquanto 40 mg/kg foi adotada pela União Europeia (BUTCHKO et al., 2002). Primeiramente, em 1981, o aspartame foi regulamentado como edulcorante de uma série de preparações secas (adoçante de mesa, cereais,

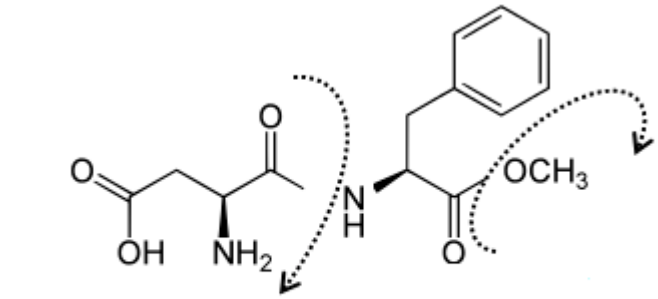
gelatinas e pudins). Tal aprovação foi ampliada em 1983 para incluir bebidas carbonatadas, e mais tarde, em 1996, o aspartame foi regulamentado como “um edulcorante de uso geral” para utilização em todos os alimentos e bebidas (AMERICAN DIETETIC ASSOCIATION, 2004).

Desde sua aprovação, o consumo do aspartame representa 62% do total dos edulcorantes de alta intensidade disponíveis no mercado mundial (FRY, 1999). Ele é aproximadamente 200 vezes mais doce que a sacarose (MAGNUSON et al., 2007) e tem sido usado em mais de 6000 produtos por centenas de milhões de pessoas em todos os países do mundo, podendo ser encontrado em uma ampla variedade de alimentos industrializados (refrigerantes, gomas de mascar, doces, gelatinas, misturas para sobremesa, pudins e recheios, sobremesas congeladas e iogurtes), adoçantes de mesa e alguns medicamentos (vitaminas e pastilhas para tosse sem açúcar) (WHITEHOUSE et al., 2008). Sua introdução no mercado modificou a qualidade de vida de indivíduos diabéticos, permitindo-lhes desfrutar de alimentos saborosos e bebidas, e ainda, cumprir as exigências alimentares. Além disso, os produtos dietéticos contendo aspartame têm sido úteis como parte de programas multidisciplinares para promover perda de peso e controle de peso corporal em indivíduos obesos (BUTCHKO et al., 2002).

O aspartame é único entre os edulcorantes de alta intensidade, uma vez que é absorvido e completamente hidrolisado no intestino (HOOPER et al., 1994), sendo, posteriormente, metabolizado da mesma forma em roedores, primatas e humanos, resultando em três constituintes alimentares comuns: fenilalanina (50%), aspartato (40%) e metanol (10%) (RANNEY et al., 1976) (Figura 1). Os aminoácidos que o compõe são metabolizados para fornecer 4 kcal/g. Dessa forma, o aspartame fornece energia, no entanto, por causa de sua intensa doçura, apenas quantidades mínimas precisam ser adicionadas, e a quantidade de energia derivada é insignificante (AMERICAN DIETETIC ASSOCIATION, 2004).

A fenilalanina é um aminoácido essencial necessário para o crescimento normal, desenvolvimento e manutenção da vida. Entretanto, portadores de fenilcetonúria devem evitar o aumento das concentrações plasmáticas deste aminoácido, o qual está associado com o retardo mental e com a deficiência cognitiva. Para tanto, estes indivíduos são submetidos a uma dieta especial restrita em fenilalanina e desencorajados a consumir produtos que contenham aspartame,

os quais devem ser rotulados para indicar a presença deste aminoácido (BUTCHKO et al., 2002).



Fonte: Adaptado de BUTCHKO et al. (2002).

Figura 1- Metabolismo do aspartame em três constituintes, o aspartato, a fenilalanina e o metanol.

A fenilalanina é metabolizada pela enzima fenilalanina hidroxilase, que a converte em tirosina, podendo, ambos, serem oxidados a dióxido de carbono (CO_2) via ciclo do ácido tricarboxílico. Tanto a fenilalanina quanto a tirosina são constituintes normais do plasma e podem sofrer incorporação em proteínas (RANNEY et al., 1976). A razão da conversão da fenilalanina em tirosina em roedores é cinco vezes mais rápida do que a razão da conversão em humanos (FERNSTROM et al., 1989). Sendo assim, em humanos, a maior parte da fenilalanina da dieta passa para a circulação sistêmica inalterada, podendo ser transportada através da barreira hematoencefálica ao Sistema Nervoso Central (SNC) por um sistema de transporte que é relativamente específico para aminoácidos neutros grandes (FERNSTROM E WURTMAN, 1972). Em roedores, a maior porção da fenilalanina proveniente da dieta passa para a circulação sistêmica como tirosina, o que pode resultar em um aumento da captação da tirosina através da barreira hematoencefálica e, assim, aumentar a sua concentração e disponibilidade no SNC (BUTCHKO et al., 2002).

Embora a comercialização do aspartame tenha sido aprovada, ele foi implicado em numerosos relatos de casos em decorrência de dores de cabeça, tonturas e alterações de humor (MASSACHUSETTS MEDICAL SOCIETY, 1984), os quais foram associados aos efeitos potenciais da fenilalanina sobre as funções cerebrais, uma vez que a tirosina é precursora da família das catecolaminas, que

inclui a dopamina, a norepinefrina, e a epinefrina (NELSON E COX, 2002). No entanto, não foi observado na literatura um padrão de resultados consistentes a respeito dos efeitos adversos do aspartame sobre as funções neuroquímicas do cérebro. Por exemplo, Coulombe e Sharma (1986) demonstraram que os níveis de catecolaminas, principalmente, norepinefrina, estavam elevados em diversas regiões cerebrais de camundongos 3 h depois da administração de aspartame por gavagem (13, 130 e 650 mg/kg); enquanto Fernstrom et al. (1983) evidenciaram que, embora os níveis de fenilalanina e tirosina estivessem aumentados no plasma e no cérebro de ratos 1h depois da administração de aspartame por gavagem (200 mg/kg), a razão da formação da dopamina e a norepinefrina sofreu mínimos efeitos.

O aspartato é um aminoácido não-essencial em mamíferos, sendo um dos mais comuns encontrados na dieta (BUTCHKO et al., 2002). Ranney e Oppermann (1979) mostraram que a porção aspartato da molécula de aspartame é metabolizada de maneira similar ao aspartato da dieta, sendo, portanto, rapidamente convertido em alanina e oxaloacetato por descarboxilação e transaminação, respectivamente, os quais são oxidados a CO_2 ao entrar no ciclo do ácido tricarboxílico. O aspartato também pode ser incorporado em constituintes corporais, tais como em outros aminoácidos, proteínas, pirimidinas, na asparagina e no ácido N-acetilaspártico. O aspartato e o glutamato, o qual possui similaridade estrutural com o aspartato, são encontrados em concentrações bem altas no cérebro, onde apresentam efeitos excitatórios nos neurônios em praticamente todas as regiões do SNC (BLOOM, 2005), sendo ambos conhecidos por induzir necrose neuronal em camundongos recém-nascidos (DAABEES et al., 1985). Reynolds et al. (1976) verificaram que a administração de aspartame em doses variando de 1.000 a 2.500 mg/kg de peso corporal a camundongos recém-nascidos também produziu necrose neuronal hipotalâmica, possivelmente, devido aos elevados níveis de aspartato encontrados no plasma destes animais.

O metanol é cada vez mais reconhecido como uma substância que causa dano às células hepáticas, onde ele é oxidado a formaldeído e, então, a formato (TEPHLY, 1991). Em humanos, o metanol é inicialmente metabolizado a formaldeído via álcool desidrogenase. Os roedores, por sua vez, metabolizam o metanol através da atividade peroxidativa da enzima catalase (CAT), resultando na liberação de peróxido de hidrogênio (H_2O_2) (CEDERBAUM E QURESHI, 1982). O formaldeído produzido é convertido a formato, um metabólito altamente tóxico, via formaldeído

desidrogenase, uma enzima dependente de glutationa reduzida (GSH), cujo mecanismo é similar em ambas as espécies (HARRIS et al., 2004). O formato é eliminado na urina ou nas fezes como formato, ou via respiratória como CO_2 e água (H_2O) através de uma via dependente de tetrahidrofolato (JOHLIN et al., 1987).

Os primatas não-humanos e os humanos são as espécies mais sensíveis à intoxicação por metanol, uma vez que apresentam baixo conteúdo hepático de folato (JOHLIN et al., 1987) e metabolizam o formato duas vezes mais lentamente do que os ratos (MCMARTIN et al., 1979). Sendo assim, o acúmulo de formato em primatas não-humanos e nos humanos tem sido considerado o responsável pelo clássico padrão de toxicidade por metanol, o qual inclui a depressão do SNC e posterior desenvolvimento de deficiência visual e acidose metabólica, podendo levar, inclusive, à morte (BUTCHKO et al., 2002).

Um estudo realizado por Trocho et al. (1998) sugeriu que o consumo de aspartame pode constituir um risco à saúde humana, afetando as funções celulares mesmo quando os consumidores não tenham experimentado nenhum sintoma, uma vez que a quantidade de adutos de formaldeído formados em proteínas e ácidos nucléicos de diferentes tecidos foi cumulativa em ratos. O efeito citotóxico do formaldeído também foi demonstrado por Saito et al. (2005), os quais revelaram que o formaldeído induziu um incremento na formação de espécies ativas de oxigênio (EAO), levando à morte celular. Além disso, diversas pesquisas indicaram que o conteúdo de GSH e a atividade das enzimas relacionadas ao seu metabolismo estavam diminuídos em diferentes tecidos de ratos com intoxicação por metanol, resultando em uma situação conhecida como estresse oxidativo (SKRZYDLEWSKA E FARBISZEWSKI, 1997; SKRZYDLEWSKA et al., 1998; PARTHASARATHY et al., 2006).

Portanto, passados 30 anos da aprovação do aspartame, persistem os debates relacionados ao potencial efeito tóxico exercido por cada um dos componentes provenientes de sua metabolização sobre a saúde humana. Butchko et al. (2002) afirmaram que este edulcorante não é tóxico, enquanto Schernhammer et al. (2012) indicaram evidências de que sua ingestão está associada ao possível risco de câncer no sistema hematopoiético em humanos. Uma pesquisa *in vitro* realizada por Tsakiris et al. (2006) indicou que a incubação de altas concentrações de fenilalanina, aspartato e metanol diminuiu marcadamente a atividade da enzima acetilcolinesterase na membrana de eritrócitos humanos, relacionando alguns

sintomas neurológicos, como a perda de memória e deficiência de aprendizagem, com as elevadas concentrações destes metabólitos do aspartame. Além disso, diversos estudos têm demonstrado que o consumo de aspartame está relacionado ao estresse oxidativo (RUIZ et al., 2008; ABHILASH et al. 2011; MOURAD, 2011; MOURAD E NOOR, 2011; ABDEL-SALAM et al., 2012; IYYASWAMY E RATHINASAMY, 2012; ABHILASH et al., 2013; ASHOK E SHEELADEVI, 2014; PROKIC et al., 2014; ASHOK et al., 2014).

O estresse oxidativo é definido como o desequilíbrio entre os antioxidantes e os pró-oxidantes e ocorre devido ao aumento na velocidade de geração de EAO e/ou diminuição na atividade do sistema de defesa antioxidante, resultando em um aumento sustentado das concentrações em estado estacionário de EAO (SIES, 1991). O desenvolvimento e a existência de um organismo em presença de oxigênio molecular (O_2) estão associados à geração de EAO, sob condições fisiológicas (HALLIWELL E GUTTERIDGE, 1999). As EAO são formadas, principalmente, durante os processos de oxidação biológica, dentre os quais está a respiração celular acoplada à fosforilação oxidativa, para formação de adenosina trifosfato na mitocôndria (CHANCE et al., 1979).

Neste processo, o O_2 é reduzido até H_2O recebendo quatro elétrons de uma só vez pela citocromo oxidase. Entretanto, em razão de sua configuração eletrônica o O_2, que é um birradical, tem uma forte tendência a receber um elétron de cada vez, gerando compostos intermediários muito reativos, conhecidos como EAO. A formação dessas espécies ocorre em aproximadamente 5% de todo o processo de redução do O_2 até H_2O. Dentre esses compostos intermediários estão o radical ânion superóxido ($O_2^{\bullet -}$), o H_2O_2 e o radical hidroxila ($OH^{\bullet}$) (HALLIWELL E GUTTERIDGE, 1999). Tais EAO podem ser radicais ou espécies não-radicalares, os quais são capazes de oxidar biomoléculas. Dessa forma, esses intermediários também são chamados de oxidantes ou pró-oxidantes (SIES, 1991). Quando as EAO apresentam pelo menos um elétron desemparelhado são denominadas radicais livres. Os radicais livres são definidos como qualquer espécie química capaz de existir independentemente e que contenha um ou mais elétrons desemparelhados (HALLIWELL E GUTTERIDGE, 1999).

No organismo humano existem várias fontes específicas de formação de EAO. O $O_2^{\bullet -}$ é o primeiro intermediário formado pela redução monovalente do O_2 a H_2O pela ação enzimática da xantina oxidase, NADPH oxidase ou pela quebra da

cadeia respiratória, e a partir dele serão formadas, através de reações sequenciais, as demais EAO (HALLIWELL E GUTTERIDGE, 1999). O H_2O_2 é o segundo intermediário gerado neste processo. Ele é uma espécie reativa não-radicalar e pode facilmente se difundir entre as células vivas, podendo gerar o $OH^{\bullet}$ em processos catalisados por metais de transição (reação de Fenton e Reação de Haber-Weiss). O H_2O_2 desempenha um papel fisiológico na transdução de sinal, entretanto, quando em excesso, ele pode contribuir para o mecanismo de diversos distúrbios através da desregulação da transdução de sinal, causando, consequente dano oxidativo às macromoléculas, o qual excede a capacidade celular de reparo (WELLS et al., 2009). O $OH^{\bullet}$, por sua vez, é um dos mais potentes oxidantes do sistema biológico e atua reagindo com biomoléculas. A alta reatividade deste radical implica a reação imediata no local onde ele foi formado (DIPLOCK et al., 1998).

O potencial reativo das EAO pode ser evidenciado através dos processos que elas desencadeiam, incluindo a peroxidação lipídica da membrana, que sofre alterações na sua permeabilidade e função secretora, além das reações oxidativas em proteínas, que levam a alterações dos seus grupamentos carbonila, modificando suas estruturas e estados conformacionais, e também o ataque ao ácido desoxirribonucleico (DNA), induzindo mutações e formação de 8-hidroxideoxiguanosina (HALLIWELL E GUTTERIDGE, 1999).

Para neutralizar a carga pró-oxidante e manter baixo os níveis intracelulares das EAO em concentrações de estado estacionário, uma diversidade de defesas antioxidantes foi desenvolvida em sistemas biológicos. Um antioxidante é definido como qualquer substância que, quando presente em baixas concentrações comparadas às do substrato oxidável, retarda significamente ou impede a oxidação daquele substrato. Os antioxidantes podem atuar prevenindo a formação das EAO, interceptando-as quando formadas, e reparando os danos macromoleculares. Os sistemas de defesas antioxidantes são compostos por antioxidantes enzimáticos e não-enzimáticos, que atuam conjuntamente na proteção celular (HALLIWELL E GUTTERIDGE, 1999).

O sistema de defesa enzimático é composto, dentre outras enzimas, pela superóxido dismutase (SOD), CAT, glutationa peroxidase (GPx), glutationa redutase (GR) e glutationa-S-transferase (GST). A SOD é responsável pela dismutação do $O_2^{\bullet -}$ em H_2O_2, o qual é convertido a O_2 e H_2O pela ação da enzima CAT. A GPx, por sua vez, além de remover o H_2O_2, também catalisa a conversão de hidroperóxidos

orgânicos a produtos menos reativos, empregando a GSH como substrato, impedindo, assim, a formação de $OH^{\bullet}$ e, o consequente dano celular. A glutationa oxidada (GSSG) produzida neste processo é reciclada a moléculas de GSH pela ação da GR, a qual utiliza a nicotinamida adenina dinucleotídeo fosfato reduzido (NADPH) como substrato (HALLIWELL E GUTTERIDGE, 1999). Por fim, a GST constitui uma família de enzimas de detoxificação de fase II, que catalisa a conjugação da GSH a compostos eletrofílicos, incluindo radicais livres e xenobióticos (HABIG et al., 1974).

O sistema de defesa não-enzimático é formado por antioxidantes lipossolúveis, como, por exemplo o α-tocoferol, assim como por hidrossolúveis, tais como o ácido ascórbico e a GSH (HALLIWELL E GUTTERIDGE, 1999). O α-tocoferol é capaz de inibir as reações de propagação desencadeadas durante a peroxidação lipídica, enquanto o ácido ascórbico além de desempenhar um papel muito importante nos processos de regeneração do α-tocoferol (HUANG E MAY, 2003), é capaz de reagir diretamente com as EAO, como o $O_2^{\bullet-}$, o H_2O_2 e o $OH^{\bullet}$, e vários produtos formados durante a peroxidação lipídica (NIKI, 1991). A GSH, por sua vez, está implicada na reciclagem do ácido ascórbico a partir de sua forma oxidada, o ácido dehidroascórbico (LINSTER E SCHAFTINGEN, 2007).

A GSH é considerada o tiol de baixo peso molecular mais abundante nas células, sendo sua maior parte presente no citosol (85-90%) e o restante distribuído em muitas organelas, incluindo a mitocôndria, a matriz nuclear e os peroxissomos (LU, 2000). Sua síntese, a partir da glicina, do glutamato e da cisteína, ocorre em todos os tipos celulares, por meio da ação da γ-glutamil-cisteina-sintetase e glutationa-sintetase, sendo o fígado o principal produtor e exportador de GSH (GRIFFITH, 1999), e a cisteína, o aminoácido limitante para a síntese deste composto (LYONS et al., 2000). Sendo assim, os fatores que estimulam a captação de cisteína pelas células, como a insulina e os fatores de crescimento, geralmente, aumentam as concentrações intracelulares de GSH (LU, 2000).

Dentre outras funções vitais que pode exercer, a GSH é capaz de atuar como *scavenger*, reagindo diretamente com as EAO, incluindo o H_2O_2 e o $OH^{\bullet}$, entre outras; ela também pode agir indiretamente sobre tais espécies através de reações enzimáticas catalisadas pela GPx e GST, bem como servir de substrato para a enzima formaldeído desidrogenase (TOWNSEND et al., 2003). Dessa forma, a GSH é um antioxidante essencial que ajuda a reduzir o impacto do estresse oxidativo e a

proteger os componentes celulares vitais, sendo a sua diminuição particularmente prejudicial às células, uma vez que aumenta a vulnerabilidade das mesmas ao dano oxidativo (MEISTER E ANDERSON, 1983).

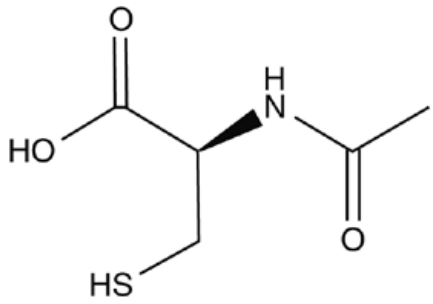
Recentemente, distintas pesquisas têm mostrado de maneira unânime que a depleção da GSH, e o dano oxidativo (principalmente peroxidação lipídica) estão associados tanto ao consumo agudo (ASHOK et al., 2014) quanto ao crônico de doses altas (RUIZ et al., 2008; ABHILASH et al., 2011; IYYASWAMY E RATHINASAMY, 2012; ABHILASH et al., 2013), aceitáveis (MOURAD, 2011; MOURAD E NOOR, 2011; ASHOK E SHEELADEVI, 2014) e até mesmo baixas de aspartame (ABDEL-SALAM et al., 2012). Essa depleção têm sido relacionada à formação do metanol e seus metabólitos (formaldeído e formato), cujo metabolismo é dependente de GSH (IYYASWAMY E RATHINASAMY, 2012; ASHOK E SHEELADEVI, 2014; ASHOK et al., 2014). Além disso, o metanol tem sido responsabilizado pela perda de atividade enzimática da GR (ABHILASH et al., 2011; ABHILASH et al., 2013; ASHOK E SHEELADEVI, 2014), causando, assim, um déficit ainda maior na produção de GSH, e o estresse oxidativo (ABHILASH et al., 2011; ABHILASH et al., 2013; ASHOK E SHEELADEVI, 2014). Tais alterações decorrentes do consumo de aspartame também têm sido relacionadas com: a hiperglicemia, a hiperlipidemia e a maior produção de EAO, como $O_2^{\bullet-}$ e H_2O_2; o dano hepático (infiltração leucocitária) (ABHILASH et al., 2011) e cerebral (leve congestão vascular) (ABHILASH et al., 2013); a menor disponibilidade de glicose no cérebro e prejuízo de memória, (ABDEL-SALAM et al., 2012); e mudanças apoptóticas cerebrais (ASHOK E SHEELADEVI 2014).

O estresse oxidativo decorrente, principalmente devido à deficiência de GSH, também tem sido associado à outros numerosos distúrbios, tais como convulsões, doenças neurodegenerativas e psiquiátricas, doenças hepáticas, fibrose cística, doenças cardiovasculares, *diabetes mellitus* e câncer (WU et al., 2004), sendo este fato a base das terapias antioxidantes (SIES, 1999). Essas terapias têm sido extensivamente estudadas e propostas como um método para conter os danos celulares relacionados ao estresse oxidativo. Sendo assim, diversos antioxidantes são utilizados nestes casos e diferentes níveis de sucesso são alcançados com estes tratamentos. Embora os antioxidantes mais utilizados sejam o ácido ascórbico, o α-tocoferol e o ácido lipóico, e estes possam atuar diretamente sobre as EAO, eles não são capazes de restabelecer a cisteína necessária para a síntese e reposição

da GSH (HALLIWELL, 1996). Nesse contexto, destaca-se a N-acetilcisteína, cuja administração aumenta o fornecimento de cisteína, eleva a síntese de GSH e previne a deficiência da mesma em uma ampla diversidade de distúrbios em humanos e animais (SAMUNI et al., 2013; RUSHWORTH E MEGSON, 2014; SHAHRIPOUR et al., 2014).

A N-acetilcisteína (Figura 2) tem sido utilizada na prática clínica por muitas décadas, sendo os seus efeitos benéficos relatados primeiramente no início dos anos 1960, quando Hurst et al. (1967) demonstraram que ela era um eficiente agente mucolítico em pacientes com fibrose cística. Posteriormente, durante a década de 1970, uma sequência substancial de estudos envolvendo potenciais candidatos de compostos doadores de cisteína foi testada em intoxicação por acetaminofeno, sendo que diferentemente da cisteamina, metionina e penicilamina, que não foram efetivas ou provocaram efeitos adversos (PRESCOTT et al., 1976), a N-acetilcisteína apresentou potencial terapêutico no tratamento de tal intoxicação (PRESCOTT et al., 1977; PRESCOTT et al., 1980). A partir dos anos 1980, a N-acetilcisteína passou a ser pesquisada no tratamento de vários distúrbios relacionados ao estresse oxidativo (RUSHWORTH E MEGSON, 2014), sendo, atualmente utilizada no tratamento de várias doenças neurológicas (SHAHRIPOUR et al., 2014); *diabetes mellitus*; síndrome da imunodeficiência adquirida; toxicidade induzida pela quimioterapia; nefropatia induzida por contraste radiográfico; e toxicidade por metais pesados (SAMUNI et al., 2013).

A N-acetilcisteína é um precursor acetilado do aminoácido cisteína e está farmaceuticamente disponível sob a forma intravenosa, oral e inalatória. Ela apresenta uma baixa toxicidade, estando associada com leves efeitos colaterais, tais como náuseas, vômitos, rinorréia, prurido e taquicardia (ATKURI et al., 2007). Sua meia-vida é de 5,6 h depois da administração de uma única dose via intravenosa, sendo 30% eliminada por excreção renal. A N-acetilcisteína possui uma biodisponibilidade relativamente baixa (abaixo de 5%), a qual está relacionada com sua N-desacetilação na mucosa intestinal e metabolização de primeira passagem no fígado (HOLDINESS, 1991). Este último processo ocasiona a liberação da cisteína (WHILLIER et al., 2009; RADTKE et al., 2012), a qual pode atravessar as membranas das células epiteliais e manter a síntese de GSH (SIES, 1999).



N-acetilcisteína

Fonte: TEPEL (2007).

Figura 2- Estrutura química da N-acetilcisteína.

Embora a N-acetilcisteína, possa reagir diretamente com o H_2O_2 e com o $OH^{\cdot}$, ela é considerada um antioxidante fraco, uma vez que além de não atuar como *scavenger de* $O_2^{\bullet -}$ (ARUOMA et al., 1989), concentrações 10 vezes maiores desse antioxidante são necessárias para exercer atividade equivalente à da GSH como *scavenger* de EAO (GIBSON et al., 2009). Portanto, é provável que a vasta maioria dos efeitos antioxidantes atribuídos diretamente à N-acetilcisteína seja mediada pelo aumento dos níveis intracelulares de GSH (RUSHWORTH E MEGSON, 2014), uma vez que é difícil discernir os efeitos diretos exercidos pela N-acetilcisteína daqueles desempenhados pela GSH (SAMUNI et al., 2013).

Apesar disso, diversos estudos indicaram que a N-acetilcisteína exerceu efeito protetor contra o estresse oxidativo na intoxicação por metanol não só por promover a síntese de GSH, mas também por atuar como *scavanger* de EAO e do formaldeído, atenuando assim, a toxicidade deste metabólito do metanol sobre o sistema antioxidante de diferentes tecidos de ratos intoxicados (SKRZYDLEWSKA E FARBISZEWSKI, 1999; DOBRZYÑSKA et al., 2000; FARBISZEWSKI et al., 2000). Estes autores afirmam que, embora as vias metabólicas da intoxicação por metanol sejam diferentes em humanos e ratos, os metabólitos produzidos durante este processo são os mesmos, sugerindo que estes resultados podem ter relevância clínica. O etanol usado como um antídoto na intoxicação por metanol diminui o metabolismo do metanol, prevenindo, assim, as ações tóxicas do formaldeído. Entretanto, o metabolismo do etanol é acompanhado por formação de EAO e não pode prevenir as modificações no balanço de oxirredução. Sendo assim, a N-

acetilcisteína pode ser considerada como um composto adjuvante na terapia da intoxicação por metanol.

O metanol, embora constitua apenas 10% da molécula do aspartame, é um subproduto do metabolismo deste edulcorante (RANNEY et al., 1976). Diferentes pesquisas evidenciaram que a L-cisteína e a GSH reverteram *in vitro* a inibição da enzima Na^+ K^+-ATPase induzida pelos metabólitos do aspartame que correspondiam às doses de ingestão comum, abusiva ou tóxica (34, 150, 200 mg/kg, respectivamente) na membrana de eritrócitos humanos (SCHULPIS et al., 2006), hipocampo (SIMINTZI et al., 2007) e córtex cerebral (SIMINTZI et al., 2008) de ratos, atribuindo tais efeitos benéficos à ação *scavenger* destes compostos, bem como à melhora da depleção da GSH induzida pelo metanol proveniente do aspartame.

Sendo assim, visto que o consumo do aspartame é paradoxal, essencial e prejudicial, se torna evidente a necessidade de buscar compostos com propriedades antioxidantes para combater o estresse oxidativo causado aos seus usuários, comumente, indivíduos diabéticos e aqueles que buscam perda de peso. No entanto, nenhum composto foi pesquisado e nem apontado como uma possível alternativa para melhorar esta condição. Nesse contexto, a N-acetilcisteína aparece como uma opção a ser estudada, uma vez que ela é capaz de promover a síntese de GSH e modular a atividade das enzimas relacionadas ao seu metabolismo.

2 OBJETIVOS

2.1 Objetivo geral

Investigar o efeito da N-acetilcisteína sobre o estresse oxidativo causado pela administração oral crônica de aspartame em diferentes tecidos de ratos saudáveis.

2.2 Objetivos específicos

- Analisar o peso corporal, ganho de peso, relação hepatossomática, bem como razão de peso renal e peso corporal dos ratos dos grupos controle, controle tratado com N-acetilcisteína, aspartame e aspartame tratado com N-acetilcisteína;
- Avaliar os níveis de glicose, triglicerídeos, colesterol total, lipoproteína de alta densidade (HDL), lipoproteína de baixa densidade (LDL), creatinina e ácido úrico, bem como as atividades das enzimas alanina aminotransferase (ALT), aspartato aminotransferase (AST) e fosfatase alcalina (FA) em soro de ratos dos diferentes grupos experimentais;
- Determinar o conteúdo de glicose em homogenatos de tecido hepático de ratos dos diferentes grupos experimentais;
- Verificar o dano oxidativo através da determinação da concentração de hidroperóxidos lipídicos (LOOH) em homogenatos de tecido encefálico e suas diferentes estruturas: córtex cerebral, cerebelo, tronco encefálico e hipotálamo, bem como nos de hepático e renal de ratos dos diferentes grupos experimentais;
- Analisar o dano oxidativo pela medida dos níveis de substâncias reativas ao ácido tiobarbitúrico (TBARS) e proteínas carbonilas em homogenatos de tecido encefálico, hepático e renal de ratos dos diferentes grupos experimentais;

- Verificar a concentração de H_2O_2 em fatias de tecido hepático e renal de ratos dos diferentes grupos experimentais;
- Determinar a atividade das enzimas antioxidantes SOD total e CAT em homogenatos de tecido encefálico, hepático e renal de ratos dos diferentes grupos experimentais;
- Avaliar a atividade das enzimas GPx, GR e GST em homogenatos de tecido encefálico e suas diferentes estruturas: córtex cerebral, cerebelo, tronco encefálico e hipotálamo, bem como nos de hepático e renal de ratos dos diferentes grupos experimentais;
- Analisar o conteúdo de tiois não-proteicos (NPSH) em homogenatos de tecido encefálico e suas diferentes estruturas: córtex cerebral, cerebelo, tronco encefálico e hipotálamo, bem como nos de hepático e renal de ratos dos diferentes grupos experimentais;
- Verificar os níveis de ácido ascórbico e o potencial antioxidante reativo total (TRAP) em homogenatos de tecido encefálico, hepático e renal de ratos dos diferentes grupos experimentais.

3 ARTIGOS

Nesta tese foram executados dois experimentos, que utilizaram o mesmo protocolo e originaram três artigos. Cada artigo apresenta seus respectivos Materiais e Métodos, Resultados, Discussão e Referências.

O primeiro e o segundo artigos foram obtidos a partir da realização do primeiro experimento. Neste experimento, o encéfalo, o fígado e os rins dos ratos foram removidos para a análise dos biomarcadores de estresse oxidativo. O primeiro artigo é composto de resultados relativos ao encéfalo. Este artigo é intitulado "*The protective effect of N-acetylcysteine on oxidative stress in the brain caused by the long-term intake of aspartame by rats*" e foi publicado na "*Neurochemical Research*". Esta revista possui fator de impacto "2,551" e é classificada pelo Sistema WebQualis da CAPES como "B2" na categoria Ciências Biológicas II. O segundo artigo é constituído de dados referentes ao fígado e aos rins destes animais. Este artigo é intitulado "*N-acetylcysteine in aspartame-induced oxidative stress: its beneficial effects in the rat liver and kidney*" e foi submetido para o "*European Journal of Pharmacology"*. Esta revista possui fator de impacto "2,684" e é classificada pelo Sistema WebQualis da CAPES como "B1" na categoria Ciências Biológicas II.

No segundo experimento, diferentes estruturas encefálicas de ratos foram extraídas e utilizadas para a determinação dos biomarcadores de estresse oxidativo. Este experimento forneceu dados para a elaboração do terceiro artigo intitulado "*N-acetylcysteine attenuates the toxic effects of aspartame on the glutathione-related antioxidant system in the rat brain regions*", que será submetido para o "*Journal of Biosciences*". Esta revista possui fator de impacto "1,939" e é classificada pelo Sistema WebQualis da CAPES como "B2" na categoria Ciências Biológicas II.

O artigo "*The protective effect of N-acetylcysteine on oxidative stress in the brain caused by the long-term intake of aspartame by rats*" está disponível em http://link.springer.com/article/10.1007%2Fs11064-014-1360-9.

N-acetylcysteine in aspartame-induced oxidative stress: its beneficial effects in the rat liver and kidney

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Abstract

This research examined the effects of N-acetylcysteine on the aspartame-induced oxidative stress in the rat liver and kidney. So, the rats were divided into four groups: control - received both aspartame and N-acetylcysteine vehicles; NAC - received aspartame vehicle and N-acetylcysteine treatment (150 mg kg^{-1}, i.p.); ASP - received aspartame treatment (40 mg kg^{-1}, v.o.) and N-acetylcysteine vehicle; ASP-NAC - received both aspartame and N-acetylcysteine treatments. Aspartame was administrated for six weeks; whilst N-acetylcysteine was injected from the fifth to the sixth week. After N-acetylcysteine treatment, we observed a reduction in the thiobarbituric acid reactive substances, lipid hydroperoxides, and hydrogen peroxide levels in both hepatic and renal tissues of the aspartame-treated rats; whilst it resulted in decreased protein carbonyl levels only in their kidney. It was linked to an elevation in the glutathione peroxidase, glutathione reductase, and glutathione S-transferase activities and non-protein thiols, ascorbic acid, and total reactive antioxidant potential levels in both tissues of the aspartame-treated animals. N-acetylcysteine administration occasioned an increase in the catalase activity only in the kidney of aspartame-treated rats. In summary, N-acetylcysteine was useful for the protection of the rat liver and kidney against aspartame-induced oxidative stress.

Keywords: N-acetylcysteine, protection, aspartame, oxidative stress, liver, kidney.

1 Introduction

Aspartame (L-aspartyl-L-phenylalanine methyl ester) is unique among the high-intensity sweeteners because it is absorbed from the intestine and rapidly metabolized similarly in rodents, primates, and humans to aspartic acid, phenylalanine, and methanol (Ranney et al., 1976); methanol is the only one that causes systemic toxicity (Kruse, 1992). There are species differences in the metabolism of methanol because humans metabolize methanol to formaldehyde primarily using alcohol dehydrogenase, whereas rodents rely on the peroxidative activity of the enzyme catalase (CAT), which also has antioxidative activity (Cederbaum and Qureshi, 1982). Formaldehyde is converted to the acutely toxic metabolite formate through a similar mechanism in both species via reduced glutathione (GSH)-dependent enzyme formaldehyde dehydrogenase (Harris et al., 2004).

GSH depletion and changes in the GSH-related enzymes are markedly evident in both rat liver (Abhilash et al., 2011; Mourad, 2011) and kidney (Mourad, 2011) following aspartame intake. GSH is involved in the cell membrane integrity, being its deficiency linked to increased vulnerability to reactive oxygen species (ROS) damage (Halliwell and Gutteridge, 1999). In fact, GSH lack due to the formation of methanol and its derivative metabolites (formaldehyde and formate) is one of the reasons why the aspartame usage has been the subject of much debate about its suspected adverse effects on the health (Ashok and Sheeladevi, 2014). Nevertheless, aspartame, since its regulatory approval, has been consumed in more than 6000 products by hundreds of millions of people around the world who want to enjoy the sweet taste of sugar without ingesting the associated calories (Butchko et al., 2002). Thus, since aspartame consumption is paradoxical, essential (Butchko et al., 2002) and harmful (Humphries et al., 2008), the need to focus attention in antioxidant compounds to protect cells becomes evident.

Since the 1980s, there has been a growing interest about the therapeutic potential of N-acetylcysteine for a range of diseases where oxidative stress is seen to be a driver and in which antioxidant effects may convey a benefit (Rushworth and Megson, 2014). N-acetylcysteine is the acetylated precursor of the amino acid L-cysteine, which sustains the synthesis of GSH (Samuni et al., 2013). Indeed, N-acetylcysteine administration (150 mg kg^{-1}, i.p.) during one week to treat methanol-

intoxicated rats, increased GSH production and stimulated its related enzymes, mitigating lipid peroxidation (Skrzydlewska and Farbiszewski, 1999). So, the purpose of the present research was to examine the effects of N-acetylcysteine on the serum biochemical perturbation and some oxidative stress biomarkers in both rat liver and kidney after long-term administration of aspartame.

2 Materials and Methods

2.1 Chemicals

All reagent-grade chemicals were obtained from Sigma (St. Louis, Missouri, USA).

2.2 Animals

Male Wistar rats (120-150 g) were kept in a controlled temperature (23±2°C) and a light-dark cycle of 12 h with access to water *ad libitum* and about 30 g daily per animal of rodent laboratory chow (Supra, São Leopoldo, Rio Grande do Sul, Brazil). The investigation was approved by the Comissão de Ética no Uso de Animais (CEUA) of the Universidade Federal de Santa Maria, Rio Grande do Sul, Brazil (#020/2012).

2.3 Experimental protocol

The rats (*n*=40) were randomly divided into four groups with ten animals.

- Control group;
- NAC (control group treated with N-acetylcysteine);
- ASP (aspartame group);
- ASP-NAC (aspartame group treated with N-acetylcysteine).

Control group and NAC received aspartame vehicle by gavage for six weeks; whereas ASP and ASP-NAC received aspartame (40 mg kg^{-1}, prepared in 0.9% NaCl solution) under the same conditions as the control groups. From fifty to sixty week, immediately after aspartame or its vehicle administration, the control group and ASP received N-acetylcysteine vehicle (0.9% NaCl solution) via intraperitoneal; whereas NAC and ASP-NAC received N-acetylcysteine (150 mg kg-1, pH 6.8-7.2) under the same conditions as described to its vehicle injection. All treatments were freshly

prepared and administrated on a daily basis. The body weight of the rats was recorded weekly.

2.4 Blood collection and euthanasia

At the end of sixth week and 3 h after the last treatment, the rats were weighted, anesthetized with xylazine and ketamine for the blood collection by cardiac puncture. After it, they were euthanized by exsanguination for the removal of their liver and kidney, which were immediately weighted, cleaned and washed in an ice-cold 0.9% NaCl solution.

2.5 Blood analysis

The blood was collected in tubes and it was immediately centrifuged at 1800 *g* for 15 min at 4ºC. Serum was separated for alanine aminotransferase (ALT), aspartate aminotransferase (AST), alkaline phosphatase (ALP), creatinine and uric acid measurements by using commercial kits (Labtest, Lagoa Santa, Minas Gerais, Brazil).

2.6 Tissue homogenate preparation

The liver and kidney were homogenized as described by Finamor et al. (2012).

2.7 Lipid peroxidation

Lipid peroxidation, as indicated by the amount of lipid hydroperoxides (LOOH), was measured by the xylenol orange method (Jiang et al., 1991). This technique can detect the primary products of peroxidation using the oxidation of Fe^{2+} by LOOH in an acidic medium with xylenol orange dye, which forms a complex with Fe^{3+}. For the assay the following reagents were added sequentially at a final concentration: 20 mol l^{-1} methanol, 100 µmol l^{-1} xylenol orange, 25 mmol l^{-1} sulfuric acid (H_2SO_4), 4 mmol l^{-1} butylated hydroxytoluene, 250 µmol l^{-1} ferrous sulfate, to a total of 0.9 ml. The sample aliquots (0.1 ml) were then added and incubated at room temperature for 30 min, and the absorbance at 560 nm was then read. The results were reported as nmol mg protein^{-1} using ε_{560} = 43 mmol^{-1}cm^{-1}. Lipid peroxidation was also estimated based on the formation of thiobarbituric reactive substances (TBARS) (Buege and Aust, 1978). Briefly, the sample aliquots (0.25 ml) were added to a tube containing 0.75 ml of 1.22 mol l^{-1} trichloroacetic acid (TCA) and 0.5 ml of 46 mmol l^{-1}

thiobarbituric acid. The mixture was heated for 15 min at 100ºC. After cooling, the precipitate was removed by centrifugation. The absorbance of the organic phase was measured at 535 nm, and the results were expressed as nmol mg protein^{-1} using ε_{535} = 156 mmol^{-1}cm^{-1}.

2.8 Carbonyl protein

Protein carbonyl content was assayed by a method based on the reaction of carbonyls with 2,4-dinitrophenylhydrazine (DNPH) forming dinitrophenylhydrazone, a yellow compound. The sample aliquots (0.2 ml) were incubated with 0.8 ml of 10 mmol l^{-1} DNPH in 2.5 mol l^{-1} HCl solution or 2.5 mol l^{-1} HCl solution (blank) for 1 h at room temperature, in the dark. Samples were vortexed every 15 min. Then, 1 ml of 1.2 mol l^{-1} TCA solution was added in the tube samples, left in ice for 10 min and centrifuged for 5 min at 1000 *g* to collect the protein precipitates. Another wash was performed with 0.8 ml of 0.61 mol l^{-1} TCA. The pellet was washed three times with 0.8 ml of ethanol:ethyl acetate (1:1). The final precipitates were dissolved in 0.4 ml of 6 mol l^{-1} guanidine hydrochloride solution, left for 10 min at 37ºC and read at 360 nm (Reznick and Packer, 1994). The standard curve was prepared by using different bovine serum albumin concentrations (0.5 to 1.5 mg ml^{-1}) and the slope was used to express the levels of carbonyl protein as nmol mg protein^{-1}.

2.9 Hydrogen peroxide

The assay was based on the horseradish peroxidase (HRPO)-mediated oxidation of phenol red by hydrogen peroxide (H_2O_2), leading to the formation of a compound that absorbs at 610 nm. Liver and kidney slices were incubated for 30 min at 37ºC in 1 ml of 10 mmol l^{-1} phosphatase buffer (140 mmol l^{-1} NaCl and 5 mmol l^{-1} dextrose). The supernatant (0.2 ml) were transferred to tubes with 1.275 ml of phenol red buffer (0.28 mmol l^{-1} phenol red, 8.5 U ml^{-1} HRPO and 5 mmol l^{-1} dextrose). After 5 min incubation, 0.025 ml of 1 mmol l^{-1} NaOH was added, and the samples were read at 610 nm. The standard curve was prepared by using different H_2O_2 concentrations (0.1 to 1000 nmol l^{-1}) and the slope was used to express the levels of H_2O_2 as nmol mg tissue^{-1} (Pick and Keisari, 1980).

2.10 Antioxidant enzymes

Total superoxide dismutase (SOD) activity was determined as the inhibition rate of autocatalytic adrenochrome generation at 480 nm. Sample aliquots (0.01 ml) were added to 2.99 ml of an assay mixture containing 1 mmol l^{-1} epinephrine and 50 mmol l^{-1} glycine (pH 10.2) at a final concentration. The enzyme activity was expressed as USOD mg $protein^{-1}$ using ε_{480} = 4 $mmol^{-1}cm^{-1}$. One SOD unit was defined as the amount of enzyme required for 50% inhibition of the adrenochrome formation (Misra and Fridovich, 1972).

CAT activity was evaluated by measuring the decrease in the absorption at 240 nm. Sample aliquots (0.05 ml) were added to 1.95 ml of a reaction medium consisting of 50 mmol l^{-1} phosphate buffer (pH 7.4) and 2 mmol l^{-1} H_2O_2 at a final concentration, thereby providing information to determine the pseudo-first-order reaction constant (k´) of the decrease in H_2O_2 absorption (Chance et al., 1979). The results were reported as pmol mg $protein^{-1}$ using ε_{240} = 40 $mol^{-1}cm^{-1}$.

Glutathione peroxidase (GPx) activity was measured by using a coupled reaction with glutathione reductase (GR) in the presence of GSH and NADPH (Flohé and Gunzler, 1984). The oxidized glutathione (GSSG) produced upon reduction of t-butyl hydroperoxide by GPx is reduced by GR using NADPH as a cofactor. The oxidation of NADPH to $NADP^+$ results in a decrease in the absorbance at 340 nm. Sample aliquots (0.05 ml) were added to 1.25 ml of the assay mixture containing 100 mmol l^{-1} phosphate buffer (pH 7.7), 1 mmol l^{-1} EDTA, 0.2 U ml^{-1} of GR, 2 mmol l^{-1} of GSH, 0.2 mmol l^{-1} NADPH at a final concentration. Subsequently t-butylhydroperoxide was added to a final concentration of 0.5 mmol l^{-1} and the change in the absorbance was recorded at regular intervals of a period of 3 minutes. The enzyme activity was expressed as µmol min^{-1} mg $protein^{-1}$ using ε_{340} = 6.22 $mmol^{-1}cm^{-1}$.

GR is an NADPH-dependent enzyme that regenerates GSH from GSSG. Its activity was expressed as µmol min^{-1} mg $protein^{-1}$ and measured by the rate of NADPH consumption at 340 nm using ε_{340} = 6.22 $mmol^{-1}cm^{-1}$. Samples aliquots (0.05 ml) were added to 0.95 ml of a reaction medium consisted of phosphate buffer (pH 8.0), 10 mmol l^{-1} EDTA, 0.25 mmol l^{-1} GSSG and 0.1 mmol l^{-1} NADPH in 238 mmol l^{-1} $NaHCO_3$ at a final concentration (Carlberg and Mannervik, 1985).

Glutathione-S-transferase (GST) activity was assayed based on the conjugation reaction with GSH, using 1-chloro-2,4-dinitrobenzene (CDNB) as a substrate (Habig et al., 1974). Sample aliquots (0.01 ml) were added to 1.29 ml of the assay mixture containing 100 mmol l^{-1} phosphate buffer (pH 6.5), GSH and CDNB at a final concentration of 1mmol l^{-1} each. The activity was calculated from the changes in absorbance at 340 nm. It was expressed as pmol min^{-1} mg $protein^{-1}$ using ε_{340} = 9.6 $mmol^{-1}cm^{-1}$.

2.11 Non-enzymatic antioxidants

Non-protein thiols (NPSH) represent an indirect measure of GSH. Proteins were eliminated by adding 0.5 mmol l^{-1} perchloric acid to the homogenates and centrifuging the mixture at 700 *g* for 5 min. To the supernatants (0.1 ml), 0.9 ml of 0.2 mol l^{-1} phosphate buffer (pH 8.0) and 0.18 mmol l^{-1} 5,5'-dithiobis-(2-nitrobenzoic acid) (DTNB) at a final concentration were added and vortexed. The DTNB formed a yellow complex with GSH, and the absorbance was measured at 412 nm. NPSH content was expressed as nmol mg $protein^{-1}$ using ε_{412} = 13.6 $mmol^{-1}cm^{-1}$ (Ellman, 1959).

The ascorbic acid measurement was carried out according to the method of Roe and Kuether (1942). Tissue homogenates were prepared in 240 mmol l^{-1} TCA and then centrifuged 700 *g* for 5 min. The samples aliquots (0.3 ml) were treated with charcoal and filtered. It was added to 0.1 ml of the assay mixture containing 0.15 mol l^{-1} thiourea and 89 mmol l^{-1} DNPH at a final concentration. It was incubated at 37ºC for 3 h. Thereafter, color was produced by adding 0.5 ml of 16 mol l^{-1} H_2SO_4. The absorbance was read at 540 nm. The standard curve was prepared by using different ascorbic acid concentrations (0.005 to 0.05 mg ml^{-1}) and the slope was used to express the amount of ascorbic acid as µM mg $protein^{-1}$.

2.12 Total reactive antioxidant potential

Total reactive antioxidant potential (TRAP) was measured based on the capacity of the sample to scavenge luminol luminescence induced by thermolysis of 2,2´-azo-bis (2-amidinopropane) (ABAP). ABAP is a source of free radicals that react with luminol, yielding chemiluminescence. The addition of sample aliquots (0.01 ml) to 0.29 ml of the reaction medium consisting of 50 mmol l^{-1} phosphate buffer (pH

7.4), 2 mmol l^{-1} ABAP, 1.42 µmol l^{-1} luminol at a final concentration decreases chemiluminescence to basal levels for a period proportional to the total load of antioxidants present in the samples. The standard curve was prepared by using different Trolox concentrations (1 to 4 µmol l^{-1}). The comparison of the induction time after the addition of Trolox and the sample allowed calculation of TRAP as the equivalent of the Trolox concentration necessary to produce the same induction time (Evelson et al., 2001). The results were expressed as µmol mg $protein^{-1}$.

2.13 Protein assay

Protein was determined by the method of Lowry et al. (1951) by using bovine serum albumin as standard.

2.14 Statistical analysis

Levene's test was used to verify whether the data were parametric. One-way analysis of variance followed by Duncan's test was performed to assess the differences among the groups. The results are expressed as mean ± standard error. The differences were considered significant at $P < 0.05$. The statistical analysis was performed by using the software Statistica® 7.0 (StatSoft, Inc., Tulsa, Oklahoma, USA).

3 Results

3.1 Physiological data

The final body weight, the weight gain and the ratio of liver weight to body weight and the ratio of kidney weight to body weight were reported in Table 1. All data showed no differences across the experimental groups ($P > 0.05$).

3.2 Blood analysis

The ALT, AST and ALP activities and the creatinine levels were higher in ASP than in the control group ($P < 0.05$). The ASP-NAC animals showed a lower ALT, AST and ALP activities when compared to ASP ($P < 0.05$). The creatinine levels as well as the ALT, AST and ALP activities in ASP-NAC were similar to those found in the control group and NAC ($P > 0.05$). The uric acid levels exhibited no differences in

their serum levels across the experimental groups (*P* >0.05). All data were illustrated in Table 2.

3.3 Oxidative stress biomarkers in the liver

The TBARS (Fig. 1A), LOOH (Fig. 1B), carbonyl protein (Fig. 1C) and H_2O_2 (Fig. 1D) levels were higher in ASP than in control group (*P* <0.05). The ASP-NAC animals showed a lower TBARS (Fig. 1A), LOOH (Fig. 1B) and H_2O_2 (Fig. 1D) levels when compared to ASP (*P* <0.05). The SOD activity was lower in ASP than in the control group (Fig. 2A) (*P* <0.05). The CAT activity was lower in ASP than in the control group (*P* <0.05) (Fig. 2B). The ASP-NAC animals showed a lower CAT activity when compared to the control group and NAC (*P* <0.05) (Fig. 2B). The GPx, GR and GST activities were lower in ASP than in the control group (*P* <0.05) (Figs. 2C, 2D and 2E, respectively). The ASP-NAC animals showed a higher GPx, GR and GST activities when compared to ASP (*P* <0.05) (Figs. 2C, 2D and 2E, respectively). Regarding the non-enzymatic antioxidants, both NPSH and ascorbic acid levels were lower in ASP than in the control group (*P* <0.05) (Figs. 2F and 2G, respectively). The ASP-NAC group showed a higher NPSH and ascorbic acid levels when compared to ASP (*P* <0.05) (Figs. 2F and 2G, respectively). The TRAP levels were lower in ASP than in the control group (*P* <0.05) (Fig. 2H). The ASP-NAC rats showed a higher TRAP levels when compared to ASP (*P* <0.05) (Fig. 2H).

3.4 Oxidative stress biomarkers in the kidney

The TBARS (Fig. 1E), LOOH (Fig. 1F), carbonyl protein (Fig. 1G) and H_2O_2 (Fig. 1H) levels were higher in ASP than in control group (*P* <0.05). The ASP-NAC animals showed a lower TBARS (Fig. 1F), LOOH (Fig. 1G), carbonyl protein (Fig. 1G) and H_2O_2 (Fig. 1H) levels when compared to ASP (*P* <0.05). SOD activity did not vary across the different experimental groups (Fig. 3A) (*P* <0.05). The CAT activity was lower in ASP than in the control group (*P* <0.05) (Fig. 3B). The ASP-NAC animals showed a higher CAT activity when compared to ASP (*P* <0.05) (Fig. 3B). The GPx activity was lower in ASP than in the control group (*P* <0.05) (Fig. 3C). The ASP-NAC animals showed a higher GPx activity when compared to NAC and ASP (*P* <0.05) (Fig. 3C). The GR activity was lower in ASP than in the control group (*P* <0.05) (Fig. 3D). The ASP-NAC group showed a higher GR activity when compared to the control group, NAC and ASP (*P* <0.05) (Fig. 3D). The GST activity was lower

in ASP than in the control group (P <0.05) (Fig. 3E). The ASP-NAC rats showed a higher GST activity when compared to the control group (P <0.05) (Fig. 3E). The NPSH levels were lower in ASP than in the control group (P <0.05) (Fig. 3F). The ASP-NAC group showed a higher NPSH levels when compared to NAC and ASP (P <0.05) (Fig. 3F). The ascorbic acid content was lower in ASP than in the control group (P <0.05) (Fig. 3G). The ASP-NAC rats showed a higher ascorbic acid content when compared to the control group, NAC and ASP (P <0.05) (Fig. 3G). The TRAP levels were lower in ASP than in the control group (P <0.05) (Fig. 3H). The ASP-NAC rats showed a higher TRAP levels when compared to the control group, NAC and ASP (P <0.05) (Fig. 3H).

4 Discussion

Aspartame has been shown to be safe for individuals with renal disease and for those with liver disorders (Butchko et al., 2002). However, recent researches reported that its chronic administration at 40 mg kg^{-1} was toxic to rat erythrocytes (Prokic et al., 2014), brain (Ashok and Sheeladevi, 2014; Finamor et al., 2014), liver (Abhilash et al., 2011; Mourad, 2011) and kidney (Mourad, 2011). It is crucial to point out that such dose (40 mg kg^{-1}) is the acceptable daily intake (ADI) for aspartame, which was approved by the European Food Safety Authority (2006). Even though, we also exhibited that the long-term intake of aspartame at the ADI resulted in elevation of the ALT, AST and ALP activities; and creatinine levels in the serum. The aspartame byproduct, methanol, could be the responsible for such changes, since it was shown that methanol caused the same changes in the serum of methanol-intoxicated rats (Dobrzyñza et al., 2000; Parthasarathy et al., 2006). Dobrzyñza et al. (2000) explained that N-acetylcysteine exerted a protective role against such alterations, preventing the leakage of hepatic enzymes (ALT, AST and ALP) from liver cells into the intracellular space and into the blood due to the membrane liver cell damage. Alike, our data revealed that liver damage was avoided after N-acetylcysteine administration. Furthermore, our research shows that besides N-acetylcysteine protects the rat liver and kidney against such biochemical perturbation, it also prevents the aspartame-induced oxidative stress.

The aspartame administration at the ADI to our rats occasioned protein carbonylation; and, the initiation and termination of the lipid peroxidation process in the hepatic and renal tissues, since LOOH are the first byproducts of the lipid peroxidation and degrade into different cytotoxic aldehydes, such as malondialdehyde, which were detected through TBARS (Finamor et al., 2012). In accordance, it has been shown that aspartame triggers lipid peroxidation in the rat liver (Abhilash et al., 2011; Mourad, 2011) and kidney (Mourad, 2011). Such organs were chosen as targets of our research because the aspartame constituents (phenylalanine, aspartic acid and methanol) are metabolized in the liver; and, even in small proportions, their metabolism products are excreted in the urine (Ranney et al., 1976). So, it seems that beyond the aspartame utilization impaired the liver function, which could not be able to cope the properties of the amino acid components, or effectively metabolize methanol; it also declined the kidney performance, perhaps due the ROS formation (as H_2O_2) through the methanol action, thereby reducing the membrane fluidity, which is essential for proper functioning of the cell.

H_2O_2, when in excess, can contribute to the mechanisms of several disorders through the dysregulation of signal transduction and by causing oxidative damage to cellular macromolecules, as proteins and lipids (Wells et al., 2009). As our data, H_2O_2 was accumulated in both rat tissues after aspartame treatment; we ascribe it to the probable role played by CAT in the conversion of methanol to formaldehyde, which resulted in the H_2O_2 release (Cederbaum and Qureshi, 1982). Such H_2O_2 accumulation could have caused CAT and GPx inactivation, as described by Skrzydlewska et al. (1998) in the liver of methanol-treated rats; thus, occasioning an even greater accretion of this ROS, and its conversion to hydroxyl radical, which contributed to the lipid peroxidation and protein carbonylation processes.

Besides converts H_2O_2, GPx also catalyzes the conversion of organic hydroperoxides to less reactive products utilizing GSH as a substrate (Halliwell and Gutteridge, 1999). Furthermore, GSH also serves as a substrate for the GST activity, which constitutes a family of phase II detoxification enzymes involved in the detoxification of xenobiotics (Habig et al., 1974). So, we suggested that their reduced activities in both organs of the aspartame-treated rats resulted from the GSH depletion (represented as NPSH), causing even more H_2O_2 accumulation and xenobiotic accretion, respectively. It has been revealed in a common manner that

GSH depletion in rat liver (Abhilash et al., 2011; Mourad, 2011) and kidney (Mourad, 2011) is linked to the aspartame consumption.

GSH is a non-enzymatic antioxidant that is involved in the methanol metabolism, playing a critical role in cellular defense against toxic chemicals (Johlin et al., 1987). GSH levels were found diminished in the rat brain after the aspartame long-term exposure at the ADI. It was attributed to the elevation in the plasma methanol levels (Ashok and Sheeladevi, 2014). So, for this reason and also because we detected a reduced GR activity in both tissues of the aspartame-treated rats, we believe that in our research the GSH levels (represented as NPSH) were found reduced. In accordance, Abhilash et al. (2011) proposed that the decreased GR activity in the liver of their aspartame-treated rats indicates a deficit in the production of GSH from GSSG.

Additionally, GSH is involved in the ascorbic acid metabolism, since ascorbic acid enters into the cell in the oxidized form and is reduced through the GSH action (Niki, 1991). Thus, it could explain the resultant depletion in the ascorbic acid concentration in both tissues of the aspartame-treated rats. Likewise GSH, ascorbic acid reacts with ROS and also with various LOOH, so its depletion could provide the oxidative damage processes too (Niki, 1991). Furthermore, GSH and ascorbic acid are known as the main water-soluble antioxidants in the cells; thus, their insufficiencies could explain the reduction in the TRAP levels in both organs of the aspartame-treated rats.

N-acetylcysteine is the derivative of the amino acid L-cysteine (Samuni et al., 2013), which can cross the cell membrane and sustains the GSH synthesis in the liver, the major producer and exporter of this compound (Griffith, 1999). Schulpis et al. (2006) showed that L-cysteine and GSH prevented the inhibition of Na^+/K^+-ATPase activity in human erythrocyte membranes, which was caused by their exposure to aspartame metabolites corresponding to the intake of common, abusive or toxic doses (34, 150 or 200 mg kg^{-1}, respectively). Moreover, it has been exhibited that N-acetylcysteine protected the rat liver from methanol intoxication because it acts as a ROS and formaldehyde scavenger and a GSH precursor too (Dobrzyñska et al., 2000; Skrzydlewska and Farbiszewski, 1999). Hence, we proposed that N-acetylcysteine protected the rat hepatic and renal tissues from the aspartame toxic effects through the GSH synthesis, which restored the activities of the GSH-related enzymes, as GPx, GR and GST; and also, the ascorbic acid and TRAP levels.

However, after N-acetylcysteine treatment, we found a little difference between the antioxidant responses of these two tissues of the aspartame-treated rats: in the liver, only GPx acted in the H_2O_2 removal; whereas in the kidney, both GPx and CAT catalyzed reactions for the H_2O_2 elimination. Thus, even in the absence of an adequate CAT activity to degrade H_2O_2 in the rat liver, GPx was able to play this protective role. Furthermore, the N-acetylcysteine itself seemed to help in the H_2O_2 removal, acting as a ROS scavanger, diminishing H_2O_2 effects on the lipid peroxidation and protein oxidation. Indeed, it has been described that N-acetylcysteine can directly react with H_2O_2 and $OH^{\cdot}$, thus, weakening the cumulative consequences of the oxidative stress (Aruoma et al., 1989).

In conclusion, N-acetylcysteine alleviated the aspartame-induced biochemical perturbation in the rat serum and oxidative stress biomarkers in its hepatic and renal tissues through the GSH production. The increased GSH levels (measured as NPSH) modulated the GSH-related enzyme activities, thus, causing the decrease in the H_2O_2 accumulation; and, therefore, diminishing the lipid peroxidation and protein oxidation.

Conflict of Interest

The authors declare that there are no conflict of interest.

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Figure captions

Figure 1 Effects of N-acetylcysteine on TBARS (A), LOOH (B), carbonyl protein (C), and H_2O_2 (D) levels in the liver, and on TBARS (E), LOOH (F), carbonyl protein (G), and H_2O_2 (H) levels in the kidney of control and aspartame-treated rats. TBARS, thiobarbituric reactive substances; LOOH, lipid hydroperoxides; H_2O_2, hydrogen peroxide. Each bar represents the mean ± standard error (*n*=10). [a] Denotes that the data are significantly different from the control group at *P* <0.05. [b] Denotes that the data are significantly different from ASP at *P* <0.05.

Figure 2 Effects of N-acetylcysteine on SOD (A), CAT (B), GPx (C), GR (D), and GST (E) activities; NPSH (F), ascorbic acid (G), and TRAP (H) levels in the liver of control and aspartame-treated rats. SOD, superoxide dismutase; CAT, catalase; GPx, glutathione peroxidase; GR, glutathione reductase; GST, glutathione S-transferase; NPSH, non-protein thiols; TRAP, total reactive antioxidant potential. Each bar represents the mean ± standard error (*n*=10). [a] Denotes that the data are significantly different from the control group at *P* <0.05. [b] Denotes that the data are significantly different from ASP at *P* <0.05. [c] Denotes that the data are significantly different from NAC at *P* <0.05.

Figure 3 Effects of N-acetylcysteine on SOD (A), CAT (B), GPx (C), GR (D), and GST (E) activities; NPSH (F), ascorbic acid (G), and TRAP (H) levels in the kidney of the control of control and aspartame-treated rats. SOD, superoxide dismutase; CAT, catalase; GPx, glutathione peroxidase; GR, glutathione reductase; GST, glutathione S-transferase; NPSH, non-protein thiols; TRAP, total reactive antioxidant potential. Each bar represents the mean ± standard error (*n*=10). [a] Denotes that the data are significantly different from the control group at *P* <0.05. [b] Denotes that the data are significantly different from ASP at *P* <0.05. [c] Denotes that the data are significantly different from NAC at *P* <0.05.

Table 1 Effects of N-acetylcysteine on the final body weight, weight gain, the ratio of liver weight to body weight, and the ratio of kidney weight to body weight of control and aspartame-treated rats

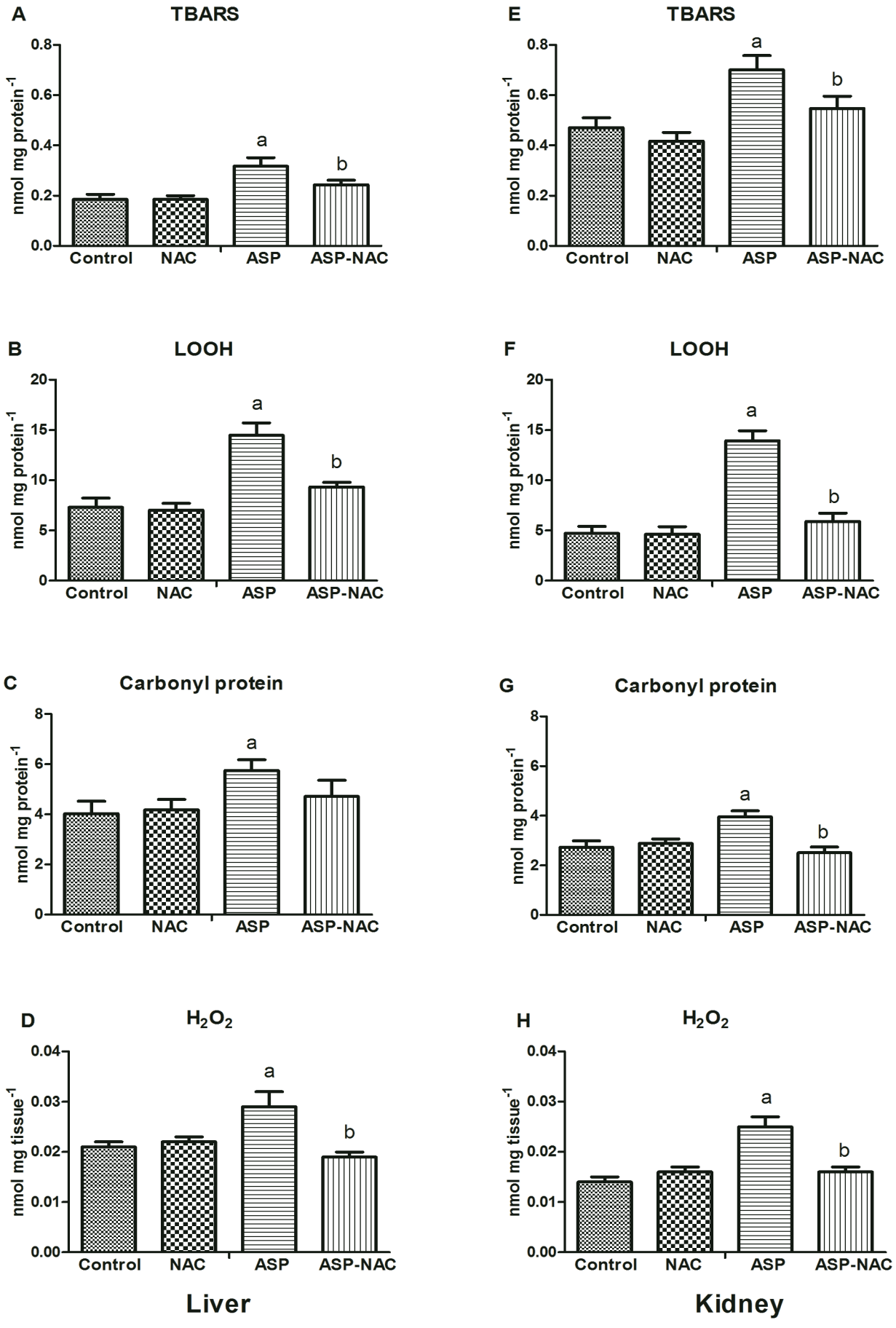
Variable	Control	NAC	ASP	ASP-NAC
Final body weight (g)	326.5±6.1	322.1±5.4	318.4±4.7	331.5±7.3
Weight gain (%)	119.9±7.2	127.1±5.6	118.8±6.3	122.4±5.4
Ratio of liver weight to body weight (mg g^{-1})	32.5±0.9	32.0±0.8	33.3±1.2	33.3±0.7
Ratio of kidney weight to body weight (mg g^{-1})	6.77±0.15	6.81±0.47	6.62±0.28	6.89±0.14

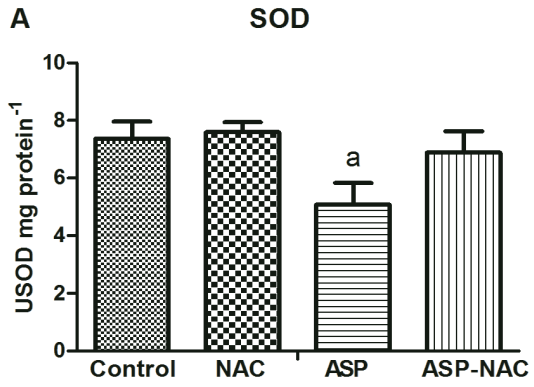
The data appear as the mean ± standard error (*n*=10).

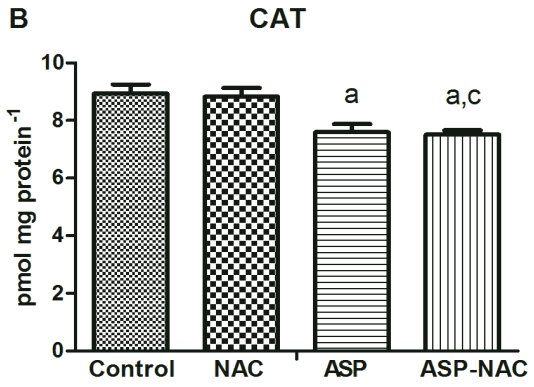
Table 2 Effects of N-acetylcysteine on the ALT, AST and ALP activities; and creatinine, and uric acid levels in the serum of control and aspartame-treated rats

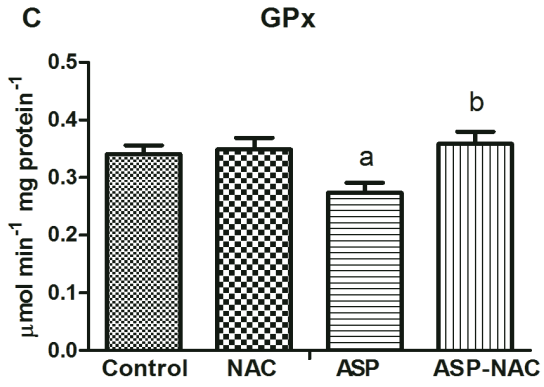
Variable	Control	NAC	ASP	ASP-NAC
ALT (U l^{-1})	40.0±1.8	37.6±1.8	45.9±2.4[a]	37.6±2.4[b]
AST (U l^{-1})	159.4±5.9	156.5 ±5.3	210.0±5.9[a]	162.9±4.1[b]
ALP (U l^{-1})	131.2±4.7	127.6±5.3	182.9±4.7[a]	135.3±4.1[b]
Creatinine (mg dl^{-1})	1.21±0.02	1.27±0.04	1.36±0.03[a]	1.28±0.05
Uric acid (mg dl^{-1})	1.29±0.09	1.28±0.08	1.34±0.07	1.23±0.07

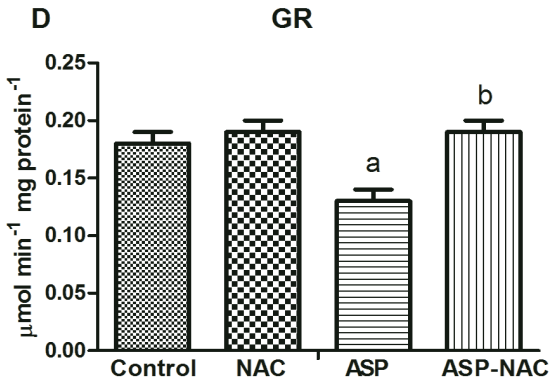
The data appear as the mean ± standard error (*n*=10). ALT, alanine aminotransferase; AST, aspartate aminotransferase; ALP, alkaline phosphatase. [a] Denotes that the data are significantly different from the control group at $P < 0.05$. [b] Denotes that the data are significantly different from ASP at $P < 0.05$.

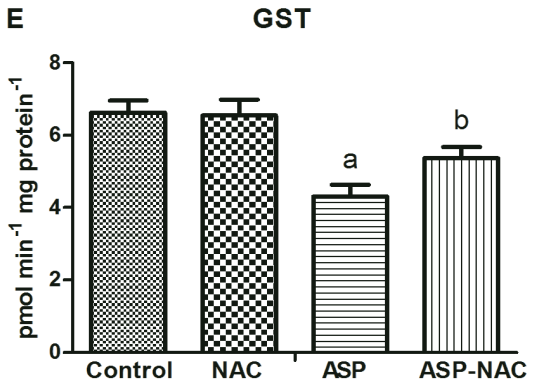

A
TBARS
nmol mg protein-1
0.0
0.2
0.4
0.6
0.8
a
b
Control
NAC
ASP
ASP-NAC
E
TBARS
nmol mg protein-1
0.0
0.2
0.4
0.6
0.8
a
b
Control
NAC
ASP
ASP-NAC
B
LOOH
nmol mg protein-1
0
5
10
15
20
a
b
Control
NAC
ASP
ASP-NAC
F
LOOH
nmol mg protein-1
0
5
10
15
20
a
b
Control
NAC
ASP
ASP-NAC
C
Carbonyl protein
nmol mg protein-1
0
2
4
6
8
a
Control
NAC
ASP
ASP-NAC
G
Carbonyl protein
nmol mg protein-1
0
2
4
6
8
a
b
Control
NAC
ASP
ASP-NAC
D
H2O2
nmol mg tissue-1
0.00
0.01
0.02
0.03
0.04
a
b
Control
NAC
ASP
ASP-NAC
Liver
H
H2O2
nmol mg tissue-1
0.00
0.01
0.02
0.03
0.04
a
b
Control
NAC
ASP
ASP-NAC
Kidney

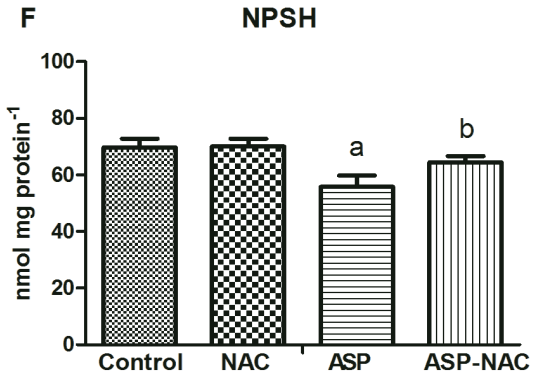

A
SOD
USOD mg protein-1
10
8
6
4
2
0
a
Control
NAC
ASP
ASP-NAC

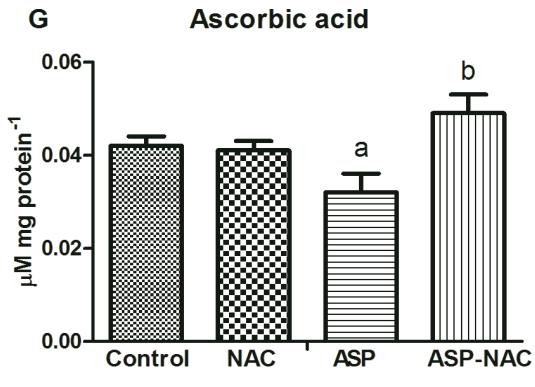

B
CAT
pmol mg protein-1
10
8
6
4
2
0
a
a,c
Control
NAC
ASP
ASP-NAC

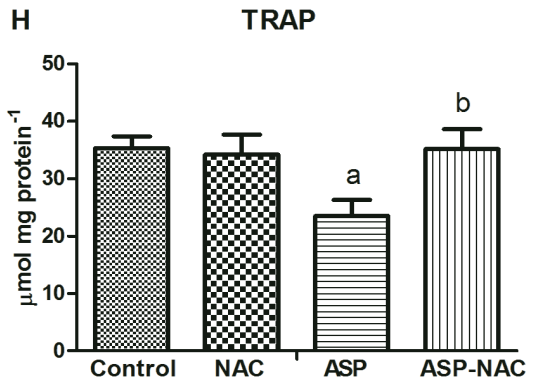

C
GPx
µmol min-1 mg protein-1
0.5
0.4
0.3
0.2
0.1
0.0
a
b
Control
NAC
ASP
ASP-NAC

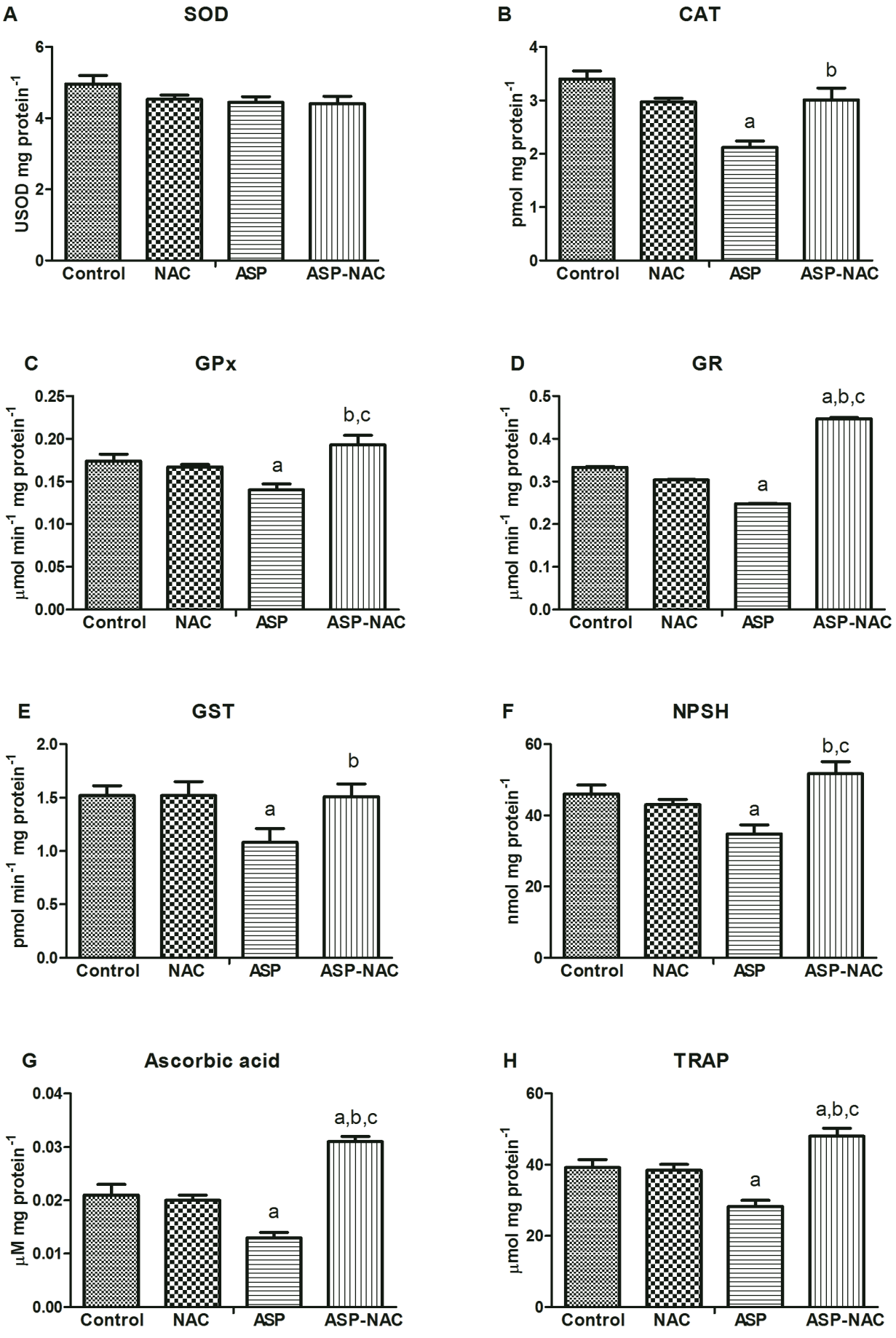

D
GR
µmol min-1 mg protein-1
0.25
0.20
0.15
0.10
0.05
0.00
a
b
Control
NAC
ASP
ASP-NAC


E
GST
pmol min-1 mg protein-1
8
6
4
2
0
a
b
Control
NAC
ASP
ASP-NAC


F
NPSH
nmol mg protein-1
100
80
60
40
20
0
a
b
Control
NAC
ASP
ASP-NAC


G
Ascorbic acid
µM mg protein-1
0.06
0.04
0.02
0.00
a
b
Control
NAC
ASP
ASP-NAC


H
TRAP
µmol mg protein-1
50
40
30
20
10
0
a
b
Control
NAC
ASP
ASP-NAC


A
SOD
USOD mg protein-1
0
2
4
6
Control
NAC
ASP
ASP-NAC
B
CAT
pmol mg protein-1
0
1
2
3
4
a
b
Control
NAC
ASP
ASP-NAC
C
GPx
µmol min-1 mg protein-1
0.00
0.05
0.10
0.15
0.20
0.25
a
b,c
Control
NAC
ASP
ASP-NAC
D
GR
µmol min-1 mg protein-1
0.0
0.1
0.2
0.3
0.4
0.5
a
a,b,c
Control
NAC
ASP
ASP-NAC
E
GST
pmol min-1 mg protein-1
0.0
0.5
1.0
1.5
2.0
a
b
Control
NAC
ASP
ASP-NAC
F
NPSH
nmol mg protein-1
0
20
40
60
a
b,c
Control
NAC
ASP
ASP-NAC
G
Ascorbic acid
µM mg protein-1
0.00
0.01
0.02
0.03
0.04
a
a,b,c
Control
NAC
ASP
ASP-NAC
H
TRAP
µmol mg protein-1
0
20
40
60
a
a,b,c
Control
NAC
ASP
ASP-NAC

N-acetylcysteine attenuates the toxic effects of aspartame on the glutathione-related antioxidant system in the rat brain regions

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Abstract

This study evaluated the effects of N-acetylcysteine on the impairment in the glutathione (GSH)-related antioxidant system in the cerebral cortex, cerebellum, brainstem and hypothalamus after aspartame intake. The rats were divided into four groups: control - received both aspartame and N-acetylcysteine vehicles; NAC - received aspartame vehicle and N-acetylcysteine treatment (150 mg kg^{-1}, i.p.); ASP - received aspartame treatment (40 mg kg^{-1}, v.o.) and N-acetylcysteine vehicle; ASP-NAC - received both aspartame and N-acetylcysteine treatments. Aspartame was administrated for six weeks; whilst N-acetylcysteine was injected from the fifth to the sixth week. N-acetylcysteine reduced the aspartame-induced lipid hydroperoxides formation in the cerebral cortex, cerebellum, brainstem and hypothalamus. It also increased the glutathione reductase activity and non-protein thiols levels in all these rat brain regions, which were diminished due to aspartame exposure. N-acrtylcysteine treatment resulted in augmented glutathione peroxidase activity in the cerebral cortex, brainstem and hypothalamus, reduced due to aspartame administration. N-acetylcysteine restored to the control values the glutathione S-transferase, whose activity was elevated in the cerebral cortex, cerebellum and brainstem; and decreased in hypothalamus after aspartame administration. In conclusion, N-acetylcysteine protected the cerebral cortex, cerebellum, brainstem and hypothalamus through GSH production; and the modulation of GSH-related enzymes, triggering different defensive responses according each brain region.

Keywords: N-acetylcysteine; aspartame; glutathione; antioxidant; brain.

1 Introduction

N-acetylcysteine is a precursor of L-cysteine and supports glutathione (GSH) synthesize pathway (Aruoma *et al.*, 1989). GSH is a trypeptide formed by glutamate, glycine and cysteine, being the cysteine availability the limiting for the GSH production during oxidative stress (Aruoma *et al.*, 1989). GSH acts in antioxidant defense, detoxification of electrophilic xenobiotics, and others (Sies, 1999). It is also involved in the methanol metabolism. Methanol is metabolized to formaldehyde and then to formate (Tephly, 1991). The conversion of formaldehyde to formate is catalyzed by formaldehyde dehydrogenase, a GSH-dependent enzyme (Harris *et al.*, 2004).

Methanol is a metabolite of the aspartame metabolism. Besides methanol, aspartame metabolism also yields phenylalanine and aspartate (Ranney *et al.*, 1976). Aspartame (L-aspartyl-L-phenylalanine methyl ester), a low calorie artificial sweetener, is about 200 times sweeter than sucrose, being highly consumed by the people worldwide, including diabetic individual and persons in weight loss regime. It is available commercially since its approval in 1981 by regulatory agencies as a sweetener and flavor enhancer (Butchko *et al.*, 2002). However, immediately after aspartame was marketed, there were a large number of reports concerning its adverse reactions, as neurological and behavioral disturbances: headaches, dizziness and mood alterations (Massachusetts Medical Society, 1984), which were related to alterations in regional brain levels of catecholamines (Coulombe and Sharma, 1986). Nowadays, even after an update of its acceptable daily intake (ADI) for humans, established as 40 mg kg^{-1} (European Food Safety Authority, 2006), aspartame safety is still doubtful (Humphries *et al.*, 2008). As Trocho *et al.* (1998), aspartame consumption constitutes a hazard to human health because formaldehyde adducts could accumulate in nucleic acids and brain proteins. It has been shown that aspartame ingestion (40 mg kg^{-1}) induces oxidative stress in the rat brain (Finamor *et al.* 2014) and in its different regions too (Mourad and Noor, 2011; Ashok and Sheeladevi, 2014) due to methanol action, whose levels were found elevated in the rat plasma (Ashok and Sheeladevi, 2014). All these studies shared a common finding, the diminution in the GSH levels. Therefore, since the introduction of the aspartame changed the quality of life of diabetic individuals, being also useful as a part of program to promote weight loss (Butchko *et al.*, 2002), a search for

antioxidant substances that can reduce the harmful effects of aspartame, such as oxidative stress, would help them.

N-acetylcysteine is used to treat different neurological disorders and brain dysfunctions in both animals and humans (Shahripour *et al.*, 2014). It stands out among the other antioxidants because its therapeutic actions are directly related with reactive oxygen species (ROS) scavenger; and indirectly with the intracellular GSH restoration. A recent research from our laboratory has noticed that N-acetylcysteine (150 mg kg^{-1}, i.p.) attenuated the lipid peroxidation and protein carbonylation in the rat brain through enhancing GSH levels and GSH-related enzymes; nevertheless such antioxidative treatment not normalized hyperglycemia in the aspartame-treated rats (40 mg kg^{-1}) (Finamor *et al.*, 2014). Thus, now we seek to evaluate the effects of N-acetylcysteine on the lipid hydroperoxides (LOOH) formation, GSH levels and glutathione peroxidase (GPx) and glutathione reductase (GR), glutathione S-transferase (GST) activities in order to identify whether the pattern of antioxidant response raised by this antioxidative treatment was uniform in the different brain regions of aspartame-treated rats: cerebral cortex, cerebellum, brainstem and hypothalamus. Furthermore, we researched the mechanism responsible for the aspartame-induced hyperglycemia; and the N-acetylcysteine actions on the serum lipid profile of aspartame-treated rats through the estimation of triglycerides, total cholesterol, high-density lipoprotein cholesterol (HDL) and low-density lipoprotein cholesterol (LDL).

2 Materials and Methods

2.1 Chemicals

All the reagents were purchased from Sigma-Aldrich (St. Louis, Missouri, USA).

2.2 Animals

Male Wistar rats (120-150 g) were maintained under standard laboratory conditions: 23± 2°C and light-dark cycle of 12 h; with free access to tap water and

about 30 g daily per animal of rodent chow (Supra, São Leopoldo, Rio Grande do Sul, Brazil). The study was approved by the Comissão de Ética no Uso de Animais (CEUA) of the Universidade Federal de Santa Maria, Rio Grande do Sul, Brazil (#020/2012).

2.3 Experimental design

2.3.1 Groups

The rats (*n=32*) were divided into four groups composed by eight animals each one:

- Control group: received both aspartame and N-acetylcysteine vehicles;
- NAC (control group treated with N-acetylcysteine): received aspartame vehicle and N-acetylcysteine treatment;
- ASP (aspartame group): received aspartame treatment and N-acetylcysteine vehicle;
- ASP-NAC (aspartame group treated with N-acetylcysteine): received both aspartame and N-acetylcysteine treatments.

2.3.2 Aspartame and N-acetylcysteine doses

The aspartame was prepared in a 0.9% NaCl solution and it was administrated by gavage at a dose of 40 mg kg^{-1} during six weeks. The N-acetylcysteine was dissolved in a 0.9% NaCl solution and its pH was adjusted within the range of 6.8 - 7.2 by using 6N NaOH. In the fifth and sixth weeks, immediately after the aspartame administration, N-acetylcysteine was injected via intraperitoneal at a dose of 150 mg kg^{-1} during two weeks. All treatments (aspartame, N-acetylcysteine or their vehicles) were freshly prepared, being administrated on a daily basis. The rat's body weight was recorded weekly.

2.3.3 Blood collection and euthanasia

At the end of the experimental period (six weeks) and 3 h after the last treatment, the animals were weighed and anesthetized with xylazine and ketamine. So, the blood was collected through cardiac puncture. After it, they were euthanized by exsanguination for the liver and brain removals.

2.4 Brain dissection

After its removal, the brain was immediately washed with ice 0.9% NaCl solution. The different brain regions (cerebral cortex, cerebellum, brainstem and hypothalamus) were dissected in an ice-cold glass plate as Glowinski and Iverson (1996).

2.5 Sample preparation

Blood was collected in tubes and it was separated using centrifugation (1800 *g,* 15 min). The serum was stored at -20°C for further analysis. Liver was homogenized in a tissue homogenizer (20%, w/v), using 100 mmol L^{-1} citrate buffer (pH 5.0). Brain regions were homogenized as described in Finamor *et al.* (2014). For glucose measurement, liver homogenate were heated for 10 min at 100°C and, then centrifuged at 6000 *g* for 15 min at 4°C. Differently, brain regions homogenate were centrifuged at 700 *g* for 10 min at 4°C to eliminate nuclei and cell debris; their supernatants were frozen at -70°C for further measurements.

2.6 Assessment of glucose in serum and liver

Liver glucose levels were measured as Carr and Neff (1984). The results were reported as μg mg $tissue^{-1}$. Serum glucose contents were determined through commercial kits (Labtest, Lagoa Santa, Minas Gerais, Brasil). The results were expressed as mmol L^{-1}.

2.7 Determination of serum lipid profile

Triglycerides, total cholesterol and HDL cholesterol levels were measured by using commercial kits (Labtest, Lagoa Santa, Minas Gerais, Brasil). LDL was calculated through the formula: LDL = total cholesterol - HDL - (triglycerides/5). All results were reported as mmol L^{-1}.

2.8 Estimation of lipid hydroperoxides

Lipid peroxidation, as indicated by the amount of LOOH, was measured by the xylenol orange method (Jiang *et al.*, 1991). This technique can detect the primary products of peroxidation using the oxidation of Fe^{2+} by LOOH in an acidic medium with xylenol orange dye, which forms a complex with Fe^{3+}. For the assay the

following reagents were added sequentially at a final concentration: 20 mol L^{-1} methanol, 100 µmol L^{-1} xylenol orange, 25 mmol L^{-1} sulfuric acid, 4 mmol L^{-1} butylated hydroxytoluene, 250 µmol L^{-1} ferrous sulfate, to a total of 0.45 mL. The sample aliquots (0.05 mL) were then added and incubated at room temperature for 30 min, and the absorbance at 560 nm was then read. The results were reported as nmol mg protein^{-1} using ε_{560} = 43 mmol^{-1}cm^{-1}.

2.9 Assay of glutathione peroxidase

GPx activity was measured by using a coupled reaction with GR in the presence of GSH and NADPH, as described previously by Flohé and Gunzler (1984). The oxidized glutathione (GSSG) produced upon reduction of t-butyl hydroperoxide by GPx is reduced by GR using NADPH as a cofactor. The oxidation of NADPH to $NADP^+$ results in a decrease in the absorbance at 340 nm. Sample aliquots (0.05 mL) were added to 0.6 mL of the assay mixture containing 100 mmol L^{-1} phosphate buffer (pH 7.7), 1 mmol L^{-1} EDTA, 0.2 U mL^{-1} of GR, 2 mmol L^{-1} of GSH, 0.2 mmol L^{-1} NADPH at a final concentration. Subsequently t-butylhydroperoxide was added to a final concentration of 0.5 mmol L^{-1} and the change in the absorbance was recorded at regular intervals of a period of 3 minutes. The enzyme activity was expressed as µmol min^{-1} mg protein^{-1} using ε_{340} = 6.22 mmol^{-1}cm^{-1}.

2.10 Assay of glutathione reductase

GR is an NADPH-dependent enzyme that regenerates GSH from GSSG. Its activity was expressed as nmol min^{-1} mg protein^{-1} and measured by the rate of NADPH consumption at 340 nm using ε_{340} = 6.22 mmol^{-1}cm^{-1}. Samples aliquots (0.05 mL) were added to 0.45 mL of a reaction medium consisted of phosphate buffer (pH 8.0), 10 mmol L^{-1} EDTA, 0.25 mmol L^{-1} GSSG and 0.1 mmol L^{-1} NADPH in 238 mmol L^{-1} $NaHCO_3$ at a final concentration (Carlberg and Mannervick, 1985).

2.11 Assay of glutathione S-transferase

GST activity was assayed based on the conjugation reaction with GSH, using 1-chloro-2,4-dinitrobenzene (CDNB) as a substrate (Habig *et al.*, 1974). Sample aliquots (0.05 mL) were added to 0.6 mL of the assay mixture containing 100 mmol L^{-1} phosphate buffer (pH 6.5), GSH and CDNB at a final concentration of 1mmol L^{-1}

each. The activity was calculated from the changes in absorbance at 340 nm. It was expressed as pmol min^{-1} mg $protein^{-1}$ using ε_{340} = 9.6 $mmol^{-1}cm^{-1}$.

2.12 Assay of non-protein thiols

Non-protein thiols (NPSH) represent an indirect measure of GSH. Proteins were eliminated by adding 0.25 mmol L^{-1} perchloric acid to the homogenates and centrifuging the mixture at 700 *g* for 5 min. To the supernatants (0.05 mL), 0.45 mL of 0.2 mol L^{-1} phosphate buffer (pH 8.0) and 0.18 mmol L^{-1} 5,5'-dithiobis-(2-nitrobenzoic acid) (DTNB) at a final concentration were added and vortexed. The DTNB formed a yellow complex with GSH, and the absorbance was measured at 412 nm. NPSH content was expressed as nmol mg $protein^{-1}$ using ε_{412} = 13.6 $mmol^{-1}cm^{-1}$ (Ellman, 1959).

2.13 Estimation of protein

Protein was determined by the method of Lowry *et al.* (1951) by using bovine serum albumin as standard.

2.14 Statistical analysis

The results are expressed as mean ± standard error. Levene's test was used to attest whether the data were parametric. One-way analysis of variance followed by Duncan post-hoc test was done to evaluate the differences among the groups. The statistical analyses were performed using the software Statistica 7.0 (StatSoft, Inc., Tulsa, Oklahoma, USA) and differences were considered significant at $p < 0.05$.

3 Results

3.1 Body weight

The final body weight and the weight gain did not differ among all studied groups (data not shown) ($p > 0.05$).

3.2 Glucose levels in liver and serum

The ASP group exhibited higher glucose levels in both liver (1.5-folder) and serum (1.3-folder) than control rats (p <0.05). Even after N-acetylcysteine treatment, the glucose levels remained higher in liver (about 1.4-folder) and serum (about 1.1-folder) of the ASP-NAC rats than in both control and NAC groups (p <0.05). All data are illustrated in the Figure 1.

3.3 Lipid profile

All results are shown in the Table 1. The ASP group exhibited higher triglycerides, total cholesterol, LDL cholesterol; and also lower HDL levels than control rats (p <0.05). ASP-NAC rats revealed lower triglycerides and also higher HDL cholesterol levels when compared to the ASP group (p <0.05). Although N-acetylcysteine did not restore the total cholesterol and LDL cholesterol levels in the serum of ASP-NAC rats to the control and NAC values (p <0.05), such values were reduced about 1.2-fold and 3.8-fold, respectively, when compared to the ASP group (p <0.05).

3.4 Lipid hydroperoxides

The ASP group showed higher LOOH levels in the cerebral cortex (Fig. 2A), cerebellum (Fig. 2B), brainstem (Fig. 2C) and hypothalamus (Fig. 2D) than control animals (p <0.05). The ASP-NAC rats exhibited lower LOOH levels in the cerebral cortex (Fig. 2A), cerebellum (Fig. 2B), brainstem (Fig. 2C) and hypothalamus (Fig. 2D) when compared to ASP group (p <0.05).

3.5 Glutathione peroxidase

The ASP group showed lower GPx activity in the cerebral cortex (Fig. 3A), cerebellum (Fig. 3B), brainstem (Fig. 3C) and hypothalamus (Fig. 3D) than control animals (p <0.05). The ASP-NAC rats exhibited higher GPx activity in the cerebral cortex (Fig. 3A), brainstem (Fig. 3C) and hypothalamus (Fig. 3D) when compared to ASP group; whilst in the cerebellum, they revealed lower GPx activity than both control and NAC animals (Fig. 3B) (p <0.05).

3.6 Glutathione reductase

The ASP group showed lower GR activity in the cerebral cortex (Fig. 4A), cerebellum (Fig. 4B), brainstem (Fig. 4C) and hypothalamus (Fig. 4D) than control animals (p <0.05). The ASP-NAC rats exhibited higher GR activity in the cerebral cortex (Fig. 4A), cerebellum (Fig. 4B), brainstem (Fig. 4C) and hypothalamus (Fig. 4D) when compared to ASP group (p <0.05). In the cerebellum, the ASP-NAC animals also revealed higher GR activity than both control and NAC groups (Fig. 4B) (p <0.05); whilst in their brainstem, the GR activity remained lower when compared to both control and NAC rats (Fig. 4C) (p <0.05).

3.7 Glutathione S-transferase

The ASP group showed higher GST activity in the cerebral cortex (Fig. 5A), cerebellum (Fig. 5B) and brainstem (Fig. 5C) than control animals (p <0.05). Unlike, in the hypothalamus, the ASP rats revealed lower GST activity when compared to the control group (Fig. 5D) (p <0.05). The ASP-NAC animals exhibited lower GST activity in the cerebral cortex (Fig. 5A), cerebellum (Fig. 5B) and brainstem (Fig. 5C) when compared to ASP group (p <0.05). Contrasting, in the hypothalamus, the ASP-NAC rats revealed higher GST activity than ASP group (Fig. 5D) (p <0.05). Furthermore, in the brainstem, ASP-NAC animals also demonstrated lower GST activity when compared to both control and NAC rats (Fig. 5C) (p <0.05).

3.8 Non-protein thiols

The ASP group showed lower NPSH levels in the cerebral cortex (Fig. 6A), cerebellum (Fig. 6B), brainstem (Fig. 6C) and hypothalamus (Fig. 6D) than control animals (p <0.05). In the cerebral cortex, cerebellum, brainstem and hypothalamus of the ASP-NAC rats, these values returned to the control and NAC levels (Figs. 6A, 6B, 6C and 6D) (p >0.05).

4 Discussion

Aspartame is a synthetic nonnutritive sweetener used in over 90 countries worldwide in about 6,000 products (Magnuson *et al.,* 2007). It is considered a sweetener that plays a main role in nutritional guidance for the diabetes mellitus and obesity (Fitch and Keim, 2012). However, our research exhibited that the chronic

aspartame administration led to a rise in the serum glucose, triglycerides, total cholesterol levels and its fraction, LDL; whilst the other portion, HDL, was reduced. As observed now, a previous investigation from our laboratory also reported that long-term intake of aspartame resulted in a mild state of hyperglycemia even after N-acetylcysteine treatment. The authors suggested that augment in the glucose levels was not generated by the aspartame-induced oxidative stress (Finamor *et al.*, 2014). In such work, it was hypothesized that the hyperglycemia occurred due to an aspartame metabolite (phenylalanine), which could induce raised glucose production in the liver, as revealed in the literature by Piccardo *et al.*, 1983. Now, we confirm these results and indicate that, in fact, an elevation in the hepatic glucose synthesis could be one of the reasons for the aspartame-induced hyperglycemia.

In agreement with our results, Prokic *et al.* (2014) reported that their rats suffered hyperglycemia, hypertriglyceridemia and hypercholesterolemia after the aspartame treatment at the ADI (40 mg kg^{-1}) for six weeks. Such changes were responsible for trigger the oxidative stress in the erythrocytes, since they occasioned a neutrophil activation through the ROS production. Moreover, as Kim *et al.* (2011), under presence of hyperlipidemia, aspartame-fed zebra fish exhibited lowest survival, increase in brain inflammation and severe ROS formation. Thus, we believe that the alterations observed in the serum of our aspartame-treated rats could be important factors in the oxidative stress generation in their cerebral cortex, cerebellum, brainstem and hypothalamus.

After N-acetylcysteine usage, a reestablishment of the triglycerides and HDL cholesterol to the control levels was evidenced in the serum of our aspartame-treated animals. Nevertheless, although N-acetylcysteine has not been able to restore the total and LDL cholesterol concentrations to the control quantities, it significantly improved these changes. Such partial protective effect could be connected to the increased cholesterol catabolism to give bile acids and/or the inhibition of both cholesterol production and LDL receptor action (Dietschy, 1997). Similar results showed that a combination of N-acetylcysteine and folic acid restored to the control values the triglycerides, total cholesterol quantities and its fraction, LDL and HDL, in the rat serum after chronic aspartame administration (Shaheen and Afifi, 2014). Our research also supports the evidence of the protective role of N-acetylcysteine on aspartame-induced oxidative stress in several rat brain regions, as cerebral cortex, cerebellum, brainstem and hypothalamus.

Brain is more vulnerable to oxidative processes because it is rich in polyunsaturated fatty acids (Floyd and Carney, 1992); consumes high oxygen levels and exhibits a poor antioxidant system (Samuel *et al.*, 2005). Our investigation found that the aspartame consumption for an extended period increased LOOH formation in the rat cerebral cortex, cerebellum, brainstem and hypothalamus. LOOH are the first stable product of the lipid peroxidation reaction (Halliwell and Gutteridge, 1999). So, we propose that it perhaps occurred due to the impairment in the rat blood-brain barrier. Lipid peroxidation causes higher membrane permeability, changing its barrier functions (Fridovich and Porter, 1981). Under such condition, aspartame metabolites can penetrate more easily across it (Humphries *et al.*, 2008), making the brain even more susceptible to the ROS attack. Recent report attributed to the aspartame metabolite action (methanol) the reason of ROS generation in the different brain regions (cerebral cortex, cerebellum, midbrain, pons medulla, hippocampus and hypothalamus) of aspartame-treated rats (40 mg kg^{-1}) (Ashok and Sheeladevi, 2014). As these authors, the ROS are responsible to the lipid peroxidation, apoptotic changes and degeneration in brain function.

In order to avoid the lipid peroxidation of biological membranes caused by ROS, enzymatic and non-enzymatic antioxidant defense systems are present in the cell (Halliwell and Gutteridge, 1999). GSH is the most important ROS scavenging in neurons (Rice and Russo-Menna, 1998). GSH itself protects against oxidative insults and also acts as substrate for the GPx and GST activities; the latter is involved in the detoxification of xenobiotic and signaling cascades (Habig *et al.*, 1974); whilst GPx converts hydrogen peroxide (H_2O_2) and organic hydroperoxides to less reactive products**.** In the latter process GSH is oxidized to form GSSG, which is recycled back to GSH through the GR action (Halliwell and Gutteridge, 1999). Several reports have been attributed the aspartame toxic effects in the rodent brain (Abdel-Salam *et al.*, 2012; Finamor *et al.*, 2014) and the different brain regions (Mourad and Noor, 2011; Iyyaswamy and Rathinasamy, 2012; Abhilash *et al.*, 2013; Ashok and Sheeladevi, 2014) to the GSH depletion because methanol possess a GSH-dependent metabolism (Harris *et al.*, 2004). Our data corroborate with these findings, since we revealed a diminished GSH levels (represented as NPSH) in the rat cerebral cortex, cerebellum, brainstem and hypothalamus. It was linked to reduced GPx and GR activities. Otherwise, although we have also found a reduced GST activity in the

hypothalamus, such enzymatic activity was augmented in the other brain regions of the aspartame-treated animals.

As suggested in a previous research, a GSH depletion detected after methanol intoxication occurs due to the GSH binding with accumulated formaldehyde, a derivate product from methanol metabolism. It also results from the inactivation of GR activity by the methanol action and/or LOOH formed after methanol intake (Skrzydlewska and Farbiszewski, 1998). These two explanations could justify the declined GSH levels found in the cerebral cortex, cerebellum, brainstem and hypothalamus of the aspartame-treated rats, and their consequent vulnerability to the injury by ROS. Moreover, it has obvious implications because GSH is a substrate for GPx and GST activities too (Halliwell and Gutteridge, 1999). In our investigation, a combined deficit of both enzymes could be the responsible for potentiate aspartame harmfulness through increasing oxidative stress and reducing the antioxidative potential in the hypothalamus. Coulombe and Sharma (1986) exhibited that aspartame causes a profound enhance in the norepinephrine synthesis basically in the hypothalamus. For this reason, we believe that GST activity was diminished in the hypothalamus due to its several attempts to detoxify the brain against the noxiousness caused by norepinephrine accumulation, which perhaps resulted in its functional exhaustion. Unlike, in the other brain regions of the aspartame-treated rats, it is possible that GST synthesis was induced by aspartame in response to methanol action, which resulted in the enhancement of its activity. Mourad and Noor (2011) observed such increment in GST activity in the rat cerebral cortex after long-term aspartame administration (40 mg kg^{-1}).

N-acetylcysteine is an acetylated cysteine residue able to increase cell protection to oxidative stress in neurological disorders (Shahripour *et al.*, 2014). In a previous research from our laboratory, we have shown that N-acetylcysteine prevented against the LOOH production in the brain of aspartame-treated animals through restoring GSH levels and all the GSH-related enzymes (Finamor *et al.*, 2014). Now, we revealed that N-acetylcysteine was capable to protect different cerebral regions (cerebral cortex, cerebellum, brainstem and hypothalamus) against the LOOH formation. It perhaps happened through its direct reaction with ROS and formaldehyde, thus reducing their toxicity; and/or due to its reaction with the membrane protein sulfhydryl groups, thereby stabilizing membrane lipid-protein and

improving the brain structures and functions, as reported in a previous investigation (Farbiszewski *et al.*, 2000).

Furthermore, now we have identified that N-acetylcysteine triggered different protective responses according each brain region. Such antioxidative treatment restored to the control values the activities of all the GSH-related enzymes and GSH levels (represented as NPSH) in the cerebral cortex and hypothalamus of the aspartame-treated rats. N-acetylcysteine is a precursor of L-cysteine and GSH (Aruoma *et al.*, 1989). Both L-cysteine and GSH restored to normal values the Na^+K^+-ATPase activity in cerebral cortex after aspartame metabolites exposure due to its ROS scavanger effects and/or the amelioration of cellular GSH, which was depleted from the methanol action (Simintzi *et al.*, 2008). So, it is probable that N-acetylcysteine enhanced GSH levels because it could act as source of cysteine for GSH production; other hypothesis for the increasing in the GSH levels is its recycling back from GSSG through the GR action, whose activity also was greater in the cerebral cortex and hypothalamus of the aspartame-treated rats. GR is an enzyme that regenerates GSH from GR using NADPH as cofactor (Halliwell and Gutteridge, 1999). Moreover, we proposed these elevated GSH levels could be the responsible for the increase in the GPx and GST activities, since it is a substrate for their action. Subsequently, GSH looks act as antioxidant itself; in turn, GPx appears eliminated H_2O_2 and subsequent hydroxyl radical production (Halliwell and Gutteridge, 1999); and, finally, GST detoxified the cells (cerebral cortex and hypothalamus) from xenobiotic elements (Habig *et al.*, 1974), thereby reducing the toxic action of the methanol.

In our research, N-acetylcysteine administration resulted in the GR reestablishment in the cerebellum of the aspartame-treated rats, where its activity was even more elevated than the control values. Yang *et al.* (2006) suggested that GR is an important component of the mice antioxidant system, since its activity was higher in their cerebellum than in their cerebral cortex and liver. Thus, it is possible that, when restoring GSH levels, N-acetylcysteine induced an increment in the GR activity, which performed a key role in the protection of the rat cerebellum. Nevertheless, N-acetylcysteine exerted no effect on the GPx activity, which remained reduced. It probable happened in an attempt to remove the H_2O_2, leading to GPx exhaustion and excessive GSSG formation; in turn, GR increment resulted in minor GSSG accumulation, recycling it back to GSH. GSH is a critical antioxidant that

supports the GST in its detoxifying activity; thus limiting the impact of oxidative stress and protecting vital cellular components against harmful lipid peroxidation (Rushworth and Megson, 2014).

Finally, in the brainstem of the aspartame-treated rats, the antioxidant effect of N-acetylcysteine seems to be dependent, in especial, on both GPx activity and GSH mobilization to keep lower the ROS levels. Since GSH acts as a substrate to GPx (Halliwell and Gutteridge, 1999), it might elucidate the small increase found in the GSH levels. Other reason for it could be the less GSH recycle from GSSG through the reduced action of the GR. So, GPx seems to act as the main antioxidant in the protection of the brainstem; thus, making the GST mobilization not required to perform the defensive function.

In conclusion, in accordance with a previous report, we reaffirm that aspartame intake induced hyperglycemia, which remained even after N-acetylcysteine administration. Now, in this research, we revealed that aspartame also stimulated an elevation in the glucose production in the liver, which was maintained after N-acetylcysteine exposure; suggesting that hepatic glucose synthesis could be a cause for the aspartame-induced hyperglycemia. Furthermore, we evidenced that aspartame resulted in lipid peroxidation, GSH depletion and changes in its related enzymes (GPx, GR and GST) in the rat cerebral cortex, cerebellum, brainstem and hypothalamus. N-acetylcysteine treatment reduced the lipid peroxidation, increased the GR activity and GSH levels (represented as NPSH) in the cerebral cortex, cerebellum, brainstem and hypothalamus. It also enhanced GPx activity in the rat cerebral cortex, brainstem and hypothalamus; and restored to the control values the GST activity in the cerebral cortex, cerebellum, brainstem and hypothalamus. Therefore, N-acetylcysteine produced different defensive responses according each brain region. However, in general, N-acetylcysteine exerted its protective action in the cerebral cortex, cerebellum, brainstem and hypothalamus through GSH production and the modulation of GSH-related enzymes.

Conflict of Interest

The authors declare that there are no conflict of interest.

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Figure captions

Figure 1 Effects of N-acetylcysteine on glucose levels in the liver (A) and serum (B) of control and aspartame-treated rats. Each bar represents the mean ± standard error (*n*=8). [a] Denotes that the data are significantly different from the control group at $p < 0.05$. [c] Denotes that the data are significantly different from NAC at $p < 0.05$.

Figure 2 Effects of N-acetylcysteine on LOOH levels in the cerebral cortex (A), cerebellum (B), brainstem (C), and hypothalamus (D) of control and aspartame-treated rats. LOOH, lipid hydroperoxides. Each bar represents the mean ± standard error (*n*=8). [a] Denotes that the data are significantly different from the control group at $p < 0.05$. [b] Denotes that the data are significantly different from ASP at $p < 0.05$.

Figure 3 Effects of N-acetylcysteine on GPx activity in the cerebral cortex (A), cerebellum (B), brainstem (C), and hypothalamus (D) of control and aspartame-treated rats. GPx, glutathione peroxidase. Each bar represents the mean ± standard error (*n*=8). [a] Denotes that the data are significantly different from the control group at $p < 0.05$. [b] Denotes that the data are significantly different from ASP at $p < 0.05$. [c] Denotes that the data are significantly different from NAC at $p < 0.05$.

Figure 4 Effects of N-acetylcysteine on GR activity in the cerebral cortex (A), cerebellum (B), brainstem (C), and hypothalamus (D) of control and aspartame-treated rats. GR, glutathione reductase. Each bar represents the mean ± standard error (*n*=8). [a] Denotes that the data are significantly different from the control group at $p < 0.05$. [b] Denotes that the data are significantly different from ASP at $p < 0.05$. [c] Denotes that the data are significantly different from NAC at $p < 0.05$.

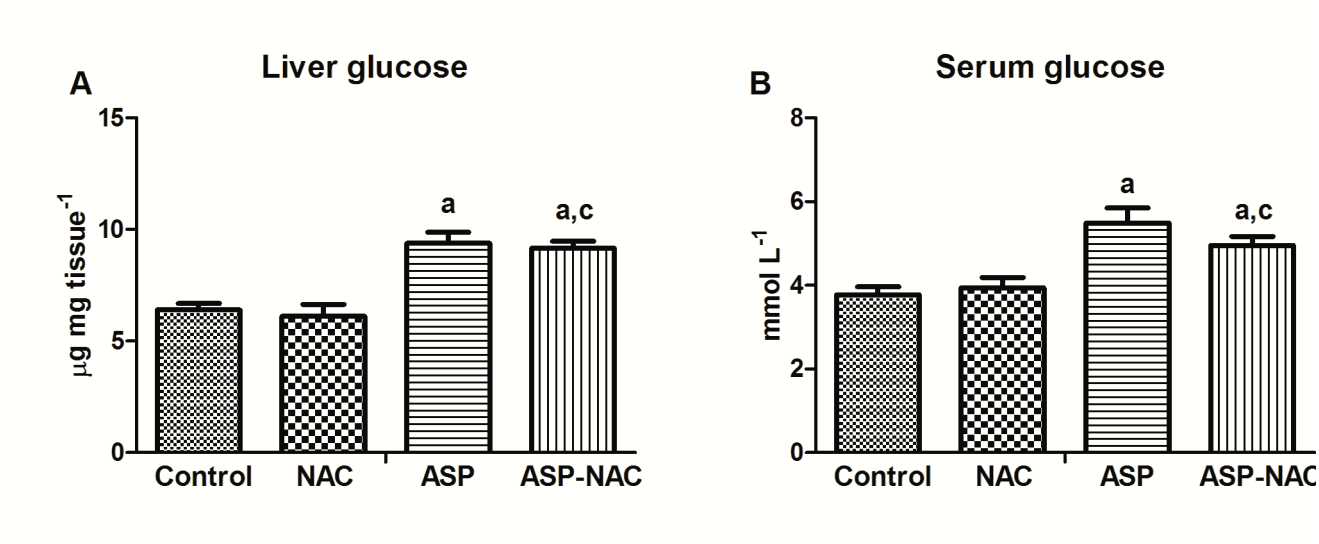
Figure 5 Effects of N-acetylcysteine on GST activity in the cerebral cortex (A), cerebellum (B), brainstem (C), and hypothalamus (D) of control and aspartame-treated rats. GST, glutathione-S-transferase. Each bar represents the mean ± standard error (*n*=8). [a] Denotes that the data are significantly different from the control group at $p < 0.05$. [b] Denotes that the data are significantly different from ASP at $p < 0.05$. [c] Denotes that the data are significantly different from NAC at $p < 0.05$.

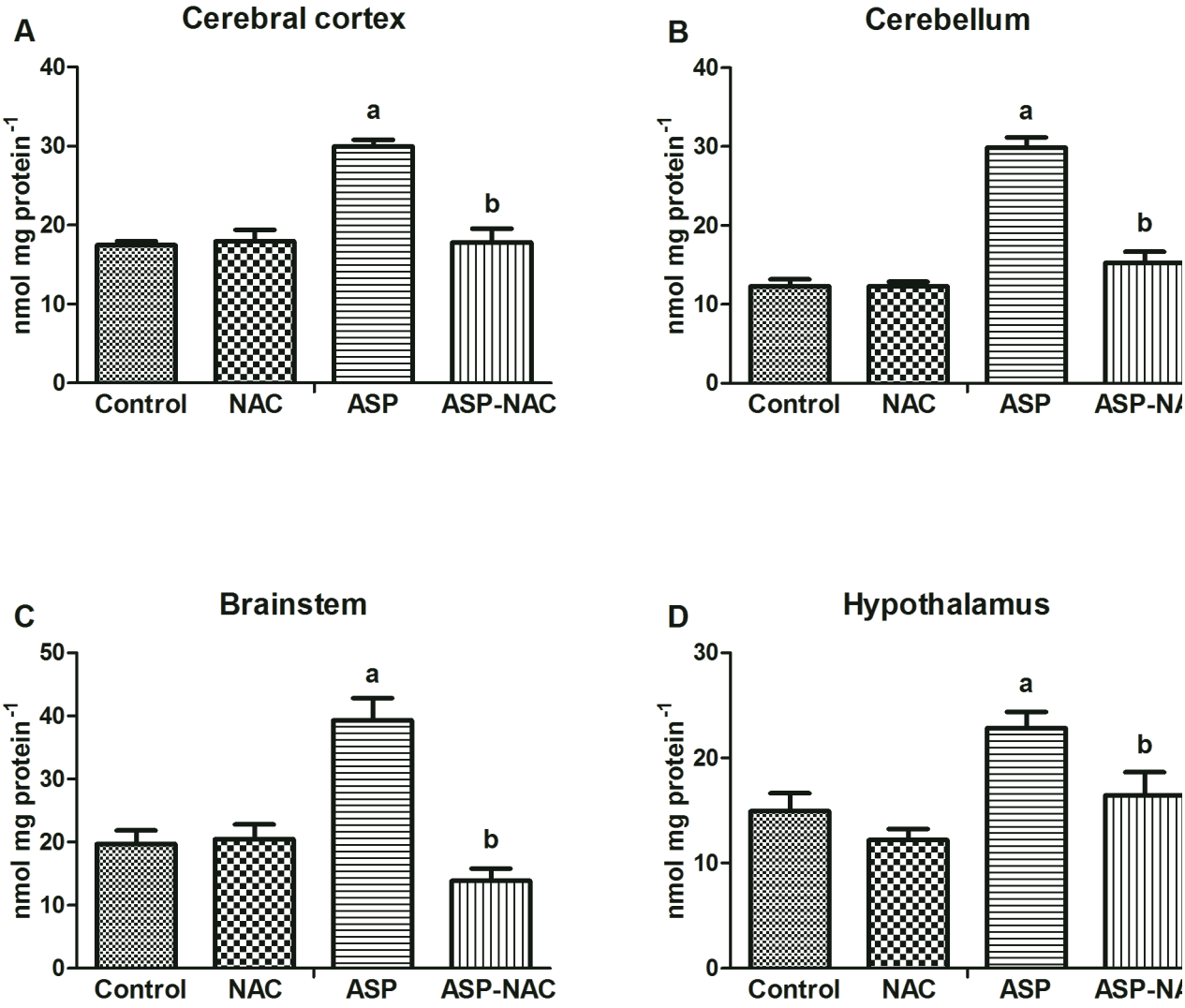
Figure 6 Effects of N-acetylcysteine on NPSH levels in the cerebral cortex (A), cerebellum (B), brainstem (C), and hypothalamus (D) of control and aspartame-treated rats. NPSH, non-protein thiols. Each bar represents the mean ± standard error (*n*=8). [a] Denotes that the data are significantly different from the control group at $p < 0.05$. [b] Denotes that the data are significantly different from ASP at $p < 0.05$.

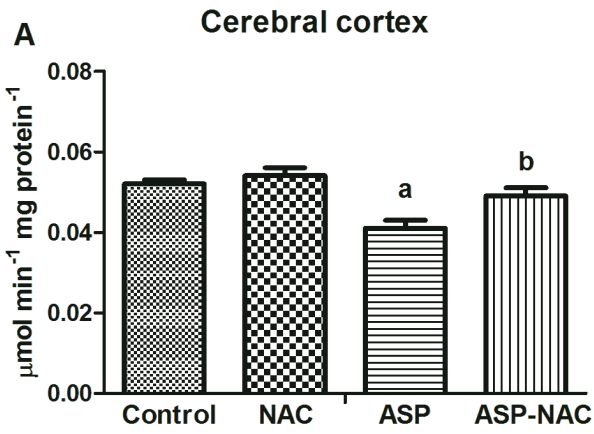
Table 1. Effects of N-acetylcysteine on the triglycerides, total cholesterol and its fractions, HDL and LDL, in the serum of control and aspartame-treated rats

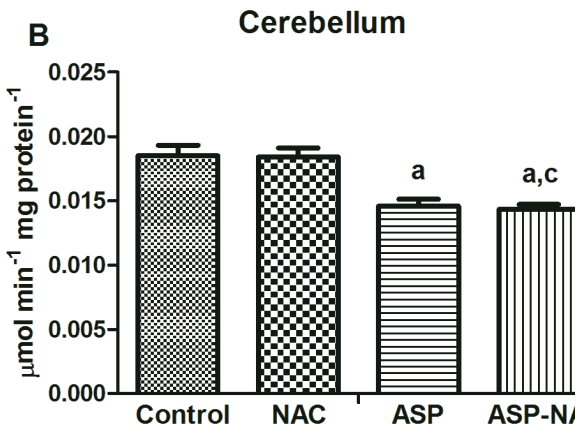
Variable	Control	NAC	ASP	ASP-NAC
Triglycerides (mg dL^{1})	131.8±8.8	110.6±18.5	203.5±27.4[a]	123.9±9.7[b]
Total cholesterol (mg dL^{-1})	103.1±6.6	98.8±5.0	172.6±8.1[a]	137.8±13.1[a,b,c]
HDL (mg dL^{-1})	84.2±8.1	82.2±6.9	54.4±5.0[a]	99.6±9.3[b]
LDL (mg dL^{-1})	7.72±1.93	7.72±1.54	101.93±2.31[a]	27.4±2.70[a,b,c]

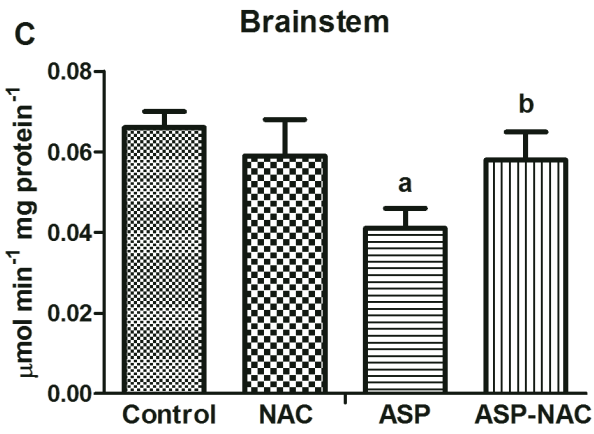
HDL, high-density lipoprotein cholesterol; LDL, low-density lipoprotein cholesterol. The data appear as the mean ± standard error (n=8). [a] Denotes that the data are significantly different from the control group at p <0.05. [b] Denotes that the data are significantly different from ASP at p <0.05. [c] Denotes that the data are significantly different from NAC at p <0.05.

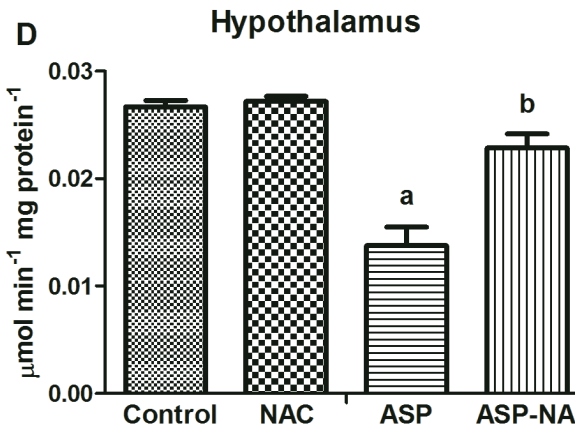

A
Liver glucose
μg mg tissue-1
15
10
5
0
a
a,c
Control
NAC
ASP
ASP-NAC
B
Serum glucose
mmol L-1
8
6
4
2
0
a
a,c
Control
NAC
ASP
ASP-NAC

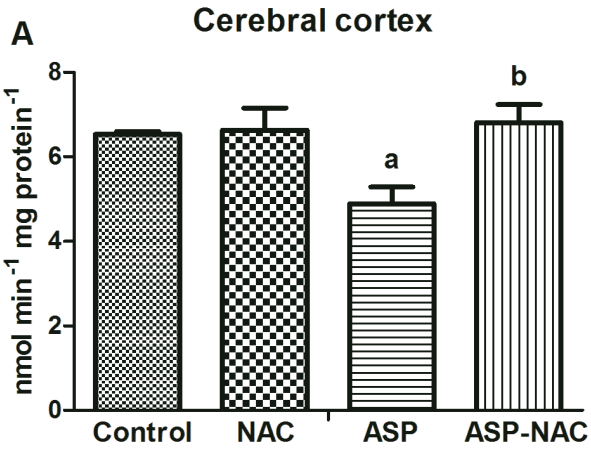

A
Cerebral cortex
nmol mg protein-1
40
30
20
10
0
a
b
Control
NAC
ASP
ASP-NAC
B
Cerebellum
nmol mg protein-1
40
30
20
10
0
a
b
Control
NAC
ASP
ASP-NA
C
Brainstem
nmol mg protein-1
50
40
30
20
10
0
a
b
Control
NAC
ASP
ASP-NAC
D
Hypothalamus
nmol mg protein-1
30
20
10
0
a
b
Control
NAC
ASP
ASP-NA

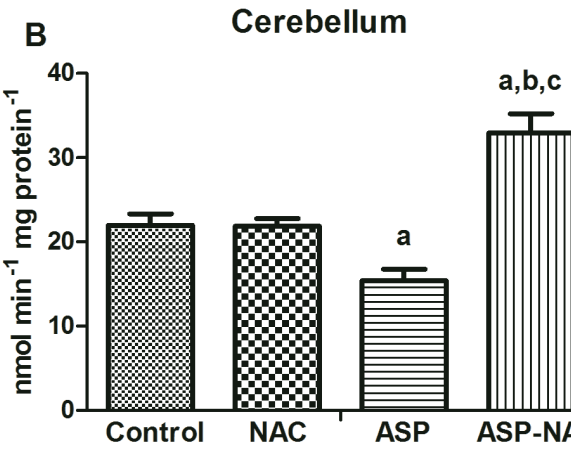

A
Cerebral cortex
µmol min-1 mg protein-1
0.08
0.06
0.04
0.02
0.00
a
b
Control
NAC
ASP
ASP-NAC

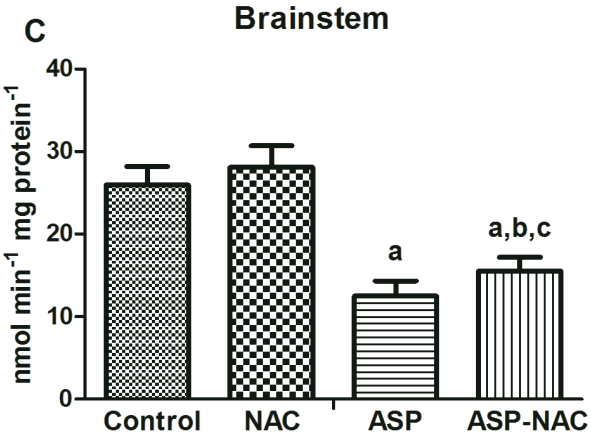

B
Cerebellum
µmol min-1 mg protein-1
0.025
0.020
0.015
0.010
0.005
0.000
a
a,c
Control
NAC
ASP
ASP-NA

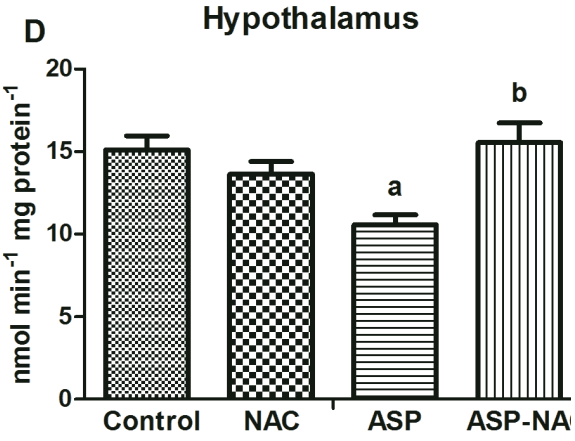

C
Brainstem
µmol min-1 mg protein-1
0.08
0.06
0.04
0.02
0.00
a
b
Control
NAC
ASP
ASP-NAC

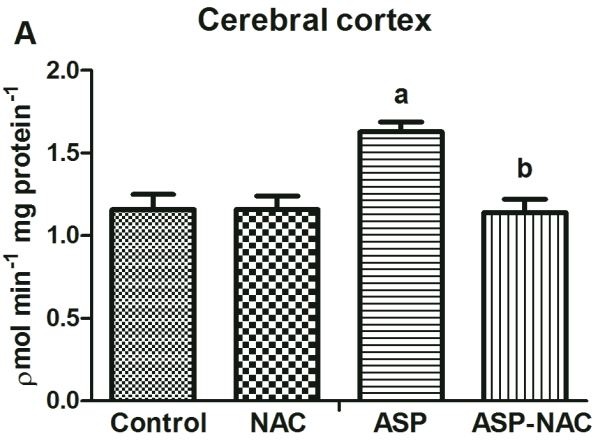

D
Hypothalamus
µmol min-1 mg protein-1
0.03
0.02
0.01
0.00
a
b
Control
NAC
ASP
ASP-NA

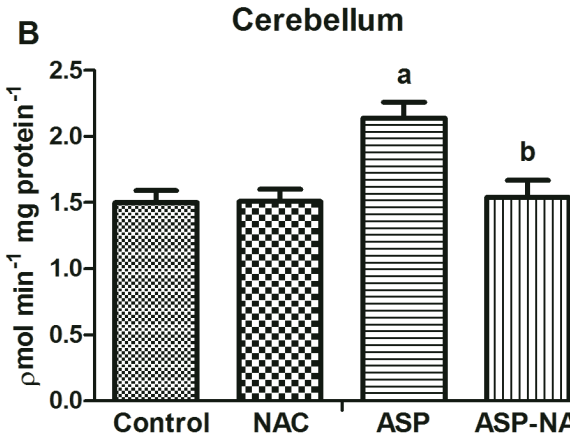

A
Cerebral cortex
nmol min-1 mg protein-1
0
2
4
6
8
a
b
Control
NAC
ASP
ASP-NAC

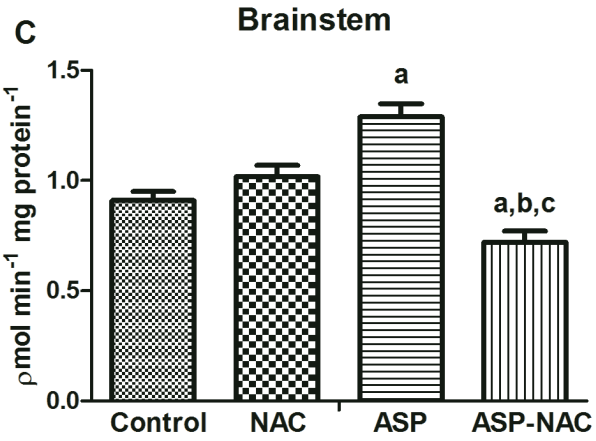

B
Cerebellum
nmol min-1 mg protein-1
0
10
20
30
40
a
a,b,c
Control
NAC
ASP
ASP-NA

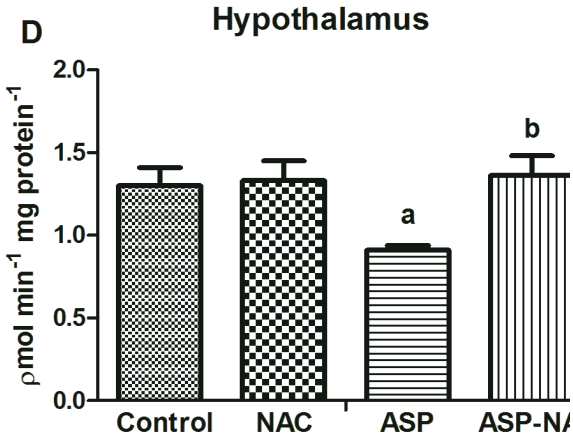

C
Brainstem
nmol min-1 mg protein-1
0
10
20
30
40
a
a,b,c
Control
NAC
ASP
ASP-NAC

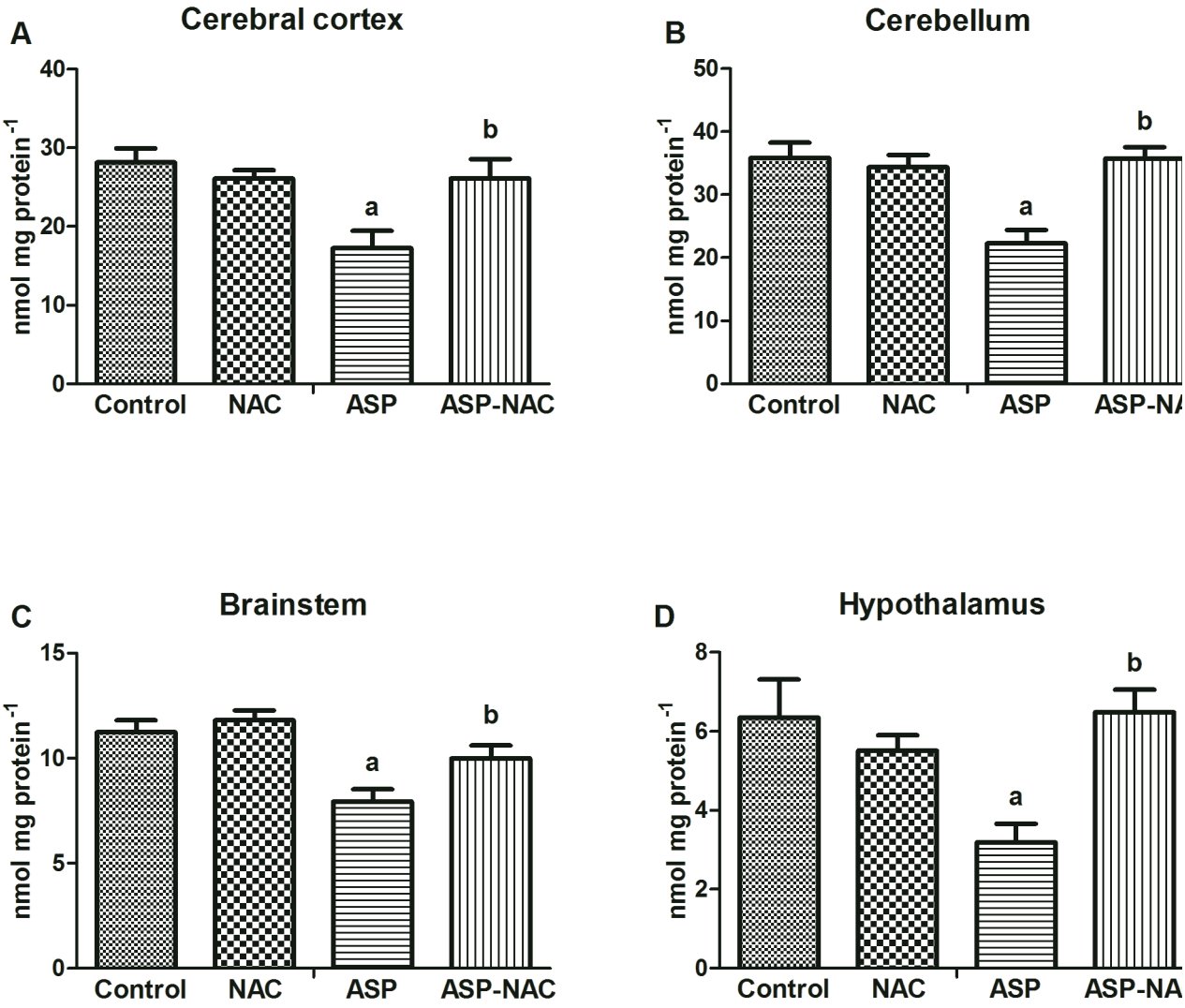

D
Hypothalamus
nmol min-1 mg protein-1
0
5
10
15
20
a
b
Control
NAC
ASP
ASP-NA


A
Cerebral cortex
ρmol min-1 mg protein-1
2.0
1.5
1.0
0.5
0.0
a
b
Control
NAC
ASP
ASP-NAC


B
Cerebellum
ρmol min-1 mg protein-1
2.5
2.0
1.5
1.0
0.5
0.0
a
b
Control
NAC
ASP
ASP-NA


C
Brainstem
ρmol min-1 mg protein-1
1.5
1.0
0.5
0.0
a
a,b,c
Control
NAC
ASP
ASP-NAC


D
Hypothalamus
ρmol min-1 mg protein-1
2.0
1.5
1.0
0.5
0.0
a
b
Control
NAC
ASP
ASP-NA


A
Cerebral cortex
nmol mg protein-1
40
30
20
10
0
a
b
Control
NAC
ASP
ASP-NAC
B
Cerebellum
nmol mg protein-1
50
40
30
20
10
0
a
b
Control
NAC
ASP
ASP-NA
C
Brainstem
nmol mg protein-1
15
10
5
0
a
b
Control
NAC
ASP
ASP-NAC
D
Hypothalamus
nmol mg protein-1
8
6
4
2
0
a
b
Control
NAC
ASP
ASP-NA

4 DISCUSSÃO

Os experimentos desta tese foram realizados com a finalidade de avaliar os efeitos da N-acetilcisteína frente às alterações bioquímicas e oxidativas ocasionadas pelo consumo crônico de 40 mg/kg aspartame por ratos. Esta dose foi selecionada, pois, apesar de a União Europeia ter recentemente confirmado a mesma como IDA para humanos (EUROPEAN FOOD SAFETY AUTHORITY, 2006), alguns estudos encontrados na literatura utilizando modelos animais questionam sua segurança (MOURAD, 2011; MOURAD E NOOR, 2011; ASHOK E SHEELADEVI, 2014; PROKIC et al., 2014). Sendo assim, a realização deste trabalho confirma a necessidade de buscar compostos que minimizem a toxicidade produzida pelo aspartame, visto que sua introdução no mercado melhorou a qualidade de vida de indivíduos diabéticos, obesos, entre outros, tornando-o um dos mais populares edulcorantes artificiais não-calóricos (BUTCHKO et al., 2002). Nos Estados Unidos, por exemplo, estima-se que dentre os mais de 6.000 novos produtos contendo edulcorantes artificiais não-calóricos que estão disponíveis, o aspartame está presente em mais de 974, especialmente em bebidas carbonatadas (YANG, 2010), as quais contém cerca de 180 mg de aspartame em um volume 355 ml (BUTCHKO E STARGEL, 2001).

Inicialmente, esta investigação avaliou se a ingestão de aspartame causaria um aumento no peso corporal dos animais, pois este efeito foi previamente relatado em ratos (FEIJÓ et al., 2013) e *zebra fish* (COLLISON et al., 2012). Contrariamente, foi detectado que este edulcorante não induziu um maior ganho de peso corporal, que se manteve semelhante em todos os grupos examinados. O presente estudo verificou se o consumo de aspartame resultaria na alteração do índice glicêmico dos ratos, uma vez que foram encontrados relatos na literatura que indicaram que sua ingestão prolongada coincide com o aparecimento de hiperglicemia, e *diabetes mellitus* (KIM et al., 2011; ABDEL-SALAM et al., 2012; COLLISON et al., 2012; PROKIC et al., 2014). Conforme os resultados obtidos, o consumo de aspartame causou uma leve hiperglicemia; esta permaneceu mesmo depois da administração de N-acetilcisteína; sendo assim, não foi desencadeada pelo estresse oxidativo induzido por este edulcorante. Posteriormente, as possíveis causas que levaram à

hiperglicemia foram investigadas, e os dados obtidos mostraram que dentre outras razões, ela foi ocasionada pelo aumento da síntese de glicose no fígado, o qual não foi inibido pela administração de N-acetilcisteína. Piccardo (1983) relatou que a fenilalanina (um metabólito do aspartame) é capaz de induzir um aumento da produção de glicose no tecido hepático, e a consequente hiperglicemia.

Este trabalho analisou também se a N-acetilcisteína poderia causar um efeito benéfico sobre a modificação do perfil lipídico decorrente da ingestão de aspartame pelos ratos. Segundo os dados encontrados, a N-acetilcisteína desempenhou um papel protetor parcial contra essas alterações, uma vez que a mesma não reduziu os níveis de colesterol total e sua fração LDL, os quais permaneceram elevados em relação aos do controle. Shaheen e Afifi (2014) obtiveram dados similares aos desta tese ao indicarem que a administração combinada de N-acetilcisteína e ácido fólico aos ratos que ingeriram aspartame resultou em reestabelecimento de todo o perfil lipídico dos mesmos. De acordo com os dados obtidos por esta investigação, pesquisas anteriores também mostraram que estas mesmas alterações foram encontradas no soro de ratos (PROKIC et al., 2014) e *zebra fish* (KIM et al., 2011) expostos ao aspartame, as quais foram associadas com a produção de EAO e o aparecimento do estresse oxidativo.

Além destes, diversos estudos realizados em modelos animais propuseram o estresse oxidativo como uma das causas da toxicidade por aspartame em diferentes tecidos, como, por exemplo, o sanguíneo (PROKIC et al., 2014), o encefálico (RUIZ et al., 2008; ABDEL-SALAM et al., 2012; ABHILASH et al., 2013) e suas diferentes estruturas (MOURAD E NOOR, 2011; IYYASWAMY E RATHINASAMY, 2012; ASHOK E SHEELADEVI 2014: ASHOK et al., 2014); o hepático (ABHILASH et al., 2011; MOURAD, 2011); e o renal (MOURAD, 2011), sem indicar, no entanto, nenhum possível tratamento para essa condição. Por isso, como próxima etapa, foi avaliado o efeito de N-acetilcisteína sobre os diferentes marcadores indicativos de toxicidade induzida por aspartame no encéfalo, fígado e rins de ratos. Segundo os dados obtidos nesta investigação, este tratamento antioxidante reorganiza o parênquima hepático dos ratos que ingeriram aspartame, impedindo o extravasamento da ALT, AST e FA para o citoplasma, restabelecendo assim as enzimas séricas marcadoras de integridade deste tecido. Similarmente, Dobrzyñska et al. (2000) demonstraram que N-acetilcisteína reestabeleceu as atividades das enzimas relacionadas ao dano hepático: ALT, AST e FA em soro de ratos

intoxicados por metanol e impediu a formação de TBARS. De acordo com estes dados, os da presente pesquisa mostraram ainda que a N-acetilcisteína diminuiu a concentração de produtos de peroxidação lipídica e proteínas carbonilas não só no fígado, mas também nos rins e no encéfalo de ratos, provavelmente, por reagir de forma direta com as EAO e o formaldeído, atenuando a toxicidade do metanol e o processo lipoperoxidativo. Como nesta tese não foram determinados os níveis plasmáticos de metanol, esta suposição foi formulada com base nos resultados de estudos encontrados na literatura, os quais relacionaram o aumento de seu conteúdo plasmático à peroxidação lipídica derivada do consumo de aspartame (IYYASWAMY E RATHINASAMY, 2012; ASHOK E SHEELADEVI 2014; ASHOK et al., 2014).

Além de reagir diretamente com as EAO, a N-acetilcisteína atua sobretudo minimizando o efeito oxidativo das mesmas por aumentar a produção de GSH e/ou prevenir sua depleção em situações de estresse oxidativo, uma vez que este composto é precursor da L-cisteína, cuja disponibilidade pode limitar a razão de síntese de GSH (SHAHRIPOUR et al., 2014). A GSH, por sua vez, exerce um importante papel protetor nas células, atuando diretamente na defesa antioxidante e/ou agindo indiretamente como cofator não só da GPx e da GST (HALLIWELL E GUTTERIDGE, 1999), mas também da formaldeído desidrogenase, a qual é responsável pela conversão de formaldeído em formato (HARRIS et al., 2004). Sendo assim, sua depleção decorrente da exposição ao aspartame é característica comum de todos os estudos encontrados na literatura (RUIZ et al., 2008; ABHILASH et al., 2011; MOURAD, 2011; MOURAD E NOOR, 2011; ABDEL-SALAM et al., 2012; IYYASWAMY E RATHINASAMY, 2012; ABHILASH et al., 2013; ASHOK E SHEELADEVI 2014; ASHOK et al., 2014), os quais a correlacionam com o aumento da vulnerabilidade celular ao estresse oxidativo. De maneira geral, segundo os resultados obtidos a partir deste trabalho, a N-acetilcisteína promoveu a síntese de GSH (representada pela medida dos NPSH) e sua reciclagem a partir da GSSG por ação da GR. Por sua vez, a GSH conferiu proteção aos tecidos analisados por atuar diretamente, por modular as atividades de enzimas associadas a ela (GPx e GST) e/ou estimular antioxidantes não-enzimáticos relacionadas ao seu metabolismo (ácido ascórbico e TRAP).

Por fim, para compreender melhor o efeito protetor exercido pela N-acetilcisteína sobre o estresse oxidativo induzido pelo aspartame no encéfalo dos

ratos, foi realizado um experimento adicional, no qual o encéfalo foi separado nas seguintes partes: córtex cerebral, cerebelo, tronco encefálico e hipotálamo, as quais foram analisadas separadamente. Como no primeiro experimento, foram evidenciadas peroxidação lipídica e, principalmente, alterações no sistema dependente de GSH, desta vez, somente o dano, os níveis de GSH e a atividade das enzimas associadas ao seu metabolismo foram analisados. De acordo com estes achados, a N-acetilcisteína preveniu a formação de LOOH por promover a síntese de GSH em todas as estruturas encefálicas, a qual atuou diretamente e/ou suscitou diferentes respostas enzimáticas em cada tecido dos ratos que consumiram aspartame: no córtex cerebral e no hipotálamo, a atividade da GPx, GR e GST; no cerebelo, a da GR e da GST; enquanto que no tronco encefálico, a atividade da GPx e da GST foram restabelecidas a valores semelhantes aos do grupo controle.

Os resultados alcançados pela atual pesquisa podem ser considerados preocupantes, visto que, quando extrapolada a humanos, conforme conversão proposta por Reagan-Shaw et al. (2008), a dose de aspartame usada neste estudo (40mg/kg) para ser administrada nos ratos, equivaleria a 6 mg/kg em humanos, ou seja, à ingestão de 2 unidades de bebida carbonatada (355 ml) por um indivíduo adulto de 60 kg. Este valor se aproximaria da dose verdadeiramente ingerida por humanos, visto que foi estimado que o aspartame seja consumido diariamente pela população geral na dose de 2 a 3 mg/kg (BUTCHKO et al., 2002). Por outro lado, esta tese também oferece dados que revelam a N-acetilcisteína como uma alternativa a ser utilizada por consumidores de aspartame. A dose utilizada neste trabalho (150 mg/kg), quando também extrapolada a humanos, corresponde a 24 mg/kg, o que equivaleria ao consumo diário de cerca de 1 g deste composto por um indivíduo adulto de 60 kg. Ziment (1988) afirmou que essa dose de N-acetilcisteína é considerada terapêutica e recomendada diariamente a indivíduos com distúrbios relacionados ao estresse oxidativo, uma vez que a N-acetilcisteína, como qualquer nutriente antioxidante, possui potencial pró-oxidante, não sendo indicada na ausência de estresse oxidativo.

5 CONCLUSÕES

5.1 Conclusões específicas

- O consumo de aspartame não alterou o peso corporal, a relação hepatossomática, bem como a razão do peso renal e peso corporal dos ratos, não promoveu o ganho de peso, uma vez que os mesmos não variaram entre os diferentes grupos experimentais;
- A hiperglicemia resultante da ingestão de aspartame não foi desencadeada pelo estresse oxidativo, e sim, dentre outras causas, pelo aumento da produção de glicose hepática;
- Embora não tenha diminuído significativamente os níveis séricos de colesterol total e LDL, a N-acetilcisteína normalizou os de triglicerídeos e os de colesterol HDL, bem como as atividades da ALT, AST e FA alterados em decorrência do consumo de aspartame;
- A N-acetilcisteína reduziu o dano oxidativo no tecido encefálico e suas diferentes estruturas: córtex cerebral, cerebelo, tronco encefálico e hipotálamo, bem como no hepático e no renal, o qual foi induzido pela administração de aspartame;
- De maneira geral, a N-acetilcisteína não influenciou diretamente a atividade da enzima SOD, a qual também não desempenhou um papel decisivo contra a toxicidade suscitada pelo aspartame;
- Este tratamento antioxidante auxiliou na remoção do H_2O_2 acumulado nos tecidos hepático e renal, especialmente, por reestabelecer a atividade da GPx, visto que a atividade da CAT permaneceu inibida no fígado dos ratos tratados com aspartame;
- A N-acetilcisteína promoveu a síntese de GSH (representada pela medida dos NPSH), depletada devido ao consumo de aspartame, no tecido encefálico e suas diferentes estruturas: córtex cerebral, cerebelo, tronco encefálico e hipotálamo, bem como no hepático e no renal;

- No tecido encefálico e suas diferentes estruturas: córtex cerebral, cerebelo e hipotálamo, bem como no hepático e no renal dos animais tratados com aspartame, a N-acetilcisteína promoveu a reciclagem da GSH a partir da GSSG por restaurar a atividade da GR;
- No encéfalo, bem como nas suas diferentes estruturas: córtex cerebral, cerebelo, tronco encefálico e hipotálamo, a atividade da GPx foi inibida pela administração de aspartame. O tratamento com N-acetilcisteína resultou no aumento desta atividade em todos os tecidos, exceto cerebelo;
- A atividade detoxificadora desenvolvida pela GST foi inibida pela exposição ao aspartame em todos os tecidos estudados, com exceção do hipotálamo, onde a mesma foi induzida. O tratamento com N-acetilcisteína levou ao reestabelecimento de sua função no fígado, rins e em todas as estruturas encefálicas estudadas: córtex cerebral, cerebelo, tronco encefálico e hipotálamo, embora não tenha exercido o mesmo efeito sobre o encéfalo;
- Este tratamento antioxidante resultou em maiores níveis de ácido ascórbico no tecido hepático e renal, bem como causou um aumento do TRAP nos mesmos e também no encéfalo, depletados pela ingestão de aspartame.

5.2 Conclusão geral

O consumo crônico de 40 mg/kg de aspartame por ratos induz hiperglicemia, alterações no perfil lipídico sérico e estresse oxidativo no encéfalo e suas diferentes estruturas: córtex cerebral, cerebelo, tronco encefálico e hipotálamo; e nos tecidos hepático e renal. No entanto, considerando que apesar de tóxico, este edulcorante é essencial para muitos indivíduos, como os diabéticos e aqueles que buscam a perda de peso, os resultados desta tese fornecem evidências de que a N-acetilcisteína atenua o dano oxidativo causado por este edulcorante artificial. Estudos adicionais são necessários para elucidar as vias de sinalização envolvidas neste processo; bem como para determinar a quantidade de metanol presente no plasma; e, de aspartato, fenilalanina e seus metabólitos, no encéfalo destes ratos.

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