Universidade Federal do Rio Grande do Sul
Instituto de Ciências Básicas da Saúde
Programa de Pós-Graduação em Ciências Biológicas: Bioquímica

Papel do estresse oxidativo na neurotoxicidade da 5-oxoprolina e do ácido N-acetilaspártico em encéfalo de ratos

Carolina Didonet Pederzolli

Tese de Doutorado

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PARTE I

Resumo

A 5-oxoprolina (ácido L-piroglutâmico) se acumula na deficiência de glutationa sintetase, uma doença genética autossômica recessiva clinicamente caracterizada por anemia hemolítica, acidose metabólica e sintomas neurológicos severos. Considerando que os mecanismos de dano cerebral nessa doença são pouco conhecidos, e que recentemente demonstramos que a 5-oxoprolina é capaz de promover estresse oxidativo *in vitro*, resolvemos investigar os efeitos *in vivo* da 5-oxoprolina sobre parâmetros de estresse oxidativo, afim de melhor esclarecer seu papel na neurotoxicidade da 5-oxoprolina e sua participação nos mecanismos neuropatológicos da deficiência de glutationa sintetase. Para isso, os efeitos da administração subcutânea aguda de 5-oxoprolina foram estudados sobre o potencial antioxidante total (TRAP); a quimiluminescência espontânea; as substâncias reativas ao ácido tiobarbitúrico (TBA-RS); o conteúdo de carbonilas, ácido ascórbico, glutationa reduzida (GSH), peróxido de hidrogênio, tióis e dissulfetos (e a razão SH/SS), assim como sobre as atividades das enzimas antioxidantes catalase (CAT), superóxido dismutase (SOD) e glutationa peroxidase (GPx), e a atividade da glicose 6-fosfato desidrogenase (G6PD) em córtex cerebral e cerebelo de ratos de 14 dias de vida. Os resultados obtidos indicaram que *in vivo* a 5-oxoprolina causa lipoperoxidação e oxidação protéica, compromete as defesas antioxidantes cerebrais e aumenta o conteúdo de peróxido de hidrogênio, indicando que a 5-oxoprolina promove estresse oxidativo *in vivo* em córtex cerebral e cerebelo de ratos jovens, mecanismo possivelmente envolvido na neuropatologia da deficiência de glutationa sintetase, na qual a 5-oxoprolina está acumulada.

O ácido N-acetilaspártico, por outro lado, se acumula na Doença de Canavan, uma leucodistrofia severa causada pela deficiência da enzima aspartoacilase e clinicamente caracterizada por retardo mental severo, hipotonia e macrocefalia, e também por convulsões tônico-clônicas generalizadas em aproximadamente metade dos pacientes. O ácido N-acetilaspártico é um precursor imediato para a biossíntese enzimática do ácido N-acetilaspartilglutâmico, cuja concentração também está elevada nos pacientes afetados pela Doença de Canavan. Considerando que os mecanismos de dano cerebral nessa doença permanecem pouco esclarecidos, investigamos o papel do estresse oxidativo na neurotoxicidade dos ácidos N-acetilaspártico e N-acetilaspartilglutâmico, a fim de avaliar o possível envolvimento dos mesmos na neuropatologia da Doença de Canavan. Os efeitos *in vitro* dos ácidos N-acetilaspártico e N-acetilaspartilglutâmico, assim como os efeitos das administrações subcutânea e intracerebroventricular (i.c.v.) de ácido N-acetilaspártico e da administração i.c.v. do ácido N-acetilaspartilglutâmico, foram estudados sobre os seguintes parâmetros de estresse oxidativo em córtex cerebral de ratos: TRAP; TAR; quimiluminescência espontânea; TBA-RS; conteúdos de GSH, peróxido de hidrogênio, tióis totais e carbonilas; atividades das enzimas antioxidantes CAT, SOD e GPx; e atividade da G6PD. Os resultados indicaram que apenas o ácido N-acetilaspártico é capaz de aumentar o conteúdo de peróxido de hidrogênio, estimular a lipoperoxidação e a oxidação protéica e de comprometer as defesas antioxidantes em cérebro de ratos, promovendo estresse oxidativo, que pode estar envolvido nos mecanismos patofisiológicos responsáveis pelo dano cerebral observado nos pacientes afetados pela Doença de Canavan, cujo marcador bioquímico clássico é o acúmulo de ácido N-acetilaspártico.

Abstract

5-Oxoproline (L-pyroglutamic acid) accumulates in glutathione synthetase deficiency, an autossomic recessive inherited disorder clinically characterized by hemolytic anemia, metabolic acidosis and severe neurological symptoms. Considering that the mechanisms of brain damage in this disease are poorly known, and that oxidative stress is elicited by 5-oxoproline *in vitro*, we decided to study the *in vivo* effects of this metabolite on oxidative stress parameters, in order to further clarify its role in 5-oxoproline neurotoxicity and its participation on the neuropathological mechanisms of patients affected by glutathione sintetase deficiency. The effects of acute subcutaneous administration of 5-oxoproline were studied on a wide spectrum of oxidative stress parameters, such as total radical-trapping antioxidant potential (TRAP); spontaneous chemiluminescence; thiobarbituric acid-reactive substances (TBA-RS); and carbonyl, ascorbic acid, reduced glutathione (GSH), hydrogen peroxide, thiol and disulfide contents (and SH/SS ratio), as well as on the activities of the antioxidant enzymes catalase (CAT), superoxide dismutase (SOD) and glutathione peroxidase (GPx), and on the activity of glucose 6-phosphate dehydrogenase (G6PD) in cerebral cortex and cerebellum of 14-day-old rats. The results indicated that *in vivo* 5-oxoproline causes lipid peroxidation and protein oxidation, impairs brain antioxidant defenses and increases hydrogen peroxide content, indicating that 5-oxoproline elicits oxidative stress *in vivo* in cerebral cortex and cerebellum of young rats, a mechanism that may be involved in the neuropathology of gluthatione synthetase deficiency, in which this metabolite accumulates.

N-acetylaspartic acid, on the other hand, accumulates in Canavan Disease, a severe leukodystrophy characterized by swelling and spongy degeneration of the white matter of the brain. This inherited metabolic disease, caused by deficiency of the enzyme aspartoacylase, is clinically characterized by severe mental retardation, hypotonia and macrocephaly, and also generalized tonic and clonic type seizures in about half of the patients. N-acetylaspartic acid is an immediate precursor for the enzyme-mediated biosynthesis of N-acetylaspartylglutamic acid, whose concentration is also increased in urine and cerebrospinal fluid of patients affected by Canavan Disease. Considering that the mechanisms of brain damage in this disease remain poorly understood, in the present study we investigated whether oxidative stress is elicited by N-acetylaspartic acid or its metabolite, N-acetylaspartylglutamic acid. The *in vitro* effects of N-acetylaspartic acid and N-acetylaspartylglutamic acid, as well as the effect of the acute subcutaneous administration of N-acetylaspartic acid, and the intracerebroventricular administration of N-acetylaspartic acid or N-acetylaspartylglutamic acid, were studied on oxidative stress parameters: TRAP, TAR, spontaneous chemiluminescence, TBA-RS, reduced glutathione content, sufhydryl content, carbonyl content, and on enzyme activities of CAT, SOD and GPx as well as on GSH and hydrogen peroxide contents and on G6PD activity, in the cerebral cortex of rats. Our results indicated that only N-acetylaspartic acid promotes oxidative stress by stimulating lipid peroxidation, protein oxidation and by impairing antioxidant defenses and enhancing hydrogen peroxide content in rat brain. This could be involved in the pathophysiological mechanisms responsible for the brain damage observed in patients affected by Canavan Disease, in which N-acetylaspartic acid accumulation is the biochemical hallmark.

Lista de Abreviaturas

CAT	catalase
SNC	Sistema Nervoso Central
ER	Espécies Reativas
ERN	Espécies Reativas de Nitrogênio
ERO	Espécies Reativas de Oxigênio
GPx	glutationa peroxidase
G6PD	glicose 6-fosfato desidrogenase
GS	glutationa sintetase
GSH	glutationa reduzida
H_2O_2	peróxido de hidrogênio
LCR	líquido cefalorraquidiano
NAA	ácido N-acetilaspártico
NAAG	ácido N-acetilaspartilglutâmico
$NO^{\bullet}$	óxido nítrico
$O_2^{\bullet-}$	radical superóxido
$^1O_2$	oxigênio *singlet*
$OH^{\bullet}$	radical hidroxila
$ONOO^-$	peroxinitrito
ONOOH	ácido peroxinitroso
5-OP	5-oxoprolina
$RO^{\bullet}$	radical alcoxila
$RO_2^{\bullet}$	radical peroxila
SH	tiol
SS	dissulfeto
SOD	superóxido dismutase
TAR	reatividade antioxidante total
TBA-RS	substâncias reativas ao ácido tiobarbitúrico
TRAP	potencial antioxidante total

INTRODUÇÃO

1.1 – 5-Oxoprolina

1.1.1 – Papel Fisiológico

A 5-oxoprolina (5-OP), também conhecida como ácido L-piroglutâmico ou ácido pirrolidona carboxílico, é uma molécula endógena que participa do ciclo γ–glutamil, o qual por sua vez está relacionado à síntese e degradação de glutationa reduzida (GSH) e também ao transporte intracelular de aminoácidos livres (Meister, 1991) (Figura 1).

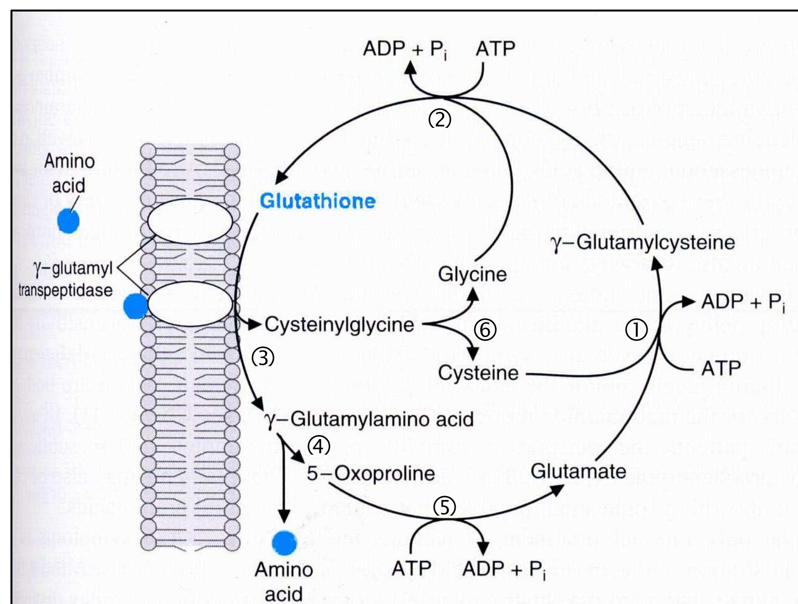

Figura 1. Ciclo γ-glutamil. Enzimas envolvidas: ① = γ-glutamilcisteína sintetase; ② = glutationa sintetase; ③ = γ-glutamil transpeptidase; ④ = γ-glutamil ciclotransferase; ⑤ = 5-oxoprolinase; ⑥ = dipeptidase. Fonte: Marks *et al.*, 1996.

Nesse ciclo, a biossíntese da GSH é catalisada pela ação consecutiva das enzimas γ-glutamilcisteína sintetase e glutationa sintetase (GS). Através de *feedback* negativo, a GSH inibe a atividade da γ-glutamilcisteína sintetase, enzima marca-passo do ciclo. O passo inicial de degradação da GSH é catalisado pela γ-glutamil transpeptidase, que transfere o

grupo γ-glutamil a um aminoácido. O γ-glutamil aminoácido formado é utilizado pela γ-glutamil ciclotransferase, que catalisa a liberação do resíduo γ-glutamil na forma de 5-OP, a qual é então convertida a glutamato por ação da 5-oxoprolinase. Em humanos, deficiências hereditárias para cinco das seis enzimas do ciclo já foram identificadas, sendo a mais comum delas a deficiência de GS (Larsson e Anderson, 2001; Ristoff e Larsson, 2007).

1.1.2 – Ações Neurotóxicas da 5-OP

Várias ações neurotóxicas foram atribuídas a 5-OP (Rieke *et al.*, 1984; Rieke *et al.*, 1989; Rothstein *et al.*, 1993), cujo acúmulo é o principal marcador bioquímico da deficiência de GS. Sua excitotoxicidade já foi demonstrada; a 5-OP se liga aos receptores glutamatérgicos (Barone e Spignoli, 1990) e também inibe a captação de glutamato por sinaptossomas (Bennet *et al.*, 1973; Dusticier *et al.*, 1985). Além disso, as membranas celulares podem ser um outro sítio de interação da 5-OP, a qual é capaz de inibir a atividade da NA^+,K^+-ATPase, enzima essencial para a função e desenvolvimento cerebrais (Escobedo e Cravioto, 1973; Rieke *et al.*, 1984). A 5-OP pode também promover um dano ao metabolismo energético cerebral, visto que inibe a produção de CO_2 e diminui os níveis de ATP e a síntese de lipídios, além de reduzir a atividade dos complexos I + III e complexo IV da cadeia respiratória, indicando que a cadeia respiratória é bloqueada em pelo menos dois pontos distintos (Silva *et al.*, 2001). A inibição da síntese de lipídios, por sua vez, pode vir a ser danosa ao sistema nervoso central (SNC), visto que prejudicaria a sinaptogênese, a mielinização e outros eventos, dependentes de lipídios, necessários para um desenvolvimento cerebral normal (Silva *et al.*, 2001).

Ainda, a infusão intracerebral aguda e crônica de 5-OP produz lesões neuronais

dose-dependentes em estriado, hipocampo, córtex cerebral e cerebelo de ratos, além de provocar alterações comportamentais, assimetria postural, ataxia, descoordenação motora, tremor e discinesias (Rieke *et al.*, 1984; Rieke *et al.*, 1989).

1.1.3 – Deficiência de GS

Descrita pela primeira vez por Jellum e colaboradores em 1970, essa desordem hereditária caracteriza-se pela presença de altos níveis de 5-OP no líquido cefalorraquidiano (LCR), sangue e outros tecidos dos pacientes afetados, juntamente com a elevada excreção urinária da mesma, sendo essa condição denominada 5-oxoprolinúria ou acidúria piroglutâmica (Larsson *et al.*, 1985; Larsson e Anderson, 2001; Njalsson, 2005).

Até o presente momento, foram descritos aproximadamente 70 pacientes com deficiência de GS (MIM 266130) (Njalsson, 2005; Ristoff e Larsson, 2007). O padrão de herança é autossômico recessivo, e a doença é monogênica; porém, mais de vinte mutações diferentes já foram identificadas, o que pode explicar a heterogeneidade fenotípica encontrada. Muitas mutações parecem afetar a capacidade de ligação do ligante ou a catálise, enquanto outras provavelmente afetam a dimerização ou o enovelamento protéico (Ristoff e Larsson, 1998; Polekhina *et al.*, 1999; Ristoff, 2002). As mutações que afetam a capacidade catalítica da GS podem fazê-lo por diminuir a afinidade pelo substrato, a velocidade máxima ou a estabilidade da enzima (Njalsson *et al.*, 2000). Supõe-se que a forma mais branda da doença seja devida a uma mutação que afeta primariamente a estabilidade da enzima, enquanto que a forma severa seja devida a mutações que afetam as propriedades catalíticas da enzima (Spielberg *et al.*, 1978). Independente do tipo de mutação, todos os pacientes apresentam algum grau de atividade enzimática residual (1-30%) (Dahl *et al.*, 1997; Ristoff e Larsson, 1998; Ristoff *et al.*, 2000).

1.1.3.1 – Manifestações Clínicas

De uma forma geral, a deficiência de GS é clinicamente caracterizada por anemia hemolítica e acidose metabólica severa; além disso, quase metade dos pacientes também desenvolve sintomas neurológicos progressivos, incluindo convulsões, retardo mental, ataxia, retardo psicomotor, espasticidade e grau variado de psicose, entre outros (Robertson *et al.*, 1991; Larsson e Anderson, 2001; Ristoff *et al.*, 2001). Aproximadamente 25% dos pacientes morrem no período neonatal (Njalsson e Norgren, 2005). O fenótipo clínico dos pacientes sobreviventes é bastante variado, podendo se apresentar de forma leve, moderada ou severa (Ristoff, 2002). A 5-oxoprolinúria ocorre em todos os pacientes, porém é mais pronunciada nos pacientes mais severamente afetados (Njalsson, 2005).

Na doença, a acidose metabólica é devida a uma diminuição na inibição por *feedback* da enzima γ–glutamilcisteína sintetase no ciclo γ-glutamil, o que levará ao final à superprodução e acúmulo de 5-OP, causando acidose metabólica (Larsson e Anderson, 2001; Ristoff, 2002).

Infecções bacterianas recorrentes também acometem esses pacientes, sendo atribuídas provavelmente a uma função granulocítica deficiente (Ristoff *et al.*, 2001). A administração de Vitamina E normaliza a função de leucócitos polimorfonucleares (granulócitos) em pacientes com deficiência de GS sem, no entanto, normalizar os níveis de GSH, sendo recomendada sua suplementação para evitar a ocorrência de infecções freqüentes nesses pacientes (Boxer *et al.*, 1979).

1.1.3.2 – Alterações Bioquímicas e Neuropatológicas

As alterações bioquímicas observadas por Marstein *et al.* (1981) em órgãos

provenientes da autópsia de um paciente com deficiência de GS incluem uma redução marcante na atividade da GS e altos níveis de 5-OP em todos os tecidos analisados, particularmente nos rins e no cérebro. O conteúdo de glutationa se encontrava muito reduzido em todas as regiões cerebrais. O conteúdo de glutamato, neurotransmissor excitatório das células granulosas, estava fortemente diminuído no córtex cerebelar, bem como o conteúdo de ácido γ-aminobutírico, refletindo provavelmente a perda de células de Purkinje, neurônios cerebelares eferentes que utilizam ácido γ-aminobutírico como seu neurotransmissor inibitório (Marstein *et al.*, 1981).

Dentre os achados neuropatológicos da deficiência de GS estão a atrofia seletiva da camada celular granulosa do cerebelo, lesões focais no córtex frontoparietal e lesões bilaterais no córtex visual e tálamo, observando-se perda neuronal e astrocitose no córtex visual (Skullerud *et al.*, 1980; Marstein *et al.*, 1981). Em pacientes que morrem no período neonatal é possível observar uma atrofia cerebelar generalizada, desmielinização e necrose cerebral (Ristoff, 2002). Cabe aqui ressaltar que as lesões cerebrais desses pacientes são bastante similares àquelas vistas após intoxicação por mercúrio, que afeta principalmente o cerebelo e algumas regiões do córtex cerebral (Skullerud, 1980; Marstein *et al.*, 1981). Nessa condição, os neurônios são presumivelmente danificados por alterações oxidativas resultantes da ação quelante do mercúrio em grupos sulfidrílicos (–SH) vitais. Assim, é possível que na deficiência de GS perdas neuronais similares possam ocorrer devido à ineficiente manutenção dos grupos –SH em sua forma reduzida (Marstein *et al.*, 1981). No entanto, o mecanismo de dano cerebral da deficiência de GS permanece ainda pouco esclarecido.

1.1.3.3 – Diagnóstico

O diagnóstico precoce de desordens metabólicas é fundamental para proporcionar uma melhora no quadro clínico dos pacientes. Geralmente suspeita-se de deficiência de GS em recém-nascidos que apresentam um quadro de anemia hemolítica juntamente com acidose metabólica. Nesses casos, o diagnóstico consiste na detecção de baixa atividade enzimática da GS e baixos níveis de GSH no sangue e/ou cultura de fibroblastos do paciente, bem como de altos níveis de 5-OP no sangue e na urina, devido à elevada excreção urinária da mesma. A 5-OP pode ser detectada através de cromatografia gasosa acoplada à espectrometria de massas (CG-EM) (Hoffmann *et al.*, 1989). Pode-se detectar também a presença de mutações no gene da GS através de técnicas de biologia molecular.

O diagnóstico pré-natal pode ser realizado através da análise de mutações em vilosidade coriônica, através da análise da 5-OP em líquido amniótico (Erasmus *et al.*, 1993; Manning *et al.*, 1994), ou ainda através da análise da atividade enzimática da GS em cultura de amniócitos ou de vilosidade coriônica.

1.1.3.4 – Tratamento

Atualmente, o tratamento primário consiste apenas na correção da acidose metabólica. Como os pacientes apresentam baixos níveis de glutationa, acredita-se que estejam mais suscetíveis ao estresse oxidativo e, por isso, tem-se sugerido a utilização de antioxidantes na terapêutica dessas desordens hereditárias do ciclo γ–glutamil (Jain *et al.*, 1994; Ristoff, 2002).

1.2 – Ácido N-acetilaspártico

1.2.1 – Papel Fisiológico

O ácido N-acetilaspártico (NAA) é um dos derivados de aminoácido mais abundantes do SNC de mamíferos, estando presente em concentrações cerebrais de até 20 mM (Baslow, 2003; Harte *et al.*, 2005). O NAA é normalmente sintetizado a partir de acetil-CoA e ácido L-aspártico por ação da enzima acetil-CoA-L-aspartato N-acetiltransferase, sendo hidrolizado a aspartato e acetato por ação da enzima aspartoacilase (Figura 2) (Beaudet, 2001).

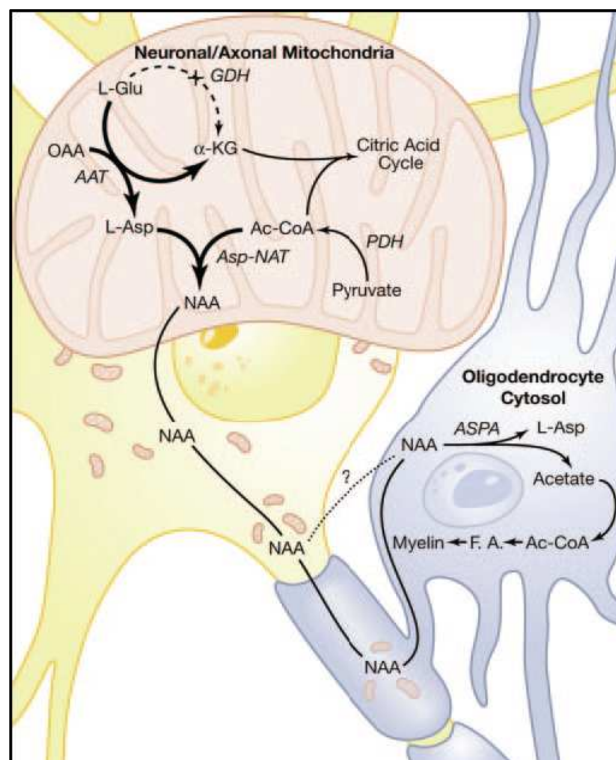

Figura 2. Síntese e degradação do ácido N-acetilaspártico. Fonte: Madhavarao *et al.*, 2005.

Apesar de sua alta concentração, o papel do NAA no metabolismo cerebral permanece ainda pouco esclarecido (Benarroch, 2008). No entanto, várias hipóteses têm sido recentemente lançadas na literatura. Dentre as possíveis funções do NAA, especula-se

que o mesmo possa apresentar um papel importante na mielinogênese, pela possibilidade de atuar como uma fonte de grupos acetil, que serão posteriormente incorporados em lipídios cerebrais (Chakraborty *et al*., 2001; Kirmani *et al*., 2002; Madhavarao *et al*., 2005; Namboodiri *et al*., 2006). Além disso, supõe-se que o NAA possa atuar também como um osmólito intracelular (Baslow, 2002); como uma forma de armazeamento de aspartato (Beaudet, 2001) ou de glutamato (Clark *et al*., 2006); e como um carreador para a remoção do excesso de nitrogênio do cérebro (Moffett *et al*., 2007).

Ainda, especula-se que uma das funções mais importantes do NAA seja a de atuar como um precursor imediato da biossíntese enzimática de N-acetilaspartilglutamato (NAAG), um dos neuropeptídeos mais abundantes do tecido nervoso de mamíferos (Gehl *et al*., 2004; Arun *et al*., 2006; Moffett *et al*., 2007), alcançando concentrações cerebrais de até 2,7 mM (Coyle, 1997; Pouwels e Frahm, 1997). O NAAG é sintetizado a partir de NAA e glutamato, havendo a produção de aproximadamente uma molécula de NAAG para cada molécula de NAA sintetizada; sob condições de *steady-state,* a relação NAA:NAAG é mantida na proporção 10:1 (Baslow e Guilfoyle, 2006). Como um composto neuroativo, o NAAG pode agir primariamente como um agonista de receptores metabotrópicos glutamatérgicos do tipo II (mGluRII), especificamente mGluR3, pré-sinápticos, levando à inibição da liberação de glutamato na fenda sináptica (Wroblewska *et al*., 1997; Benarroch, 2008); em concentrações mais altas o NAAG é um fraco agonista de receptores N-metil-D-aspartato (NMDA) (Neale, 2000; Pliss *et al*., 2000; Shave *et al*., 2001; Zhao *et al*., 2001). O NAAG pode ser hidrolizado pela enzima dipeptidase ácida α–ligada N-acetilada, produzindo glutamato e regenerando NAA (Thomas *et al*., 2000; Benarroch *et al*., 2008).

Concentrações aumentadas de NAA na urina, sangue, LCR e cérebro dos pacientes

afetados, juntamente com um aumento das concentrações de NAAG, ocorrem na Doença de Canavan, uma leucodistrofia severa e progressiva caracterizada por edema cerebral e degeneração espongiforme da substância branca (Matalon e Michals-Matalon, 2000).

1.2.2 – Ações Neurotóxicas do NAA e do NAAG

O papel do NAA na patogênese da Doença de Canavan é ainda pouco esclarecido. No entanto, a presença de concentrações aumentadas desse ácido orgânico no cérebro dos pacientes afetados por essa doença sugere a possibilidade de que o NAA ou algum metabólito relacionado possa exercer efeitos tóxicos (Beaudet, 2001).

De fato, achados recentes sugerem que o metabolismo do NAA na Doença de Canavan encontra-se alterado também na retina neural, o que pode estar relacionado à neuropatia óptica observada nessa doença (Baslow, 2003; George *et al*., 2004). No cérebro, ações neurotóxicas do NAA também foram demonstradas. A administração intracerebroventricular de NAA a ratos normais se mostrou capaz de induzir convulsões, provavelmente por uma excitação excessiva através de receptores metabotrópicos glutamatérgicos (Akimitsu *et al*., 2000; Kitada *et al*., 2000; Yan *et al*., 2003). O NAA também é capaz de aumentar a concentração intracelular de cálcio em cultura de células (Rubin *et al*., 1995). O NAAG, por sua vez, também já mostrou ações neurotóxicas no cérebro, tanto *in vitro* (Thomas *et al*., 2000) quanto *in vivo* (Pliss *et al*., 2000; Pliss *et al*., 2002; Pliss *et al*., 2003; Bubeníková-Valesová *et al*., 2006), incluindo a indução de neurodegeneração, alteração comportamental e clivagem do DNA neuronal.

1.2.3 – Doença de Canavan

A Doença de Canavan, descrita pela primeira vez em 1931 por Myrtelle Canavan, é

uma doença hereditária autossômica recessiva causada pela deficiência da enzima aspartoacilase (MIM 271900) (Matalon e Michals-Matalon, 2000), sendo mais freqüente entre a população de Judeus Ashkenazi (Matalon *et al.*, 1995a). Nessa população, a freqüência de portadores pode variar entre 1:37 e 1:40 (Matalon e Michals-Matalon, 1999; Leone *et al.*, 2000) e a incidência da doença é estimada em aproximadamente 1:6000 nascidos vivos (Matalon e Michals-Matalon, 1999; Gordon, 2000).

1.2.3.1 – Manifestações Clínicas

A Doença de Canavan é clinicamente caracterizada por retardo mental severo e progressivo, com incapacidade de aquisição de funções motoras normais durante o desenvolvimento. Apesar dos pacientes afetados geralmente parecerem normais no primeiro mês de vida, podem exibir fixação visual deficitária, irritabilidade e apatia já ao nascimento (Traeger e Rapin, 1998). Após 5-6 meses de vida, os pacientes desenvolvem também hipotonia e macrocefalia, com dificuldade de sustentar a cabeça (Matalon e Michals-Matalon, 2000; Matalon *et al.*, 2006). Esses sintomas progridem posteriormente para hipertonicidade com paralisia pseudobulbar; convulsões geralmente tônico-clônicas ocorrem em aproximadamente metade dos pacientes (Traeger e Rapin, 1998; Beaudet, 2001). Freqüentemente, os pacientes apresentam neuropatia óptica e atrofia óptica (Matalon e Michals-Matalon, 1999; Surendran *et al.*, 2003). Os pacientes afetados se tornam cada vez mais debilitados com o passar do tempo, geralmente com incapacidade de se mover voluntariamente; não desenvolvem a habilidade de andar, sentar, caminhar e falar (Surendran *et al.*, 2003). A morte tipicamente ocorre antes da adolescência, mas alguns pacientes com formas mais leves da doença podem sobreviver até seus 20 anos (Moffett *et al.*, 2007).

A Doença de Canavan é monogênica, com padrão de herança autossômico recessivo. O fenótipo é variável, de acordo com o tipo de mutação envolvida; mutações que desfaçam a conformação do sítio ativo da aspartoacilase resultarão em quase total perda de atividade e, conseqüentemente, em um fenótipo mais severo (Surendran *et al*., 2003). Enquanto apenas duas mutações são a base molecular da Doença de Canavan em 98% dos pacientes Judeus Ashkenazi, uma ampla variedade de mutações pode ser encontrada em pacientes não-judeus (Matalon e Michals-Matalon, 1999; Namboodiri *et al*., 2006).

1.2.3.2 – Alterações Bioquímicas e Neuropatológicas

Apesar de o marcador bioquímico clássico da Doença de Canavan ser o aumento das concentrações de NAA em plasma, urina, LCR e cérebro dos pacientes afetados (Tsai e Coyle, 1995; Blüml, 1999; Surendran *et al*., 2003), o NAAG também pode ser encontrado em concentrações elevadas na urina e no LCR dos pacientes afetados (Burlina *et al*., 1999; Krawczyk e Gradowska, 2003; Burlina *et al*., 2006).

Dentre as alterações neuropatológicas, pode-se observar edema cerebral e degeneração espongiforme da substância branca do cérebro; à medida que a doença progride, o cérebro se torna atrófico e a substância cinzenta é também envolvida (Matalon e Michals-Matalon, 2000). A atrofia cerebral aumenta progressivamente ao longo do tempo em pacientes com Doença de Canavan, juntamente com o aumento progressivo da concentração tecidual de NAA (Janson *et al*., 2006). Pode-se observar edema astrocitário com a presença de mitocôndrias alongadas e distorcidas (Adachi *et al*., 1972), extensiva perda de mielina, edema e vacuolização na substância branca e no tronco cerebral (Kumar *et al*., 2006; Skiranth *et al*., 2007), e um aumento no número de oligodendrócitos e de astrócitos protoplásmicos (Beaudet, 2001).

Até o presente momento existem poucas hipóteses acerca de possíveis mecanismos neuropatológicos envolvidos na Doença de Canavan. Madhavarao e colaboradores (2005) demonstraram um comprometimento na síntese de lipídios de mielina, e sugeriram o envolvimento de uma síntese de mielina deficiente, resultante de uma deficiência de acetato derivado do NAA, na patogênese da doença. Outra hipótese considera o envolvimento do NAA como um osmólito intracelular na patogênese dessa desordem, à medida que o aumento patológico de NAA levaria a um desequilíbrio osmótico com acúmulo de excesso de fluido no cérebro (Baslow, 2002). Apesar das presentes hipóteses, o papel do NAA na patogênese da Doença de Canavan permanece ainda pouco esclarecido.

1.2.3.3 – Diagnóstico

O diagnóstico da Doença de Canavan pode ser confirmado através da detecção de níveis aumentados de NAA na urina, sangue e LCR dos pacientes (Surendran *et al*., 2003). No cérebro, o aumento de NAA pode ser detectado *in vivo* de forma não-invasiva através de ressonância nuclear magnética de prótons (^{1}H-RMN) (Tsai e Coyle, 1995; Blüml, 1999; Gordon, 2000; Harte *et al*., 2005). O diagnóstico de escolha consiste na detecção de altos níveis de NAA na urina (aumento de 10 a 100 vezes) (Surendran *et al*., 2003).

O diagnóstico pré-natal da doença pode ser feito através da medida da concentração de NAA no fluido amniótico, quando o aumento da concentração é expressivo (devendo ser maior que 5 a 10 vezes o normal), ou por análise de DNA, quando as mutações são conhecidas na família (Matalon *et al*., 1995b; Gordon, 2000). No entanto, a diversidade de mutações associadas à Doença de Canavan em pacientes não-judeus limita o uso da análise de DNA para diagnóstico pré-natal nesses pacientes (Namboodiri *et al*., 2006).

1.2.3.4 – Tratamento

Até o presente momento não há nenhum tratamento específico para a Doença de Canavan, sendo atualmente apenas sintomático, devendo-se principalmente controlar a ocorrência de convulsões (Matalon e Michals-Matalon, 1999). Recentemente foi sugerida a suplementação dietária de acetato como adjuvante no tratamento da Doença de Canavan, baseando-se na hipótese de que a alteração na síntese de lipídios por uma produção anormal de acetato no cérebro possa contribuir para a fisiopatologia dessa doença (Namboodiri *et al.*, 2006). A possibilidade de terapia gênica tem sido avaliada, mas se encontra ainda em estágio de estudos preliminares (McPhee *et al.*, 2006; Matalon *et al.*, 2006).

1.3 – Radicais Livres

1.3.1 – Definição

Um radical livre é qualquer espécie capaz de existência independente e que contenha um ou mais elétrons desemparelhados (Southorn e Powis, 1988; Halliwell e Gutteridge, 2007), situação energeticamente instável que confere alta reatividade a essas espécies. Quando um radical livre reage com um não-radical, outro radical livre pode ser formado, desencadeando reações em cadeia de transferência de elétrons (Maxwell, 1995; Boveris, 1998). Em condições fisiológicas do metabolismo celular aeróbio, o oxigênio molecular (O_2) sofre redução tetravalente, resultando na formação de duas moléculas de água (H_2O) (Bergendi *et al.*, 1999). No entanto, aproximadamente 5% do oxigênio utilizado na cadeia respiratória mitocondrial não é completamente reduzido à água, sendo convertido a intermediários reativos como radical superóxido ($O_2^{\bullet-}$), radical hidroxila ($OH^{\bullet}$), e peróxido de hidrogênio (H_2O_2) (Cohen, 1989; Cadenas e Davies, 2000; Turrens, 2003) (Figura 2), o que pode ser exacerbado em condições patológicas (Boveris e Chance, 1973).

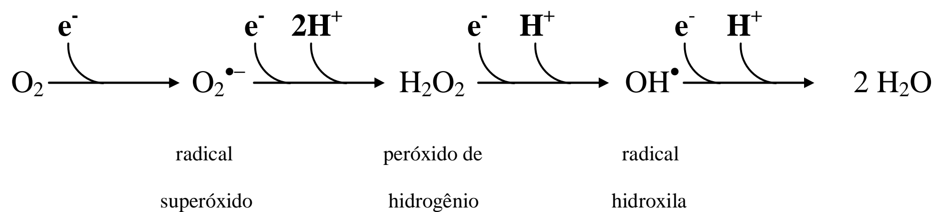

Figura 3. Redução tetravalente do oxigênio molecular (O_2) na mitocôndria até a formação de água. Adaptado de Boveris, 1998.

O termo genérico Espécies Reativas de Oxigênio (ERO) é usado para incluir não só os radicais formados pela redução do O_2 ($O_2^{\bullet-}$ e $OH^{\bullet}$), mas também alguns não-radicais derivados do oxigênio, como o peróxido de hidrogênio (H_2O_2), o oxigênio *singlet* ($^1O_2$), o ácido hipocloroso e o ozônio (Halliwell e Gutteridge, 2007). Além das ERO, existem

diversas outras espécies radicalares, como os radicais de carbonato, de enxofre, de cloreto, de brometo e ainda as Espécies Reativas de Nitrogênio (ERN), sendo as principais representantes o óxido nítrico ($NO^{\bullet}$) e o peroxinitrito ($ONOO^{-}$), formado a partir da reação do $NO^{\bullet}$ com o O_2 ou, mais comumente, a partir da combinação do $NO^{\bullet}$ com o $O_2^{\bullet -}$. De uma forma geral, o termo Espécies Reativas (ER) é usado para englobar todas essas espécies.

1.3.2 – Efeitos das Espécies Reativas (ER) em Sistemas Biológicos

As ER ocorrem tanto em processos fisiológicos quanto patológicos do organismo. Fisiologicamente, as ER apresentam diversas funções, dentre as quais a participação na fagocitose (Bergendi *et al.*, 1999), na síntese e regulação de proteínas, na plasticidade sináptica e na sinalização celular, incluindo vias envolvidas nos processos de crescimento e diferenciação celular (Ward e Peters, 1995; Serrano e Klann, 2004; Valko *et al.*, 2007).

Por outro lado, quando formadas em excesso, as ER têm potencial de oxidar as biomoléculas do organismo (Maxwell, 1995). Diversas ER podem promover diretamente lipoperoxidação na bicamada lipídica (com exceção de H_2O_2, $NO^{\bullet}$ e $O_2^{\bullet -}$), e podem reagir também com proteínas das membranas celulares, alterando as características de fluidez de membrana e levando, assim, à perda de seletividade na troca iônica e à liberação de subprodutos potencialmente tóxicos, como o malondialdeído e o 4-hidroxinonenal (Ferreira e Matsubara, 1997; Halliwell e Whiteman, 2004). Oxidando as proteínas, as ER podem levar à inativação protéica, afetando a função de enzimas, receptores e proteínas de transporte, levando conseqüentemente à alteração da função celular (Stadtman e Levine, 2003; Halliwell e Gutteridge, 2007). Na reação das ER com o DNA e RNA, o dano oxidativo pode causar alteração de bases púricas e pirimídicas, levando a mutações

somáticas e a distúrbios de transcrição (Delanty e Dichter, 1998; Halliwell e Gutteridge, 2007).

As ER apresentam diferentes reatividades; em termos gerais, pode-se dizer que H_2O_2, $NO^{\bullet}$ e $O_2^{\bullet-}$ reagem apenas com algumas poucas biomoléculas, enquanto que $OH^{\bullet}$ reage rapidamente com quase todas as biomoléculas; as demais ER apresentam reatividades intermediárias (Halliwell e Whiteman, 2004). Por não ser muito reativo, o $O_2^{\bullet-}$ promove um dano biológico altamente seletivo à inativação de algumas enzimas e oxidação de poucas moléculas (Halliwell, 2001; Liang e Patel, 2004; Halliwell e Gutteridge, 2007); o dano promovido pelo $O_2^{\bullet-}$ geralmente envolve sua reação com outros radicais, como o $NO^{\bullet}$ (gerando $ONOO^-$), ou geração de outras espécies, como o H_2O_2 (por dismutação espontânea ou catalítica). Este, por sua vez, apesar de ser também pouco reativo, é capaz de gerar $OH^{\bullet}$, altamente reativo, sempre que entrar em contato com íons Cu^{2+} ou Fe^{2+} (Reação de Fenton) (Halliwell, 2001). Ao contrário do $O_2^{\bullet-}$, que não é capaz de iniciar a lipoperoxidação por não ser suficientemente reativo para abstrair hidrogênios de lipídios, o $OH^{\bullet}$ pode reagir com todas as biomoléculas e inclusive iniciar prontamente a lipoperoxidação. O $^1O_2$ é uma forma especialmente reativa do oxigênio capaz de oxidar diversas moléculas, incluindo os lipídios de membrana, gerando com isso hidroperóxidos lipídicos (Bergendi *et al.*, 1999). A partir da reação de radicais centrados em carbono com o O_2, ou ainda a partir da decomposição de peróxidos orgânicos, podem ser formados os radicais peroxila ($RO_2^{\bullet}$) e alcoxila ($RO^{\bullet}$), excelentes agentes oxidantes que podem abstrair hidrogênios de outras moléculas, sendo também importantes na lipoperoxidação (Halliwell e Gutteridge, 2007). O $ONOO^-$, formado principalmente a partir de $NO^{\bullet}$ e $O_2^{\bullet-}$, é capaz de depletar grupos –SH e outros antioxidantes e promover dano oxidativo a lipídios, DNA e proteínas (Halliwell,

2006; Halliwell e Gutteridge, 2007). Em pH fisiológico, o $ONOO^-$ é rapidamente protonado à ácido peroxinitroso (ONOOH), extremamente reativo, capaz de oxidar e nitrar diretamente lipídios, DNA e proteínas (Alvarez e Radi, 2003). O ONOOH pode ainda causar um dano adicional, visto que pode sofrer fissão homolítica gerando $OH^{\bullet}$ (Halliwell, 2006).

1.3.3 – Defesas Antioxidantes

Em condições normais, nosso organismo é protegido contra o dano oxidativo induzido por ER pela atuação de vários antioxidantes, com diferentes funções, que constituem um sistema de defesa tanto independente quanto cooperativo, ou mesmo sinérgico. Por definição, antioxidante é qualquer substância capaz de retardar, prevenir ou remover o dano oxidativo a uma molécula alvo (Halliwell e Gutteridge, 2007). Embora diferindo de tecido para tecido em sua composição, as defesas antioxidantes estão amplamente distribuídas e compreendem principalmente as enzimas antioxidantes, que removem cataliticamente as ER, e os antioxidantes não-enzimáticos, agentes de baixo peso molecular, que são preferencialmente oxidados pelas ER a fim de preservar biomoléculas mais importantes.

1.3.3.1 – Enzimas Antioxidantes

As enzimas de defesa antioxidante operam como um sistema coordenado e equilibrado, e atuam juntamente com outros antioxidantes ditos não-enzimáticos (como α-tocoferol, ácido ascórbico e glutationa) a fim de proteger o organismo da injúria celular causada pelo dano oxidativo.

A superóxido dismutase (SOD) é uma metaloenzima que catalisa a dismutação do

$O_2^{\bullet-}$, formando H_2O_2 e O_2 ($O_2^{\bullet-} + O_2^{\bullet-} + 2H^+ \rightarrow H_2O_2 + O_2$) (Fridovich e McCord, 1969; Bergendi *et al.*, 1999; Halliwell e Gutteridge, 2007). As diferentes formas de SOD presentes em animais compreendem: a CuZnSOD, formada por duas subunidades idênticas contendo um Cu^{2+} e um Zn^{2+} em cada subunidade (Fridovich, 1975), que ocorre no citosol, lisossomos, núcleo celular, espaço mitocondrial intermembranas e peroxissomas (Fridovich, 1975; Fridovich, 1995; Ward e Peters, 1995); a SOD extracelular (EC-SOD), subtipo da CuZnSOD, presente em fluidos extracelulares (Abrahamsson *et al.*, 1992); e a MnSOD, mitocondrial, com 4 subunidades, cada qual contendo um átomo de manganês. A MnSOD mitocondrial é essencial; camundongos knock-out para MnSOD, que apresentam uma redução de 30 a 80% na atividade dessa enzima, apresentam neurodegeneração com morte celular e distúrbios motores severos, não sendo capazes de sobreviver mais do que alguns dias após o nascimento (Melov *et al.*, 1998; Gao *et al.*, 2008).

A dismutação do $O_2^{\bullet-}$ catalisada pelas SOD gera H_2O_2, que será decomposto diretamente a O_2 por ação subseqüente da enzima antioxidante catalase (CAT). O mecanismo de reação da CAT é, assim como o da SOD, essencialmente uma dismutação; uma molécula de H_2O_2 é reduzida a H_2O e a outra é oxidada a O_2 ($2\ H_2O_2 \rightarrow 2\ H_2O + O_2$). A CAT está principalmente localizada nos peroxissomas, que contêm várias enzimas produtoras de H_2O_2, e também no citosol (Chance *et al.*, 1979; Ward e Peters, 1995).

A glutationa peroxidase (GPx) também atua decompondo o H_2O_2, através do acoplamento de sua redução a H_2O com a concomitante oxidação da glutationa reduzida (GSH) ao dissulfeto de glutationa (GSSG) ($H_2O_2 + 2\ GSH \rightarrow GSSG + 2\ H_2O$). A GPx geralmente localiza-se nas membranas celulares e é específica para GSH como doador de hidrogênios, mas pode agir em outros peróxidos que não o H_2O_2, como hidroperóxidos

lipídicos, havendo a redução do grupo peróxido a um álcool, sendo por esse motivo considerada um dos principais sistemas de defesa antioxidante presentes no organismo humano, particularmente eficiente na proteção contra a lipoperoxidação (Wendel, 1981). Existem basicamente dois tipos de GPx: uma que utiliza selênio como cofator (citosólica e mitocondrial); e outra que não depende de selênio (citosólica, responsável pela metabolização de hidroperóxidos orgânicos). A primeira apresenta quatro subunidades, cada uma contendo um átomo de selênio em seu sítio ativo. Daí a importância da ingestão de traços de selênio na dieta, elemento essencial, que irá proporcionar o cofator necessário para a ação desta GPx.

1.3.3.2 – Antioxidantes Não-enzimáticos

Os antioxidantes não-enzimáticos podem ser definidos como substâncias que, em baixas concentrações em relação ao substrato oxidável, retardam ou previnem a oxidação desse substrato, dessa forma protegendo contra a oxidação de biomoléculas por ER (Halliwell e Gutteridge, 2007; Fang et al., 2002). Dentre eles, destacam-se o ácido ascórbico, o α-tocoferol e a GSH.

O ácido ascórbico, também conhecido como Vitamina C, é uma vitamina hidrossolúvel essencial, devendo ser obtida através da dieta por incapacidade de ser sintetizada (Halliwell e Gutteridge, 2007). Está presente em altas concentrações no SNC, em níveis maiores no LCR do que no plasma (Lonnrot et al., 1996). Tem como funções a regeneração do α-tocoferol, e a importante atuação como scavenger de ER, incluindo o $O_2^{\bullet-}$, $RO_2^{\bullet}$, $RO^{\bullet}$, $OH^{\bullet}$, ONOOH, $^1O_2$, além de outras espécies (Halliwell, 2001; Halliwell e Gutteridge, 2007).

Já o α-Tocoferol, por ser lipossolúvel, está presente nas membranas celulares, nas mitocôndrias e em lipoproteínas plasmáticas. Quando reage com uma ER, forma-se o radical tocoferoxil, que pode ser regenerado à α-tocoferol pelo ácido ascórbico ou pela GSH (Sokol, 1989; Ward e Peters, 1995; Halliwell e Gutteridge, 2007). É um antioxidante muito potente capaz de inibir a lipoperoxidação (Valko *et al.*, 2007), visto que seu grupamento fenólico –OH rapidamente reage com $RO_2^{\bullet}$, convertendo-os em radicais tocoferoxila, inibindo com isso a propagação dessa reação em cadeia (Halliwell, 2001).

A GSH, por sua vez, é um tripeptídio formado por glutamato, cisteína e glicina, sendo o principal tiol intracelular de baixo peso molecular presente na maioria das células. É um antioxidante endógeno que atua sinergicamente com o α-tocoferol e com o ácido ascórbico, sendo fundamental para mantê-los nas suas formas reduzidas. Atua também como um cofator enzimático, sendo particularmente importante na reação catalisada pela GPx, enzima que decompõe peróxido de hidrogênio e hidroperóxidos lipídicos. Além disso, desempenha outras funções como a proteção de grupos –SH de proteínas contra oxidação, e a manutenção da comunicação intercelular, além de servir como uma forma atóxica de depósito e armazenamento de cisteína (Meister, 1991; Anderson, 1998; Halliwell e Gutteridge, 2007). A GSH que foi oxidada será posteriormente regenerada às custas de NADPH através da ação da enzima glutationa redutase ($GSSG + NADPH + H^+ \rightarrow 2\ GSH + NADP^+$) (Chance *et al.*, 1979; Ward e Peters, 1995). Com isso garante-se que a glutationa esteja na maior parte em sua forma reduzida, o que é essencial para sua ação como redutora de outras moléculas biológicas.

1.3.4 – Estresse Oxidativo

Em condições normais, a produção de ER é em sua maior parte balanceada pelos sistemas de defesa antioxidante do organismo. No entanto, quando há um desequilíbrio entre a produção de ER e a defesa antioxidante, tem-se a condição de estresse oxidativo. Esse distúrbio no equilíbrio pró-oxidante/antioxidante, com favorecimento do primeiro, pode resultar tanto de uma diminuição das defesas antioxidantes quanto de uma produção aumentada de ER, bem como da liberação de metais de transição ou a combinação de quaisquer desses fatores (Halliwell, 2001). A diminuição dos níveis de antioxidantes pode ocorrer por mutações que afetem as atividades enzimáticas ou toxinas que as depletem, ou ainda por deficiências de minerais e/ou antioxidantes essenciais provenientes da dieta. Já a produção aumentada de ER pode ser devida à exposição a níveis aumentados de O_2 ou à toxinas que sejam elas próprias ER, ou que sejam metabolisadas gerando ER, como o pesticida Paraquat, ou ainda pela excessiva ativação de sistemas fisiológicos de geração de ER, como a hiperativação fagocitária em doenças inflamatórias crônicas (Halliwell e Whiteman, 2004). As conseqüências do estresse oxidativo podem incluir: adaptação (que geralmente resulta em *up-regulation* da síntese de sistemas de defesa antioxidante a fim de restaurar o equilíbrio oxidante/antioxidante); injúria celular (que envolve dano às biomoléculas do organismo, sendo esse dano oxidativo ou por alteração nos níveis de íons ou por ativação de proteases), que pode gerar produtos altamente neurotóxicos como o malondialdeído e o 4-hidroxinonenal; ou ainda morte celular por necrose ou apoptose (Halliwell e Gutteridge, 2007).

1.3.4.1 – Estresse Oxidativo e Doenças Neurodegenerativas

O estresse oxidativo pode ser extremamente danoso ao SNC; um dano às proteínas do citoesqueleto dos neurônios, por exemplo, pode levar à disfunção neuronal, podendo inclusive chegar à degeneração axonal e morte neuronal (Halliwell e Gutteridge, 2007). Esse potencial exacerbado de dano oxidativo ao cérebro deve-se à particular susceptibilidade do mesmo ao estresse oxidativo, especialmente devido: ao seu elevado consumo de oxigênio, já que processa uma grande quantidade de O_2 por unidade de massa tecidual; ao seu alto conteúdo de ferro, que pode favorecer a lipoperoxidação; ao seu alto conteúdo lipídico, principalmente lipídios de cadeia lateral altamente poliinsaturada, que são extremamente suscetíveis à lipoperoxidação; à sua modesta defesa antioxidante, sendo os níveis de catalase particularmente baixos em muitas regiões cerebrais; alta taxa de fluxo de cálcio através das membranas neuronais; e a presença de neurotransmissores auto-oxidáveis, entre outros fatores (Halliwell, 1996; Halliwell e Gutteridge, 2007).

De fato, as ER e o estresse oxidativo parecem estar envolvidos na patogênese dos danos neurológicos de várias doenças neurodegenerativas, como as doenças de Alzheimer, Parkinson e Huntington, a esclerose múltipla e a esclerose lateral amiotrófica, doenças cerebrovasculares, epilepsia, desmielinização e demência, entre outras (Reznick e Packer, 1993; Maxwell, 1995; Delanty e Dichter, 1998; Emerit *et al.*, 2004; Halliwell e Gutteridge, 2007). Ainda, diversos estudos realizados em nosso grupo de pesquisa revelaram que o estresse oxidativo parece estar aumentado em modelos experimentais químicos de alguns erros inatos do metabolismo, como a hiperprolinemia tipo II (Delwing *et al.*, 2003), a hiperargininemia (Wyse *et al.*, 2001), a fenilcetonúria (Kienzle-Hagen *et al.*, 2002) e a homocistinúria (Matté *et al.*, 2004), e também em um modelo experimental knock-out de acidemia glutárica tipo I (Latini *et al.*, 2007). Porém, existem ainda incertezas e

controvérsias no que diz respeito ao estresse oxidativo ser a causa ou a conseqüência das doenças nas quais está envolvido (Halliwell, 1994).

Experimentalmente, a medida de parâmetros de estresse oxidativo pode incluir:

* A avaliação das defesas antioxidantes não-enzimáticas, através das medidas do Potencial Antioxidante Total (TRAP) e da Reatividade Antioxidante Total (TAR), que avaliam, respectivamente, a quantidade e a reatividade dos antioxidantes não-enzimáticos presentes no tecido, e também através das medidas do conteúdo de antioxidantes não-enzimáticos específicos, como ácido ascórbico e glutationa;

* A avaliação das defesas antioxidantes enzimáticas, através da medida das atividades das enzimas antioxidantes catalase (CAT), superóxido dismutase (SOD) e glutationa peroxidase (GPx), e também através da medida da atividade da enzima glicose-6 fosfato desidrogenase (G6PD), que é a principal fonte celular de NADPH;

* A avaliação de dano oxidativo a lipídios, através das medidas de quimiluminescência espontânea e de Substâncias Reativas ao Ácido Tiobarbitúrico (TBA-RS);

* A avaliação de dano oxidativo protéico, através das medidas do conteúdo de carbonilas e de tióis totais;

* A avaliação do *status* redox celular, através da medida do conteúdo de tióis (SH), dissulfetos (SS), e da razão SH/SS; e

* A avaliação da formação de espécies reativas, através das medidas de fluorescência da diclorofluoresceína (DCF) e do conteúdo de peróxido de hidrogênio.

OBJETIVOS

O objetivo geral deste trabalho foi investigar o possível papel do estresse oxidativo na neurotoxicidade da 5-oxoprolina e do ácido N-acetilaspártico, bem como de seu metabólito, o ácido N-acetilaspartilglutâmico, através do estudo dos efeitos *in vitro* e *in vivo* desses ácidos orgânicos sobre diversos parâmetros de estresse oxidativo em cérebro de ratos, a fim de avaliar o envolvimento de ER nos mecanismos responsáveis pela disfunção neurológica observada nos pacientes afetados pela deficiência de GS, na qual se encontra acumulada a 5-oxoprolina, e pela Doença de Canavan, na qual se encontram acumulados os ácidos N-acetilaspártico e N-acetilaspartilglutâmico.

Os objetivos específicos deste trabalho foram:

* Estudar os efeitos *in vitro* da 5-oxoprolina sobre os conteúdos de carbonilas e de tióis totais, para avaliar o dano oxidativo protéico, e sobre a fluorescência da diclorofluoresceína (DCF), para avaliar a formação de espécies reativas, em córtex cerebral e cerebelo de ratos de 14 dias de vida.

* Estudar os efeitos da administração aguda de 5-oxoprolina em córtex cerebral e cerebelo de ratos de 14 dias de vida sobre o Potencial Antioxidante Total (TRAP), e os conteúdos de glutationa reduzida (GSH) e ácido ascórbico, para avaliar as defesas antioxidantes não-enzimáticas do tecido; a quimiluminescência espontânea e os níveis de Substâncias Reativas ao Ácido Tiobarbitúrico (TBA-RS), ambos parâmetros de lipoperoxidação; o conteúdo de carbonilas; o conteúdo de peróxido de hidrogênio; o

conteúdo de tióis e dissulfetos, para avaliar a razão SH/SS; a medida da atividade das enzimas antioxidantes catalase (CAT), superóxido dismutase (SOD) e glutationa peroxidase (GPx), para acessar as defesas antioxidantes enzimáticas do tecido; e a medida da atividade da enzima glicose 6-fosfato desidrogenase (G6PD), para avaliar a principal fonte celular de NADPH (via da pentose fosfato).

* Estudar os efeitos *in vitro* e *in vivo* do ácido N-acetilaspártico sobre TRAP, TAR e conteúdo de GSH; quimiluminescência espontânea e TBA-RS; conteúdos de tióis totais e de carbonilas; conteúdo de peróxido de hidrogênio; atividades das enzimas antioxidantes CAT, SOD e GPx; e atividade da G6PD em córtex cerebral de ratos de 14 e 30 dias de vida.

* Estudar os efeitos *in vitro* e *in vivo* do ácido N-acetilaspartilglutâmico sobre TRAP e conteúdo de GSH; quimiluminescência espontânea e TBA-RS; conteúdo de carbonilas; atividades das enzimas antioxidantes CAT, SOD e GPx; e atividade da enzima G6PD em córtex cerebral de ratos de 14 e 30 dias de vida.

PARTE II

Capítulo I

*5-oxoproline reduces non-enzymatic antioxidant defenses in vitro in rat brain**

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* Nesse capítulo, os resultados referentes à essa tese de doutorado são apenas os demonstrados nas Figuras 3, 4 e 5 desse artigo.

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ORIGINAL PAPER

5-Oxoproline Reduces Non-Enzymatic Antioxidant Defenses *in vitro* in Rat Brain

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Abstract 5-Oxoproline (pyroglutamic acid) accumulates in glutathione synthetase deficiency, an inborn metabolic defect of the γ-glutamyl cycle. This disorder is clinically characterized by hemolytic anemia, metabolic acidosis and severe neurological disorders. Considering that the mechanisms of brain damage in this disease are poorly known, in the present study we investigated whether oxidative stress is elicited by 5-oxoproline. The *in vitro* effect of (0.5–3.0 mM) 5-oxoproline was studied on various parameters of oxidative stress, such as total radical-trapping antioxidant potential, total antioxidant reactivity, chemiluminescence, thiobarbituric acid-reactive substances, sulfhydryl content, carbonyl content, and 2′,7′-dichlorofluorescein fluorescence, as well as on the activities of the antioxidant enzymes catalase, superoxide dismutase and glutathione peroxidase in cerebral cortex and cerebellum of 14-day-old rats. Total radical-trapping antioxidant potential and total antioxidant reactivity were significantly reduced in both cerebral structures. Carbonyl content and 2′,7′-dichlorofluorescein fluorescence were significantly enhanced, while sulfhydryl content was significantly diminished. In contrast, chemiluminescence and thiobarbituric acid-reactive substances were not affected by 5-oxoproline. The activities of catalase, superoxide dismutase and glutathione peroxidase were also not altered by 5-oxoproline. These results indicate that 5-oxoproline causes protein oxidation and reactive species production and decrease the non-enzymatic antioxidant defenses in rat brain, but does not cause lipid peroxidation. Taken together, it may be presumed that 5-oxoproline elicits oxidative stress that may represent a pathophysiological mechanism in the disorder in which this metabolite accumulates.

Keywords 5-Oxoproline · Pyroglutamic acid · Glutathione synthetase deficiency · Rat brain · Oxidative stress · Antioxidant defenses

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Introduction

5-Oxoproline (5-OP), also known as pyroglutamic acid, is an endogenous molecule derived from L-glutamate, being a major intermediate in the γ-glutamyl cycle. This cycle is necessary to the synthesis and breakdown of glutathione (GSH) and also to the intracellular transport of free amino acids (Meister, 1991).

High levels of 5-OP in cerebrospinal fluid, blood and urine are characteristically seen in glutathione synthetase (GS) deficiency, an inborn metabolic defect of the γ-glutamyl cycle (Jellum et al., 1970; Larsson et al., 1985; Larsson and Anderson, 2001; Njalsson, 2005). About 70 patients have been described worldwide with GS deficiency (MIM 266130) (Njalsson, 2005). This autosomal recessive inherited disorder is clinically characterized by hemolytic anemia and metabolic acidosis; moreover, almost half of the patients also develop progressive neurological symptoms, including seizures, mental retardation, failure to thrive, ataxia, psychomotor delay and spasticity (Larsson and Anderson, 2001; Ristoff et al., 2001). 5-Oxoprolinuria appears in all patients, but is more pronounced in those moderately and severely affected (Njalsson, 2005). Approximately 25% of patients with hereditary GS deficiency die during childhood (Njalsson and Norgren, 2005). The neuropathological findings of GS deficiency include selective atrophy of the granule cell layer of the cerebellum, focal lesions in frontoparietal cortex and bilateral lesions in visual cortex and (Skullerud et al., 1980; Marstein et al., 1981). The mechanisms of brain damage in this disease remain not fully established. However, 5-OP was already shown to be neurotoxic (Rieke et al., 1984; Rieke et al., 1989). This organic acid was shown to promote excitotoxicity (Bennet Jr et al., 1973; Dusticier et al., 1985; Barone and Spignoli, 1990) and inhibit brain energy metabolism (Escobedo and Cravioto, 1973; Silva et al., 2001). In addition, chronic intrastriatal infusion of 5-OP produced selective neuron sparing lesions in the rat striatum (Rieke et al., 1984), which has been assumed to be due to glutamate-induced excitotoxic damage (Rothstein et al., 1993).

In the present study we investigated the possible role of oxidative stress in 5-OP neurotoxicity in order to clarify its participation in the brain damage mechanisms responsible for the neurological impairment observed in GS-deficient patients. To accomplish that, the *in vitro* effect of 5-OP was studied on the following oxidative stress parameters in cerebral cortex and cerebellum of 14-day-old rats: total radical-trapping antioxidant potential (TRAP) and total antioxidant reactivity (TAR), to evaluate non-enzymatic antioxidant defenses; chemiluminescence and thiobarbituric acid-reactive substances (TBA-RS), to access lipid peroxidation; sulfhydryl and carbonyl contents, to evaluate protein oxidation; dichlorofluorescein (DCF) fluorescence assay, to measure RS production; and activities of the antioxidant enzymes catalase (CAT), superoxide dismutase (SOD) and glutathione peroxidase (GPx), to access enzymatic antioxidant defenses.

Experimental procedures

Materials

All chemicals were purchased from Sigma (St. Louis, MO, USA) except thiobarbituric acid, which was purchased from Merck (Darmstadt, Germany) and 2,2′-azo-bis-(2-amidinopropane) that was purchased from Wako Chemicals (USA). 5-OP solutions were prepared on the day of the experiment in 20 mM sodium phosphate buffer, pH 7.4 containing 140 mM KCl. The pH was adjusted when necessary. The acid was added to homogenates at final concentrations of 0.5, 1.0 or 3.0 mM.

Animals

Fourteen-day-old Wistar rats bred in the Department of Biochemistry, ICBS, UFRGS, were used. Sixty-four rats were used in the experiments. Eight rats were kept with dams per cage until they were sacrificed. The dams had free access to water and a 20% (w/w) protein commercial chow (Supra, Porto Alegre, RS, Brazil). They were kept in a room with 12:12 h light/dark cycle (lights on 07:00–19:00 h) and with air-conditioned controlled temperature ($22 \pm 1°C$). The NIH 'Guide for the Care and Use of Laboratory Animals' (NIH publication No. 80-23, revised 1996) were followed in all experiments.

Tissue preparation and incubation

Rats were sacrificed by decapitation, and the brain was immediately removed and kept on an ice-plate. The olfactory bulb, pons and medulla were discarded and both cerebral cortex and cerebellum were dissected, weighed and kept chilled until homogenization. These procedures lasted up to 3 min. Samples were not pooled except for measuring chemiluminescence (where 3 rats were pooled). Cerebral cortex and cerebellum were homogenized in 10 volumes (1:10, w/v) of 20 mM sodium phosphate buffer, pH 7.4 containing 140 mM KCl. Homogenates were centrifuged at 750*g* for 10 min at 4°C to discard nuclei and cell debris (Llesuy et al., 1985; Lissi et al., 1986). The pellet was discarded and the supernatant was immediately separated and used for the measurements.

Cerebral cortex and cerebellum supernatants were pre-incubated for 1 h at 37°C in the presence of 5-OP at final concentrations ranging from 0.5 to 3.0 mM. Controls were incubated only with the 20 mM sodium phosphate buffer, pH 7.4 containing 140 mM KCl, without 5-OP. After incubation, aliquots were taken to measure TRAP, TAR, chemiluminescence, TBA-RS, sulfhydryl content, carbonyl content, and DCF fluorescence. To test the action of 5-OP on the antioxidant enzymes CAT, SOD and GPx, the organic acid was added to the tissue homogenates at the time of assay of each enzyme activity without previous incubation.

Total radical-trapping antioxidant potential (TRAP)

TRAP was determined by measuring the chemiluminescence intensity of luminol induced by 2,2′-azo-bis-(2-amidinopropane) (ABAP) thermolysis (Lissi et al., 1992; Evelson et al., 2001) in a Wallac 1409 Scintillation Counter. The initial chemiluminescence value was obtained by adding 3 mL of ABAP 10 mM, dissolved in 50 mM sodium phosphate buffer pH 7.4, plus 10 μL of luminol (5.6 mM) to a glass scintillation vial. The 10 μL of 160 μM Trolox (water-soluble α-tocopherol analogue, used as standard) or 10 μL of tissue supernatant were then added to that vial, producing a decrease in the initial chemiluminescence value. This value is kept low until the antioxidants present in the sample are depleted, then chemiluminescence returns to its initial value. The time taken by the sample to keep chemiluminescence low is called induction time and is directly proportional to the antioxidant capacity of the tissue, so TRAP represents the amount (quantity) of non-enzymatic antioxidants present in the sample. The induction time of the sample was compared to that of Trolox. Results were reported as nmol Trolox/mg protein.

Total antioxidant reactivity (TAR)

TAR was determined by measuring the luminol chemiluminescence intensity induced by ABAP thermolysis (Lissi et al., 1995) using a Wallac 1409 Scintillation Counter. The

background chemiluminescence was measured by adding 4 mL of 2 mM ABAP, prepared in 0.1 mM glycine buffer, pH 8.6, plus 15 μL of luminol (4 mM) into a glass scintillation vial. This was considered to be the basal value. The 10 μL of 20 μM Trolox (used as a standard) or tissue supernatant was then added and the chemiluminescence was measured during 60 s to evaluate how fast it falls. The reduction in luminol intensity is considered a measure of TAR capacity, which reflects the tissue capacity to react in front of an enhanced free radical production. TAR represents not the amount but the reactivity (quality) of non-enzymatic antioxidants present in the sample. The results were reported as nmol Trolox/mg protein.

Chemiluminescence

Samples of three rats were pooled and assayed for chemiluminescence in a dark room (Lissi et al., 1986) using a beta liquid scintillation spectrometer Tri-Carb 2100TR. Incubation flasks contained 3.5 mL of the same buffer used for homogenization. The background chemiluminescence was measured for 5 min. An aliquot of 0.5 mL of supernatant was added and chemiluminescence was measured for 10 min at room temperature. The background chemiluminescence was subtracted from the total value. Chemiluminescence was calculated as cps/mg protein.

Thiobarbituric acid-reactive substances (TBA-RS)

TBA-RS was measured according to Esterbauer and Cheeseman (1990). Briefly, 300 μL of cold 10% trichloroacetic acid were added to 150 μL of supernatant and centrifuged at 300*g* for 10 min. Three hundred μL of the supernatant were transfered to a Pyrex® tube and incubated with 300 μL of 0.67% thiobarbituric acid in 7.1% sodium sulfate in a boiling water bath for 25 min. The mixture was allowed to cool on water for 5 min. The resulting pink stained TBA-RS were determined spectrophotometrically at 535 nm in a Beckman DU®640 Spectrophotometer. Calibration curve was performed using 1,1,3,3-tetramethoxypropane as standard, being subjected to the same treatment as that of the samples. TBA-RS were calculated as nmol TBA-RS/mg protein.

Sulfhydryl content

This assay is based on the reduction of 5,5′-dithio-bis(2-nitrobenzoic acid) (DTNB) by thyols, which in turn become oxidized (disulfide), generating a yellow derivative (TNB) whose absorption is measured spectrophotometrically at 412 nm (Aksenov and Markesbery, 2001). Briefly, 50 μL of homogenate were added to 1 mL of PBS buffer pH 7.4 containing 1 mM EDTA. Then 30 μL of 10 mM DTNB, prepared in a 0.2 M potassium phosphate solution pH 8.0, were added. Subsequently, 30 min incubation at room temperature in a dark room was performed. Absorption was measured at 412 nm using a Beckman DU®640 spectrophotometer. The sulfhydryl content is inversely correlated to oxidative damage to proteins. Results were reported as nmol TNB/mg protein.

Carbonyl content

Oxidatively modified proteins present an enhancement of carbonyl content (Stadtman, 1990). In this paper, carbonyl content was assayed by a method based on the reaction of protein carbonyls with dinitrophenylhydrazine forming dinitrophenylhydrazone, a yellow compound, measured spectrophotometrically at 370 nm [24]. Briefly, 100 μL of homogenate were added

to plastic tubes containing 400 μL of 10 mM dinitrophenylhydrazine (prepared in 2 M HCl). This was kept in the dark for 1 h and vortexed each 15 min. After that, 500 μL of 20% trichloroacetic acid were added to each tube. The moisture was vortexed and centrifuged at 14,000 rpm for 3 min. The supernatant obtained was discarded. The pellet was washed with 1 mL ethanol:ethyl acetate (1:1, v/v), vortexed and centrifuged at 14,000 rpm for 3 min. This washing procedure was repeated once again and, after centrifugation, the supernatant was discarded and the pellet re-suspended in 600 μL of 6 M guanidine (prepared in a 20 mM potassium phosphate solution pH 2.3). The sample was vortexed and incubated at 60°C for 15 min. After that, it was centrifuged at 14,000 rpm for 3 min and the absorbance was measured at 370 nm (UV) in a quartz cuvette. Results were reported as carbonyl content (nmol/mg protein).

DCF (2′,7′-dichlorofluorescein) fluorescence assay

DCF fluorescence assay is used to measure RS production (Oyama et al., 1994). The technique is based on deacetylation of 2′-7′-dichlorofluorescein diacetate (DCFH-DA), which is oxidized by intracellular reactive species to form DCF, a highly fluorescent compound. One hundred microliters of homogenate were added to 100 μl of DCFH-DA (0.02 mM) and incubated during 30 min at 37°C. A curve of DCFH-DA ranging from 0.25 to 10 μM was performed. The DCF fluorescence was measured at 525 nm emission with excitation set at 460 nm in a Hitachi fluorescence spectrophotometer (model F-2000). DCF fluorescence was calculated as nmol/mg protein.

Catalase assay

CAT activity was assayed using a double-beam spectrophotometer with temperature control (Hitachi U-2001). This method is based on the disappearance of H_2O_2 at 240 nm in a reaction medium containing 20 mM H_2O_2, 0.1% Triton X-100, 10 mM potassium phosphate buffer, pH 7.0, and 0.1–0.3 mg protein/mL (Aebi, 1984). One CAT unit is defined as one μmol of hydrogen peroxide consumed per minute and the specific activity is reported as units per mg protein.

Superoxide dismutase assay

This method for the assay of SOD activity is based on the capacity of pyrogallol to autoxidize, a process highly dependent on O_2^-, which is substrate for SOD (Marklund, 1985). The inhibition of autoxidation of this compound occurs in the presence of SOD, whose activity can be then indirectly assayed spectrophotometrically at 420 nm, using a double-beam spectrophotometer with temperature control (Hitachi U-2001). A calibration curve was performed with purified SOD as standard, in order to calculate the activity of SOD present in the samples. The results were reported as units of SOD/mg protein.

Glutathione peroxidase assay

GPx activity was measured using *tert*-butyl-hydroperoxide as substrate (Wendel, 1981). NADPH disappearance was monitored at 340 nm using a double-beam spectrophotometer with temperature control (Hitachi U-2001). The medium contained 2 mM glutathione, 0.15 U/mL glutathione reductase, 0.4 mM azide, 0.5 mM *tert*-butyl-hydroperoxide and

0.1 mM NADPH. One GPx unit is defined as one μmol of NADPH consumed per minute and the specific activity is represented as units per mg protein.

Protein determination

Protein concentration was determined in cerebral cortex and cerebellum supernatants using bovine serum albumin as standard (Lowry et al., 1951).

Statistical analysis

Statistical analysis were performed by the one-way analysis of variance (ANOVA), followed by the Tukey test for multiple comparison when the F value was significant. Linear regression analysis was also performed to verify dose-dependent effects. All analyses were performed using the Statistical Package for the Social Sciences (SPSS) software in a PC-compatible computer. A value of $p < 0.05$ was considered to be significant.

Results

Initially, we evaluated the *in vitro* effect of 5-OP on non-enzymatic antioxidant defenses in rat cerebral cortex and cerebellum. This was achieved by measuring TRAP and TAR, which evaluate non-enzymatic antioxidants quantity and reactivity, respectively, present in the samples. Figure 1 shows that TRAP was significantly reduced by the presence of 5-OP as compared to control, in a dose-dependent manner, both in cerebral cortex [$F(3, 20) = 15.911$; $p < 0.001$] [beta $= -0.79$; $p < 0.001$] and cerebellum [$F(3, 20) = 23.185$; $p < 0.001$] [beta $= -0.73$; $p < 0.001$]. In addition, TAR measurement was markedly reduced at all concentrations tested, also in a dose-dependent way, when cerebral cortex [$F(3, 20) = 53.596$; $p < 0.001$] [beta $= -0.86$; $p < 0.001$] and cerebellum [$F(3, 20) = 37.196$; $p < 0.001$] [beta $= -0.87$; $p < 0.001$] were exposed to 5-OP (Fig. 2).

Next, we investigated chemiluminescence and TBA-RS as lipid peroxidation parameters (Table 1). 5-OP caused no effect on chemiluminescence in cerebral cortex [$F(3, 12) = 2.144$; $p > 0.05$] and cerebellum [$F(3, 12) = 0.582$; $p < 0.05$] homogenates. Table 1 also shows that 5-OP did not alter TBA-RS levels in cerebral cortex [$F(3, 12) = 3.492$, $p > 0.05$] and cerebellum [$F(3, 12) = 0.204$, $p > 0.05$].

We also investigated whether tissue proteins were affected by 5-OP. To accomplish this, two different parameters of oxidative protein damage were measured–sulfhydryl and carbonyl contents. Figure 3 shows that the sulfhydryl content was significantly reduced in cerebellum by 3.0 mM 5-OP [$F(3, 12) = 6.429$; $p < 0.05$], indicating the occurrence of oxidized proteins, but not in the cerebral cortex [$F(3, 12) = 1.316$, $p > 0.05$]. These results were corroborated by those obtained with the measurement of the carbonyl content. Figure 4 shows that the carbonyl content was significantly enhanced by 3.0 mM 5-OP in cerebral cortex [$F(3, 24) = 4.067$; $p < 0.05$] and in cerebellum homogenates [$F(3, 20) = 25.005$; $p < 0.001$], indicating protein oxidation.

DCF fluorescence was also determined and showed to be enhanced in cerebral cortex homogenates exposed to 3.0 mM 5-OP [$F(3, 12) = 4.359$; $p < 0.05$], while in cerebellum this organic acid had no effect [$F(3, 12) = 0.712$; $p > 0.05$] (Fig. 5).

Finally, the activities of the antioxidant enzymes CAT, SOD and GPx were assayed in the presence of 5-OP (Table 2). This organic acid caused no effect on the activity of CAT in cerebral cortex [$F(3, 16) = 1.028$; $p > 0.05$], as well as in cerebellum [$F(3, 16) = 3.831$;

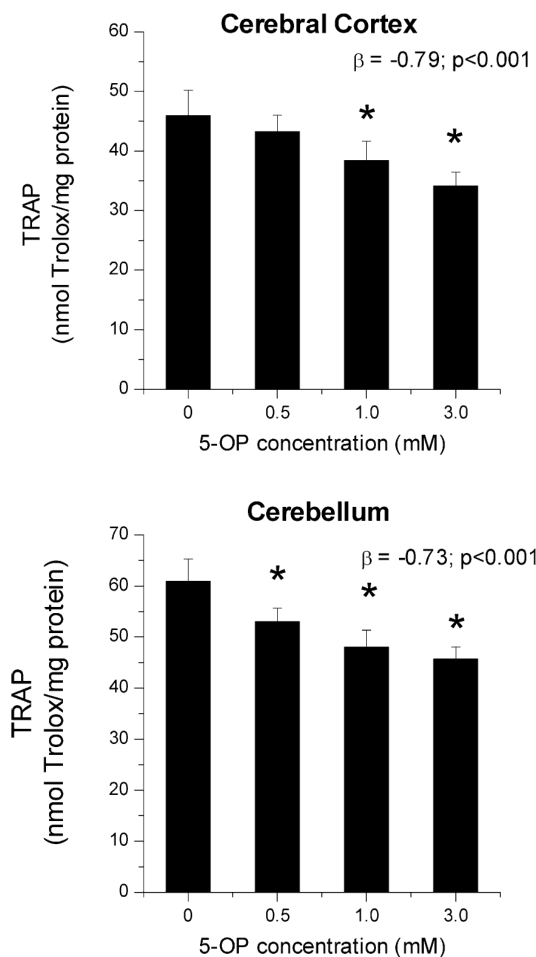


Fig. 1 *In vitro* effect of 5-oxoproline (5-OP) on total radical-trapping antioxidant potential (TRAP) in cerebral cortex and cerebellum from 14-day-old rats. Results are mean ± SD for six independent experiments performed in duplicate. The β value refers to linear regression. $^*p < 0.001$ compared to control (Tukey multiple range test)

$p > 0.05$]. Likewise, the activity of SOD was not altered by 5-OP in homogenates of cerebral cortex [$F(3, 20) = 2.791$; $p > 0.05$] and cerebellum [$F(3, 20) = 1.213$; $p > 0.05$]. The activity of GPx was also not affected by 5-OP both in cerebral cortex [$F(3, 12) = 3.471$; $p > 0.05$] and cerebellum [$F(3, 12) = 0.189$; $p > 0.05$].

Discussion

5-OP accumulation is the biochemical hallmark of patients affected by GS deficiency. Blood and CSF levels of 5-OP were reported in the range of 2–5 mM and 2–3 mM, respectively, in these patients (Meister, 1974; Jain et al., 1994) while the physiological concentrations of 5-OP are approximately 40 μM in CSF and 50 μM in plasma (Hoffmann et al., 1993). The affected individuals typically present a wide genetic and phenotypic heterogeneity including progressive neurological dysfunction with mental retardation, ataxia, spasticity,

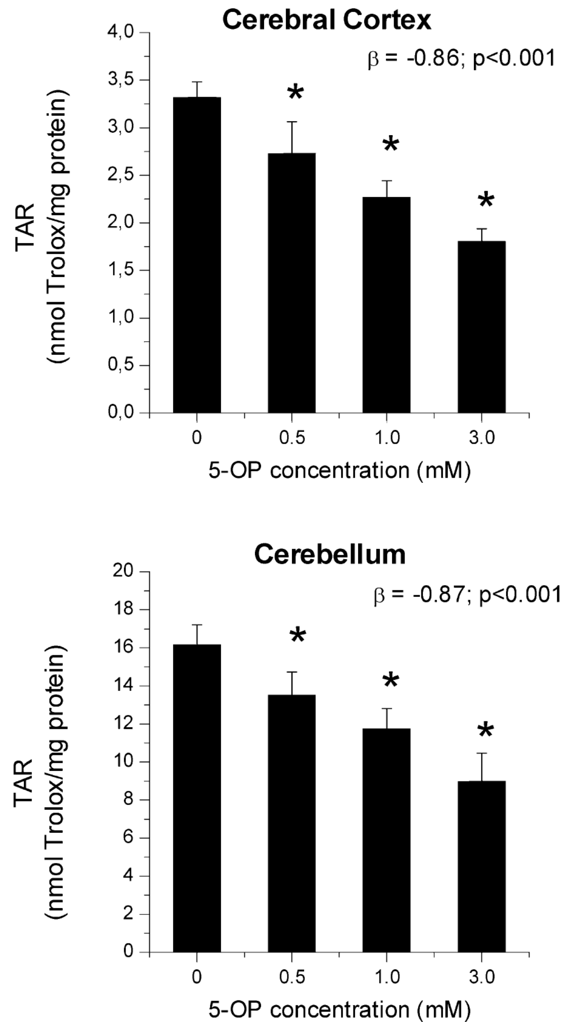


Fig. 2 *In vitro* effect of 5-oxoproline (5-OP) on total antioxidant reactivity (TAR) in cerebral cortex and cerebellum from 14-day-old rats. Results are mean ± SD for six independent experiments performed in duplicate. The β value refers to linear regression. $^{*}p < 0.001$ compared to control (Tukey multiple range test)

and seizures. Although the underlying mechanisms of brain damage in this disorder are poorly known, low GSH levels have been a postulated mechanism for cerebral injury in GSH deficiency associated to the neurological symptoms in GS-deficient patients. A recent long-term study on the outcome found no obvious correlation between GS activities, GSH levels and clinical symptoms or outcome (Ristoff et al., 2001). However, brain 5-OP levels were not determined in these patients. Moreover, the moderate and severe clinical phenotypes could not be distinguished based on enzyme activity, GSH or γ-glutamylcysteine levels in cultured fibroblasts from GS-deficient patients (Njalsson et al., 2005). Another study also found no correlation between the level of GS activity and the neurological symptoms (Dahl et al., 1997). Altogether, these findings suggest that low GSH levels alone are not the only determinant of neurodegeneration in these patients. Furthermore, individuals with 5-oxoprolinase deficiency, another inborn disorder of the γ-glutamyl cycle, also have high

Table 1 *In vitro* effect of 5-oxoproline (5-OP) on lipid peroxidation in cerebral cortex and cerebellum from 14-day-old rats

Lipid peroxidation parameters	5-oxoproline concentration (mM)			
	0	0.5	1.0	3.0
	Cerebral cortex			
Chemiluminescence (cps/mg protein)	108.4 ± 9.8	121.7 ± 18.3	126.7 ± 16.7	135.7 ± 16.0
TBA-RS (nmol/mg protein)	3.4 ± 0.05	3.7 ± 0.2	3.4 ± 0.1	3.5 ± 0.3
	Cerebellum			
Chemiluminescence (cps/mg protein)	98.3 ± 12.9	106.7 ± 16.0	107.5 ± 19.8	112.6 ± 11.8
TBA-RS (nmol/mg protein)	4.85 ± 0.6	4.69 ± 0.6	4.71 ± 0.5	4.54 ± 0.5

Note. Results are mean ± SD for four independent experiments performed in duplicate. No significant differences were detected by ANOVA.

tissue concentrations of 5-OP and neurological symptoms similar to those of GS deficiency but, in contrast, do not present reduced GSH levels (Larsson and Anderson, 2001), indicating that 5-OP may be deleterious to the CNS. In fact, there are some reports in the literature showing neurotoxic actions for 5-OP (Bennet Jr et al., 1973; Escobedo and Cravioto, 1973; Rieke et al., 1984; Dusticier et al., 1985; Rieke et al., 1989; Barone and Spignoli, 1990; Silva et al., 2001). However, the exact mechanisms underlying its toxicity remain not fully understood and to our knowledge no study investigated the role of 5-OP on oxidative stress in brain.

5-OP was shown to be excitotoxic (Bennet Jr et al., 1973; Rieke et al., 1989; Barone and Spignoli, 1990) as well as to compromise brain energy metabolism by inhibiting Na^+,K^+-ATPase (Escobedo and Cravioto, 1973; Rieke et al., 1984), reducing CO_2 production, ATP and lipid syntheses, and also respiratory chain enzyme activities (Silva et al., 2001).

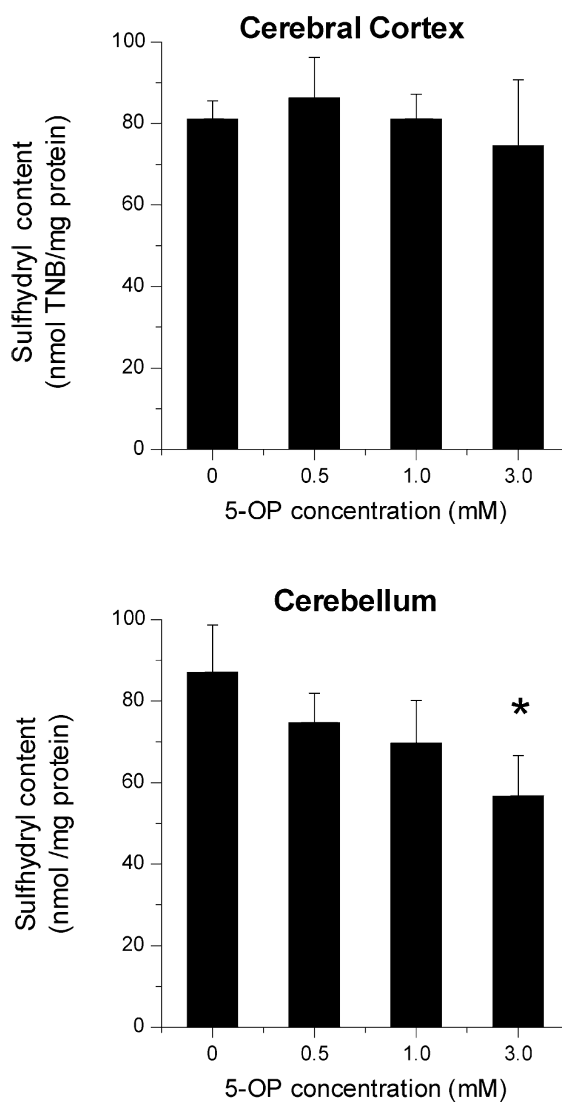
Considering that to our knowledge no study investigated so far the role of 5-OP on oxidative stress, in the present work we investigated the *in vitro* effect of this organic acid on some oxidative stress parameters in order to evaluate whether free radical generation could be elicited by this metabolite which could be possible related to the neurological damage occurring in inborn errors of the γ-glutamyl cycle.

We demonstrated that 5-OP significantly reduced both TRAP and TAR in cerebral cortex and cerebellum of 14-day-old rats in a dose-dependent manner. Considering that TRAP measures the content of non-enzymatic antioxidant defenses, while TAR reflects the capacity of a given tissue to modulate the damage associated with an increased production of RS (Lissi et al., 1995), these results indicate that 5-OP reduces the non-enzymatic antioxidant capacity in rat brain, by means of reducing non-enzymatic antioxidant content (TRAP) and also the antioxidant reactivity (TAR).

We also found that chemiluminescence and TBA-RS were not altered by the presence of 5-OP in the incubation medium, suggesting that lipid peroxidation (oxidative damage to lipids) is not induced by 5-OP neither in cerebral cortex nor in cerebellum (Esterbauer and Cheeseman, 1990).

In contrast, 5-OP was able to cause oxidative damage to proteins mainly in the cerebellum as verified by the significant decrease of sulfhydryl and carbonyl contents. Thus, it is possible

Fig. 3 *In vitro* effect of 5-oxoproline (5-OP) on sulfhydryl content in cerebral cortex and cerebellum from 14-day-old rats. Results are mean ± SD for six independent experiments performed in duplicate. $*p < 0.05$ compared to control (Tukey multiple range test)



that this mechanism may be involved in the neuropathological findings and motor delayed development observed in the patients affected by GS deficiency.

We also verified that 5-OP significantly increased reactive species, measured by the DCF fluorescence assay. DCF fluorescence is generally increased by various oxygen and nitrogen reactive species, although this probe is more sensitive to $OH^{\bullet}$ and peroxynitrite compared to other reactive species (Wardman et al., 2002; Myhre et al., 2003; Halliwell and Whiteman, 2004).

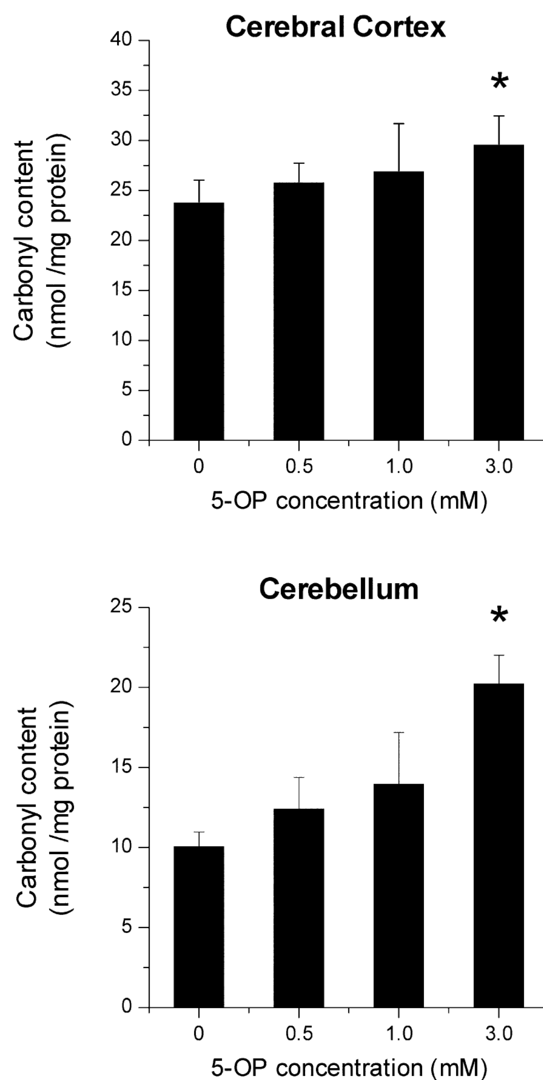


Fig. 4 *In vitro* effect of 5-oxoproline (5-OP) on carbonyl content in cerebral cortex and cerebellum from 14-day-old rats. Results are mean ± SD for six independent experiments performed in duplicate. $^{*}p < 0.05$ and $^{**}p < 0.001$ compared to control (Tukey multiple range test)

Finally, we verified that the presence of 5-OP in the incubation medium did not change the enzymatic antioxidant defenses, as determined by the activities of CAT, SOD and GPx. These results indicate that 5-OP does not directly affect the antioxidant enzyme activities.

Taken together, our present results clearly indicate that 5-OP decreases the non-enzymatic antioxidant defenses in rat brain, as shown by the significant reduction observed in TRAP and TAR measurements, and provokes oxidative damage to proteins, probable by enhancing RS production in cerebral cortex and cerebellum. At this point it should be emphasized that the central nervous system is highly sensitive to oxidative stress due to its high oxygen consumption, its high iron and lipid contents, especially polyunsaturated fatty acids, and the low activity of antioxidant defenses (Halliwell, 2001). Considering that oxidative stress

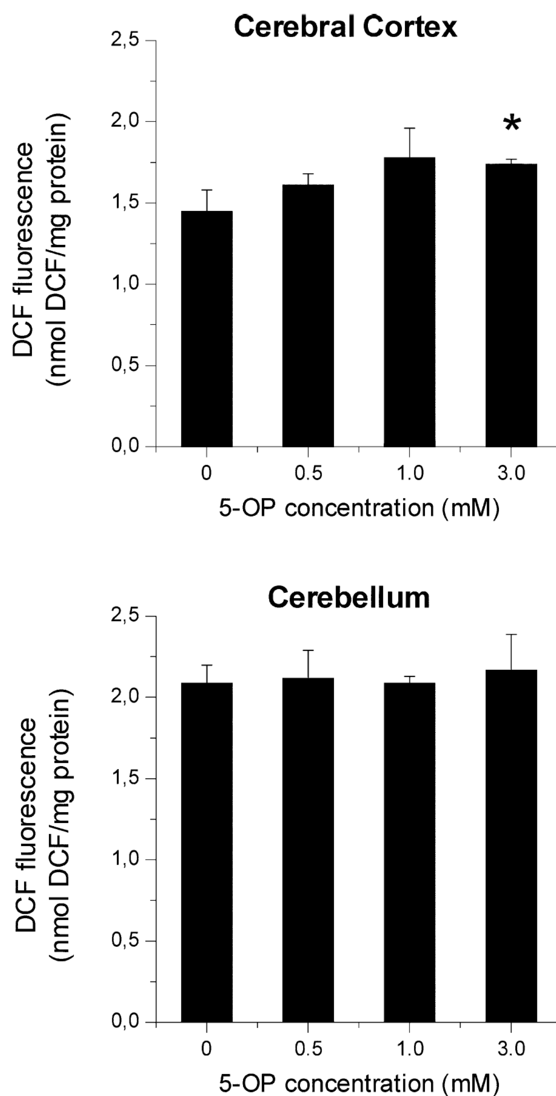


Fig. 5 *In vitro* effect of 5-oxoproline (5-OP) on dichlorofluorescein (DCF) fluorescence in cerebral cortex and cerebellum from 14-day-old rats. Results are mean ± SD for six independent experiments performed in duplicate. $^{*}p < 0.05$ compared to control (Tukey multiple range test)

can be elicit by the imbalance between free radical production and antioxidant defenses, and since 5-OP decreases the brain antioxidant defenses and provokes increase in reactive species and protein damage, it is postulated that 5-OP induces oxidative stress. Furthermore, since excitotoxicity and blockage of the respiratory chain can also indirectly lead to free radical generation (Nicholls and Budd, 1998) it is also possible that these mechanisms may act synergistically to induce significant neural cell damage. It can be therefore suggested the involvement of oxidative stress in the neuropathology of inherited disorders of the γ-glutamyl cycle, in which 5-OP accumulation is the biochemical hallmark. It is possible that *in vivo* deficiency of GSH associated with accumulation of 5-OP could act synergistically,

Table 2 *In vitro* effect of 5-oxoproline (5-OP) on catalase (CAT), superoxide dismutase (SOD) and gluthatione peroxidase (GPx) activities in cerebral cortex and cerebellum from 14-day-old rats

Enzyme activities	5-oxoproline concentration (mM)			
(units/mg protein)	0	0.5	1.0	3.0
		Cerebral cortex		
CAT ($n = 5$)	11.2 ± 0.7	11.3 ± 1.1	11.1 ± 0.5	10.4 ± 1.0
SOD ($n = 6$)	5.8 ± 0.8	5.6 ± 0.7	5.5 ± 0.8	6.6 ± 0.8
GPx ($n = 4$)	18.9 ± 1.1	18.5 ± 1.0	19.7 ± 1.8	17.0 ± 0.9
		Cerebellum		
CAT ($n = 5$)	19.1 ± 1.7	19.6 ± 1.6	17.9 ± 1.9	16.4 ± 1.2
SOD ($n = 6$)	11.9 ± 1.1	11.1 ± 1.3	10.7 ± 1.8	12.1 ± 1.6
GPx ($n = 4$)	29.4 ± 1.9	28.6 ± 2.7	28.6 ± 1.5	29.2 ± 1.7

Note. Results are mean ± SD for four to six independent experiments performed in duplicate, reported as units/mg protein. One CAT unit is defined as one μmol of hydrogen peroxide consumed per minute. One SOD unit is defined as 80% inhibition of pyrogallol autoxidation. One GPx unit is defined as one mmol of NADPH consumed per minute. No significant differences were detected by ANOVA.

enhancing RS production and decreasing non-enzymatic antioxidant defenses, thus leading to oxidative stress.

Even though the concentrations of 5-OP used in our assays are similar to those observed in plasma and cerebrospinal fluid of patients affected by GS deficiency (1–5 mM) (Eldjarn et al., 1972; Eldjarn et al., 1973; Meister, 1974; Jain et al., 1994), it is difficult to extrapolate our *in vitro* findings to the human condition. However, if these effects also occur in the brain of patients affected by GS deficiency, it is possible that they may contribute, at least in part, to the neurological dysfunction characteristic of this disease. Whether impairment of brain energy production, inhibition of Na^+, K^+-ATPase, excitotoxicity or oxidative stress is the major underlying mechanism responsible for 5-OP neurotoxicity in the brain damage of these patients remains to be elucidated.

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Capítulo II

Acute administration of 5-oxoproline induces oxidative damage to lipids and proteins and impairs antioxidant defenses in cerebral cortex and cerebellum of young rats

Artigo submetido à revista Molecular and Cellular Biochemistry

Acute administration of 5-oxoproline induces oxidative damage to lipids and proteins and impairs antioxidant defenses in cerebral cortex and cerebellum of young rats

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Abstract

5-Oxoproline accumulates in glutathione synthetase deficiency, an autossomic recessive inherited disorder clinically characterized by hemolytic anemia, metabolic acidosis, and severe neurological symptoms whose mechanisms are poorly known. In the present study we investigated the effects of acute subcutaneous administration of 5-oxoproline to verify whether oxidative stress is elicited by this metabolite *in vivo* in cerebral cortex and cerebellum of 14-day-old rats. Our results showed that the acute administration of 5-oxoproline is able to promote both lipid and protein oxidation, to impair brain antioxidant defenses, to alter SH/SS ratio and to enhance hydrogen peroxide content, thus promoting oxidative stress *in vivo*, a mechanism that may be involved in the neuropathology of gluthatione synthetase deficiency.

Keywords: **5-Oxoproline; glutathione synthetase deficiency; oxidative stress; antioxidant defenses**

Introduction

5-Oxoproline (5-OP), also known as L-pyroglutamic acid, accumulates in cerebrospinal fluid, blood and urine from patients affected by moderate and severe forms of glutathione synthetase (GS) deficiency, an autosomal recessive inherited disorder of the γ-glutamyl cycle [1-5]. About 70 patients have been described worldwide with GS deficiency (MIM 266130) [4]. Patients with the severe form of the disease present hemolytic anemia and metabolic acidosis; moreover, almost half of the patients also develop progressive neurological symptoms, including seizures, mental retardation, ataxia, psychomotor delay and spasticity [3,5,6]. Approximately 25% of all affected patients die during childhood [7]. The neuropathological findings of GS deficiency include selective atrophy of the granule cell layer of the cerebellum, focal lesions in frontoparietal cortex and bilateral lesions in visual cortex [8,9].

The mechanisms of brain damage in this disease remain not fully understood. However, 5-OP exhibits neurotoxic actions [10,11], promotes excitotoxicity [12-15] and inhibits brain energy metabolism [16,17]. Recent findings from our laboratory showed that 5-OP causes protein oxidation, reactive species production and decreases the non-enzymatic antioxidant defenses *in vitro* in rat brain homogenates [18].

Since excitotoxicity and brain energy metabolism impairment have already been related to oxidative stress [19-21], it seems feasible that altogether these processes may act sinergistically *in vivo*. So, in the present study we investigated the *in vivo* effects of 5-OP on oxidative stress parameters in rat cerebral cortex and cerebellum of 14-day-old rats. To accomplish this, the effect of acute subcutaneous administration of 5-OP was studied on the following oxidative stress parameters: spontaneous chemiluminescence and thiobarbituric

acid-reactive substances (TBA-RS), to assess lipid peroxidation; protein carbonyl content, to evaluate protein oxidation; total radical-trapping antioxidant potential (TRAP), as well as ascorbic acid and reduced glutathione (GSH) contents, to evaluate non-enzymatic antioxidant defenses; activities of the antioxidant enzymes catalase (CAT), superoxide dismutase (SOD) and glutathione peroxidase (GPx), to assess enzymatic antioxidant defenses; activity of glucose 6-phospate dehydrogenase (G6PD), to evaluate the main cellular source of NADPH (pentose phosphate pathway); thiol (SH) and disulfide (SS) contents, to evaluate SH/SS ratio; and hydrogen peroxide content.

Materials and Methods

Materials

All chemicals were purchased from Sigma (St. Louis, MO, USA) except for 2,2'-azo-bis-(2-amidinopropane) that was purchased from Wako Chemicals (USA). 5-OP solutions were freshly prepared in saline solution, and the pH was adjusted to 7.4.

Animals

Fourteen-day-old Wistar rats bred in the Department of Biochemistry, ICBS, UFRGS, from both sexes, were used. Rats were kept with dams until they were killed. The dams had free access to water and a 20% (w/w) protein commercial chow (Supra, Porto Alegre, RS, Brazil). They were kept in a room with 12:12 h light/dark cycle (lights on 07:00-19:00 h) and with air-conditioned controlled temperature (22°C ± 1°C). The NIH

‘Guide for the Care and Use of Laboratory Animals’ (NIH publication # 80-23, revised 1996) (National Institutes of Health, 1996) was followed in all experiments.

Acute administration of 5-OP and tissue preparation

Rats received subcutaneously the dose of 1g/kg body weight of 5-OP or saline solution (controls). One hour after a single injection of saline or 5-OP solution, rats were killed by decapitation, and the brain was immediately removed and kept on a Petry dish placed on ice. The olfactory bulb, pons and medulla were discarded and cerebral cortex and cerebellum were dissected, weighed and kept chilled until homogenization. These procedures lasted up to 3 min. Cerebral cortex and cerebellum were homogenized in 10 volumes (1:10, w/v) of 20 mM sodium phosphate buffer, pH 7.4 containing 140 mM KCl. Homogenates were centrifuged at 750 *g* for 10 min at 4ºC to discard nuclei and cell debris [22,23]. The pellet was discarded and the supernatant was immediately separated and used for the measurements.

5-oxoproline quantification

To ensure that the dose of 5-OP injected subcutaneously achieved satisfactory plasma and brain levels, aliquots were taken to measure the levels of this organic acid in plasma and brain of rats submitted to acute administration of 5-OP. One hour after receiving a single subcutaneous injection of 5-OP at a dose of 1g/kg body weight, rats were killed by decapitation and had their blood collected into heparinized tubes and processed for plasma separation. Their brain was also rapidly removed and homogenized in saline solution (0.9% NaCl). Plasma samples and cerebral homogenates were centrifuged at 400 *g*

for 10 min and 750 *g* for 10 min, respectively. The supernatants obtained were carefully removed for 5-OP determination. 5-OP content was determined by Gas Cromatography-Mass Spectrometry (GC/MS) [24] using margaric acid as the internal standard.

Spontaneous chemiluminescence

Samples were assayed for spontaneous chemiluminescence in a dark room [23] using a beta liquid scintillation spectrometer Tri-Carb 2100TR. The background chemiluminescence was measured for 5 min in vials containing 3.5 mL of the same buffer used for homogenization. An aliquot of 0.5 mL of supernatant was added and spontaneous chemiluminescence was measured for 10 minutes at room temperature. The background chemiluminescence was subtracted from the total value. Spontaneous chemiluminescence was calculated as counts per second (CPS)/mg protein.

Thiobarbituric acid-reactive substances (TBA-RS)

TBA-RS was measured according to Ohkawa et al. (1979) [25]. Briefly, to glass tubes were added, in order of appearence: 500 μL of tissue supernatant; 50 μL of SDS 8.1%; 1500 μL of 20% acetic acid in aqueous solution (v/v) pH 3.5; 1500 μL of 0.8 % thiobarbituric acid; and 700 μL of distilled water. The mixture was vortexed and the reaction was carried out in a boiling water bath for 1 hour. The mixture was allowed to cool on water for 5 min, and was centrifuged 750 *g* for 10 min. The resulting pink stained TBA-RS were determined spectrophotometrically at 535 nm. A calibration curve was generated using 1,1,3,3-tetramethoxypropane as a standard. TBA-RS were calculated as nmol TBA-RS/mg protein.

Protein carbonyl content

Oxidatively modified proteins present an enhancement of carbonyl content [26,27]. In this study, protein carbonyl content was assayed by a method based on the reaction of protein carbonyls with dinitrophenylhydrazine forming dinitrophenylhydrazone, a yellow compound, measured spectrophotometrically at 370 nm [28]. Briefly, 100 μL of homogenate were added to plastic tubes containing 400 μL of 10 mM dinitrophenylhydrazine (prepared in 2 M HCl). This was kept in the dark for 1 hour and vortexed each 15 minutes. After that, 500 μL of 20% trichloroacetic acid were added to each tube. The mixture was vortexed and centrifuged at 20,000 g for 3 minutes. The supernatant obtained was discarded. The pellet was washed with 1 mL ethanol:ethyl acetate (1:1, v/v), vortexed and centrifuged at 20,000 g for 3 minutes. The supernatant was discarded and the pellet re-suspended in 600 μL of 6 M guanidine (prepared in a 20 mM potassium phosphate solution pH 2.3). The sample was vortexed and incubated at 60°C for 15 minutes. After that, it was centrifuged at 20,000 g for 3 minutes and the absorbance was measured at 370 nm (UV) in a quartz cuvette. Results were represented as protein carbonyl content (nmol/mg protein).

Total radical-trapping antioxidant potential (TRAP)

TRAP was determined by measuring the chemiluminescence intensity of luminol induced by 2,2'-azo-bis-(2-amidinopropane) (ABAP) thermolysis [29,30] in a Wallac 1409 Scintillation Counter. Three mL of ABAP 10 mM, dissolved in 50 mM sodium phosphate buffer pH 7.4, plus 10 μL of luminol (5.6 mM) were added to a glass scintillation vial, and the initial chemiluminescence was measured. Ten μL of 160 μM Trolox (water-soluble α-

tocopherol analogue, used as a standard) or 10 μL of tissue supernatant were then added to that vial, producing a decrease in the initial chemiluminescence value. This value is kept low until the antioxidants present in the sample are depleted, then chemiluminescence returns to its initial value. The time taken by the sample to keep chemiluminescence low is directly proportional to the antioxidant capacity of the tissue, so TRAP represents the amount (quantity) of non-enzymatic antioxidants present in the sample. Results were represented as nmol Trolox/mg protein.

Ascorbic acid content

Ascorbic acid content was measured according to Omaye et al. (1979) [31]. The method is based on the reduction of dichlorophenolindophenol (DCIP) by ascorbic acid at pH 3-4.5, which promotes a decrease in the absorbance measured spectrophotometrically at 520 nm. The non-specific reduction of DCIP by thiolic compounds was inhibited by the addition of p-hydroxymercurobenzoic acid (pHMB) to the buffer. Initially, 600 μL of the tissue homogenates were added to plastic tubes containing 300 μL of citrate/acetate buffer pH 4.15 with pHMB. Each sample was then vortexed and reacted with 300 μL of DCIP and the absorbance at 520 nm was read after 30 seconds. A calibration curve was generated using commercial ascorbic acid solution as a standard. Ascorbic acid content was expressed as μg ascorbic acid/mg tissue.

Reduced glutathione (GSH) content

This method is based on the reaction of GSH with the fluorophore o-phtalaldeyde (OPT) after deproteinizing the samples, and was measured according to Browne and

Armstrong (1998) [32]. Initially, metaphosphoric acid was used to deproteinize the samples, which were then centrifuged at 1000 *g* for 10 min. Briefly, to 100 µL of each supernatant were added 2 mL of sodium phosphate buffer pH 8.0 and 100 µL OPT 1 mg/mL (prepared in metanol). The mixture was vortexed and allowed to stand in the dark for exactly 15 minutes. After that, the fluorescence was measured at λ_{em}= 420 nm and λ_{ex}= 350 nm. A calibration curve was also performed with a commercial GSH solution, and the results were expressed as µmol GSH/mg protein.

Catalase assay

CAT activity was assayed using a double-beam spectrophotometer with temperature control (Hitachi U-2001). This method is based on the disappearance of H_2O_2 at 240 nm in a reaction medium containing 20 mM H_2O_2, 0.1% Triton X-100, 10 mM potassium phosphate buffer pH 7.0, and 0.1-0.3 mg protein/mL [33]. One CAT unit is defined as one µmol of hydrogen peroxide consumed per minute and the specific activity is expressed as CAT units/mg protein.

Superoxide dismutase assay

This method for the assay of SOD activity is based on the capacity of pyrogallol to autoxidize, a process highly dependent on $O_2^{\bullet -}$, which is substrate for SOD [34]. The inhibition of autoxidation of this compound occurs in the presence of SOD, whose activity can be then indirectly assayed spectrophotometrically at 420 nm, using a double-beam spectrophotometer with temperature control (Hitachi U-2001). A calibration curve was generated with purified SOD as a standard, in order to calculate the activity of SOD present in the samples. The results were expressed as SOD units/mg protein.

Glutathione peroxidase assay

GPx activity was measured using *tert*-butyl-hydroperoxide as substrate [35]. NADPH disappearance was monitored at 340 nm using a double-beam spectrophotometer with temperature control (Hitachi U-2001). The medium contained 2 mM glutathione, 0.15 U/mL glutathione reductase, 0.4 mM azide, 0.5 mM *tert*-butyl-hydroperoxide and 0.1 mM NADPH. One GPx unit is defined as one μmol of NADPH consumed per minute and the specific activity is expressed as GPx units/mg protein.

Glucose 6-phosphate dehydrogenase assay

G6PD activity was measured according to Leong and Clark (1984) [36]. The method is based on the formation of NADPH at 340 nm in a rection medium containing 100 mM Tris-Hydrochloryde buffer pH 7.5, 10 mM magnesium chloryde, 0.1% triton X-100, 0.5 mM $NADP^+$, 1 mM glucose 6-phosphate and 0.1-0.3 mg protein/mL. One G6PD unit is defined as one μmol of NADPH produced per minute and the specific activity is expressed as G6PD units/mg protein.

Hydrogen peroxide content

The method used to measure hydrogen peroxide was based on the horseradish peroxidase-mediated oxidation of phenol red by hydrogen peroxide, which results in the formation of a compound with increased absorbance at 610 nm [37]. Briefly, 400 mg cerebral cortex and 100 mg cerebellum were chopped with a McIlwainn chopper and cut in two perpendicular directions to produce 400 μm wide prisms. Cortical or cerebellar prisms

were pooled and added to a glass vial containing 5.5 mM dextrose buffer pH 7.0, and the mixture was allowed to stand at room temperature for 1 hour. After that, a 60 μL supernatant aliquot was taken and reacted with 235 μL of a medium containing 50 mM sodium phosphate buffer pH 7.4, 85 μL of horseradish peroxidase and 1 mg/mL phenol red (phenolsulfonphtalein). The reaction was carried out protected from light for 10 minutes. After that, 5 μL of 1 N NaOH were added to each tube and the absorbance was read at 610 nm. A calibration curve was performed with commercial solution of hydrogen peroxide. Results were expressed as nmol hydrogen peroxide/mg tissue.

Thiol and disulfide content (SH/SS ratio)

Thiols (SH) and disulfides (SS) were determined according to Zahler and Cleland (1968) [38]. The method is based on the reaction of DTNB with the samples, producing a yellow product, thionitrobenzoic acid (TNB). For total thiol and disulfide determination, the reaction medium consisted of 25 μL of 50 mM Tris buffer pH 9.0, 25 μL of 3 mM dithiothreitol (DTT) and 100 μL of sample supernatant. After 15 min at room temperature, 50 μL of 1.0 M Tris buffer pH 8.1 and 700 μL of sodium arsenite were added. After 3 min, 25 μL of 3 mM 5,5'-dithiobis-2-nitrobenzoic acid (DTNB) in 50 mM acetate buffer pH 5.0 were added and the absorbance was recorded for 3 min at 412 nm. For thiol determination, almost the same procedure was performed, only being ommited DTT and sodium arsenite from the reaction medium. Instead of them, an equal volume of distilled water was added to the medium. The disulfide content was calculated through the difference between the two determinations. The SH/SS ratio was also calculated. Results were expressed as nmol TNB/mg protein.

Protein determination

Protein concentration was determined in cerebral cortex and cerebellum supernatants using bovine serum albumin (BSA) as a standard [39].

Statistical analysis

Statistical analysis was performed by the Student's *t* test for unpaired samples. All analyses were performed using the Statistical Package for the Social Sciences (SPSS) software in a PC-compatible computer. A value of $p < 0.05$ was considered to be significant.

Results

Initially, we measured plasmatic and cerebral levels of 5-OP after a single subcutaneous injection of 5-OP (1g/kg body weight). Plasma and brain average levels of 5-OP determined by GC-MS were 2.82 mM and 0.80 mM (number of rats = 4), respectively. Control rats receiving equal volume of saline showed 5-OP levels of 0.06 mM in plasma, whereas 5-OP was not detected in the brain.

Next we investigated spontaneous chemiluminescence and TBA-RS, considered as lipid peroxidation parameters. Figure 1A depicts that 5-OP markedly enhanced spontaneous chemiluminescence in cerebral cortex [$t(10)=8.17$; $p<0.01$] and cerebellum [$t(10)=8.47$; $p<0.01$] homogenates. Acute administration of 5-OP also caused a significant enhancement of TBA-RS levels in both cerebral cortex [$t(10)=5.19$, $p<0.01$] and cerebellum [$t(10)=5.72$, $p<0.01$] (Figure 1B). These results indicate the promotion of lipid peroxidation by 5-OP.

We also investigated whether tissue proteins were affected by acute administration of 5-OP. To accomplish that, we measured protein carbonyl content. Figure 1C shows that protein carbonyl content was significantly enhanced in cerebral cortex [$t(10)=5.64$; $p<0.01$] and in cerebellum [$t(10)=5.01$; $p<0.01$], indicating the occurrence of oxidized proteins.

Next, we evaluated the *in vivo* effect of 5-OP on non-enzymatic antioxidant defenses in rat cerebral cortex and cerebellum. Initially we measured TRAP, which evaluates the quantity of non-enzymatic antioxidants present in the samples. Figure 1D shows that TRAP was significantly reduced by 5-OP *in vivo* as compared to control both in cerebral cortex [$t(10)=4.94$; $p<0.01$] and cerebellum [$t(10)=4.30$; $p<0.01$]. After that, we evaluated the contents of the specific non-enzymatic antioxidants ascorbic acid and GSH. Their contents were not affected by acute administration of 5-OP (Table 1). Ascorbic acid content was not altered both in cerebral cortex [$t(6)=0.37$; $p>0.05$] and in cerebellum of young rats [$t(6)=0.84$; $p>0.05$]. GSH content was equally not affected by acute administration of 5-OP neither in cerebral cortex [$t(14)=0.36$; $p>0.05$] nor in cerebellum [$t(14)=0.99$; $p>0.05$].

Then we decided to study the effect of acute administration of 5-OP on the brain enzymatic antioxidant defenses. The activities of the antioxidant enzymes CAT, SOD and GPx were assayed, as well as the activity of glucose 6-phosphate dehydrogenase (G6PD). Acute administration of 5-OP significantly inhibited the activity of CAT (Figure 2A) in cerebral cortex of young rats [$t(6)=4.08$; $p<0.01$]. However, this organic acid caused no effect on this activity in the cerebellum [$t(10)=1.29$; $p>0.05$]. Likewise, the activity of GPx (Figure 2B) was also inhibited by 5-OP in cerebral cortex [$t(12)=2.55$; $p<0.05$] but not in the cerebellum [$t(10)=1.59$; $p>0.05$]. The activity of SOD (Figure 2C) was not altered by acute administration of 5-OP in cerebral cortex [$t(6)=0.28$; $p>0.05$] and cerebellum

[$t(10)=0.30$; $p>0.05$] (Figure 7B). The activity of G6PD (Figure 2D), on the other hand, was inhibited by 5-OP *in vivo* only in cerebral cortex [$t(6)=4.36$; $p<0.01$] and not in cerebellum [$t(6)=1.84$; $p>0.05$]. These results clearly show an impairment in the enzymatic antioxidant defenses in cerebral cortex of rats submitted to acute administration of 5-OP.

Since hydrogen peroxide is a substrate for both CAT and GPx and the activities of these enzymes were inhibited by 5-OP, we decided to measure hydrogen peroxide content. We found that acute administration of 5-OP was able to enhance hydrogen peroxide content both in cerebral cortex [$t(14)=2.52$; $p<0.05$] and cerebellum [$t(10)=8.11$; $p<0.01$] (Figure 3).

Finally we measured thiol and disulfide content, as well as the SH/SS ratio in brain homogenates. Table 2 shows that 5-OP acute administration caused no alteration on SH content neither in cerebral cortex [$t(8)=1.63$; $p>0.05$] nor in the cerebellum [$t(10)=0.07$; $p>0.05$]. In contrast, SS content was interestingly enhanced in cerebral cortex [$t(8)=3.21$; $p<0.05$] and also in cerebellum [$t(10)=3.19$; $p<0.05$]. In fact, the increase of SS content was high enough to reduce SH/SS ratio both in cerebral cortex [$t(7)=3.41$; $p<0.05$] and in the cerebellum [$t(8)=7.55$; $p<0.01$].

Discussion

Patients affected by severe forms of GS deficiency present 5-OP accumulation as a biochemical hallmark and are clinically characterized by progressive neurological dysfunction with mental retardation, ataxia, spasticity and seizures. The underlying mechanisms of brain damage in this disorder remain far poorly known. Although low GSH levels were postulated as a mechanism of cerebral injury in GS deficiency, recent studies

suggest that low GSH levels are not the only determinant of neurodegeneration in these patients, and that 5-OP itself may be deleterious to the CNS [4,6,40,41].

Along with the already reported neurotoxic actions for 5-OP of excitotoxicity [11,12,14] and impairment of brain energy metabolism [10,16,17], we have recently reported a role of oxidative stress in 5-OP neurotoxicity [18].

In the present study we investigated the *in vivo* effects of 5-OP on oxidative stress parameters in rat cerebral cortex and cerebellum of 14-day-old rats. Initially, we measured plasmatic and cerebral levels of 5-OP induced by a single dose of 1 g/kg body weight of 5-OP injected subcutaneously to rats. Blood and CSF levels of 5-OP in GS-deficient patients reported in the literature are in the range of 2-5 mM and 1-3 mM, respectively [42,43] while physiological concentrations of 5-OP are approximately 40 μM in CSF and 50 μM in plasma [44]. The values obtained in our study were similar to those present in GS-deficient patients, ensuring that our acute model could be further used to study the *in vivo* effects of 5-OP on oxidative stress parameters.

We then investigated the effect of acute administration of 5-OP on the lipid peroxidation parameters spontaneous chemiluminescence and TBA-RS. We observed that 5-OP administration significantly enhanced spontaneous chemiluminescence and TBA-RS levels in cerebral cortex and cerebellum indicating that 5-OP promotes lipid peroxidation *in vivo* in rat cerebral cortex and cerebellum. Light in the chemiluminescence assay can arise mainly from excited carbonyls, $^1O_2$, $ONOO^-$ and from peroxidizing lipids as a result of increased reactive species production; TBA-RS levels, on the other hand, reflects the amount of malondialdehyde, which is end product of lipid peroxidation [45]. Therefore, the enhancement of these parameters promoted by 5-OP *in vivo* indicates an induction of lipid

oxidative damage by this compound. Since these results were not observed previously *in vitro* [18], it is suggested that 5-OP may not directly elicit lipid peroxidation but rather induce an indirect effect, probably involving enhanced generation of reactive species that trigger lipid peroxidation. It should be pointed out that some reactive species, such as H_2O_2, $O_2^{\bullet -}$, and $NO^{\bullet}$, are not directly able to trigger lipid peroxidation whereas $OH^{\bullet}$ and $ONOO^{-}$ can directly initiate oxidative damage to lipids [45].

We also verified that protein carbonyl content was significantly enhanced in cerebral cortex and cerebellum by 5-OP *in vivo* administration, indicating protein oxidative damage. These results corroborate our previous *in vitro* findings [18], and so it seems feasible to conclude that 5-OP is able to promote oxidation of proteins both *in vitro* and *in vivo*.

With regard to the antioxidant defense system, 5-OP significantly reduced the overall content of non-enzymatic antioxidants, measured as TRAP, both in cerebral cortex and cerebellum, which is in line with our previous *in vitro* findings [18]. However, ascorbic acid and GSH levels were not affected by acute administration of 5-OP, suggesting that TRAP reduction observed *in vivo* is not due to a decrease of GSH or ascorbic acid. In the brain, there are other non-enzimatic antioxidants that may account for the TRAP values, including α-tocopherol, metal-binding proteins, melatonine, and carnosine [46,47]. Considering that antioxidant proteins account for aproximately 40% of the TRAP values [30] and that in the present study 5-OP promoted oxidative damage to proteins, it seems possible that the TRAP reduction observed could be, at least in part, due to a reduction in the antioxidant action of proteins, which could be impaired by the oxidative damage promoted by 5-OP.

On the other hand, with respect to the enzymatic antioxidant defenses, the acute administration of 5-OP significantly inhibited the activities of CAT and GPx in cerebral cortex of young rats, with no alteration of these enzyme activities in cerebellum. As CAT and GPx activities were not affected *in vitro* by 5-OP [18], it is presumed that the inhibition of these enzymes *in vivo* occurred through an indirect effect of this organic acid. In contrast, the activity of SOD was not altered by acute administration of 5-OP in cerebral cortex and cerebellum. Altogether, these results show an impairment in the enzymatic antioxidant defenses in cerebral cortex of rats subjected to acute administration of 5-OP. Interestingly, absolute and relative activities of the antioxidant enzymes are higher in cerebellum than in cerebral cortex, which could generate different responses to oxidative stress [48,49].

The activity of G6PD was also measured in cerebral cortex and cerebellum homogenates from rats subjected to acute administration of 5-OP. G6PD is the key regulatory enzyme of the pentose phosphate pathway and, as such, controls the flow of carbon through the oxidative phase of this pathway and produces reducing equivalents in the form of NADPH to meet cellular needs for reductive biosynthesis and maintenance of the cellular redox state [50]. In mammals, most of the NADPH is produced by the pentose phosphate pathway [51]. In the present study, we also found that the acute administration of 5-OP was able to inhibit G6PD activity in cerebral cortex from young rats, which could promote an impairment in the production of NADPH and a dysruption in the cellular redox balance. This is probably in line with the observed inhibition of GPx activity, since the activity of this enzyme depends on the regeneration of reduced glutathione by glutathione reductase, which in turn relies on NADPH that is dependent on a normal activity of G6PD [51,52]. It has also been reported that G6PD is strongly inactivated by 4-hydroxy-2-

nonenal, a toxic product of membrane peroxidation [53].

We measured thiol and disulfide contents, as well as the SH/SS ratio. Acute administration of 5-OP caused no alteration of SH content but enhanced the SS content and reduced SH/SS ratio in cerebral cortex and in cerebellum. Enhanced generation of reactive species and/or impaired antioxidant detoxification functions provokes an imbalance between oxidative and reductive reactions, altering the thiol/disulfide redox status [54]. Reduction of the SH/SS ratio reflects an oxidized redox state of the cell that may eventually lead to oxidative stress [55]. Therefore, considering that disulfide content was significantly enhanced by acute administration of 5-OP and that SH/SS ratio was significantly reduced, it is suggested that 5-OP is able to disturb the thiol redox status in cerebral cortex and cerebellum of young rats *in vivo*, which could also be contributing to the brain damage observed in GS-deficient patients.

The present study showed greater alterations of oxidative stress parameters in cerebral cortex than in cerebellum, suggesting that cerebral cortex may be more susceptible to oxidative stress. This hypothesis is corroborated by reports showing greater reductive potential (calculated as $NADPH/NADP^{+}$ and GSH/GSSG ratios) [49] and greater antioxidant enzyme activities [48,56] in the cerebellum compared to the cerebral cortex, suggesting a greater resistance of cerebellum to oxidative stress. Prooxidant factors that directly or indirectly induce free radical generation also differ among brain structures. For instance, levels of iron, which catalyses $OH^{\bullet}$ formation from hydrogen peroxide through the Fenton reaction, are higher in cerebral cortex than in cerebellum [57]. So, the higher iron levels along with increased hydrogen peroxide content may contribute to the higher susceptibility of cerebral cortex to oxidative stress promoted by 5-OP.

Together, the present results demonstrated that 5-OP elicits oxidative stress *in vivo* which may act synergistically with excitotoxicity [11,12,14] and impairment of energy metabolism [10,16] to induce more significant neural cell damage in inherited disorders of the γ-glutamyl cycle in which 5-OP accumulates. Understanding the underlying mechanisms of brain damage that occurs in these disorders may be helpful in the development of effective therapy to ameliorate the neurological dysfunction of the affected patients.

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Table 1. Effect of acute administration of 5-oxoproline (5-OP) on ascorbic acid and reduced glutathione contents in cerebral cortex and cerebellum from 14-day-old rats.

	Cerebral cortex	
	Ascorbic acid (µg/mg tissue)	Glutathione (µmol/mg protein)
Control	2.10 ± 0.43	8.35 ± 1.40
5-OP	2.29 ± 0.94	8.62 ± 1.60
	Cerebellum	
	Ascorbic acid (µg/mg tissue)	Glutathione (µmol/mg protein)
Control	4.70 ± 0.33	8.01 ± 0.91
5-OP	4.31 ± 0.88	8.52 ± 1.14

Results are mean ± SD (n=4-8) for independent experiments performed in duplicate. No significant differences were detected (Student's *t* test for unpaired samples).

Table 2. Effect of acute administration of 5-oxoproline (5-OP) on thiol (SH) and disulfide (SS) contents, as well as on SH/SS ratio, in cerebral cortex and cerebellum from 14-day-old rats.

	Cerebral cortex		
	SH content (nmol TNB/mg protein)	SS content (nmol TNB/mg protein)	SH/SS ratio
Control	59.98 ± 5.05	3.65 ± 1.47	14.10 ± 4.37
5-OP	67.44 ± 8.91	13.82 ± 6.92 *	6.00 ± 2.77 *
	Cerebellum		
	SH content (nmol TNB/mg protein)	SS content (nmol TNB/mg protein)	SH/SS ratio
Control	81.53 ± 4.08	9.74 ± 2.48	7.60 ± 0.95
5-OP	81.82 ± 8.80	17.02 ± 5.02 *	4.35 ± 0.17 **

Results are mean ± SD (n=5-6) for independent experiments performed in duplicate.

* $p<0.05$ and ** $p<0.01$ compared to control (Student's *t* test for unpaired samples).

Figure Captions

Fig. 1 Effect of acute administration of 5-oxoproline (5-OP) on various parameters of oxidative stress in cerebral cortex and cerebellum from 14-day-old rats: spontaneous chemiluminescence (A), thiobarbituric acid-reactive substances (TBA-RS) (B), protein carbonyl content (C) and total radical-trapping antioxidant potential (TRAP) (D). Results are mean ± SD (n=6) for independent experiments performed in duplicate. ** $p<0.01$ compared to control (Student's *t* test for unpaired samples)

Fig. 2 Effect of acute administration of 5-oxoproline (5-OP) enzyme activities in cerebral cortex and cerebellum from 14-day-old rats: catalase (CAT) (A), glutathione peroxidase (GPx) (B) superoxide dismutase (SOD) (C) and glucose 6-phosphate dehydrogenase (G6PD) (D). Results are mean ± SD (n=4-6) for independent experiments performed in duplicate. * $p<0.05$ and ** $p<0.01$ compared to control (Student's *t* test for unpaired samples)

Fig. 3 Effect of acute administration of 5-oxoproline (5-OP) on hydrogen peroxide content in cerebral cortex and cerebellum from 14-day-old rats. Results are mean ± SD (n=6-8) for independent experiments performed in duplicate. * $p<0.05$ and ** $p<0.01$ compared to control (Student's *t* test for unpaired samples)

Figure 1

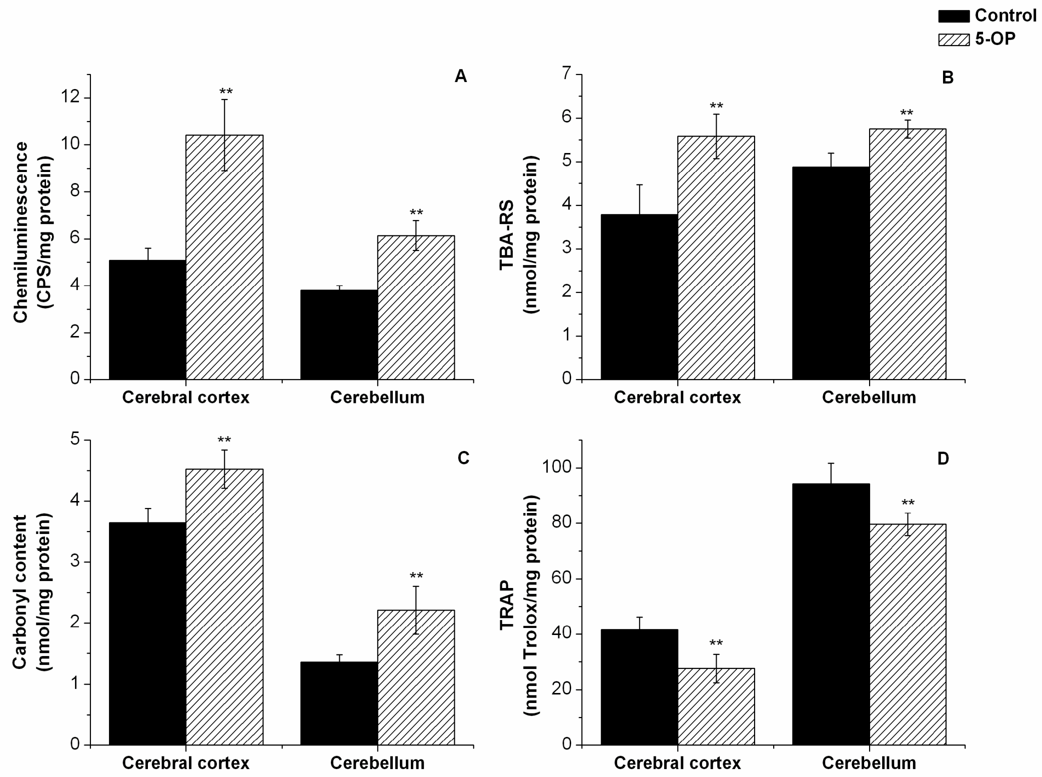


Figure 2

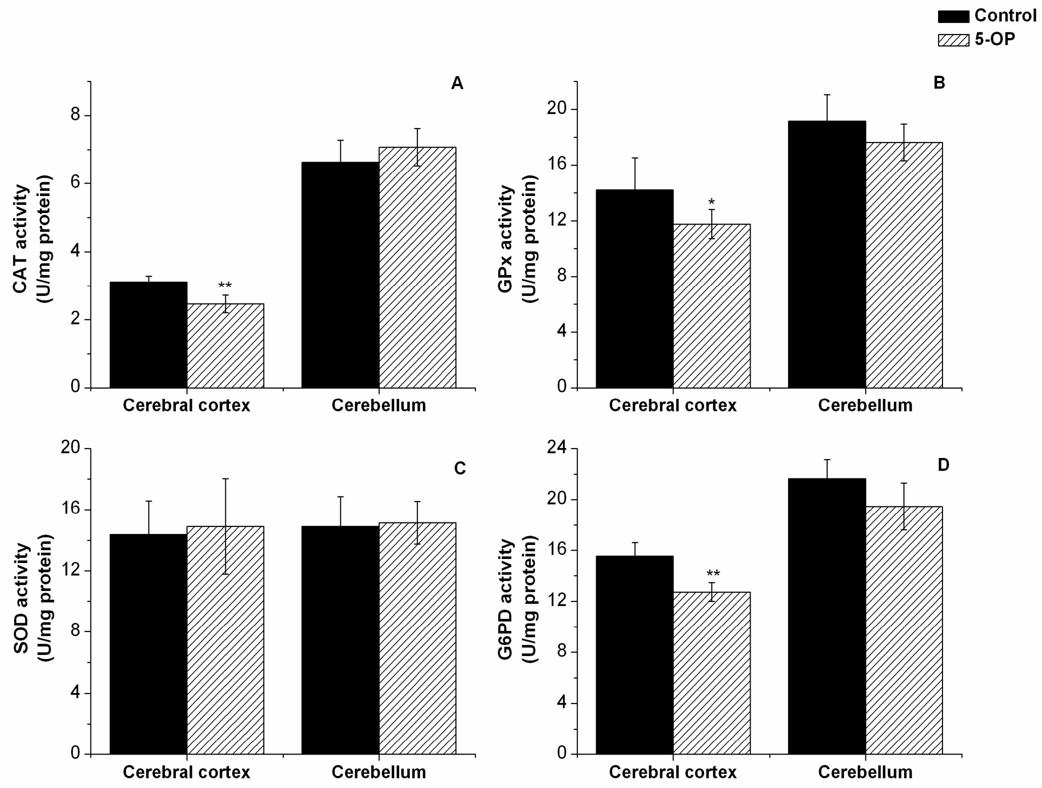
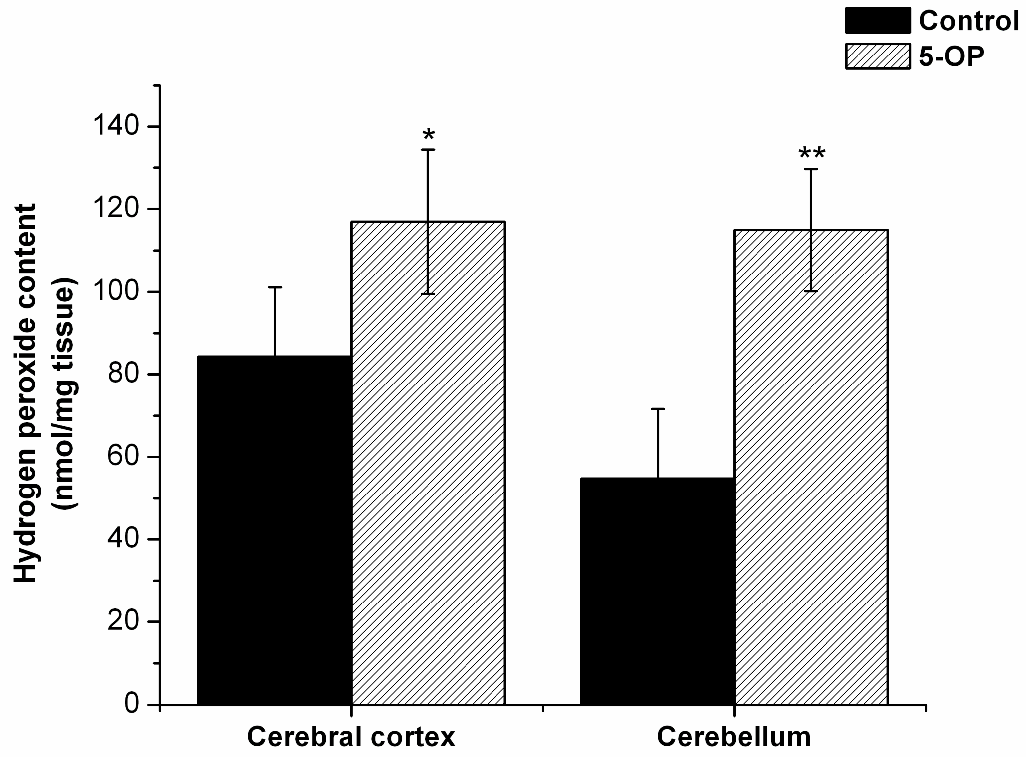


Figure 3



Capítulo III

N-acetylaspartic acid promotes oxidative stress in cerebral cortex of rats

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N-Acetylaspartic acid promotes oxidative stress in cerebral cortex of rats

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Abstract

N-Acetylaspartic acid accumulates in Canavan Disease, a severe leukodystrophy characterized by swelling and spongy degeneration of the white matter of the brain. This inherited metabolic disease, caused by deficiency of the enzyme aspartoacylase, is clinically characterized by severe mental retardation, hypotonia and macrocephaly, and also generalized tonic and clonic type seizures in about half of the patients. Considering that the mechanisms of brain damage in this disease remain not fully understood, in the present study we investigated whether oxidative stress is elicited by *N*-acetylaspartic acid. The *in vitro* effect of *N*-acetylaspartic acid (10–80 mM) was studied on oxidative stress parameters: total radical-trapping antioxidant potential (TRAP), total antioxidant reactivity (TAR), chemiluminescence, thiobarbituric acid-reactive substances (TBA-RS), reduced glutathione content, sufhydryl content and carbonyl content in the cerebral cortex of 14-day-old rats. The effect of the acute administration of *N*-acetylaspartic acid (0.1–0.6 mmol/g body weight) was studied on TRAP, TAR, carbonyl content, chemiluminescence and TBA-RS. TRAP, TAR, reduced glutathione content and sulfhydryl content were significantly reduced, while chemiluminescence, TBA-RS and carbonyl content were significantly enhanced by *N*-acetylaspartic acid *in vitro*. The enhancement in TBA-RS promoted by *N*-acetylaspartic acid was completely prevented by ascorbic acid plus Trolox, and partially prevented by glutathione and dithiothreitol. The acute administration of *N*-acetylaspartic acid also significantly reduced TRAP and TAR, and significantly enhanced carbonyl content, chemiluminescence and TBA-RS. Our results indicate that *N*-acetylaspartic acid promotes oxidative stress by stimulating lipid peroxidation, protein oxidation and by decreasing non-enzymatic antioxidant defenses in rat brain. This could be another pathophysiological mechanism involved in Canavan Disease.
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Keywords: *N*-Acetylaspartic acid; Aspartoacylase deficiency; Rat brain; Canavan Disease; Oxidative stress

N-Acetylaspartic acid (NAA) is one of the most abundant free amino acid derivatives in the mammalian central nervous system, largely neuron specific, present in the brain in concentrations up to 20 mM (Baslow, 2003). It is second only to glutamate in its abundance in the mammalian brain (Matalon and Michals-Matalon, 2000). NAA is normally synthesized from acetyl-CoA and L-aspartic acid by acetyl-CoA-L-aspartate *N*-acetyltransferase, and hydrolyzed to aspartate and acetate by aspartoacylase (Beaudet, 2001). In spite of its high concentration, the role of NAA in brain metabolism remains unclear (Matalon and Michals-Matalon, 1999). Speculations regarding the function of NAA include the possibilities that the acetyl group is incorporated into brain lipids (Chakraborty et al., 2001; Kirmani et al., 2002; Madhavarao et al., 2005; Namboodiri et al., 2006), that NAA serves as an intracellular osmolite (Baslow, 2002), that it might serve as a precursor of *N*-acetyl-aspartyl-glutamate (NAAG) (Gehl et al., 2004), that it might stabilize the concentration of acetyl-CoA and that it might serve as a storage form of aspartate (Beaudet, 2001).

Abbreviations: NAA, *N*-acetylaspartic acid; CD, Canavan Disease; TRAP, total radical-trapping antioxidant potential; TAR, total antioxidant reactivity; TBA-RS, thiobarbituric acid-reactive substances; GSH, reduced glutathione; AA, ascorbic acid; DTT, dithiothreitol; L-NAME, L$^{\varpi}$nitro-L-arginine methyl ester; SOD, superoxide dismutase; CAT, catalase; ABAP, 2,2′-azo-bis-(2-amidinopropane); DTNB, 5,5′-dithio-bis-(2-nitrobenzoic acid); TNB, thionitrobenzoic acid

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Massive excretion of NAA in the urine is the biochemical marker for Canavan Disease (CD), which is caused by deficiency of the enzyme aspartoacylase (Matalon and Michals-Matalon, 2000). CD, an autosomal recessive disease found more frequently among the Ashkenazi Jews, is a severe progressive leukodystrophy characterized by swelling and spongy degeneration of the white matter of the brain (Matalon and Michals-Matalon, 2000). This disorder is clinically characterized by severe mental retardation with inability to gain developmental milestones. Although infants are usually normal in the first month of life, they may exhibit poor visual fixation, irritability, and poor suckling at birth (Traeger and Rapin, 1998). After 5–6 months of age, the patients also develop hypotonia, head lag and macrocephaly (Matalon and Michals-Matalon, 2000). The patients progress to extreme hypertonicity with pseudobulbar palsy and decorticate posturing. Seizures, usually generalized tonic and clonic types, occur in about half of patients (Traeger and Rapin, 1998; Beaudet, 2001). When the condition is suspected, the diagnosis can be confirmed by elevated NAA levels in the urine, blood and spinal fluid and in the brain *in vivo* by the use of proton nuclear magnetic resonance spectroscopy (Gordon, 2000). The increased levels of NAA lead to "swelling" or sponginess of the brain and disruption of the white matter. As CD progresses, the brain becomes atrophic, and the gray matter becomes involved as well (Matalon and Michals-Matalon, 2000). Swollen astrocytes can be seen, and electron microscopy shows distorted and elongated mitochondria (Adachi et al., 1972). The neuropathological findings of CD also include an extensive loss of myelin, maintenance or slight increase in numbers of oligodendroglia, and a prominent increase in protoplasmic astrocytes (Beaudet, 2001).

The mechanisms of brain damage in this disease remain not fully established. Madhavarao et al. (2005) demonstrated that myelin lipid synthesis is significantly compromised in CD and provide direct evidence that defective myelin synthesis, resulting from a deficiency of NAA-derived acetate, is involved in the pathogenesis of CD. Evidence supporting a role for NAA as a molecular water pump may also be involved in the pathogenesis of CD, as disrupted NAA metabolism leads to the pathological build up of NAA, and consequently to osmotic imbalance with a build up of excessive fluid in the brain, and demyelination characteristic of this disease (Baslow, 2002).

Although the role of NAA in the pathogenesis of CD is still unclear, increased concentrations of NAA in tissues and fluids would suggest the possibility that NAA or related metabolites might have toxic effects (Beaudet, 2001). Patients with CD often present with optic neuropathy (Matalon and Michals-Matalon, 1999). The presence of NAA has already been demonstrated in the retina (Baslow, 2003). Recent findings suggest that the metabolism of NAA is disrupted in CD not only in the brain but also in the neural retina, thus providing the molecular basis for the retinal and optic nerve dysfunction in this disease (George et al., 2004).

In the brain, NAA has been shown to induce seizures after intracerebroventricular administration to normal rats, probably by neuronal overexcitation through metabotropic glutamate receptors (Akimitsu et al., 2000; Kitada et al., 2000; Yan et al., 2003). Indeed, epilepsy has already been related to oxidative stress (Patel, 2004). In addition, as occurs in CD, it was found that tardive dyskinesia patients also have significantly higher concentrations of NAA in their cerebrospinal fluid (Tsai et al., 1998). Interestingly, these authors studied the association between oxidative stress and tardive dyskinesia.

In the present study we investigated the possible role of oxidative stress in NAA neurotoxicity in order to clarify its participation in the brain damage mechanisms responsible for the neurological impairment observed in CD patients. To accomplish that, the *in vitro* effect of NAA was studied on the following oxidative stress parameters in cerebral cortex of 14-day-old rats: total radical-trapping antioxidant potential (TRAP) and total antioxidant reactivity (TAR), to evaluate non-enzymatic antioxidant defenses; chemiluminescence and thiobarbituric acid-reactive substances (TBA-RS), to assess lipid peroxidation; sulfhydryl and carbonyl contents, to evaluate protein oxidation; and reduced glutathione (GSH) content. We also evaluated the possible reversal of the effects of NAA by some antioxidants on TBA-RS. The effect of the acute administration of NAA on TRAP, TAR, carbonyl content, chemiluminescence and TBA-RS in cerebral cortex of 14-day-old rats was also studied.

1. Experimental procedures

1.1. Materials

All chemicals were purchased from Sigma (St. Louis, MO, USA) except 2,2′-azo-bis-(2-amidinopropane) that was purchased from Wako Chemicals (USA). NAA solutions were freshly prepared in 20 mM sodium phosphate buffer, pH 7.4 containing 140 mM KCl. The pH was adjusted when necessary. The acid was added to homogenates at final concentrations of 10, 20, 40 or 80 mM.

1.2. Animals

Fourteen-day-old Wistar rats bred in the Department of Biochemistry, ICBS, UFRGS, from both sexes and different litters, were used. Rats were kept with dams until they were killed. The dams had free access to water and a 20% (w/w) protein commercial chow (Supra, Porto Alegre, RS, Brazil). They were kept in a room with 12 h light:12 h dark cycle (lights on 07:00–19:00 h) and with air-conditioned controlled temperature (22 ± 1 °C). The NIH 'Guide for the Care and Use of Laboratory Animals' (NIH publication #80-23, revised 1996) was followed in all experiments.

1.3. Tissue preparation

Rats were killed by decapitation, and the brain was immediately removed and kept on an ice-plate. The olfactory bulb, pons and medulla were discarded and the cerebral cortex was dissected, weighed and kept chilled until homogenization. These procedures lasted up to 3 min. Cerebral cortex was homogenized in 10 volumes (1:10, w/v) of 20 mM sodium phosphate buffer, pH 7.4 containing 140 mM KCl. Homogenates were centrifuged at 750 × *g* for 10 min at 4 °C to discard nuclei and cell debris (Llesuy et al., 1985; Lissi et al., 1986). The pellet was discarded and the supernatant was immediately separated and used for the measurements. The homogenates used were from individual animals, and they were never pooled. All experiments were repeated with different animals.

1.4. In vitro experiments

Cerebral cortex supernatants were pre-incubated for 1 h at 37 °C in the presence of NAA at final concentrations ranging from 10 to 80 mM. Controls were incubated only with 20 mM sodium phosphate buffer, pH 7.4 containing 140 mM KCl, without NAA. After incubation, aliquots were taken to measure TRAP, TAR, chemiluminescence, TBA-RS, sulfhydryl content, carbonyl content and GSH content.

The effect of some antioxidants on the enhanced TBA-RS levels promoted by NAA was also investigated. All antioxidants were prepared in 20 mM sodium phosphate buffer, pH 7.4, containing 140 mM KCl, and the final concentrations were similar to those described previously (Latini et al., 2003; Bridi et al., 2005), as follows: 200 μM ascorbic acid, 10 μM Trolox (a water soluble α-tocopherol mimic), 400 μM dithiothreitol (DTT); 400 μM reduced glutathione (GSH), 200 μM N^{ω}nitro-L-arginine methyl ester (L-NAME), 50 mU/mL superoxide dismutase (SOD) (purified from bovine erythrocytes, EC 1.15.1.1) and 50 mU/mL catalase (CAT) (purified from bovine erythrocytes, EC 1.11.1.6).

1.5. Acute administration of NAA

NAA was dissolved in saline solution and the pH was adjusted to 7.4. NAA was administered subcutaneously in the following doses: 0.1, 0.3 or 0.6 mmol/g body weight. Controls received saline solution. One hour after injections, rats were killed by decapitation and tissue was prepared as mentioned above. Cerebral cortex supernatants were directly used to measure TRAP, TAR, carbonyl content, chemiluminescence and TBA-RS.

1.6. Total radical-trapping antioxidant potential (TRAP)

TRAP was determined by measuring the chemiluminescence intensity of luminol induced by 2,2′-azo-bis-(2-amidinopropane) (ABAP) thermolysis (Lissi et al., 1992; Evelson et al., 2001) in a Wallac 1409 Scintillation Counter. Three milliliters of ABAP 10 mM, dissolved in 50 mM sodium phosphate buffer pH 7.4, plus 10 μL of luminol (5.6 mM) were added to a glass scintillation vial, and the initial chemiluminescence was measured. Ten microliters of 160 μM Trolox (water-soluble α-tocopherol analogue, used as standard) or 10 μL of tissue supernatant were then added to that vial, producing a decrease in the initial chemiluminescence value. This value is kept low until the antioxidants present in the sample are depleted, then chemiluminescence returns to its initial value. The time taken by the sample to keep chemiluminescence low is directly proportional to the antioxidant capacity of the tissue, so TRAP represents the amount (quantity) of non-enzymatic antioxidants present in the sample. The results were calculated as nanomoles Trolox per milligram of protein.

1.7. Total antioxidant reactivity (TAR)

TAR was determined by measuring the luminol chemiluminescence intensity induced by ABAP thermolysis (Lissi et al., 1995) using a Wallac 1409 Scintillation Counter. The background chemiluminescence was measured by adding 4 mL of 2 mM ABAP, prepared in 0.1 mM glycine buffer, pH 8.6, plus 15 μL of luminol (4 mM) into a glass scintillation vial. Ten microliters of 20 μM Trolox or tissue supernatant was then added and the chemiluminescence was measured during 60 s to evaluate how fast it falls. This velocity of reduction in luminol intensity reflects the tissue capacity to promptly react against an enhanced free radical production. TAR represents not the amount but the reactivity (quality) of non-enzymatic antioxidants present in the sample. The results were calculated as nanomoles Trolox per milligram of protein.

1.8. Reduced glutathione content

To determine reduced glutathione, cerebral cortex homogenates were deproteinized with 2 M perchloric acid and centrifuged 10 min at 1000 × *g*. Supernatants were neutralized with 2 M potassium hydroxide to pH 7.0 and centrifuged at 1000 × *g* for 10 min, as previously described (Araujo et al., 2006). The supernatant obtained reacted with 6 mM 5,5′-dithiobis(2-nitrobenzoic acid) and was read at 420 nm (Akerboom and Sies, 1981). The results were expressed in nanomoles GSH per milligram of protein.

1.9. Carbonyl content

Oxidatively modified proteins present an enhancement of carbonyl content (Stadtman, 1990). In this paper, carbonyl content was assayed by a method based on the reaction of protein carbonyls with dinitrophenylhydrazine forming dinitrophenylhydrazone, a yellow compound, measured spectrophotometrically at 370 nm (Reznick and Packer, 1994). Briefly, 100 μL of homogenate were added to plastic tubes containing 400 μL of 10 mM dinitrophenylhydrazine (prepared in 2 M HCl). This was kept in the dark for 1 h and vortexed each 15 min. After that, 500 μL of 20% trichloroacetic acid were added to each tube. The mixture was vortexed and centrifuged at 14,000 rpm for 03 min. The supernatant obtained was discarded. The pellet was washed with 1 mL ethanol:ethyl acetate (1:1, v/v), vortexed and centrifuged at 14,000 rpm for 3 min. The supernatant was discarded and the pellet re-suspended in 600 μL of 6 M guanidine (prepared in a 20 mM potassium phosphate solution pH 2.3). The sample was vortexed and incubated at 60 °C for 15 min. After that, it was centrifuged at 14,000 rpm for 03 min and the supernatant was used to measure absorbance at 370 nm (UV) in a quartz cuvette. Results were reported as carbonyl content (nmol/mg protein).

1.10. Sulfhydryl content

This assay is based on the reduction of DTNB by thiols, which in turn become oxidized (disulfide), generating a yellow derivative (TNB) whose absorption is measured spectrophotometrically at 412 nm (Aksenov and Markesbery, 2001). Briefly, 50 μL of homogenate were added to 1 mL of PBS buffer pH 7.4 containing 1 mM EDTA. Then 30 μL of 10 mM DTNB, prepared in a 0.2 M potassium phosphate solution pH 8.0, were added. Subsequently, 30 min incubation at room temperature in a dark room was performed. Absorption was measured at 412 nm using a Beckman DU® 640 spectrophotometer. The sulfhydryl content is inversely correlated to oxidative damage to proteins. Results were reported as nanomole TNB per milligram of protein.

1.11. Chemiluminescence

Samples were assayed for chemiluminescence in a dark room (Lissi et al., 1986) using a beta liquid scintillation spectrometer Tri-Carb 2100TR. The background chemiluminescence was measured for 5 min in vials containing 3.5 mL of the same buffer used for homogenization. An aliquot of 0.5 mL of supernatant was added and chemiluminescence was measured for 10 min at room temperature. The background chemiluminescence was subtracted from the total value. Chemiluminescence was calculated as counts per second (CPS) per milligram of protein.

1.12. Thiobarbituric acid-reactive substances (TBA-RS)

For the *in vitro* experiments, TBA-RS was measured according to Esterbauer and Cheeseman, 1990. Briefly, 300 μL of cold 10% trichloroacetic acid were added to 150 μL of supernatant and centrifuged at 300 × *g* for 10 min. Three hundred microliters of the supernatant were incubated with 300 μL of 0.67% thiobarbituric acid in 7.1% sodium sulfate in a boiling water bath for 25 min. For the *in vivo* experiments, TBA-RS was measured according to Ohkawa et al., 1979. Briefly, to glass tubes were added, in order of appearance: 500 μL of tissue supernatant; 50 μL of SDS 8.1%; 1500 μL of 20% acetic acid in aqueous solution (v/v) pH 3.5; 1500 μL of 0.8% thiobarbituric acid; and 700 μL of distilled water. The mixture was vortexed and the reaction was carried out in a boiling water bath for 1 h. The next procedures are the same for *in vitro* and *in vivo* techniques. The mixture was allowed to cool on water for 5 min, and was centrifuged 750 × *g* for 10 min. The resulting pink stained TBA-RS obtained with both methods were determined spectrophotometrically at 535 nm in a Beckman DU® 640 Spectrophotometer. A calibration curve was generated using 1,1,3,3-tetramethoxypropane as a standard, being subjected to the same treatment as that of the samples. TBA-RS were calculated as nanomole per milligram of protein.

1.13. Protein determination

Protein concentration was determined in cerebral cortex supernatants using bovine serum albumin as a standard (Lowry et al., 1951).

1.14. Statistical analysis

Statistical analysis was performed by the one-way analyses of variance (ANOVA), followed by the Tukey test for multiple comparison when the F value was significant. Linear regression analysis was also performed to verify dose-dependent effects. All analyses were performed using the Statistical Package for the Social Sciences (SPSS) software in a PC-compatible computer. A value of $p < 0.05$ was considered to be significant.

2. Results

Initially, we evaluated the *in vitro* effect of NAA on non-enzymatic antioxidant defenses in rat cerebral cortex. This was achieved by measuring TRAP and TAR, which evaluate non-enzymatic antioxidants quantity and reactivity, respectively. Fig. 1A shows that TRAP was significantly reduced by NAA as compared to control [$F(4,35) = 5.15$; $p < 0.05$]. In addition, TAR measurement was also significantly reduced [$F(4,25) = 9.50$; $p < 0.05$] in a dose-dependent manner [$\beta = -0.89$; $p < 0.01$] (Fig. 1B) when cerebral cortex was exposed to NAA. Next we measured GSH content, which is one of the major cerebral non-enzymatic antioxidants, to evaluate whether TRAP and TAR reduction occurred by means of GSH content reduction. Fig. 2 shows that GSH content was significantly reduced in the presence of NAA [$F(4,15) = 4.20$; $p < 0.05$].

We also investigated whether tissue proteins were affected by NAA. To accomplish this, two different parameters of oxidative protein damage were measured: sulfhydryl and carbonyl contents. Fig. 3A shows that the carbonyl content was significantly enhanced by NAA in cerebral cortex homogenates [$F(4,15) = 6.29$; $p < 0.05$] in a dose-dependent manner [$\beta = 0.69$; $p < 0.001$], indicating protein oxidation. This result was corroborated by those obtained with the measurement of the sulfhydryl content. Fig. 3B shows that the sulfhydryl content was significantly reduced in cerebral cortex by NAA [$F(4,15) = 20.30$; $p < 0.001$], indicating the occurrence of oxidized proteins.

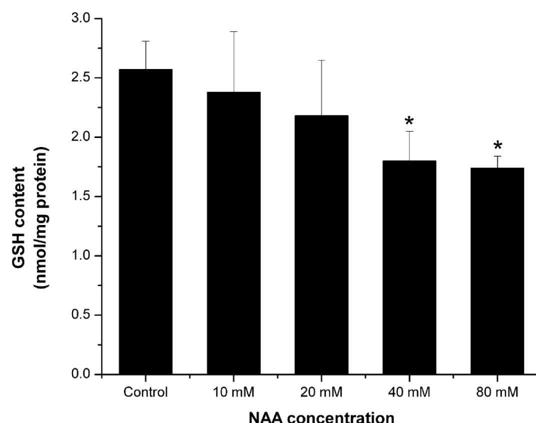

Fig. 2. *In vitro* effect of *N*-acetylaspartic acid (NAA) on reduced glutathione (GSH) content in cerebral cortex from 14-day-old rats. Results are mean ± S.D. ($n = 4$) for independent experiments performed in duplicate. $^{*}p < 0.05$ compared to control (Tukey test).

Next, we investigated chemiluminescence and TBA-RS as lipid peroxidation parameters. Chemiluminescence was significantly enhanced by NAA in cerebral cortex homogenates [$F(4,35) = 13.37$; $p < 0.05$] in a dose-dependent way [$\beta = 0.75$; $p < 0.001$] (Fig. 4A). Fig. 4B shows that NAA was also able to alter TBA-RS levels in cerebral cortex

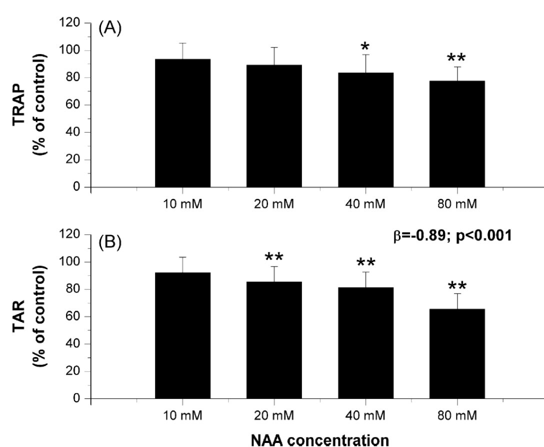

Fig. 1. *In vitro* effect of *N*-acetylaspartic acid (NAA) on non-enzymatic antioxidant defenses: total radical-trapping antioxidant potential (TRAP) (A) and total antioxidant reactivity (TAR) (B) in cerebral cortex from 14-day-old rats. Results are mean ± S.D. ($n = 6–8$) for independent experiments performed in duplicate. $^{*}p < 0.05$ and $^{**}p < 0.01$ compared to control (Tukey test).

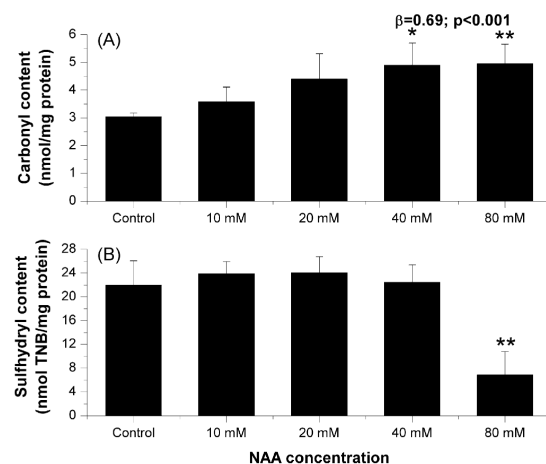

Fig. 3. *In vitro* effect of *N*-acetylaspartic acid (NAA) on protein oxidation indices: carbonyl content (A) and sulfhydryl content (B) in cerebral cortex from 14-day-old rats. Results are mean ± S.D. ($n = 4$) for independent experiments performed in duplicate. $^{*}p < 0.05$ and $^{**}p < 0.01$ compared to control (Tukey test).

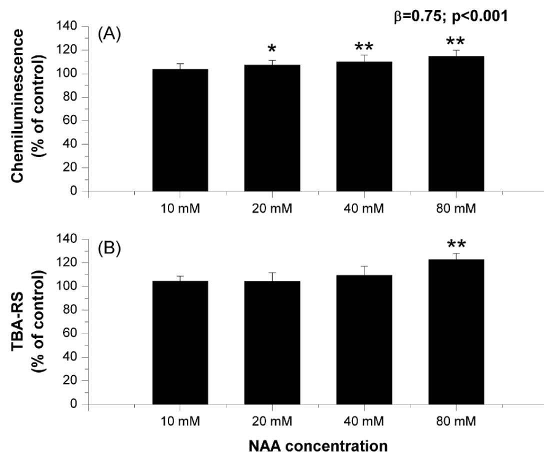

Fig. 4. *In vitro* effect of *N*-acetylaspartic acid (NAA) on lipid peroxidation parameters: chemiluminescence (A) and thiobarbituric acid-reactive substances (TBA-RS) (B) in cerebral cortex from 14-day-old rats. Results are mean ± S.D. ($n = 4–8$) for independent experiments performed in duplicate. $^{**}p < 0.01$ compared to control (Tukey test).

$[F(4,15) = 9.99; p < 0.05]$. Both results indicate that NAA may stimulate lipid peroxidation *in vitro*. Finally we studied the role of various antioxidants on the effect produced by NAA on TBA-RS levels. To accomplish that, cerebral cortex homogenates were incubated for 1 h with 80 mM NAA alone or combined with the free radical scavengers ascorbic acid, Trolox, DTT, GSH, CAT, SOD or L-NAME and the TBA-RS levels were measured afterwards. Fig. 5 shows that the NAA-induced increase of TBA-RS measurement was completely prevented by 200 μM ascorbic acid plus 10 μM Trolox. It was also observed that 400 μM DTT and 400 μM GSH were able to partially prevent the increase of TBA-RS levels caused by NAA. In contrast, the use of 50 mU/mL CAT, 50 mU/mL SOD or 200 μM of the nitric oxide synthase inhibitor L-NAME did not reduce the increased TBA-RS values observed in NAA-treated homogenates $[F(13,42) = 13.76; p < 0.01]$. It is important to note that the addition of any of these antioxidants alone did not significantly alter TBA-RS values.

We also studied the effect of the acute administration of NAA on TRAP, TAR, carbonyl content, chemiluminescence and TBA-RS in order to evaluate if the effects observed on these parameters *in vitro* were confirmed *in vivo*. TRAP (Fig. 6A) was significantly reduced by NAA as compared to control $[F(4,25) = 18.74; p < 0.01]$ in a dose-dependent manner $[\beta = -0.89; p < 0.01]$. In addition, TAR was also significantly reduced $[F(4,25) = 13.49; p < 0.01]$ (Fig. 6B) in cerebral cortex from rats submitted to acute administration of NAA. Carbonyl content was significantly enhanced by the acute administration of NAA $[F(3,14) = 5.75; p < 0.01]$ (Fig. 7), indicating protein oxidation. Lipid peroxidation was also demonstrated, as chemiluminescence was significantly enhanced by the acute administration of NAA $[F(4,24) = 7.24; p < 0.01]$ (Fig. 8A). Fig. 8B shows that the acute administration of NAA was also able to alter TBA-RS levels $[F(4,25) = 4.52; p < 0.01]$. These results indicate that NAA may impair non-enzymatic antioxidant defenses and promote both protein oxidation and lipid peroxidation *in vivo*, in agreement with our *in vitro* results.

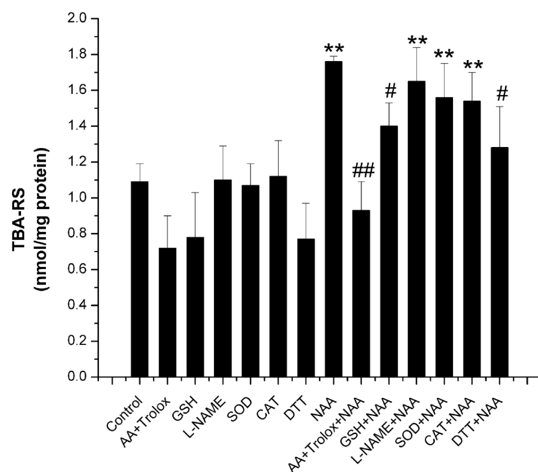

Fig. 5. *In vitro* effect of 80 mM *N*-acetylaspartic acid (NAA) on thiobarbituric acid-reactive substances (TBA-RS) in cerebral cortex from 14-day-old rats in the presence of various antioxidants: ascorbic acid (AA) plus Trolox; reduced glutathione (GSH); N^{ϖ}nitro-L-arginine methyl ester (L-NAME); superoxide dismutase (SOD); catalase (CAT) and dithiothreitol (DTT). Results are mean ± S.D. ($n = 4$) for independent experiments performed in duplicate. $^{**}p < 0.01$ compared to control; $^{\#}p < 0.05$ and $^{\#\#}p < 0.01$ compared to NAA (Tukey test).

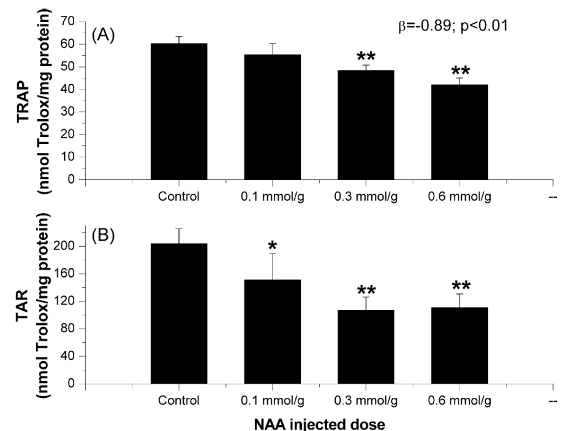

Fig. 6. Effect of the acute administration of *N*-acetylaspartic acid (NAA) on non-enzymatic antioxidant defenses: total radical-trapping antioxidant potential (TRAP) (A) and total antioxidant reactivity (TAR) (B) in cerebral cortex from 14-day-old rats. Results are mean ± S.D. ($n = 6$) for independent experiments performed in duplicate. $^{*}p < 0.05$ and $^{**}p < 0.01$ compared to control (Tukey test). Rats were killed 1 h after injection.

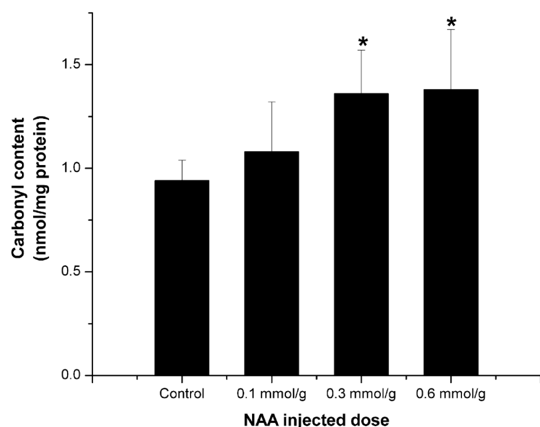

Fig. 7. Effect of the acute administration of *N*-acetylaspartic acid (NAA) on carbonyl content in cerebral cortex from 14-day-old rats. Results are mean ± S.D. ($n = 4$–5) for independent experiments performed in duplicate. $^{*}p < 0.05$ compared to control (Tukey test). Rats were killed 1 h after injection.

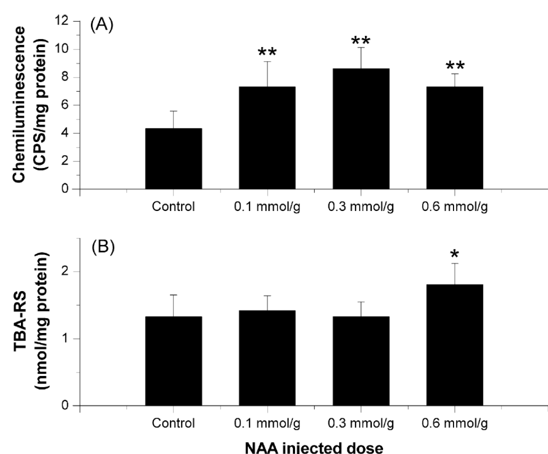

Fig. 8. Effect of the acute administration of *N*-acetylaspartic acid (NAA) on lipid peroxidation parameters: chemiluminescence (A) and thiobarbituric acid-reactive substances (TBA-RS) (B) in cerebral cortex from 14-day-old rats. Results are mean ± S.D. ($n = 5$–6) for independent experiments performed in duplicate. $^{**}p < 0.01$ compared to control (Tukey test). Rats were killed 1 h after injection. CPS = counts per second.

3. Discussion

NAA accumulation is the biochemical hallmark of patients affected by CD. The affected individuals typically present progressive neurological dysfunction with mental retardation, hypotonia, macrocephaly and seizures. Although the underlying mechanisms of brain damage in this disorder remain unclear, increased concentrations of NAA in tissues and fluids would suggest the possibility that NAA or related metabolites might have toxic effects (Beaudet, 2001), and that excess NAA may be deleterious to the CNS. Although the exact mechanisms underlying its toxicity remain not fully understood, there are some reports in the literature showing neurotoxic actions for NAA. In isolated hippocampal neurons, NAA was found to induce an inward current, acting on the G protein-coupled metabotropic glutamate receptors, resulting in excitation of the neurons, thereby contributing to the occurrence of epileptic seizures (Yan et al., 2003). According to Akimitsu et al. (2000), NAA was strongly suggested to be a novel excitatory amino acid involved in the appearance of seizures in some types of epilepsy. Recent findings reported that tremor rats (a genetic model for CD) receiving adenovirus-based aspartoacylase gene transfer showed partial reduction of NAA levels in the brain which resulted in modulation of both seizure length and frequency. These data strongly suggest that increased NAA levels in the tremor rat brain participate in the course of epilepsy (Klugmann et al., 2005). Animal studies show that epileptic seizures result in free radical production and oxidative damage to cellular proteins, lipids and DNA (Bruce and Baudry, 1995; Liang et al., 2000). Conversely, recent work suggests that chronic mitochondrial oxidative stress and resultant dysfunction can render the brain more susceptible to epileptic seizures (Trotti et al., 1998; Liang and Patel, 2004). It seems that there is a role for oxidative stress both as a cause and a consequence of epileptic seizures (Patel, 2004).

Considering that to our knowledge no study has investigated so far the role of oxidative stress on NAA neurotoxicity, in the present work we investigated the *in vitro* effect of this organic acid as well as the effect of the acute administration of NAA on some oxidative stress parameters in order to evaluate whether free radical generation could be elicited by this metabolite, which could be possibly related to the neurological damage occurring in CD.

We demonstrated that NAA significantly reduced both TRAP and TAR in cerebral cortex of 14-day-old rats. Considering that TRAP measures the content of non-enzymatic antioxidant defenses, while TAR reflects the capacity of a given tissue to modulate the damage associated with an increased production of RS (Lissi et al., 1995), these results indicate that NAA reduces the non-enzymatic antioxidant capacity in rat brain both *in vitro* and *in vivo*, by reducing non-enzymatic antioxidant content (TRAP) and also the antioxidant reactivity (TAR). GSH is a major non-enzymatic antioxidant present in mammalian brain, so we evaluated whether the reduction of TRAP and TAR promoted by NAA was in fact caused by a reduction in GSH content of the brain. We demonstrated that NAA significantly reduced GSH content *in vitro*, which suggests that the TRAP and TAR reduction observed in the presence of NAA actually occurred by means of GSH content reduction.

In the present work we also showed that NAA was able to cause oxidative damage to proteins, as verified by the significant alterations of sulfhydryl and carbonyl contents. Thus, it is possible that oxidative damage to proteins may be involved in the neuropathology of CD.

We also found that chemiluminescence and TBA-RS were enhanced by the presence of NAA in the incubation medium, as well as by the acute administration of NAA, suggesting that NAA induces lipid peroxidation (oxidative damage to lipids) in

cerebral cortex of 14-day-old rats. Considering that the overproduction of reactive oxygen species gives rise to high TBA-RS levels (Halliwell and Gutteridge, 1999a), we studied the influence of various antioxidants on the increased TBA-RS levels induced by 80 mM NAA *in vitro*. We observed that ascorbic acid plus Trolox was able to completely prevent the NAA-elicited increase in TBA-RS levels in cerebral cortex homogenates. These findings may suggest the participation of hydroxyl radicals on this effect, since this reactive species is scavenged by the antioxidants used (Halliwell and Gutteridge, 1999b; Bains and Shaw, 1997). On the other hand, hydrogen peroxide and superoxide anion may not be involved because their scavenging, respectively, by CAT and SOD were not able to prevent TBA-RS levels enhancement caused by NAA. Since DTT and GSH partially prevented NAA-induced TBA-RS levels enhancement, we cannot rule out the possibility that the oxidation of SH groups is involved in the NAA neurotoxic effects. In contrast, nitric oxide was not probably involved in the increased TBA-RS levels caused by NAA since L-NAME was not able to reduce the increase of TBA-RS levels promoted by NAA.

Taken together, our results indicate that NAA may promote oxidative stress *in vitro* and *in vivo* in cerebral cortex of 14-day-old rats by decreasing non-enzymatic antioxidant defenses and stimulating oxidative damage to both lipids and proteins, probably by enhancing reactive species in cerebral cortex. Our study introduces another possible pathological mechanism to the brain damage observed in affected patients, and it can be therefore suggested that oxidative stress may be involved in the neuropathology of CD, in which NAA accumulation is the biochemical hallmark.

Even though the concentrations of NAA used in our assays are similar to those observed in plasma and cerebrospinal fluid of patients affected by CD (up to four-fold elevated in plasma and cerebrospinal fluid) (Tsai and Coyle, 1995; Surendran et al., 2003), it is difficult to extrapolate our findings to the human condition. However, our results are in line with those of Tsai et al. (1998), who studied the association between oxidative stress and tardive dyskinesia. Interestingly, it was found that tardive dyskinesia patients also have significantly elevated concentrations of NAA in their cerebrospinal fluid (100 mM), as occurs in CD. Tardive dyskinesia symptoms correlated positively with markers of excitatory neurotransmission and protein carbonyl group (Tsai et al., 1998). Elevated levels of conjugated dienes and thiobarbituric acid-reactive products in the CSF of tardive dyskinesia patients have also been reported (Pall et al., 1987; Lohr et al., 1990). The hypothesis of oxidative damage in tardive dyskinesia is supported by reports that vitamin E reverses the symptoms of tardive dyskinesia patients (Adler et al., 1993; Lohr and Caligiuri, 1996; Zhang et al., 2004). Notably, patients are more responsive to treatment with vitamin E earlier in the course of their disorder, consistent with the model that the oxidative damage is cumulative over time and would involve functional impairment before frank degeneration (Tsai et al., 1998). Those findings of enhanced carbonyl content and enhanced lipid peroxidation in the CSF of tardive dyskinesia patients, who also present dramatic elevations of NAA levels, are in line with our findings of enhanced protein oxidation and lipid peroxidation in cerebral cortex homogenates exposed to NAA.

In the present study we demonstrated that NAA decreases non-enzymatic antioxidant defenses and stimulates oxidative damage to both lipids and proteins, promoting oxidative stress. If the oxidative stress elicited by NAA in our study also occurs in the brain of patients affected by CD, it is possible that it may contribute, along with other mechanisms, to the neurological dysfunction characteristic of this disease. Based on the results presented here, it is proposed that the administration of antioxidants, especially vitamins E and C, should be considered as a potential adjuvant therapy for patients affected by CD. However, the underlying mechanisms responsible for NAA neurotoxicity in the brain damage of these patients remains to be further elucidated.

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Capítulo IV

N-acetylaspartic acid impairs enzymatic antioxidant defenses in rat brain: relevance to Canavan Disease

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N-acetylaspartic acid impairs enzymatic antioxidant defenses in rat brain: relevance to Canavan Disease

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Running title: N-acetylaspartic acid impairs antioxidant defenses

Abstract

N-Acetylaspartic acid accumulates in Canavan Disease, a severe inherited neurometabolic disease clinically characterized by severe mental retardation, hypotonia, macrocephaly and generalized tonic and clonic type seizures. Considering that the mechanisms of brain damage in this disease remain poorly understood, in the present study we investigated the effect of N-acetylaspartic acid on the enzymatic antioxidant status in rat brain. The in vitro effect of N-acetylaspartic acid (10-80 mM) was studied on the activities of catalase, superoxide dismutase and glutathione peroxidase, as well as on hydrogen peroxide content in cerebral cortex of 14-day-old rats. The effect of acute administration of 0.6 mmol N-acetylaspartic acid/g body weight on the same parameters was also studied. Catalase and glutathione peroxidase activities were significantly inhibited, while hydrogen peroxide content was significantly enhanced by N-acetylaspartic acid both in vitro and in vivo. In contrast, superoxide dismutase activity was not altered by N-acetylaspartic acid. Our results clearly show that N-acetylaspartic acid impairs the enzymatic antioxidant defenses in rat brain. This could be involved in the pathophysiological mechanisms responsible for the brain damage observed in patients affected by Canavan Disease.

Keywords: N-Acetylaspartic acid; Canavan Disease; hydrogen peroxide; catalase; glutathione peroxidase; rat brain

N-acetylaspartic acid (NAA) is normally synthesized from acetyl-CoA and L-aspartic acid by acetyl-CoA:L-aspartate *N*-acetyltransferase (EC 2.3.1.17) and hydrolyzed to aspartate and acetate by N-acyl-L-aspartate amidohydrolase (EC 3.5.1.15, aspartoacylase) (Beaudet, 2001). In spite of reaching cerebral concentrations up to 20 mM (Baslow, 2003), the role of NAA on brain metabolism remains unclear (Matalon and Michals-Matalon, 1999).

Massive excretion of NAA in the urine is the biochemical hallmark of Canavan Disease (CD), an autossomal recessive inherited metabolic disease caused by deficiency of the enzyme aspartoacylase (Matalon and Michals-Matalon, 2000). CD, found more frequently among the Ashkenazi Jews, is a severe progressive leukodystrophy characterized by swelling and spongy degeneration of the white matter of the brain; as the disease progresses, the brain becomes atrophic and the gray matter becomes involved as well (Matalon and Michals-Matalon, 2000). Brain atrophy progressively increases over time in CD patients and this occurs in parallel with NAA rise (Janson et al., 2006). Diagnosis is confirmed by elevated NAA levels in the urine, blood and spinal fluid and also in the brain in vivo by the use of proton nuclear magnetic resonance spectroscopy (Gordon, 2000).

Patients affected by CD present severe mental retardation with inability to gain developmental milestones, as well as hypotonia and macrocephaly (Traeger and Rapin, 1998; Matalon and Michals-Matalon, 2000). About half of the patients also develop generalized tonic and clonic convulsions (Traeger and Rapin, 1998; Beaudet, 2001). The affected children become increasingly debilitated with age, often with inability to move voluntarily or swallow. Death typically occurs before adolescence, but some Canavan patients with milder forms survive into their 20s or beyond (Moffett et al., 2007).

Neuropathological findings of CD include extensive loss of myelin with intramyelinic splitting, edema and vacuolation in the white matter and brain stem (Kumar, et al., 2006; Skiranth et al., 2007) increase of oligodendroglia and protoplasmic astrocytes (Beaudet, 2001), the presence of swollen astrocytes, and distorted and elongated mitochondria (Adachi et al., 1972).

Although the role of NAA in the pathogenesis of CD is still unclear, increased concentrations of NAA in the brain would suggest the possibility that NAA or related metabolites might have toxic effects (Beaudet, 2001). Indeed neurotoxic actions for NAA in the brain have already been demonstrated, as it is able to induce seizures after intracerebroventricular administration to normal rats, probably by neuronal overexcitation through metabotropic glutamate receptors (Akimitsu et al., 2000; Kitada et al., 2000; Yan et al., 2003). It was also proposed that the defective myelin synthesis results from a deficiency of NAA-derived acetate (Madhavarao et al., 2005; Namboodiri et al., 2006), and that an osmotic imbalance with a build up of excessive fluid in the brain is a consequence of the pathological accumulation of NAA, which was suggested to act as a 'molecular water pump', leading to demyelination (Baslow, 2002).

Recent work from our research group demonstrated the role of NAA inducing oxidative stress, and it was hypothesized the participation of this accumulating metabolite in the brain damage pathomechanisms responsible for the neurological impairment observed in CD patients (Pederzolli et al., 2007). Our data indicated that NAA may promote oxidative stress in vitro and in vivo in cerebral cortex of young rats by decreasing non-enzymatic antioxidant defenses and stimulating oxidative damage to both lipids and proteins, probably by enhancing reactive species in cerebral cortex.

In the present study we investigated the effect of NAA on the enzymatic antioxidant status in rat brain in order to further clarify the role of oxidative stress in NAA neurotoxicity and to try to better understand its participation in the mechanisms of brain damage responsible for the neurological impairment observed in CD patients. To accomplish this, the in vitro effect of N-acetylaspartic acid (10-80 mM) was studied on the activities of the antioxidant enzymes hydrogen-peroxide:hydrogen-peroxide oxidoreductase (EC 1.11.1.6; catalase, CAT), superoxide:superoxide oxidoreductase (EC 1.15.1.1; superoxide dismutase, SOD) and glutathione:hydrogen-peroxide oxidoreductase (EC 1.11.1.9; glutathione peroxidase, GPx), as well as on the hydrogen peroxide content in cerebral cortex of 14-day-old rats. The effect of acute administration of NAA was also studied on the same parameters in the brain.

Experimental Procedures

Materials

All chemicals were purchased from Sigma (St. Louis, MO, USA). N-acetylaspartic acid solutions were freshly prepared in 20 mM sodium phosphate buffer with 140 mM KCl pH 7.4 or saline solution, and the pH was adjusted to 7.4.

Animals

Fourteen-day-old Wistar rats bred in the Department of Biochemistry, ICBS, UFRGS, from both sexes and different litters, were used. Rats were kept with dams until

they were killed. The dams had free access to water and a 20% (w/w) protein commercial chow (Supra, Porto Alegre, RS, Brazil). They were kept in a room with 12:12 h light/dark cycle (lights on 07:00-19:00 h) and with air-conditioned controlled temperature (22°C ± 1°C). The NIH Guide for the Care and Use of Laboratory Animals (NIH publication # 80-23, revised 1996) was followed in all experiments.

Tissue preparation

Rats were killed by decapitation, and the brain was immediately removed and kept on an ice-plate. The olfactory bulb, pons and medulla were discarded and the cerebral cortex was dissected, weighed and kept chilled until homogenization. These procedures lasted up to 3 min. Cerebral cortex was homogenized in 10 volumes (1:10, w/v) of 20 mM sodium phosphate buffer, pH 7.4 containing 140 mM KCl. Homogenates were centrifuged at 750 g for 10 min at 4 ºC to discard nuclei and cell debris (Llesuy et al., 1985; Lissi et al., 1986). The pellet was discarded and the supernatant was immediately separated and used for the measurements. The homogenates used were from individual animals and they were never pooled. All experiments were repeated with different animals.

In vitro experiments

Cerebral cortex supernatants were used for the measurements in the presence or absence of NAA, with and without pre-incubation. In the experiments without pre-incubation, NAA was tested at final concentrations ranging from 10 to 80 mM. In the experiments with pre-incubation, 80 mM NAA was pre-incubated with cerebral cortex supernatants for 1 hour at 37°C. Controls were incubated only with 20 mM sodium

phosphate buffer, pH 7.4 containing 140 mM KCl, without NAA.

Acute administration of NAA

NAA was dissolved in saline solution and the pH was adjusted to 7.4. NAA at a dose of 0.6 mmol/g body weight was administered subcutaneously. Controls received saline solution. One hour after injections, rats were killed by decapitation and tissue was prepared as mentioned above. Cerebral cortex supernatants were used to measure antioxidant enzyme activities and hydrogen peroxide content.

Catalase assay

CAT activity was assayed using a double-beam spectrophotometer with temperature control (Hitachi U-2001). This method is based on the disappearance of H_2O_2 at 240 nm in a reaction medium containing 20 mM H_2O_2, 0.1% Triton X-100, 10 mM potassium phosphate buffer pH 7.0, and 0.1-0.3 mg protein/mL (Aebi, 1984). One CAT unit is defined as one μmol of hydrogen peroxide consumed per minute and the specific activity is calculated as CAT units/mg protein.

Superoxide dismutase assay

SOD activity was determined using the RANSOD kit from RANDOX (Antrim, UK). The method is based on the formation of red formazan from the reaction of 2-(4-iodophenyl)-3-(4-nitrophenol)-5-phenyltetrazolium chloride and superoxide radical produced in the incubation medium from xanthine-xanthine oxidase reaction system, which is assayed spectrophotometrically at 505 nm. The inhibition of the produced chromogen is proportional to the activity of the SOD present in homogenates. A 50% inhibition is defined

as one unit of SOD and the specific activity is calculated as SOD units/mg protein.

Glutathione peroxidase assay

GPx activity was measured using tert-butyl-hydroperoxide as substrate (Wendel, 1981). NADPH disappearance was monitored at 340 nm using a double-beam spectrophotometer with temperature control (Hitachi U-2001). The medium contained 2 mM glutathione, 0.15 U/mL glutathione reductase, 0.4 mM azide, 0.5 mM tert-butyl-hydroperoxide and 0.1 mM NADPH. One GPx unit is defined as one μmol of NADPH consumed per minute and the specific activity is represented as GPx units/mg protein.

Hydrogen peroxide content

This method is based on the horseradish peroxidase-mediated oxidation of phenol red by hydrogen peroxide, which results in the formation of a compound demonstrating increased absorbance at 610 nm (Pick and Keisari, 1980). Briefly, 400 mg cerebral cortex were chopped with a McIlwainn chopper in cortical prisms (400 μm), which were added to a glass vial containing 5.5 mM dextrose buffer pH 7.0, and the mixture was allowed to stand at room temperature for 1 hour. After that, a 60 μL supernatant aliquot was taken and reacted with 235 μL of a medium containing 50 mM sodium phosphate buffer pH 7.4, 85 μL of horseradish peroxidase and 1 mg/mL phenol red (phenolsulfonphtalein). The reaction was carried out in the dark for 10 minutes. After that, 5 μL of 1 N NaOH was added to each tube, and the absorbance was read at 610 nm. A calibration curve was performed with commercial solution of hydrogen peroxide. Results were calculated as nmol hydrogen peroxide/mg tissue.

Protein determination

Protein concentration was determined in cerebral cortex supernatants using bovine serum albumin as a standard (Lowry et al., 1951).

Statistical analysis

For the experiments with more than two groups in comparison, statistical analysis was performed by the one-way analysis of variance (ANOVA), followed by the Tukey test for multiple comparisons when the F value was significant. For the experiments with only two groups, statistical analysis was performed by the Student's t test. All analyses were performed using the Statistical Package for the Social Sciences (SPSS) software in a PC-compatible computer. A value of $p < 0.05$ was considered to be significant. The enzyme kinetic parameters were calculated using the GraphPad Prism 5 software.

Results

In the present study we evaluated the in vitro and in vivo effects of NAA on the enzymatic antioxidant defenses and on hydrogen peroxide content in cerebral cortex of 14-day-old rats.

NAA markedly inhibits CAT activity in an uncompetitive manner

We started measuring the effect of NAA on CAT activity in vitro by measuring the enzyme activity in cerebral cortex homogenates in the presence or the absence of NAA at

final concentrations ranging from 10 to 80 mM, without and with 1 hour pre-incubation. Figures 1A and 1B show that CAT activity was markedly inhibited by the presence of NAA in the reaction medium at all concentrations tested with no pre-incubation [$F(4,15)=14.87$; $p<0.01$] and with 1 hour pre-incubation [$t(6)=17.28$; $p<0.01$], showing a strong inhibition of up to 44% compared to control. Similar results were obtained with a purified commercial CAT preparation (EC 1.11.1.6, from bovine liver) (Figure 1C), in the absence of cerebral cortex homogenates, indicating a possible direct interaction of NAA with CAT. We also tested the effect of acute administration of 0.6 mmol NAA/g body weigth to 14-day-old rats on CAT activity in order to verify if the effect observed in vitro was also detected in vivo. We found that CAT activity was significantly reduced by acute administration of NAA [$t(14)=3.19$; $p<0.01$] (Figure 2). Next, we performed kinetic studies on the interaction between NAA and CAT. The Hanes-Woolf plot (Figure 3) was analyzed over the range of 2.5-20 mM hydrogen peroxide as substrate in the absence and presence of 1-20 mM NAA. K_m value was calculated as the intersection of inhibitor line with x-axis, whereas V_{max} was calculated as the slope of the inhibitor line. Considering that the different inhibitor curves intercepted the x-axis at different points and that those lines presented different slopes, our data indicated that the inhibition of CAT activity caused by NAA was uncompetitive, because both K_m values and maximal velocity were altered with increasing NAA concentrations. Apparent K_m for hydrogen peroxide as substrate and V_{max} of CAT were 15.09 mM and 9.69 µmol H_2O_2 consumed per minute per mg protein, respectively. The K_i' value (uncompetitive inhibition constant) was 15.19 mM. A similar inhibition profile was obtained with the Lineweaver-Burk double reciprocal plot, which was also analyzed in the same conditions.

SOD activity is not affected by NAA

The effect of NAA on SOD activity was also studied. Results are depicted in Figure 4. We evaluated the in vitro effect of NAA on SOD activity in cerebral cortex homogenates in the presence or absence of NAA, at final concentrations ranging from 10 to 80 mM, without and with 1 hour pre-incubation. NAA caused no effect on this enzyme activity at all concentrations tested without pre-incubation [$F(4,25)=1.11$; $p>0.05$] (Figure 4A) or after 1 hour pre-incubation [$t(6)=0.08$; $p>0.05$] (Figure 4B). The in vivo acute administration of NAA also caused no effect on SOD activity [$t(14)=0.26$; $p>0.05$] (Figure 5), which is in line with the in vitro results.

NAA strongly inhibits GPx activity

Next, we evaluated the in vitro effect of NAA on GPx activity in cerebral cortex homogenates in the presence or absence of NAA. GPx activity was inhibited (27% to 34% compared to control) in vitro by the presence of NAA in the reaction medium at concentrations as low as 20 mM without pre-incubation [$F(4,15)=6.60$; $p<0.01$] (Figure 6A) and with 1 hour pre-incubation [$t(6)=5.03$; $p<0.01$] (Figure 6B). Similar results were obtained with a purified commercial GPx preparation (EC 1.11.1.9, from bovine erythrocytes) (Figure 6C), in the absence of cerebral cortex homogenates, indicating a possible direct acting effect of NAA with GPx. GPx activity was also significantly reduced by acute administration of NAA [$t(14)=2.97$; $p<0.05$] (Figure 7).

Hydrogen peroxide content is enhanced by NAA

Altogether, the inhibition of CAT and GPx activities by NAA points to the possible involvement of hydrogen peroxide in NAA neurotoxicity mechanism, since this reactive

species is the substrate for both enzymes. To confirm this hypothesis, we measured hydrogen peroxide content both in vitro and in vivo (Figure 8). After 1 hour pre-incubation of 80 mM NAA with cerebral cortex slices, hydrogen peroxide content was significantly higher in the presence of NAA as compared to control [$t(6)=3.77$; $p<0.01$] (Figure 8A). The acute administration of NAA was also able to significantly enhance hydrogen peroxide content in cerebral cortex of rats [$t(12)=3.41$; $p<0.01$] (Figure 8B). These results suggest that an increase of hydrogen peroxide content may be involved in NAA neurotoxicity.

Discussion

NAA brain accumulation is the biochemical hallmark of patients affected by CD, which is clinically characterized by severe neurological dysfunction with progressive mental retardation, hypotonia, macrocephaly and seizures. Although the underlying mechanisms of brain damage in this disorder remain unclear, increased concentrations of NAA in tissues and fluids suggest the possibility that NAA or related metabolites might have toxic effects (Beaudet, 2001) and that excess NAA may be deleterious to the CNS. Although the exact mechanisms underlying its toxicity remain not fully understood, there are some reports in the literature showing neurotoxic actions for NAA. In this context, NAA was able to induce an inward current, acting on the G protein-coupled metabotropic glutamate receptors, resulting in excitation of the neurons, thereby contributing to the occurence of epileptic seizures (Akimitsu et al., 2000; Yan et al., 2003). In addition, Klugmann et al. (2005) showed that a partial reduction of NAA levels in the brain of genetically aspartoacylase-deficient rats (tremor rats) results in modulation of both seizure length and frequency, suggesting that increased NAA levels in the tremor rat brain

participate in the course of epilepsy. Interestingly, epileptic seizures have already been related to oxidative stress (Bruce and Baudry, 1995; Trotti et al., 1998; Liang et al., 2000; Liang and Patel., 2004; Patel, 2004), a condition that occurs when the delicate balance between production of reactive species and antioxidant defense systems seen in healthy aerobes is upset, or repair system fails. This can be due both from diminished antioxidant defenses and/or increased production of reactive species, which can potentially lead to biomolecular oxidative damage (Halliwell and Gutteridge, 2007a).

In fact, a recent study from our research group has demonstrated a possible role of oxidative stress on NAA neurotoxicity, as the results obtained indicated that NAA may promote oxidative stress in vitro and in vivo in cerebral cortex of 14-day-old rats by decreasing the non-enzymatic antioxidant defenses and stimulating oxidative damage to both lipids and proteins, probably by enhancing reactive species in cerebral cortex (Pederzolli et al., 2007). By that time, we demonstrated that NAA significantly reduces the non-enzymatic antioxidant capacity in rat brain both in vitro and in vivo, by reducing tissue non-enzymatic antioxidant content (TRAP), the antioxidant reactivity (TAR) and GSH content. We also showed that NAA was able to cause oxidative damage to proteins (increased carbonyl content) and lipids (increased chemiluminescence and TBA-RS levels) both in vitro and in vivo. Interestingly, it was observed that ascorbic acid plus Trolox completely prevented the NAA-elicited increase in TBA-RS levels in cerebral cortex homogenates.

In order to identify mechanisms by which oxidative stress plays a role in NAA neurotoxicity, in the present work we investigated the in vitro and in vivo effects of this organic acid on major enzymatic antioxidant defenses and on hydrogen peroxide content in cerebral cortex of 14-day-old rats.

We started measuring the effect of NAA on the activity of CAT, the antioxidant enzyme that catalyses direct decomposition of hydrogen peroxide to ground-state O_2. We found that CAT activity from cerebral cortex homogenates was markedly inhibited (44% to 69% inhibition compared to control) in vitro by the presence of NAA in the reaction medium at all concentrations tested (10 mM and higher) without pre-incubation. We also showed that 80 mM NAA also markedly decreased CAT activity after 1 hour exposition of cerebral cortex homogenates to this metabolite. Furthermore, similar results were obtained with a purified commercial CAT preparation, indicating a possible direct interaction of NAA with CAT. Finally, we observed that acute administration of 0.6 mmol NAA/g body weigth to 14-day-old rats significantly inhibited cortical CAT activity, corroborating our in vitro findings. In order to investigate the mechanism of NAA inhibition on CAT activity, we performed kinetic studies on the interaction between NAA and CAT from cerebral cortex homogenates. Values of K_m and V_{max} obtained (15.09 mM and 9.69 µmol.min^{-1}.mg prot^{-1}, respectively) were similar to the ones reported previously in rat brain (Somani and Husain, 1996). Our results have also shown that NAA inhibits CAT activity in an uncompetitive manner and that K_i' for NAA (15.19 mM) was lower than the concentrations found in the brain of patients affected by CD.

Next, we studied the effect of NAA on activity of SOD, the antioxidant enzyme which dismutates superoxide to produce hydrogen peroxide and water, being highly efficient in the catalytic removal of superoxide radicals. NAA caused no effect on this enzyme activity in vitro and in vivo.

We then evaluated the effect of NAA on GPx activity, which removes hydrogen peroxide by coupling its reduction to H_2O with oxidation of reduced glutathione (GSH). Although being more specific for GSH as a hydrogen donor, it can also act on peroxides

other than hydrogen peroxide, where the peroxide group is reduced to an alcohol. GPx activity from cerebral cortex homogenates was also inhibited in vitro by NAA in the reaction medium without previous incubation and after 1 hour of pre-incubation. NAA was also able to inhibit commercial GPx preparation, indicating a possible direct interaction of NAA with GPx. Similarly to CAT, GPx activity was also significantly reduced by the acute administration of NAA, reinforcing our in vitro findings. Taken together, the inhibition of CAT and GPx activities may indicate an impairment of detoxification of its shared substrate hydrogen peroxide. So, we then measured the effect of NAA on hydrogen peroxide content both in vitro and in vivo. We found that exposition of cerebral cortex homogenates to 80 mM NAA for 1 hour significantly enhanced hydrogen peroxide content. The acute administration of NAA was also able to significantly enhance hydrogen peroxide content in cerebral cortex of 14-day-old rats, in accordance with our in vitro findings. These results suggest that an enhancement in hydrogen peroxide content is probably involved in NAA neurotoxicity and may be secondary to the reduction of CAT and GPx activities. In this context, hydrogen peroxide mixes readily with water and can diffuse within and between cells and is toxic to many cells in the 10 to 100 μM range, causing senescence and apoptosis, and at higher levels it promotes necrotic cell death (Halliwell and Gutteridge, 2007b). Considering that in our previous work we showed that ascorbic acid plus Trolox, which are scavengers of $OH^{\bullet}$, were able to completely prevent the NAA-elicited increase in TBA-RS levels in cerebral cortex homogenates (Pederzolli et al., 2007) and that in the present work we found a NAA-mediated increase in hydrogen peroxide content it may be suggested the participation of hydrogen peroxide, and probably of $OH^{\bullet}$ that is produced from the former by the Fenton reaction, on NAA neurotoxicity.

Altogether, our present findings clearly show that NAA may promote an impairment of enzymatic antioxidant defenses in cerebral cortex of young rats both in vitro and in vivo, by inhibiting CAT and GPx activities, compromising the eficiency of reactive species detoxification, which could lead to oxidative damage to biomolecules. Moreover, NAA promotes an enhancement of hydrogen peroxide content in vitro and in vivo, which could be possibly involved in the progression and maintenance of the neurodegeneration characteristic of CD.

Regarding to the pathophysiology relevance of our present data, it must be emphasized that the alteration of the oxidative stress parameters occurred with concentrations of NAA observed in plasma and cerebrospinal fluid of patients affected by CD (up to 4-fold elevated) (Tsay and Coyle, 1995; Surendran et al., 2003). Although it is difficult to extrapolate our findings to the human condition, in case the antioxidant defense impairment elicited by NAA in our study also occurs in the brain of patients affected by CD, it is possible that it may contribute, along with other mechanisms, to the neurological dysfunction characteristic of this disease. Finally, our present results showing an impairment in enzymatic antioxidant defenses and an enhancement of hydrogen peroxide content, together with our previous results of NAA decreasing non-enzymatic antioxidant defenses and stimulating protein and lipid oxidative damage in cerebral cortex of 14-day-old rats, indicate that NAA may promote oxidative stress *in vitro* and *in vivo* both by enhancing reactive species (hydrogen peroxide, and possibly hydroxyl radical) and by diminishing antioxidant defenses. Based on these results, it is reinforced here the proposition that administration of antioxidants, especially vitamins E and C, should be considered as a potential and beneficial adjuvant therapy for patients affected by CD. However, the exact underlying mechanisms of NAA neurotoxicity and its participation in

the brain damage of these patients remain to be further elucidated.

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FIGURE LEGENDS

Figure 1. In vitro effect of N-acetylaspartic acid (NAA) on catalase (CAT) activity: (A) in cerebral cortex, without previous incubation; (B) in cerebral cortex, with 1 hour pre-incubation at 37°C; and (C) in a purified commercial CAT preparation, without previous incubation. Results are mean ± SD (n=4) for independent experiments performed in duplicate. ** $p<0.01$ compared to control (Tukey test or Student's t test).

Figure 2. Effect of acute administration of 0.6 mmol NAA/g body weigth to 14-day-old rats on catalase (CAT) activity in cerebral cortex homogenates. Results are mean ± SD (n=8) for independent experiments performed in duplicate. ** $p<0.01$ compared to control (Student's t test).

Figure 3. Kinetic analysis of the inhibition of catalase (CAT) caused by N-acetylaspartic acid (NAA) in vitro in cerebral cortex homogenates from 14-day-old rats. The figure shows the Hanes-Woolf plot of the CAT activity for hydrogen peroxide concentrations (2.5-20 mM) in the absence of NAA and in the presence of 1 mM, 5 mM, 10 mM and 20 mM NAA. All experiments were performed at least four independent times, and similar results were obtained. Data presented were from individual experiments.

Figure 4. In vitro effect of N-acetylaspartic acid (NAA) on superoxide dismutase (SOD) activity: (A) in cerebral cortex, without previous incubation; and (B) in cerebral cortex,

with 1 hour pre-incubation at 37°C. Results are mean ± SD (n=4-6) for independent experiments performed in duplicate. No significant differences were detected from control (Tukey test or Student's t test).

Figure 5. Effect of acute administration of 0.6 mmol NAA/g body weigth to 14-day-old rats on superoxide dismutase (SOD) activity in cerebral cortex homogenates. Results are mean ± SD (n=8) for independent experiments performed in duplicate. No significant differences were detected from control (Student's t test).

Figure 6. In vitro effect of N-acetylaspartic acid (NAA) on glutathione peroxidase (GPx) activity: (A) in cerebral cortex, without previous incubation; (B) in cerebral cortex, with 1 hour pre-incubation at 37°C; and (C) in a purified commercial GPx preparation, without previous incubation. Results are mean ± SD (n=4) for independent experiments performed in duplicate. * $p<0.05$ and ** $p<0.01$ compared to control (Tukey test or Student's t test).

Figure 7. Effect of acute administration of 0.6 mmol NAA/g body weigth to 14-day-old rats on glutathione peroxidase (GPx) activity in cerebral cortex homogenates. Results are mean ± SD (n=8) for independent experiments performed in duplicate. ** $p<0.01$ compared to control (Student's t test).

Figure 8. In vitro and in vivo effects of N-acetylaspartic acid (NAA) on hydrogen peroxide content from 14-day-old rats: (A) in vitro in cerebral cortex homogenates, with 1 hour pre-incubation at 37°C; and (B) in vivo in cerebral cortex homogenates from rats subjected to

an acute administration of 0.6 mmol NAA/g body weigth. Results are mean ± SD (n=4-7) for independent experiments performed in duplicate. ** $p<0.01$ compared to control (Student's t test).

Figure 1

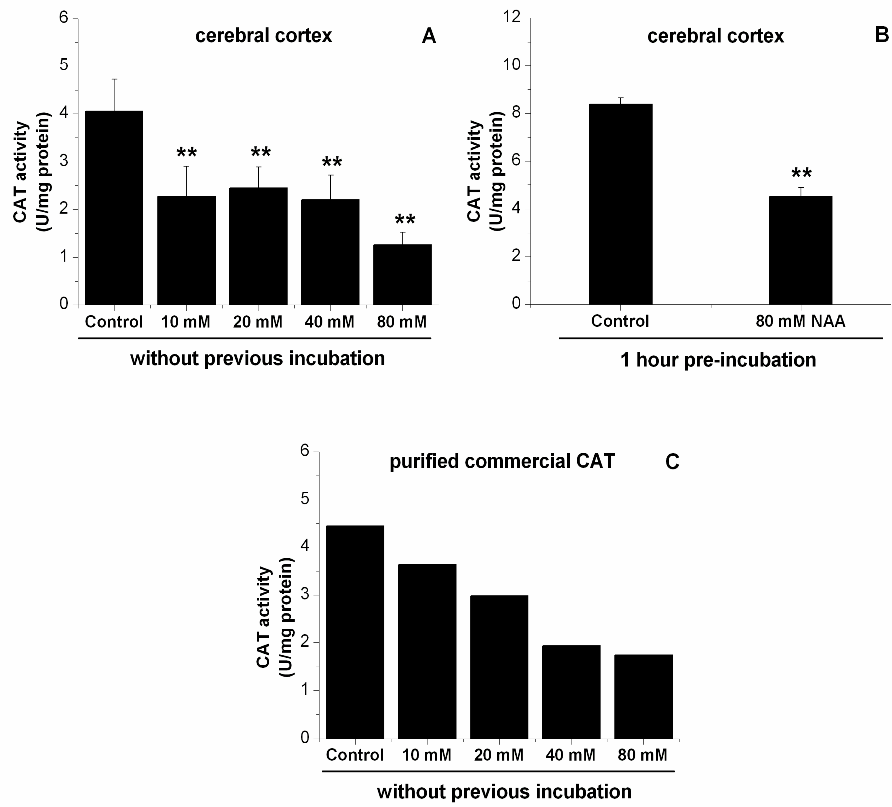


Figure 2

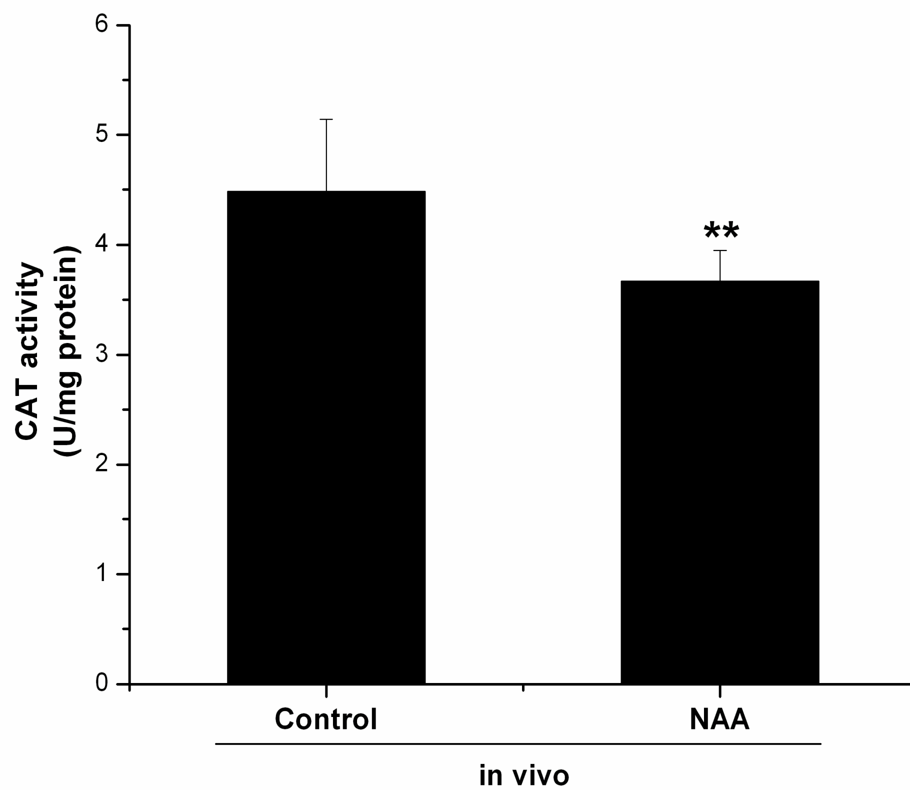


Figure 3

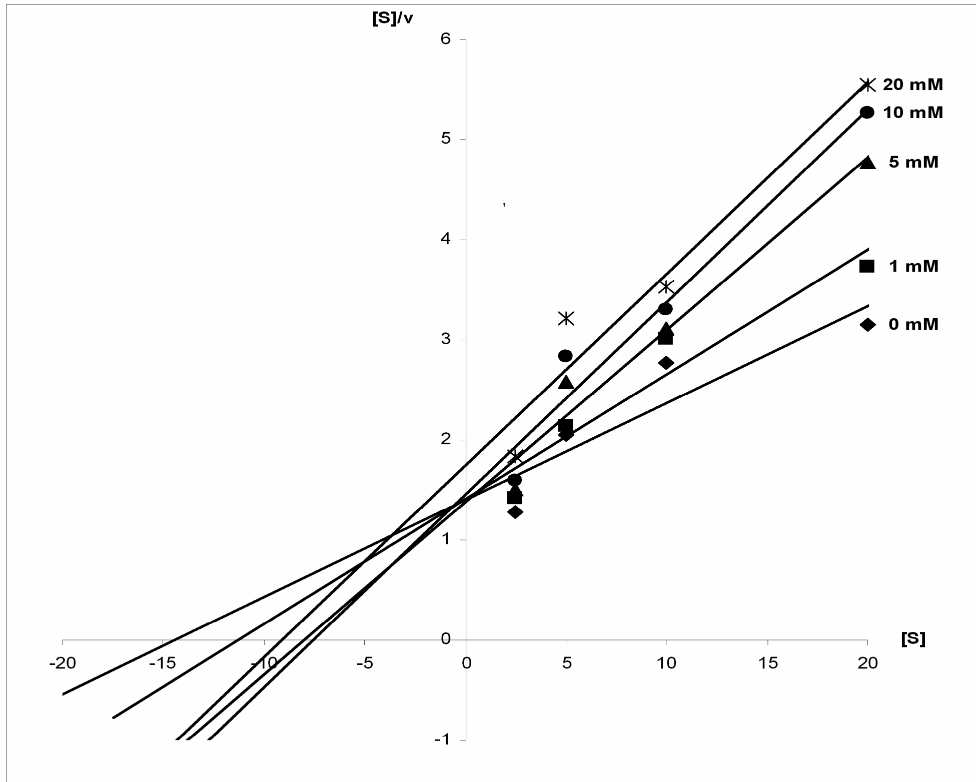


Figure 4

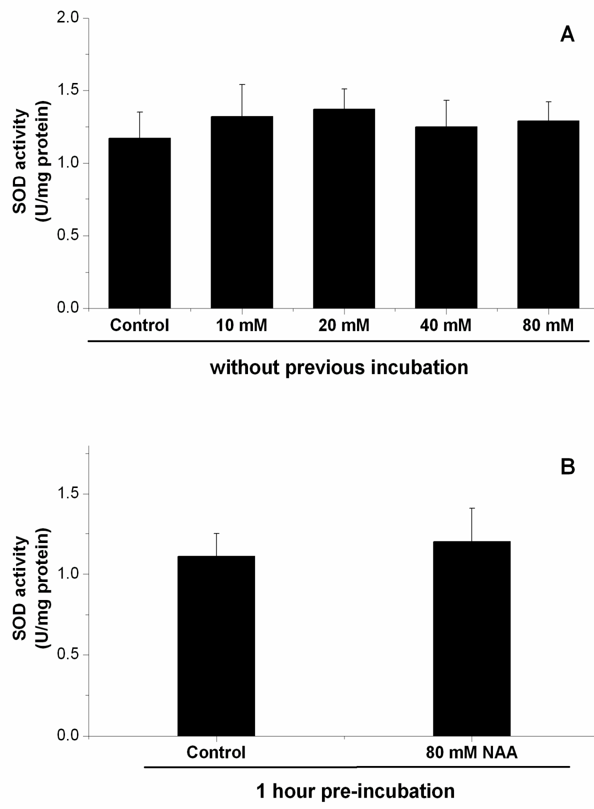


Figure 5

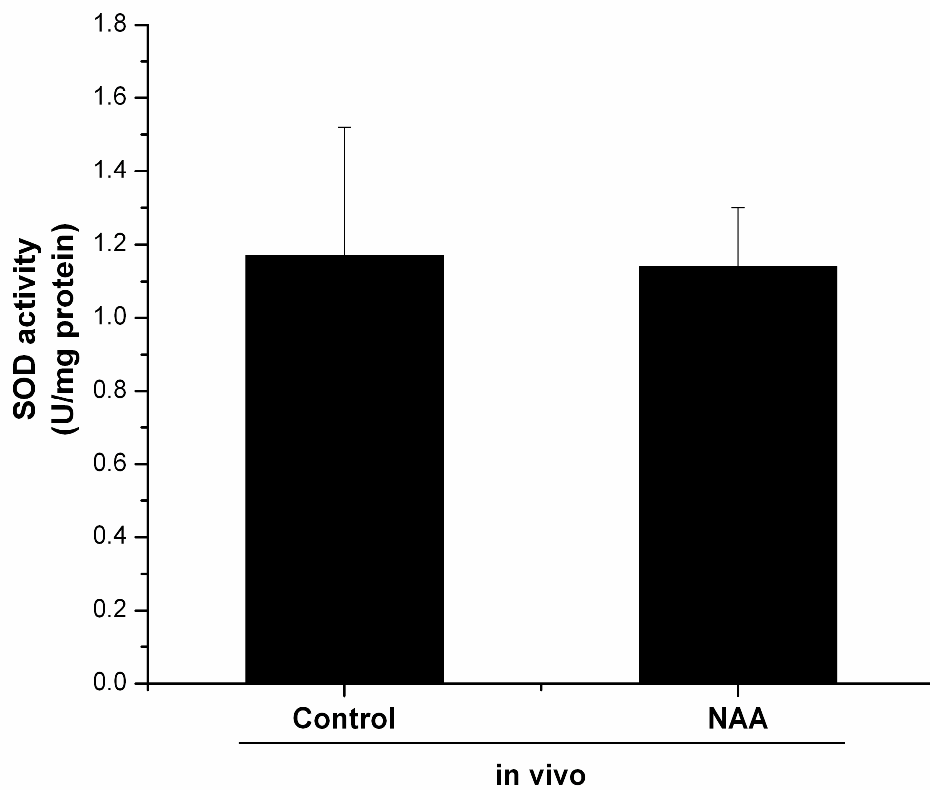


Figure 6

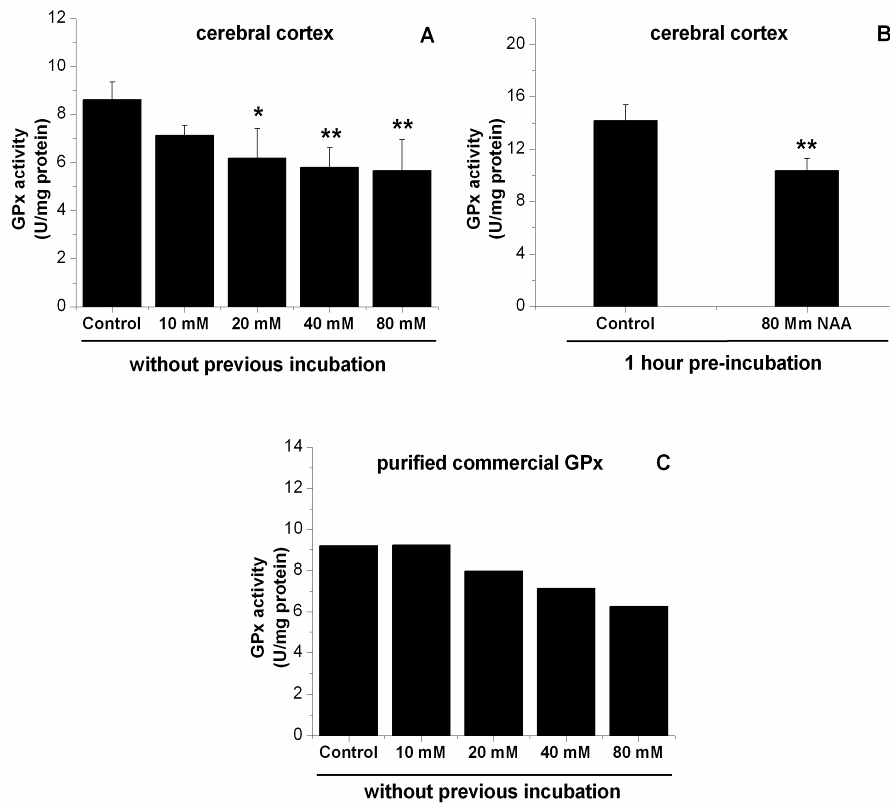


Figure 7

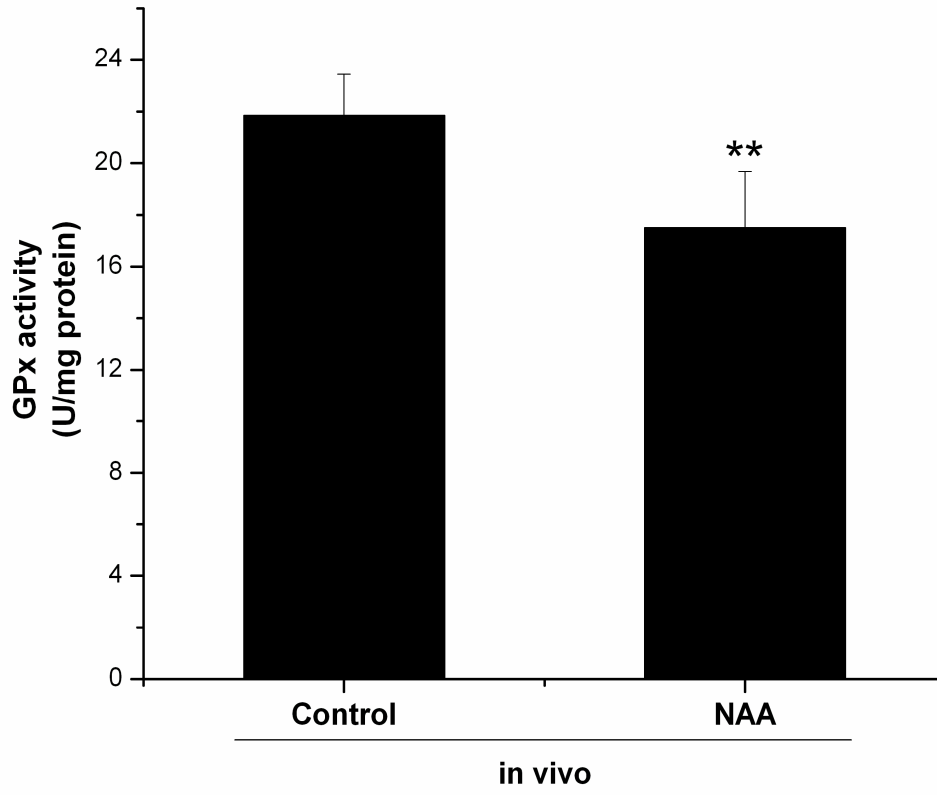
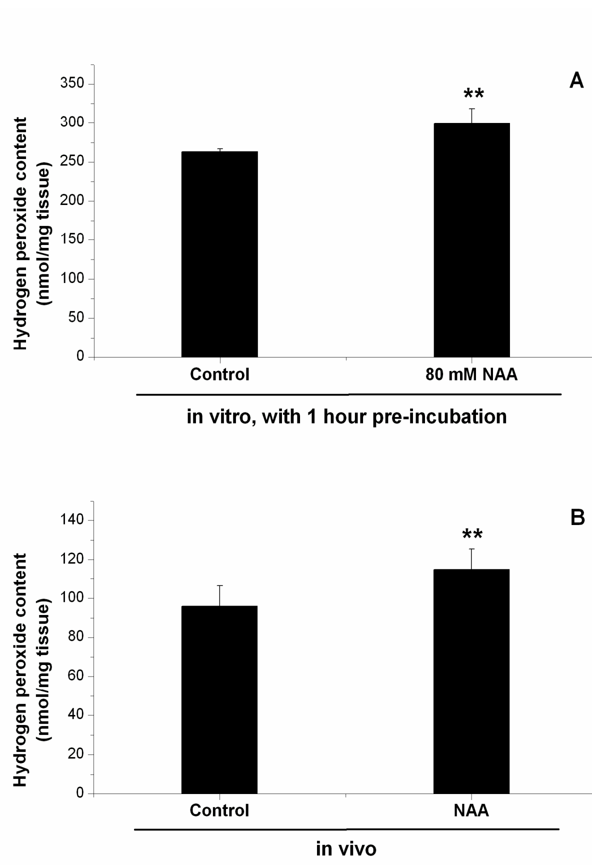


Figure 8



Capítulo V

Intracerebroventricular administration of N-acetylaspartic acid impairs antioxidant defenses and promotes protein oxidation in cerebral cortex of rats

Artigo submetido à revista Metabolic Brain Disease

Intracerebroventricular administration of N-acetylaspartic acid impairs antioxidant defenses and promotes protein oxidation in cerebral cortex of rats

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Abstract

N-acetylaspartic acid (NAA) is the biochemical hallmark of Canavan Disease, an inherited metabolic disease caused by deficiency of aspartoacylase activity. NAA is an immediate precursor for the enzyme-mediated biosynthesis of N-acetylaspartylglutamic acid (NAAG), whose concentration is also increased in urine and cerebrospinal fluid of patients affected by CD. This neurodegenerative disorder is clinically characterized by severe mental retardation, hypotonia and macrocephaly, and generalized tonic and clonic type seizures. Considering that the mechanisms of brain damage in this disease remain not fully understood, in the present study we investigated whether intracerebroventricular administration of NAA or NAAG elicits oxidative stress in cerebral cortex of 30-day-old rats. NAA significantly reduced total radical-trapping antioxidant potential, catalase and glucose 6-phosphate dehydrogenase activities, whereas protein carbonyl content and superoxide dismutase activity were significantly enhanced. Lipid peroxidation indices and glutathione peroxidase activity were not affected by NAA. In contrast, NAAG did not alter any of the oxidative stress parameters tested. Our results indicate that intracerebroventricular administration of NAA impairs antioxidant defenses and induces oxidative damage to proteins, which could be involved in the neurotoxicity of NAA accumulation in CD patients.

Keywords: N-acetylaspartic acid; N-acetylaspartylglutamic acid; aspartoacylase deficiency; Canavan Disease; oxidative stress; rat brain

Introduction

N-acetylaspartic acid (NAA) is present at exceptionally high concentrations in mammalian brain, reaching up to 20 mM (Baslow, 2003) but its metabolic and neurochemical functions remain unclear (Moffett et al., 2007). However, it has been postulated that NAA may serve as a source of the acetyl group to be incorporated into brain lipids (Chakraborty et al., 2001; Kirmani et al., 2002; Madhavarao et al., 2005; Namboodiri et al., 2006), as an intracellular osmolite (Baslow, 2002) and as a storage form of aspartate (Beaudet, 2001). In addition, NAA is an immediate precursor for the biosynthesis of N-acetylaspartylglutamate (NAAG), which is one of the most abundant neuropeptides in mammalian nervous tissue (Gehl et al., 2004; Arun et al., 2006; Moffet et al., 2007) with brain concentrations ranging from 0.5 to 2.7 mM (Pouwels and Frahm, 1997). In rat brain, NAAG is synthesized from NAA and glutamate at a rate of about one molecule of NAAG for every 10 molecules of NAA synthesized and under steady-state conditions this ratio is maintained (Baslow and Guilfoyle, 2006). As a neuroactive compound, NAAG acts primarily as an agonist at metabotropic glutamate receptors of group II (mGluR II), and, at higher concentrations, NAAG is a weak agonist of N-methyl-D-aspartate receptors (NMDA-R) (Neale, 2000; Pliss et al., 2000; Shave et al., 2001; Zhao et al., 2001). Interestingly, NAAG can be hydrolized by N-acetylated α-linked acidic dipeptidase (NAALADase), regenerating NAA (Thomas et al., 2000).

Canavan Disease (CD) is an autossomal recessive inherited neurometabolic disorder in which NAA accumulation is the biochemical hallmark. This severe and progressive leukodystrophy is caused by deficiency of the enzyme aspartoacylase, which hydrolyzes NAA to acetate and aspartate (Beaudet, 2001; Matalon and Michals-Matalon, 2000).

Patients with CD present severe mental retardation, hypotonia and macrocephaly, and about half of them also present generalized tonic and clonic seizures (Traeger and Rapin, 1998; Matalon and Michals-Matalon, 2000; Beaudet, 2001). The diagnosis of CD can be established by the detection of increased NAA levels in patient urine, blood and spinal fluid and in the brain *in vivo* by the use of proton nuclear magnetic resonance spectroscopy (Gordon, 2000). Increased NAAG concentrations have also been reported in urine and cerebrospinal fluid of patients affected by CD (Burlina et al., 1999; Krawczyk and Gradowska, 2003). Neuropathological findings of CD include an extensive loss of myelin (Kumar et al., 2006) with intramyelinic splitting, edema and vacuolation in the white matter and the brain stem (Skiranth et al., 2007), leading to brain "swelling" or sponginess characteristic of this disease, an increase in numbers of oligodendroglia and protoplasmic astrocytes (Beaudet, 2001), and the presence of swollen astrocytes, as well as distorted and elongated mitochondria (Adachi et al., 1972). As CD progresses, the brain becomes atrophic, and the gray matter becomes involved as well (Matalon and Michals-Matalon, 2000). Brain atrophy progressively increases over time in CD patients, and whole-brain NAA rises linearly and continuously (Janson et al., 2006).

Possible underlying mechanisms of brain damage in CD include a defective myelin synthesis resulting from a deficiency of NAA-derived acetate (Madhavarao et al., 2005; Namboodiri et al., 2006; Kumar et al., 2006) and a osmotic imbalance due to the pathological accumulation of NAA, which lead to a build up of excessive fluid in the brain (Baslow, 2002). Although the role of NAA in the pathogenesis of CD is still unclear, increased concentrations of NAA in tissues and fluids would suggest the possibility that NAA or related metabolites might have toxic effects (Beaudet, 2001). In fact,

intracerebroventricular administration of NAA to normal rats was able to induce seizures, probably by neuronal overexcitation through metabotropic glutamate receptors (Akimitsu et al., 2000; Kitada et al., 2000; Yan et al., 2003). In this scenario, epilepsy has already been related to oxidative stress (Bruce and Baudry, 1995; Trotti et al., 1998; Liang et al., 2000; Liang and Patel, 2004; Patel, 2004). In addition, recent data from our laboratory indicated that subcutaneous administration of NAA promotes oxidative stress in cerebral cortex of young rats by decreasing non-enzymatic antioxidant defenses and stimulating oxidative damage to both lipids and proteins (Pederzolli et al., 2007), but it was not clear whether these results were caused by NAA or by its derivative NAAG.

In the present study we investigated the effect of intracerebroventricular administration of NAA and NAAG to 30-day-old rats on various parameters of oxidative stress in cerebral cortex, in order to evaluate whether these accumulating metabolites could induce oxidative stress in the brain, that could be related to the mechanisms of brain damage responsible for the neurological impairment observed in CD patients. To accomplish this, the following oxidative stress parameters were studied: spontaneous chemiluminescence and thiobarbituric acid-reactive substances (TBA-RS), to assess lipid peroxidation; carbonyl content, to evaluate protein oxidation; total radical-trapping antioxidant potential (TRAP), to evaluate non-enzymatic antioxidant defenses; glucose 6-phosphate dehydrogenase (G6PD) activity, to evaluate the main cellular source of NADPH (pentose phosphate pathway); and the activities of the antioxidant enzymes glutathione peroxidase (GPx), catalase (CAT) and superoxide dismutase (SOD), to assess enzymatic antioxidant defenses.

Materials and Methods

Materials

All chemicals were purchased from Sigma (St. Louis, MO, USA) except 2,2’-azo-bis-(2-amidinopropane) that was purchased from Wako Chemicals (USA). NAA and NAAG solutions were freshly prepared in artificial cerebrospinal fluid containing 12 mM NaCl, 0.35 mM KCl, 0.125 mM NaH_2PO_4, 0.13 mM $MgCl_2$, 2.6 mM $NaHCO_3$, 0.2 mM $CaCl_2$ and 1.1 mM glucose, prepared as previously described by Zielke et al. (2002). The pH was adjusted to 7.4 when necessary.

Animals

Thirty-day-old male Wistar rats bred in the Animal House of Universidade Federal de Ciências da Saúde de Porto Alegre were used. Rats had free access to water and a 20% (w/w) protein commercial chow (Supra, Porto Alegre, RS, Brazil). They were kept in a room with 12:12 h light/dark cycle (lights on 07:00-19:00 h) and with air-conditioned controlled temperature (22°C ± 1°C). The NIH Guide for the Care and Use of Laboratory Animals (NIH publication # 80-23, revised 1996) was followed in all experiments.

Intracerebroventricular (i.c.v.) administration of NAA and NAAG

After deeply anesthetized with sodium pentobarbithal (Nembutal, 45 mg/kg body weight, i.p.), tricotomy was done and the rats were fixed in a stereotaxic apparatus. An incision of about 12-15 mm long was made through the scalp to expose the bone. Using the

stereotaxic apparatus (David Kopf Instruments, California, USA), the needle of a Hamilton syringe was inserted into the lateral cerebral ventricule through a drilled opening using the following coordinates, according to Paxinos and Watson (2004): -0.1 mm caudally from bregma (anteroposterior); 1.4 mm from the sagittal suture (lateral); and 3.9 mm from the skull surface (dorsoventral). The volume of 5 μL of artificial cerebrospinal fluid containing 8 μmol NAA (Akimitsu et al., 2000) or 0.8 μmol NAAG (Pliss et al., 2003) was slowly infused over two minutes into the lateral cerebral ventricule. Control rats received the same volume of artificial cerebrospinal fluid alone. The needle was left in situ for another 2 minutes before withdrawal. Sham rats were subjected to the same surgical procedure, but received no i.c.v. administration, and showed no significant difference from control (data not shown).

Tissue preparation

Rats were killed by decapitation 15 or 60 minutes after i.c.v. administration, and the brain was immediately removed and kept on an ice-plate. The olfactory bulb, pons and medulla were discarded and the cerebral cortex was dissected, weighed and kept chilled until homogenization. These procedures lasted up to 3 min. Cerebral cortex was homogenized in 10 volumes (1:10, w/v) of 20 mM sodium phosphate buffer, pH 7.4 containing 140 mM KCl. Homogenates were centrifuged at 750 *g* for 10 min at 4ºC to discard nuclei and cell debris (Llesuy et al., 1985; Lissi et al., 1986). The pellet was discarded and the supernatant was immediately separated and used for the measurements. The homogenates used were from individual animals, and they were never pooled. All experiments were repeated with different animals.

Spontaneous chemiluminescence

Samples were assayed for spontaneous chemiluminescence in a dark room (Lissi et al., 1986) using a beta liquid scintillation spectrometer Tri-Carb 2100TR in the out of coincidence mode. The background chemiluminescence was measured for 5 min in vials containing 3.5 mL of the same buffer used for homogenization. An aliquot of 0.5 mL of supernatant was added and spontaneous chemiluminescence was measured for 10 minutes at room temperature. The background chemiluminescence was subtracted from the total value. Spontaneous chemiluminescence was represented as counts per second (CPS)/mg protein.

Thiobarbituric acid-reactive substances (TBA-RS)

TBA-RS was measured according to Ohkawa et al., 1979. Briefly, to glass tubes were added, in order of appearence: 500 μL of tissue supernatant; 50 μL of SDS 8.1%; 1,500 μL of 20% acetic acid in aqueous solution (v/v) pH 3.5; 1,500 μL of 0.8 % tiobarbituric acid; and 700 μL of distilled water. The mixture was vortexed and the reaction was carried out in a boiling water bath for 1 hour. The mixture was allowed to cool on water for 5 min, and was centrifuged at 750 *g* for 10 min. The resulting pink stained TBA-RS obtained were determined spectrophotometrically at 535 nm in a Beckman DU®640 Spectrophotometer. A calibration curve was generated using 1,1,3,3-tetramethoxypropane as a standard, being subjected to the same treatment as that of the samples. TBA-RS were represented as nmol/mg protein.

Carbonyl content

Oxidatively modified proteins present an enhancement of carbonyl content (Stadtman and Levine, 2003). In this paper, carbonyl content was assayed by a method based on the reaction of protein carbonyls with dinitrophenylhydrazine forming dinitrophenylhydrazone, a yellow compound, measured spectrophotometrically at 370 nm (Reznick and Packer, 1994). Briefly, 100 µL of homogenate were added to plastic tubes containing 400 µL of 10 mM dinitrophenylhydrazine (prepared in 2 M HCl). This was kept in the dark for 1 hour and vortexed each 15 minutes. After that, 500 µL of 20 % trichloroacetic acid were added to each tube. The mixture was vortexed and centrifuged at 20,000 g for 3 minutes. The supernatant obtained was discarded. The pellet was washed with 1 mL ethanol:ethyl acetate (1:1, v/v), vortexed and centrifuged at 20,000 g for 3 minutes. The supernatant was discarded and the pellet re-suspended in 600 µL of 6 M guanidine (prepared in a 20 mM potassium phosphate solution pH 2.3). The sample was vortexed and incubated at 60 ^{o}C for 15 minutes. After that, it was centrifuged at 20,000 g for 3 minutes and the supernatant was used to measure absorbance at 370 nm. Results were represented as carbonyl content (nmol/mg protein).

Total radical-trapping antioxidant potential (TRAP)

TRAP was determined by measuring the chemiluminescence intensity of luminol induced by 2,2'-azo-bis-(2-amidinopropane) (ABAP) thermolysis (Lissi et al., 1992; Evelson et al., 2001) in a Wallac 1409 Scintillation Counter working in the out of coincidence mode. Three mL of the reaction mixture containing 10 mM ABAP and 0.02 mM luminol in 50 mM sodium phosphate buffer pH 7.4 were added to a glass scintillation

vial and the initial chemiluminescence was measured. Ten μL of 160 μM Trolox (water-soluble α-tocopherol analogue, used as standard) or 10 μL of tissue supernatant were then added to that vial, producing a decrease in the initial chemiluminescence value. This value is kept low until the antioxidants present in the sample are depleted, then chemiluminescence returns to its initial value. The time taken by the sample to keep chemiluminescence low is directly proportional to the antioxidant capacity of the tissue, so TRAP represents the amount (quantity) of non-enzymatic antioxidants present in the sample. The results were represented as nmol Trolox/mg protein.

Glucose 6-phosphate dehydrogenase assay

G6PD activity was measured according to Leong and Clark (1984). The method is based on the formation of NADPH at 340 nm in a rection medium containing 100 mM Tris-Hydrochloryde buffer pH 7.5, 10 mM magnesium chloryde, 0.1% triton X-100, 0.5 mM $NADP^+$, 1 mM glucose 6-phosphate and 0.1-0.3 mg protein/mL. One G6PD unit is defined as one μmol of NADPH produced per minute and the specific activity is represented as G6PD units/mg protein.

Glutathione peroxidase assay

GPx activity was measured using tert-butyl-hydroperoxide as substrate (Wendel, 1981). NADPH disappearance was monitored at 340 nm using a double-beam spectrophotometer with temperature control (Hitachi U-2001). The medium contained 2 mM glutathione, 0.15 U/mL glutathione reductase, 0.4 mM azide, 0.5 mM tert-butyl-hydroperoxide and 0.1 mM NADPH. One GPx unit is defined as one μmol of NADPH

consumed per minute and the specific activity is represented as GPx units/mg protein.

Catalase assay

CAT activity was assayed using a double-beam spectrophotometer with temperature control (Hitachi U-2001). This method is based on the disappearance of H_2O_2 at 240 nm in a reaction medium containing 20 mM H_2O_2, 0.1% Triton X-100, 10 mM potassium phosphate buffer pH 7.0, and 0.1-0.3 mg protein/mL (Aebi, 1984). One CAT unit is defined as one μmol of hydrogen peroxide consumed per minute and the specific activity is represented as CAT units/mg protein.

Superoxide dismutase assay

This method for the assay of SOD activity is based on the capacity of pyrogallol to autoxidize, a process highly dependent on O_2^-, which is substrate for SOD (Marklund, 1985). The inhibition of autoxidation of this compound occurs in the presence of SOD, whose activity can be then indirectly assayed spectrophotometrically at 420 nm, using a double-beam spectrophotometer with temperature control (Hitachi U-2001). A calibration curve was generated with purified SOD as a standard, in order to calculate the activity of SOD present in the samples. The results were represented as SOD units/mg protein.

Protein determination

Protein concentration was determined in cerebral cortex supernatants using bovine serum albumin as a standard (Lowry et al., 1951).

Statistical analysis

Statistical analysis was performed by the Student's *t* test. All analyses were performed using the Statistical Package for the Social Sciences (SPSS) software in a PC-compatible computer. A value of $p < 0.05$ was considered to be significant.

Results

In the present work, the oxidative stress parameters were analyzed in cerebral cortex from 30-day-old rats after 15 or 60 minutes of a single i.c.v. injection of NAA or NAAG.

Initially, we evaluated the effect of these compounds on the lipid peroxidation parameters spontaneous chemiluminescence and TBA-RS. As it can be seen in Figure 1A, NAA was not able to alter spontaneous chemiluminescence in cerebral cortex homogenates obtained from 30-day-old rats after 15 minutes [$t(14)=0.52$; $p>0.05$] and after 60 minutes [$t(10)=0.48$; $p>0.05$] after i.c.v. injection. Similar results were obtained with NAAG at 15 minutes [$t(10)=0.39$; $p>0.05$] or 60 minutes [$t(10)=0.58$; $p>0.05$] after i.c.v. injection (Figure 1B). TBA-RS levels were equally not affected by i.c.v. administration of NAA (15 minutes after injection, [$t(14)=0.70$; $p>0.05$]; 60 minutes after injection, [$t(10)=0.42$; $p>0.05$]; Figure 2A) or of NAAG (15 minutes after injection, [$t(10)=1.81$; $p>0.05$]; 60 minutes after injection, [$t(10)=1.20$; $p>0.05$]; Figure 2B). Altogether, the data demonstrated that i.c.v. administration of NAA or NAAG does not promote lipid oxidative damage.

We also investigated whether oxidation of tissue proteins were affected by i.c.v. administration of NAA or NAAG, by measuring carbonyl content. Figure 3A shows that carbonyl content was significantly enhanced by NAA in cerebral cortex homogenates after

15 minutes [$t(14)=5.60$; $p<0.01$] and 60 minutes of i.c.v. injection [$t(10)=4.43$; $p<0.01$], indicating protein oxidative damage. However, i.c.v. administration of NAAG was not able to affect carbonyl content at 15 minutes [$t(10)=1.21$; $p>0.05$] or 60 minutes [$t(10)=0.80$; $p>0.05$] after injection, as depicted in Figure 3B. These results show that NAA, but not NAAG, was able to induce oxidative damage to proteins.

The next set of experiments was performed to evaluate the effect of i.c.v. administration of NAA and NAAG on non-enzymatic antioxidant defenses, by measuring TRAP. Figure 4A shows that TRAP was significantly reduced by NAA, as compared to control at 15 minutes [$t(14)=4.46$; $p<0.01$] and 60 minutes [$t(10)=2.84$; $p<0.05$] after injection, suggesting that this organic acid reduces the non-enzymatic antioxidant defenses. However, TRAP was not affected by i.c.v. administration of NAAG at 15 minutes [$t(10)=0.51$; $p>0.05$] and 60 minutes [$t(10)=1.29$; $p>0.05$] after injection (Figure 4B).

We also studied the effect of i.c.v. administration of NAA or NAAG on G6PD activity, which is is the key regulatory enzyme of the pentose phosphate pathway (Table I). We found that G6PD activity was significantly reduced by i.c.v. administration of NAA only at 60 minutes after injection [$t(10)=5.90$; $p<0.01$], whereas at 15 minutes no effect was observed [$t(14)=0.18$; $p>0.05$]. NAAG, on the other hand, did not alter G6PD activity at 15 minutes [$t(10)=1.22$; $p>0.05$] or 60 minutes [$t(10)=0.79$; $p>0.05$] after injection.

The effect of i.c.v. administration of NAA or NAAG was also tested on the antioxidant enzyme activities of GPx, CAT and SOD (Table I). We observed that at 15 minutes after NAA injection, CAT activity was significantly reduced [$t(14)=7.83$; $p<0.01$], while SOD [$t(14)=1.30$; $p>0.05$] and GPx [$t(14)=1.34$; $p>0.05$] activities were not affected by i.c.v. NAA administration. Furthermore, 60 minutes after NAA administration, CAT

activity was also significantly reduced [$t(10)=2.95$; $p<0.05$], GPx activity not affected [$t(10)=0.42$; $p>0.05$] and, interestingly, SOD activity was significantly enhanced [$t(10)=4.51$; $p<0.01$]. In contrast, i.c.v. administration of NAAG was not able to induce alterations in CAT activity (15 minutes, [$t(10)=0.02$; $p>0.05$]; 60 minutes, [$t(10)=1.05$; $p>0.05$]), SOD activity (15 minutes, [$t(10)=1.52$; $p>0.05$]; 60 minutes, [$t(10)=0.93$; $p>0.05$]) and GPx activity (15 minutes, [$t(10)=1.59$; $p>0.05$]; 60 minutes, [$t(10)=1.95$; $p>0.05$]). These results clearly indicate that i.c.v. administration of NAA impairs enzymatic antioxidant defenses, whereas NAAG causes no effect.

Discussion

We have previously reported that NAA at concentrations similar to those found in CD patients promotes oxidative stress *in vitro* and *in vivo* (acute subcutaneous administration) in cerebral cortex of 14-day-old rats (Pederzolli et al., 2007). NAAG also accumulates in the brain of CD patients and recent findings showed that intracerebroventricular administration of NAAG to rats is able to alter their behaviour and to induce neurodegeneration, with significant changes in cell morphology and cleavage of DNA (Pliss et al., 2003; Bubeníková-Valesová et al., 2006) which in turn can be produced by oxidative damage (Halliwell and Gutteridge, 2007).

Therefore, in the present study, we extended our previous investigation by evaluating the influence of i.c.v. administration of NAA or also of NAAG on oxidative stress parameters in cerebral cortex of rats. We found that spontaneous chemiluminescence and TBA-RS were not affected by NAA or NAAG, suggesting that oxidative damage to

lipids is not elicited by i.c.v. administration of these compounds to 30-day-old rats. This effect is not in accordance with our previous results, which clearly showed a promotion of oxidative damage to lipids by NAA *in vitro* or when subcutaneously administered to 14-day-old rats (Pederzolli et al., 2007). The lack of effect of NAA on 30-day-old rats may possibly be due to the better antioxidant status present at this age compared to younger rats (Mavelli et al., 1982; Schreiber et al., 1995; Driver et al., 2000) or to other unknown mechanisms.

On the other hand, NAA, but not NAAG, was able to cause oxidative damage to proteins, as verified by the significant increase of carbonyl content. These results corroborate our previous findings showing that NAA *in vitro* and *in vivo* (subcutaneous administration) increased carbonyl content in cerebral cortex of rats (Pederzolli et al., 2007).

We also demonstrated here that NAA significantly reduced TRAP in cerebral cortex of rats subjected to i.c.v. administration of NAA. Considering that TRAP measures the content of non-enzymatic antioxidants (Lissi et al., 1995), these results indicate that NAA reduces the non-enzymatic antioxidant capacity in rat brain by reducing non-enzymatic antioxidant content (TRAP), which is in line with our previous results.

We observed that NAA was able to significantly reduce G6PD activity in cerebral cortex after 60 minutes of i.c.v. injection, while NAAG produced no effect on this enzyme activity. G6PD is the key regulatory enzyme of the pentose phosphate pathway, which is regulated by a number of factors including hormones, nutrients and oxidative stress (Kletzien et al., 1994). G6PD is the rate-limiting enzyme in the GSH- and NADPH-dependent H_2O_2 elimination, suggesting the importance of G6PD in the antioxidant function of brain and pathogenesis of oxidative stress-related diseases (Hashida et al.,

2002). Thus, G6PD activity may provide an early marker of oxidative stress since it is able of responding rapidly to the increased demand for NADPH necessary for the maintenance of the cellular redox state (Kletzien et al., 1994). The reduction of G6PD activity caused by NAA could elicit an impairment of NADPH production.

With regard to the effects of NAA and NAAG on the antioxidant enzymes CAT, SOD and GPx from cerebral cortex, NAAG did not alter these activities. However, CAT activity was significantly reduced by NAA i.c.v. injection, whereas GPx activity was not affected and SOD activity was significantly increased in cerebral cortex. CAT is a ferric heme protein that directly catalyses the decomposition of H_2O_2 to water, being the major defense to remove H_2O_2 when in excess (Halliwell and Gutteridge, 2007). On the other hand, the increased activity of SOD may reflect a rebout effect of higher de novo synthesis of this enzyme in an effort to remove the superoxide radical.

Our data on the effects of NAA on the major antioxidant enzymes reinforce the view that oxidative stress responses do not always involve a coordinated expression of all antioxidant enzymes and that their expression is regulated by different mechanisms (Röhrdanz et al., 2000; Wilson and Johnson, 2000). Our results showing an enhancement of SOD activity and a reduction of CAT and G6PD activities by NAA administered intracerebrocentricularly suggest a possible impairment in H_2O_2 detoxification. Considering that SOD expression is readily induced after 1 hour exposition of cell cultures to H_2O_2 (Yoo et al., 1999; Rojo et al., 2004), our finding of an increase of SOD activity may also involve enhanced expression of this antioxidant enzyme in response to oxidative stress promoted by NAA.

Altogether, our present findings show that i.c.v. administration of NAA promotes impairment of antioxidant defenses in cerebral cortex of 30-day-old rats, by reducing

TRAP, CAT and G6PD activities, compromising therefore the efficiency of reactive species detoxification, which could eventually lead to the deleterious consequences of oxidative damage to biomolecules, and also to an impairment in NADPH production and a dysruption in the cellular redox balance. Considering that oxidative stress can be elicited by the imbalance between free radical production and antioxidant defenses, and since NAA promoted protein damage, as well as diminished antioxidant defenses, it might be postulated that oxidative stress is induced in cerebral cortex of 30-day-old rats by i.c.v. administration of NAA, the major metabolite accumulating in CD.

Pliss and coworkers (2003) recently found that NAAG i.c.v. was able to induce DNA cleavage, which was thought that could be mediated by oxidative stress. In the present study we found that i.c.v. administration of NAAG is not able to promote oxidative stress up to 60 minutes after being administered, since it was unable to alter any of the parameters tested. Thus, it seems that the neurotoxic effects observed earlier by other investigators (Pliss et al., 2000; Pliss et al., 2002; Pliss et al., 2003; Bubeníková-Valesová et al., 2006) may not be mediated by oxidative stress.

Even though the i.c.v. administration doses of NAA and NAAG used in our assays are similar to those reported previously that caused neurotoxic effects (Akimitsu et al., 2000; Pliss et al., 2003), it is difficult to extrapolate our findings to the human condition. However, if the effects observed here, together with previous findings (Pederzolli et al., 2007), also occur in brain of patients affected by CD, it is possible that they may contribute, at least in part, to the mechanisms responsible for the brain damage observed in those patients and the therapeutic use of antioxidants should be considered.

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Table I. Effect of intracerebroventricular (i.c.v.) administration of N-acetylaspartic acid (NAA) and N-acetylaspartylglutamic acid (NAAG) on the activities of glucose 6-phosphate dehydrogenase (G6PD), catalase (CAT), superoxide dismutase (SOD) and glutathione peroxidase (GPx) in cerebral cortex of 30-day-old rats.

NAA

	G6PD activity (U/mg protein)		**CAT activity** (U/mg protein)		**SOD activity** (U/mg protein)		**GPx activity** (U/mg protein)	
	15 min[a]	**60 min**	**15 min**	**60 min**	**15 min**	**60 min**	**15 min**	**60 min**
Control	11.45 ± 0.21	17.00 ± 0.72	4.40 ± 0.36	5.42 ± 0.24	6.04 ± 0.68	6.91 ± 0.71	16.38 ± 1.88	19.24 ± 0.58
NAA	11.30 ± 1.29	14.42 ± 0.79 **	3.04 ± 0.34 **	4.52 ± 0.71 *	5.68 ± 0.48	8.35 ± 0.34 **	15.40 ± 0.88	18.98 ± 1.38

NAAG

	G6PD activity (U/mg protein)		**CAT activity** (U/mg protein)		**SOD activity** (U/mg protein)		**GPx activity** (U/mg protein)	
	15 min[a]	**60 min**	**15 min**	**60 min**	**15 min**	**60 min**	**15 min**	**60 min**
Control	14.39 ± 1.64	11.65 ± 0.87	3.36 ± 0.47	3.58 ± 0.45	6.07 ± 0.55	5.93 ± 0.59	16.78 ± 2.39	14.79 ± 1.82
NAAG	13.45 ± 0.95	12.13 ± 1.18	3.35 ± 0.45	3.88 ± 0.52	5.61 ± 0.49	5.70 ± 0.20	14.89 ± 1.69	12.96 ± 1.40

Results are mean ± SD for six-to-eight independent experiments (animals) performed in duplicate. *$p<0.05$ and **$p<0.01$ compared to control (Student's *t* test for unpaired samples). [a] Time after i.c.v. injection.

Figure 1. Effect of intracerebroventricular (i.c.v.) administration of N-acetylaspartic acid (NAA) (A) or N-acetylaspartylglutamic acid (NAAG) (B) on spontaneous chemiluminescence in cerebral cortex from 30-day-old rats. Rats were killed 15 or 60 minutes after injection. Results are mean ± SD (n=6-8) for independent experiments (animals) performed in duplicate. No significant differneces were detected from control (Student's *t* test).

Figure 2. Effect of intracerebroventricular (i.c.v.) administration of N-acetylaspartic acid (NAA) (A) or N-acetylaspartylglutamic acid (NAAG) (B) on thiobarbituric acid reactive substances (TBA-RS) in cerebral cortex from 30-day-old rats. Rats were killed 15 or 60 minutes after injection. Results are mean ± SD (n=6-8) for independent experiments (animals) performed in duplicate. No significant differences were detected from control (Student's *t* test).

Figure 3. Effect of intracerebroventricular (i.c.v.) administration of N-acetylaspartic acid (NAA) (A) or N-acetylaspartylglutamic acid (NAAG) (B) on carbonyl content in cerebral cortex from 30-day-old rats. Rats were killed 15 or 60 minutes after injection. Results are mean ± SD (n=6-8) for independent experiments (animals) performed in duplicate. * $p<0.05$ and ** $p<0.01$ compared to control (Student's *t* test).

Figure 4. Effect of intracerebroventricular (i.c.v.) administration of N-acetylaspartic acid (NAA) (A) or N-acetylaspartylglutamic acid (NAAG) (B) on non-enzymatic antioxidant defenses: total radical-trapping antioxidant potential (TRAP) in cerebral cortex from 30-day-old rats. Rats were killed 15 or 60 minutes after injection. Results are mean ± SD (n=6-8) for independent experiments (animals) performed in duplicate. * $p<0.05$ and ** $p<0.01$ compared to control (Student's *t* test).

FIGURE 1

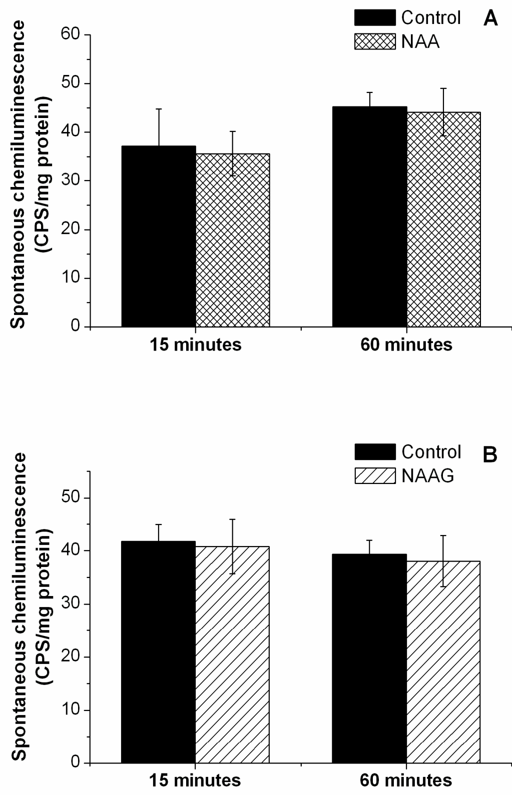
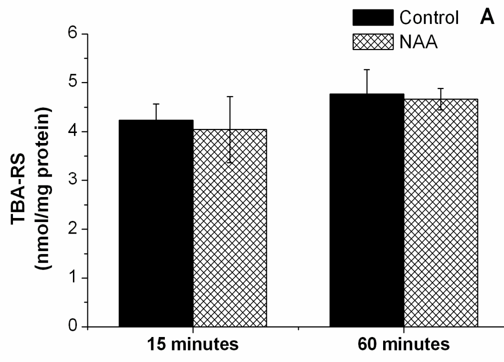


FIGURE 2



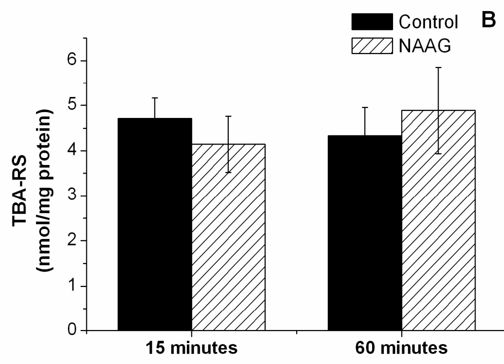


FIGURE 3

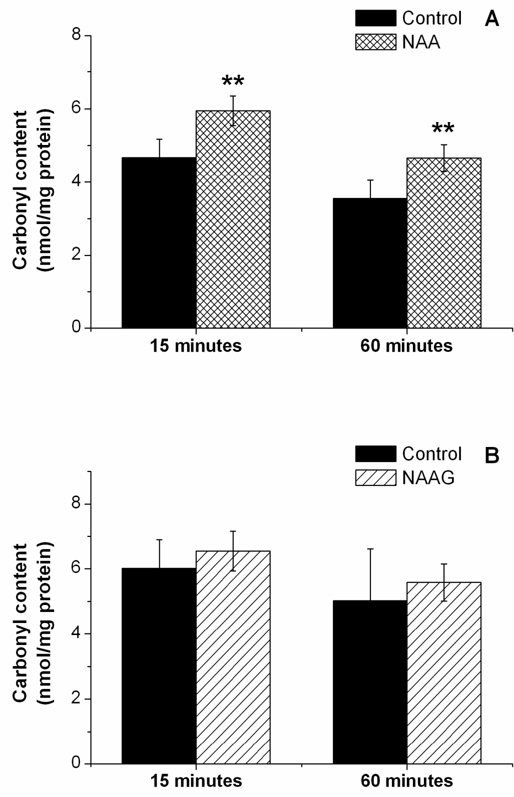
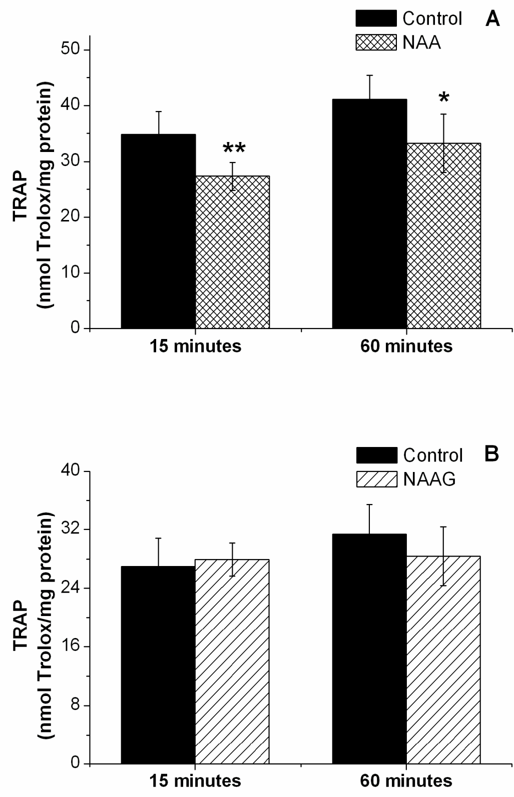


FIGURE 4



Capítulo VI

Efeitos in vitro do ácido N-acetilaspartilglutâmico sobre parâmetros de estresse oxidativo em córtex cerebral de ratos

Avaliamos também no presente trabalho os efeitos *in vitro* do ácido N-acetilaspartilglutâmico, outro metabólito acumulado na doença de Canavan, sobre alguns parâmetros de estresse oxidativo em córtex cerebral de ratos. Considerando que o pico máximo de concentração do ácido N-acetilaspartilglutâmico se dá entre os dias 8 e 14 de vida pós-natal em ratos, e que esse ácido orgânico apresenta maior toxicidade em ratos de aproximadamente 15 dias de vida do que em ratos adultos (Bubeníková-Valesová *et al.*, 2006), neste experimento foram utilizados ratos de 14 dias de vida. Os homogeneizados de córtex cerebral foram incubados por 1 hora a 37°C na presença ou ausência de ácido N-acetilaspartilglutâmico nas concentrações de 1, 5 e 10 mM. Após a incubação foram medidos os seguintes parâmetros de estresse oxidativo: Potencial Antioxidante Total (TRAP), para avaliar as defesas antioxidantes não-enzimáticas; quimiluminescência espontânea e Substâncias Reativas ao Ácido Tiobarbitúrico (TBA-RS), para avaliar dano oxidativo a lipídios; e conteúdo de carbonilas, para avaliar o dano oxidativo protéico. Observamos nesse estudo que o ácido N-acetilaspártico *in vitro* não foi capaz de alterar significativamente os parâmetros de estresse oxidativo avaliados em córtex cerebral de ratos de 14 dias de vida (Tabela I), não sendo, portanto, capaz de promover estresse oxidativo *in vitro*.

Tabela I. Efeitos *in vitro* do ácido N-acetilaspartilglutâmico (NAAG) sobre parâmetros gerais de estresse oxidativo: potencial antioxidante total (TRAP), quimiluminescência espontânea, substâncias reativas ao ácido tiobarbitúrico (TBA-RS) e conteúdo de carbonilas em córtex cerebral de ratos de 14 dias de vida.

NAAG *in vitro*

	TRAP (nmol Trolox/mg proteína)	**Quimiluminescência espontânea** (CPS/mg proteína)	**TBA-RS** (nmol/mg proteína)	**Conteúdo de carbonilas** (nmol/mg proteína)
Controle	43,56 ± 0,86	62,01 ± 1,87	4,00 ± 0,58	2,61 ± 0,42
NAAG 1 mM	42,80 ± 3,54	61,45 ± 5,15	3,99 ± 0,35	2,82 ± 0,17
NAAG 5 mM	42,42 ± 1,50	62,63 ± 4,08	4,11 ± 0,49	2,88 ± 0,30
NAAG 10 mM	41,66 ± 4,16	62,44 ± 2,94	4,24 ± 0,64	2,90 ± 0,49

Resultados expressos como média ± desvio padrão para um *n* de quatro experimentos independentes realizados em duplicata. Não foram detectadas diferenças significativas em relação ao controle (ANOVA).

PARTE III

DISCUSSÃO

1. 5-Oxoprolina

Apesar de apresentarem ampla heterogeneidade genética e fenotípica, os pacientes afetados pela deficiência de GS, na qual há um acúmulo de 5-OP, geralmente manifestam um quadro de disfunção neurológica progressiva caracterizada por retardo mental, ataxia e convulsões, entre outros sintomas. No entanto, os mecanismos responsáveis pelo dano cerebral observado nesses pacientes permanecem ainda pouco conhecidos.

Sabe-se que esses pacientes apresentam, além de altos níveis de 5-OP, níveis diminuídos do antioxidante GSH no cérebro, e por esse motivo tem sido postulado que pacientes com deficiência de GS poderiam ter uma sensibilidade aumentada ao estresse oxidativo (Ristoff *et al.*, 2001; Larsson e Anderson, 2001). Apesar de os baixos níveis de GSH terem sido até então postulados como o principal mecanismo responsável pelos danos cerebrais nesses pacientes, estudos recentes realizados em pacientes afetados por essa desordem revelaram não haver correlação entre a atividade da GS, os níveis de GSH, os níveis de γ–glutamilcisteína e a presença ou ausência de sintomas neurológicos (Dahl *et al.*, 1997; Ristoff *et al.*, 2001; Njalsson *et al.*, 2005; Nygren *et al.*, 2005), não tendo sido avaliados os níveis sangüíneos de 5-OP em nenhum desses estudos. Nygren e colaboradores (2005) observaram ausência de dano oxidativo ao DNA nuclear em fibroblastos de pacientes afetados pela deficiência de GS, e ausência de correlação entre os níveis de GSH ou de γ–glutamilcisteína e a proteção contra dano oxidativo ao DNA. Esses pesquisadores sugerem, então, que a oxidação de outros compostos celulares, a alteração do potencial redox celular ou efeitos tóxicos de precursores de GSH, como a 5-OP, sejam explicações mais plausíveis para os mecanismos responsáveis pelos sintomas clínicos

associados à deficiência de GS (Nygren *et al*., 2005).

Ainda, é interessante observar que indivíduos com deficiência de 5-oxoprolinase, que constitui outro erro inato do ciclo γ–glutamil, também apresentam altas concentrações teciduais de 5-OP e manifestações clínicas neurológicas similares às encontradas na deficiência de GS; no entanto, não apresentam níveis diminuídos de GSH (Larsson e Anderson, 2001).

Em conjunto, essas evidências sugerem que os níveis reduzidos de GSH talvez não sejam os únicos determinantes da neurodegeneração nesses pacientes. Considerando que a 5-OP apresenta diversas ações neurotóxicas (Bennet *et al*., 1973; Escobedo e Cravioto, 1973; Rieke *et al*., 1984; Dusticier *et al*., 1985; Rieke *et al*., 1989; Barone e Spignoli, 1990; Silva *et al*., 2001), é possível que a 5-OP por si só tenha, ao menos em parte, algum papel no dano cerebral observado nesses pacientes. No entanto, os mecanismos responsáveis pela sua toxicidade permanecem ainda pouco esclarecidos.

Dentre os efeitos neurotóxicos da 5-OP, tem-se sua ligação a receptores glutamatérgicos e inibição da captação de glutamato, promovendo excitotoxicidade (Bennet *et al*., 1973; Rieke *et al*., 1984; Barone e Spignoli, 1990); a inibição, tanto em neurônios quanto em células gliais, da Na^+,K^+-ATPase, enzima fundamental na manutenção do gradiente iônico de Na^+ e K^+ através das membranas neuronais e da excitabilidade neuronal (Escobedo e Cravioto, 1973; Rieke *et al*., 1984; Stahl, 1984; Lees, 1991); e o comprometimento do metabolismo energético cerebral, pela redução da produção de CO_2, dos níveis de ATP e da síntese de lipídios, além da inibição da atividade dos complexos I + III e complexo IV da cadeia respiratória (Silva *et al*., 2001). Cabe salientar que tanto o mecanismo de excitotoxicidade, quanto a inibição da Na^+,K^+-ATPase

e a inibição dos complexos da cadeia respiratória já foram relacionados a ER (Hexum e Fried, 1979; Kukreja *et al*., 1990; Pellegrini-Giampietro, 1990; Cleeter *et al*., 1992; Bondy e Le Bel, 1993; Lees, 1993; Nicholls e Budd, 1998; Halliwell e Gutteridge, 2007), sendo por isso possível que todas essas ações promovidas pela 5-OP sejam de fato mediadas pela produção de ER. Além disso, cabe salientar que pacientes com deficiência de GS apresentam alterações neuropatológicas muito semelhantes àquelas observadas nos casos de intoxicação por mercúrio, na qual o envolvimento de uma produção aumentada de ER já foi também demonstrado (Marstein *et al*., 1981). Em conjunto, todas essas evidências sugerem o possível envolvimento do estresse oxidativo na neurotoxicidade da 5-OP.

Recentemente demonstramos que a 5-OP pode comprometer *in vitro* as defesas antioxidantes não-enzimáticas em cérebro de ratos, visto que diminuiu significativamente e de forma dose-dependente tanto o TRAP quanto o TAR, que medem quantidade e reatividade dos antioxidantes não-enzimáticos do tecido, respectivamente (Pederzolli *et al*., 2007). No presente trabalho, avaliamos os efeitos *in vitro* da 5-OP sobre parâmetros de dano oxidativo a proteínas e sobre a produção de ER em córtex cerebral e cerebelo de ratos. Observamos que a 5-OP pode promover *in vitro* dano oxidativo protéico, já que alterou os conteúdos de carbonilas e de tióis totais, provavelmente através do aumento da produção de ER no córtex cerebral e cerebelo de ratos jovens, visto que aumentou a fluorescência do DCF, ensaio que mede a produção generalizada, e não específica, de ER (Pederzolli *et al*., 2007). Dessa forma, considerando que o estresse oxidativo pode ser gerado pelo desequilíbrio entre a produção de ER e as defesas antioxidantes, e que a 5-OP diminui as defesas antioxidantes não-enzimáticas, aumenta a produção de ER e promove dano oxidativo protéico, postula-se que a 5-OP é capaz de induzir estresse oxidativo *in*

vitro, sendo esse mais um possível mecanismo envolvido em sua neurotoxicidade.

Considerando que tanto a excitotoxicidade quanto o dano ao metabolismo energético cerebral são igualmente promovidos pela 5-OP, e que ambos já foram relacionados ao estresse oxidativo, parece possível que esses processos possam agir de forma sinérgica *in vivo*. Assim, resolvemos investigar os efeitos *in vivo* da 5-OP sobre parâmetros de estresse oxidativo a fim de melhor esclarecer seu papel na neurotoxicidade desse ácido orgânico. Para isso, ratos de 14 dias de vida foram submetidos à administração aguda de solução salina (ratos controle) ou de 5-OP, administradas por via subcutânea, sendo os animais mortos 1 hora após a injeção. Efetuamos em nosso estudo a medida dos níveis plasmáticos e cerebrais atingidos por uma dose única de 5-OP (1g/kg de peso corporal). A escolha da dose de 5-OP baseou-se em estudo prévio realizado por Caccia e colaboradores (1982), que mostrou que a administração oral de igual dose de 5-OP a ratos produziu concentrações plasmáticas similares às encontradas nos pacientes afetados pela deficiência de GS. Cabe ressaltar que os níveis de 5-OP no plasma e LCR dos pacientes afetados pela deficiência de GS se encontram na faixa de 2 a 5 mM e de 1 a 3 mM, respectivamente (Meister, 1974; Jain *et al.*, 1994), enquanto que as concentrações fisiológicas de 5-OP são aproximadamente 50 μM no plasma e 40 μM no LCR (Hoffmann *et al.*, 1993). Os níveis plasmáticos e cerebrais de 5-OP obtidos em nosso estudo (2,82 mM e 0,80 mM, respectivamente) são similares aos encontrados no plasma e LCR de pacientes com deficiência de GS, assegurando que nosso modelo agudo atinge concentrações satisfatórias para avaliar os efeitos *in vivo* da 5-OP.

Foram avaliados os efeitos da administração aguda de 5-OP sobre a quimiluminescência espontânea e sobre os níveis de substâncias reativas ao ácido

tiobarbitúrico (TBA-RS), sendo ambos considerados parâmetros de lipoperoxidação. A quimiluminescência espontânea mede a luz emitida pelos lipídios peroxidados resultantes de um aumento na produção de ER (Halliwell e Gutteridge, 2007), enquanto o TBA-RS reflete a quantidade de malondialdeído formado na degradação de lipídios promovida pela lipoperoxidação (Esterbauer e Cheeseman, 1990). Observamos que a administração aguda de 5-OP aumenta significativamente a quimiluminescência espontânea e os níveis de TBA-RS em córtex cerebral e cerebelo, indicando que a 5-OP é capaz de promover lipoperoxidação *in vivo* em cérebro de ratos, resultados que não haviam sido observados anteriormente *in vitro* (Pederzolli *et al.*, 2007). Cabe lembrar que nem todas as ER são capazes de iniciar a lipoperoxidação; $OH^{\bullet}$, $RO^{\bullet}_2$, $NO^{\bullet}_2$ e ONOOH podem prontamente fazê-lo. Por outro lado, H_2O_2, $NO^{\bullet}$ e $O_2^{\bullet-}$ não são suficientemente reativos para abstrair um hidrogênio de um lipídio poliinsaturado, não sendo capazes de iniciar diretamente a reação em cadeia de lipoperoxidação (Halliwell e Whiteman, 2004; Halliwell e Gutteridge, 2007). Considerando que o estímulo à lipoperoxidação promovido pela 5-OP foi observado apenas *in vivo*, e não *in vitro*, sugere-se que a 5-OP possa apresentar um efeito indireto sobre esse parâmetro, provavelmente envolvendo produção *in vivo* de ER capazes de iniciar a lipoperoxidação (Halliwell e Gutteridge, 2007). Além disso, é importante ressaltar que nos experimentos *in vitro,* o córtex cerebral e o cerebelo foram expostos à 5-OP somente após a homogeneização desses tecidos, processo que promove o rompimento de membranas celulares, excluindo-se nesse tipo de experimento o envolvimento de mecanismos como o da excitotoxicidade, por exemplo, que exige a presença de membranas e sinapses neuronais íntegras. Cabe lembrar que a promoção de excitotoxicidade pela 5-OP já foi demonstrada (Bennet et al., 1973; Rieke et al., 1989; Barone e Spignoli, 1990), e que a excitotoxicidade

por si só já foi relacionada a estresse oxidativo (Lees, 1993; Hazel, 2007). O excesso de liberação neuronal de glutamato pode ativar diversos receptores glutamatérgicos pré- e pós-sinápticos, gerando um conseqüente aumento intracelular de cálcio, que pode levar à disfunção mitocondrial, superprodução de ER e ativação de proteases, fosfolipases e endonucleases, culminando com a morte celular (Halliwell e Gutteridge, 2007; Hazel *et al.*, 2007). Assim, a possível contribuição da excitotoxicidade para os efeitos promovidos pela 5-OP *in vivo* não deve ser desconsiderada, podendo ter havido um sinergismo de efeitos para a promoção de lipoperoxidação pela 5-OP *in vivo*.

Em relação ao dano oxidativo protéico, sabe-se que diversas ER podem oxidar resíduos de aminoácidos em proteínas formando produtos contendo grupos carbonila, que podem ser quantificados através de sua reação com a dinitrofenilhidrazina (Halliwell e Gutteridge, 2007). Verificamos no presente trabalho que o conteúdo de carbonilas é significativamente aumentado em córtex cerebral e cerebelo de ratos submetidos à administração aguda de 5-OP, indicando dano oxidativo protéico. Esses resultados corroboram nossos resultados anteriores *in vitro (*Pederzolli *et al.*, 2007*)*, sugerindo que a 5-OP é capaz de promover a oxidação de proteínas tanto *in vitro* quanto *in vivo*.

Observamos ainda que o TRAP, que avalia a quantidade de antioxidantes não-enzimáticos presentes no tecido, é significativamente reduzido pela 5-OP *in vivo* tanto em córtex cerebral quanto em cerebelo de ratos, o que está de acordo com nossos resultados *in vitro* (Pederzolli *et al.*, 2007). No entanto, os níveis de ácido ascórbico e de GSH, dois importantes antioxidantes não-enzimáticos cerebrais, não são afetados pela administração aguda de 5-OP, sugerindo que a redução do TRAP observada *in vivo* não seja especificamente devida à redução de ácido ascórbico ou GSH. No cérebro, outros

importantes antioxidantes não-enzimáticos incluem o α-tocoferol (Vitamina E, principal antioxidante lipofílico cerebral), proteínas quelantes de metais como a ceruloplasmina e a metalotioneína, e ainda a carnosina (neuropeptídeo) e a melatonina (hormônio pineal) (Stvolinski *et al*., 1999; Coyle *et al*., 2002; Boldyrev *et al*., 2004; Guelman *et al*., 2004; Vassiliev *et al*., 2005; Halliwell, 2006; Anisimov *et al*., 2006; Jimenez-Jorge *et al*., 2007; Carpenè *et al*., 2007), entre outros. Dessa forma, além do ácido ascórbico e da GSH, diversos outros antioxidantes não-enzimáticos podem contribuir para os valores do TRAP (Halliwell e Gutteridge, 2007). Especificamente as proteínas com funções antioxidantes contribuem para aproximadamente 40% dos valores medidos do TRAP em homogeneizados de cérebro de ratos (Evelson *et al*., 2001). Considerando que no presente estudo observamos um significativo dano oxidativo protéico promovido pela 5-OP *in vivo*, parece possível que a redução do TRAP promovida pela 5-OP possa ter sido ao menos em parte devida a uma redução de proteínas com ações antioxidantes, que podem ter tido seu funcionamento normal comprometido pelo dano oxidativo promovido pela 5-OP.

Quanto às defesas antioxidantes enzimáticas, a administração aguda de 5-OP reduz significativamente as atividades das enzimas antioxidantes CAT e GPx apenas em córtex cerebral de ratos jovens. A atividade da SOD, por sua vez, não é alterada em nenhuma das estruturas cerebrais estudadas. Os diferentes padrões de alteração encontrados em nosso estudo para as atividades enzimáticas estão de acordo com o fato de que o sistema antioxidante endógeno é complexo (Halliwell e Gutteridge, 2007), e com o fato de que as atividades de enzimas antioxidantes (defesas enzimáticas) e o conteúdo de antioxidantes de baixo peso molecular (defesas não-enzimáticas) apresentam diferentes padrões de alteração entre tecidos periféricos e estruturas cerebrais, alterando-se também com a idade (Hussain

et al., 1995; Ferri *et al.*, 2005; Yang *et al.*, 2006; Campese *et al.*, 2007). Ainda, estudos mostram que as regiões cerebrais apresentam diferentes atividades enzimáticas e padrões de alteração das mesmas, a fim de reestabelecer a homeostase redox; especificamente as atividades das enzimas antioxidantes são maiores no cerebelo do que no córtex cerebral, o que pode gerar respostas distintas de cada região ao estresse oxidativo (Hussain *et al.*, 1995; Yang *et al.*, 2006). Essas variações de resposta podem ocorrer em parte devido às características específicas de cada região cerebral, como o consumo de oxigênio, a taxa de atividade metabólica, a susceptibilidade aos oxidantes, entre outros (Kaushik e Kaur, 2003). Além disso, fatores pró-oxidantes que direta ou indiretamente induzem a geração de ER também diferem entre órgãos e entre estruturas cerebrais; o conteúdo de ferro, por exemplo, é maior no córtex cerebral do que no cerebelo de ratos em desenvolvimento (Beard *et al.*, 1993). Apesar dos diferentes efeitos encontrados após a administração aguda de 5-OP sobre as atividades das enzimas antioxidantes, os resultados obtidos mostram claramente que a 5-OP pode prejudicar *in vivo* as defesas antioxidantes enzimáticas em cérebro de ratos. Como as atividades da CAT e da GPx não haviam sido alteradas *in vitro* pela 5-OP (Pederzolli *et al.*, 2007), presume-se que a inibição dessas enzimas pela 5-OP *in vivo* tenha ocorrido através de um mecanismo indireto da 5-OP sobre as atividades enzimáticas da CAT e da GPx. Novamente, não podemos descartar um possível envolvimento do mecanismo de excitotoxicidade nesse efeito. De qualquer forma, a redução das atividades da CAT e GPx pela 5-OP *in vivo* pode indicar um comprometimento na detoxificação de seu substrato, o H_2O_2. Em baixas concentrações, o H_2O_2 é metabolisado principalmente pela GPx, que apresenta maior atividade cerebral do que a CAT; no entanto, em altas concentrações de H_2O_2, a CAT passa a ser a principal

responsável pela sua detoxificação (Kaushik e Kaur, 2003). No presente trabalho, avaliamos o conteúdo de H_2O_2 em córtex cerebral e cerebelo de ratos submetidos à administração aguda de 5-OP. Foi possível observar que a 5-OP aumenta significativamente o conteúdo de H_2O_2 *in vivo*, o que sugere que um aumento de H_2O_2 pode estar envolvido na neurotoxicidade da 5-OP. Apesar de ser pouco reativo, o H_2O_2 pode ser citotóxico (causando senescência, apoptose e morte celular necrótica) e pode ainda inativar diretamente algumas enzimas, geralmente por oxidação de grupos –SH essenciais para a atividade catalítica das mesmas (Halliwell e Gutteridge, 2007). Além disso, o H_2O_2 atravessa facilmente as membranas celulares e, apesar de ser *per se* pouco reativo, é capaz de gerar $OH^\bullet$, altamente reativo, sempre que entrar em contato com íons Cu^{2+} ou Fe^{2+} (Reação de Fenton) (Halliwell, 2001). Andreoli e colaboradores (1993) demonstraram que o H_2O_2 é capaz de reduzir significativamente os níveis de ATP em cultura de células, sendo esse efeito prevenido pela adição de CAT ao meio. Considerando que os níveis de ATP são reduzidos tanto por H_2O_2 quanto pela 5-OP (Silva *et al.*, 2001), e considerando que o conteúdo de H_2O_2 é aumentado pela 5-OP, é possível que *in vivo* haja um sinergismo entre esses efeitos, potencializando a diminuição dos níveis de ATP. É interessante observar que nossos resultados mostram um maior aumento do conteúdo de H_2O_2 pela 5-OP no córtex cerebral do que no cerebelo. Considerando que o conteúdo de ferro é também maior no córtex cerebral do que no cerebelo (Beard *et al.*, 1993), e considerando também que o ferro é um catalisador da reação de Fenton, na qual há formação de radicais $OH^\bullet$ a partir de H_2O_2, é possível que tenha havido uma maior geração de $OH^\bullet$ a partir de H_2O_2 no córtex cerebral do que no cerebelo.

A atividade da G6PD também foi medida em córtex cerebral e cerebelo de ratos

submetidos à administração aguda de 5-OP. A G6PD é a enzima regulatória da via da pentose fosfato e, como tal, controla o fluxo de carbonos através da fase oxidativa dessa via e produz equivalentes redutores na forma de NADPH para suprir as necessidades celulares para biossíntese e manutenção do *status* redox celular (Kletzien *et al.*, 1994). Em mamíferos, a maior parte do NADPH é produzida pela via da pentose fosfato (Hashida *et al.*, 2002), que é regulada por diversos fatores como hormônios, nutrientes e estresse oxidativo (Kletzien et al., 1994). Dessa forma, a atividade da G6PD pode representar um marcador precoce de estresse oxidativo, já que é capaz de responder rapidamente ao aumento na demanda de NADPH necessário para a manutenção do *status* redox celular (Kletzien *et al.*, 1994). Além disso, como a atividade da G6PD pode limitar a taxa de produção de NADPH, pode também comprometer a capacidade de detoxificação de peróxidos pela GPx, já que o funcionamento normal dessa enzima depende da regeneração da GSH pela glutationa redutase, que por sua vez depende de NADPH (Hashida *et al.*, 2002; Williams e Ford, 2004). Dessa forma, a G6PD pode ser crucial para a função antioxidante, particularmente na eliminação de peróxidos, visto que é a enzima marca-passo na regeneração de GSH (Hashida *et al.*, 2002). No presente estudo, foi possível observar que a administração aguda de 5-OP é capaz de reduzir significativamente a atividade da G6PD em córtex cerebral de ratos, o que pode causar uma alteração na produção de NADPH e no equilíbrio redox celular. Recentemente, Hashida e colaboradores (2002) sugeriram que a vulnerabilidade das células cerebrais ao dano oxidativo é em grande parte devida à baixa capacidade de fornecimento de NADPH, que é necessário para a remoção de baixas concentrações de H_2O_2. Visto que o funcionamento normal da GPx depende da regeneração de GSH pela glutationa redutase, que por sua vez depende do

NADPH produzido pela G6PD (Hashida *et al*., 2002; Williams e Ford, 2004), e que tanto a GPx quanto a G6PD têm suas atividades reduzidas pela 5-OP, é possível que *in vivo* haja um sinergismo entre esses efeitos, podendo gerar efetivamente um comprometimento na detoxificação do H_2O_2. Além disso, também já foi relatado que a G6PD é fortemente inativada por 4-hidroxinonenal, um produto tóxico da lipoperoxidação (Ninfali *et al*., 2001), processo esse que também é induzido pela 5-OP *in vivo*.

Também medimos o conteúdo de tióis (SH) e dissulfetos (SS), o que nos permitiu calcular a razão SH/SS. A administração aguda de 5-OP não causou alteração no conteúdo de SH, mas aumentou o conteúdo de SS e diminuiu a razão SH/SS em córtex cerebral e cerebelo. A produção aumentada de ER e/ou funções antioxidantes comprometidas provocam um desequilíbrio entre reações oxidativas e redutoras, alterando o *status* redox SH/SS (Moriarty-Craige e Jones, 2004). A redução da razão SH/SS reflete um *status* redox oxidado da célula que pode, por fim, levar ao estresse oxidativo (Sultana *et al*., 2008). Assim, considerando que o conteúdo de SS é significativamente aumentado pela administração aguda de 5-OP e que a razão SH/SS é significativamente reduzida, pode-se sugerir que a 5-OP é capaz de alterar *in vivo* o *status* redox tiólico em córtex cerebral e cerebelo de ratos jovens. Considerando que tanto o conteúdo de SS quanto de H_2O_2 são aumentados pela 5-OP, e que o H_2O_2 é capaz de inativar algumas enzimas diretamente pela oxidação de grupos –SH essenciais (Halliwell e Gutteridge, 2007), postula-se que a oxidação de grupos –SH, bem como o aumento do H_2O_2, possam estar de fato envolvidos nos mecanismos de neurotoxicidade da 5-OP. É interessante observar que a enzima Na^+,K^+-ATPase contém grupos –SH essenciais para a atividade catalítica, os quais são suscetíveis ao ataque oxidativo (Andreoli *et al*., 1993), e essa enzima é prontamente

inativada por ER, que promovem uma inibição irreversível de sua atividade (Hexum e Fried, 1979; Kukreja *et al.*, 1990). Andreoli e colaboradores (1993) demonstraram que o H_2O_2 é capaz de promover uma inibição direta da atividade da Na^+,K^+-ATPase em cultura de células, sendo que a adição de CAT ao meio é capaz de prevenir completamente a presença dessas alterações. Considerando que a Na^+,K^+-ATPase, que apresenta grupos –SH oxidáveis essenciais para sua atividade catalítica, é inibida tanto pela 5-OP (Escobedo e Cravioto, 1973; Rieke *et al.*, 1984) quanto por H_2O_2 (Andreoli *et al.*, 1993), e que a 5-OP é capaz de aumentar os conteúdos de H_2O_2 e de SS, é possível que *in vivo* a redução da Na^+,K^+-ATPase seja ainda mais significativa.

De uma forma geral, nossos resultados mostraram a presença de alterações mais proeminentes dos parâmetros de estresse oxidativo em córtex cerebral, com menos efeitos no cerebelo, que por sua vez apresentou maior capacidade antioxidante e maiores atividades das enzimas antioxidantes, sugerindo que o córtex cerebral parece mais suscetível ao estresse oxidativo. Diferenças regionais nas atividades dos sistemas antioxidantes e taxas metabólicas variáveis podem aumentar a vulnerabilidade ao estresse oxidativo em regiões cerebrais específicas, podendo provocar dano oxidativo com intensidades diferentes (Cardozo-Pelaez *et al.*, 2000). Yang e colaboradores (2006) demonstraram que, dentre as diferentes regiões cerebrais, a que apresenta o maior potencial redutor (calculado através da relação $NADPH/NADP^+$ e GSH/GSSG), ou seja, a estrutura mais resistente a danos oxidativos, é o cerebelo. Nossos resultados estão de acordo com alguns trabalhos que mostram menores atividades de enzimas antioxidantes (Hussain *et al.*, 1995; Campese *et al.*, 2007) e maior conteúdo de carbonilas (marcador de oxidação protéica) (El-Mohsen *et al.*, 2005) em córtex cerebral do que em cerebelo. Cabe lembrar

ainda que o conteúdo de ferro, catalisador da formação de $OH^{\bullet}$, é maior no córtex cerebral do que no cerebelo (Beard *et al.*, 1993). Todos esses fatores podem estar contribuindo para a maior sensibilidade do córtex cerebral ao estresse oxidativo promovido pela 5-OP.

Considerando que a 5-OP diminui significativamente as defesas antioxidantes não-enzimáticas, aumenta a produção de ER e promove dano oxidativo protéico tanto *in vitro* quanto *in vivo*, e considerando que, além disso, a 5-OP *in vivo* promove também dano oxidativo a lipídios e diminuição das defesas antioxidantes cerebrais enzimáticas, juntamente com o aumento do conteúdo de H_2O_2, com a redução da atividade da G6PD (possivelmente prejudicando, com isso, a produção de NADPH) e com a alteração da razão SH/SS (alterando o equilíbrio redox celular), podemos postular que a 5-OP é capaz de promover estresse oxidativo *in vitro* e *in vivo* em cérebro de ratos jovens. Vale lembrar que Nygren e colaboradores (2005), ao observarem ausência de dano oxidativo ao DNA em fibroblastos de pacientes afetados pela deficiência de GS e ausência de correlação entre os níveis de GSH e a proteção contra dano oxidativo ao DNA, sugeriram que a oxidação de outros compostos celulares, a alteração do potencial redox celular ou efeitos tóxicos de precursores de GSH, como a 5-OP, fossem explicações mais plausíveis para os mecanismos responsáveis pelos sintomas clínicos associados à deficiência de GS (Nygren *et al.*, 2005). E de fato, demonstramos no presente trabalho diversos efeitos neurotóxicos da 5-OP, incluindo o dano oxidativo a lipídios e proteínas, comprometimento das defesas antioxidantes e alteração do *status* redox celular. Se esses efeitos também ocorrerem nos pacientes afetados pela deficiência de GS, é possível que possam contribuir para a neuropatologia dessa doença. Além disso, já que tanto a excitotoxicidade (Bennet *et al.*, 1973; Rieke *et al.*, 1989; Barone e Spignoli, 1990) quanto o bloqueio da cadeia respiratória

(Silva *et al.*, 2001), ambas ações promovidas pela 5-OP, podem também levar indiretamente à geração de ER (Bondy e Le Bel, 1993; Nicholls e Budd, 1998), é possível que esses mecanismos possam agir de forma sinérgica na indução de um dano neuronal significativo. A formação excessiva de ER pode promover dano mitocondrial, incluindo a oxidação do DNA, lipídios e proteínas, e abertura do póro de transição de permeabilidade mitocondrial, evento relacionado à degeneração e morte celular (Calabrese *et al.*, 2005). Por outro lado, um dano mitocondrial, como o causado pela 5-OP através da inibição de complexos da cadeia respiratória, além de poder diminuir a produção de ATP, pode provocar a desorganização da cadeia respiratória, permitindo com isso que elétrons possam escapar mais facilmente por haver uma redução incompleta do O_2 a H_2O, podendo levar à formação aumentada de ER como $O_2^{\bullet -}$, H_2O_2 e $OH^{\bullet}$, resultando em um círculo vicioso (Milatovic *et al.*, 2001; Gupta *et al.*, 2002; Halliwell e Gutteridge, 2007). Apesar de não ser possível precisar qual seria o evento iniciador da cascata de efeitos promovida pela 5-OP, é possível que estejam todos de fato intimamente relacionados. A 5-OP promove dano oxidativo protéico, podendo estar inativando algumas enzimas; de fato, além de inibir a Na^+,K^+-ATPase (Escobedo e Cravioto, 1973) e complexos da cadeia respiratória mitocondrial (Silva *et al.*, 2001), a 5-OP á capaz de inibir as enzimas antioxidantes CAT e GPx, detoxificadoras de H_2O_2, o qual por sua vez é aumentado pela 5-OP. Considerando que a 5-OP é capaz de aumentar os conteúdos de SS e de H_2O_2, o qual por sua vez é capaz de oxidar diretamente grupos –SH, é possível que tanto o aumento do H_2O_2 quanto a oxidação de grupos –SH estejam envolvidos nos mecanismos de neurotoxicidade da 5-OP. A capacidade de detoxificação do H_2O_2 pela CAT, que atua em altas concentrações de H_2O_2, e pela GPx, que atua em baixas concentrações de H_2O_2, parece estar em grande parte

comprometida devido à inibição dessas enzimas pela 5-OP, podendo acumular H_2O_2, que pode se transformar em ER mais danosas através da reação de Fenton. O funcionamento normal da GPx, por outro lado, depende em grande parte do funcionamento normal da G6PD, que fornece NADPH para regenerar GSH, cuja oxidação ocorre acoplada à reação catalisada pela GPx. A G6PD, no entanto, também é inibida pela 5-OP, podendo realmente comprometer a eficiência da detoxificação de H_2O_2 pela GPx. A G6PD, por sua vez, é também inibida por 4-hidroxinonenal, produto tóxico da lipoperoxidação. A lipoperoxidação, também promovida pela 5-OP, só pode ser iniciada por algumas ER, excluindo-se o H_2O_2. Dessa forma, imagina-se que *in vivo* tenha ocorrido a produção de ER mais reativas, mais danosas, com real potencial de oxidar lipídios e proteínas, ambas ações promovidas pela 5-OP. Além de tudo isso, vale lembrar que a 5-OP compromete as defesas antioxidantes cerebrais, podendo com isso tornar o cérebro ainda mais suscetível ao estresse oxidativo, o qual pode então ser potencializado, levando ao dano oxidativo. Dessa forma, todos esses efeitos podem estar conjuntamente envolvidos nos mecanismos de neurotoxicidade da 5-OP, podendo contribuir para o dano cerebral observado nos pacientes com deficiência de GS.

Apesar de as concentrações de 5-OP utilizadas serem similares às observadas no plasma e LCR dos pacientes afetados pela deficiência de GS (1-5 mM) (Eldjarn *et al.*, 1972; Eldjarn *et al.*, 1973; Meister, 1974; Meister, 1991; Jain *et al.*, 1994), é difícil extrapolar nossos achados para a condição humana. No entanto, se esses efeitos também ocorrerem no cérebro dos pacientes afetados é possível que possam contribuir, ao menos em parte, para a disfunção neurológica característica da deficiência de GS, na qual a 5-OP se encontra acumulada, podendo-se sugerir o envolvimento do estresse oxidativo na

neuropatologia dessa doença. Nos pacientes afetados pela deficiência de GS, é possível que haja ainda um sinergismo entre o acúmulo de 5-OP e a deficiência de GSH, aumentando a produção de ER e diminuindo as defesas antioxidantes, levando assim ao estresse oxidativo. Não foi esclarecido ainda qual o principal mecanismo responsável pela neurotoxicidade da 5-OP no dano cerebral desses pacientes, podendo estar envolvidos o dano à produção energética cerebral, a inibição da Na^{+},K^{+}-ATPase, a excitotoxicidade, o estresse oxidativo ou uma combinação de quaisquer desses fatores. Entender os mecanismos responsáveis pelo dano cerebral que ocorre nos pacientes com deficiência de GS pode ser útil no desenvolvimento de uma terapêutica efetiva para amenizar a disfunção neurológica desses pacientes.

2. Ácido N-acetilaspártico

O acúmulo de NAA é o marcador bioquímico da Doença de Canavan, um erro inato do metabolismo clinicamente caracterizado por disfunção neurológica severa caracterizada por retardo mental progressivo, hipotonia, macrocefalia e convulsões. Até o presente momento, não há tratamento específico para essa desordem, sendo apenas sintomático, controlando-se principalmente a ocorrência de convulsões. Cabe lembrar que a presença de níveis elevados de NAAG, um metabólito do NAA, já foi também relatada na urina e LCR de pacientes afetados pela Doença de Canavan (Burlina *et al*., 1999; Krawczyk e Gradowska, 2003).

Até o presente momento existem poucas hipóteses acerca de possíveis mecanismos neuropatológicos envolvidos nessa doença. Foi sugerido que poderiam estar envolvidos: (a) o comprometimento na síntese de lipídios de mielina, resultante de uma deficiência de

acetato derivado do NAA (Madhavarao *et al*., 2005); e (b) o desequilíbrio osmótico com acúmulo de excesso de fluido cerebral promovido pelo aumento patológico de NAA, que atuaria como um osmólito intracelular (Baslow, 2002). No entanto, essas hipóteses têm sido recentemente discutidas na literatura (Baslow, 2003; Moffett *et al*., 2007), e o papel do NAA na patogênese da Doença de Canavan permanece ainda pouco esclarecido.

Apesar de os mecanismos responsáveis pelo dano cerebral nessa desordem serem ainda pouco compreendidos, a presença de concentrações aumentadas de NAA nos tecidos e fluidos corporais sugere a possibilidade de que o NAA e/ou metabólitos relacionados, como o NAAG, possam exercer efeitos tóxicos (Beaudet, 2001), e que o excesso desses ácidos orgânicos possa ser deletério ao SNC. Apesar de os mecanismos pelo qual exercem sua toxicidade não terem sido ainda completamente esclarecidos, diversos relatos na literatura mostram ações neurotóxicas tanto para o NAA quanto para o NAAG, ambos acumulados nos pacientes afetados pela Doença de Canavan. O NAA, por exemplo, é capaz de agir sobre receptores glutamatérgicos metabotrópicos acoplados à proteína G, resultando em excitação neuronal e contribuindo, assim, para a ocorrência de convulsões epiléticas (Akimitsu *et al*., 2000; Yan *et al*., 2003). Klugmann e colaboradores (2005) mostraram que a redução parcial dos níveis de NAA no cérebro de ratos geneticamente deficientes de aspartoacilase (ratos *tremor*) resulta na modulação tanto da duração quanto da freqüência das convulsões, sugerindo que os níveis cerebrais aumentados de NAA no rato *tremor* participam de fato no curso da epilepsia. Estudos em animais mostraram que convulsões epiléticas resultam em produção de ER e dano oxidativo a proteínas celulares, lipídios e DNA (Bruce e Baudry, 1995; Liang *et al*., 2000), enquanto que o estresse oxidativo mitocondrial crônico, bem como sua disfunção resultante, podem tornar o

cérebro mais suscetível a convulsões epiléticas (Trotti *et al.*, 1998; Liang e Patel, 2004); em conjunto, essas evidências sugerem que parece haver um papel para o estresse oxidativo tanto como causa quanto como conseqüência de convulsões epiléticas (Patel, 2004). Além disso, o NAA também é capaz de aumentar a concentração intracelular de cálcio em cultura de células (Rubin *et al.*, 1995). É interessante ressaltar que o estresse oxidativo é capaz de desregular o metabolismo do cálcio, geralmente causando um aumento na concentração intracelular de cálcio (Halliwell e Gutteridge, 2007). ER podem danificar o sistema de captação de cálcio pelo retículo endoplasmático e interferir no efluxo de cálcio através da membrana plasmática, por oxidarem grupos –SH essenciais de canais transmembrana, podendo promover a abertura de canais que permitam a entrada de cálcio na célula (Halliwell e Gutteridge, 2007). Por esses motivos, é possível que o aumento da concentração intracelular de cálcio promovido pelo NAA tenha sido de fato mediado por estresse oxidativo.

O NAAG, por sua vez, também apresenta diversas ações neurotóxicas relatadas na literatura. Observou-se que o NAAG pode exibir neurotoxicidade *in vitro* em culturas primárias corticais (Thomas *et al.*, 2000), e *in vivo* pode induzir morte neuronal (Pliss *et al.*, 2000) e alterar as propriedades funcionais e estruturais da barreira hemato-encefálica (Pliss *et al.*, 2002). Estudos recentes mostraram que a administração intracerebroventricular de NAAG a ratos é capaz de alterar o comportamento dos mesmos e induzir neurodegeneração hipocampal, com alterações significativas na morfologia celular e clivagem do DNA (Pliss *et al.*, 2003; Bubeníková-Valesová *et al.*, 2006). Apesar de o DNA sofrer decomposição espontânea e fisiológica diariamente, o estresse oxidativo acelera enormemente o dano ao DNA (Halliwell e Gutteridge, 2007). Com isso, parece

possível que a quebra de fitas de DNA observada após administração de NAAG possa ter sido mediada por ER, que podem ter promovido dano ao DNA.

Considerando que nenhum estudo até o presente momento havia investigado o papel do estresse oxidativo na neurotoxicidade do NAA e do NAAG, resolvemos estudar os efeitos *in vitro* e *in vivo* desses ácidos orgânicos sobre alguns parâmetros de estresse oxidativo em córtex cerebral de ratos de 14 e 30 dias de vida. O objetivo geral desse estudo foi avaliar se a produção de ER pode ser gerada por esses metabólitos, o que poderia estar relacionado ao dano neurológico observado nos pacientes com Doença de Canavan, nos quais tanto NAA quanto NAAG se encontram acumulados. Nos experimentos *in vitro*, os homogeneizados de córtex cerebral de ratos de 14 dias de vida foram incubados por 1 hora a 37°C na presença ou ausência de NAAG nas concentrações de 1; 5,0 ou 10,0 mM e na presença ou ausência de NAA nas concentrações de 10; 20; 40 ou 80 mM no meio de incubação. Apenas nos ensaios enzimáticos o efeito *in vitro* do NAA foi testado com e sem essa pré-incubação. Nos experimentos *in vivo,* ratos de 14 dias de vida foram submetidos à administração aguda de salina (ratos controle) ou de NAA nas doses de 0,1; 0,3 ou 0,6 mmol/g peso corporal, administradas por via subcutânea, sendo os animais mortos 1 hora após a injeção; e ratos de 30 dias de vida foram submetidos à administração intracerebroventricular de LCR artificial (ratos controle) ou NAA (8 μmol) ou NAAG (0,8 μmol), sendo os animais mortos em 15 ou 60 minutos após a injeção.

Demonstramos que o NAA reduz significativamente tanto o TRAP quanto o TAR em córtex cerebral *in vitro*, e reduz o TRAP também *in vivo*, tanto através da administração subcutânea quanto da intracerebroventricular de NAA a ratos de 14 e 30 dias de vida, respectivamente. Considerando que o TRAP mede o conteúdo de defesas antioxidantes

não-enzimáticas, enquanto que o TAR reflete a capacidade do tecido de modular o dano associado a uma produção aumentada de ER (Lissi *et al*., 1995), esses resultados indicam que o NAA reduz a capacidade antioxidante não-enzimática em cérebro de ratos tanto *in vitro* quanto *in vivo*, através da redução do conteúdo de antioxidantes não-enzimáticos (TRAP) e da redução da reatividade desses antioxidantes (TAR). Demonstramos ainda que o NAA reduz significativamente *in vitro* o conteúdo de GSH, o principal antioxidante não-enzimático cerebral (Rice e Russo-Menna, 1998), o que sugere que as reduções do TRAP e do TAR observadas anteriormente na presença de NAA na verdade podem ter ocorrido às expensas de uma redução de GSH. Assim, os resultados indicam claramente que o NAA é capaz de comprometer as defesas antioxidantes não-enzimáticas em cérebro de ratos, tanto *in vitro* quanto *in vivo*.

Conforme previamente citado, diversas ER podem oxidar resíduos de aminoácidos em proteínas formando produtos contendo grupos carbonila, que podem ser quantificados através de sua reação com a dinitrofenilhidrazina (Halliwell e Gutteridge, 2007). Além disso, resíduos de proteína contendo grupos –SH são alvos particularmente suscetíveis à oxidação, podendo haver a abstração de hidrogênios desses grupos por parte de ER, oxidando o grupo –SH e formando pontes dissulfeto. Dessa forma, além da alteração no conteúdo de carbonilas, a oxidação dos grupos –SH é também usada como marcador de oxidação protéica, medindo-se o conteúdo de tióis totais (Aksenov e Markesbery, 2001). Foi possível observar que *in vitro* o NAA é capaz de aumentar o conteúdo de carbonilas e diminuir o de tióis totais (havendo oxidação de grupos –SH a dissulfetos), e *in vivo* é também capaz de aumentar o conteúdo de carbonilas tanto após administração subcutânea quanto após administração intracerebroventricular de NAA. Assim, pode-se sugerir que o

NAA é capaz de causar dano oxidativo protéico *in vitro* e *in vivo* em córtex cerebral de ratos de 14 e 30 dias de vida. Portanto, parece possível que o dano oxidativo às proteínas esteja envolvido na neurotoxicidade do NAA, o que pode então estar relacionado à neuropatologia da Doença de Canavan, na qual as concentrações de NAA estão expressivamente aumentadas.

Observamos também que a quimiluminescência espontânea e o TBA-RS foram aumentados *in vitr*o pela presença de NAA no meio de incubação, assim como *in vivo* pela administração aguda de NAA, sugerindo que o NAA é capaz de induzir lipoperoxidação em córtex cerebral de ratos de 14 dias de vida. No entanto, a quimiluminescência espontânea e o TBA-RS não foram afetados pela administração intracerebroventricular de NAA a ratos de 30 dias de vida. Esses resultados sugerem que o dano oxidativo aos lipídios é estimulado pelo NAA *in vitro* e *in vivo* em córtex cerebral de ratos de 14 dias de vida, mas não é estimulado pelo NAA *in vivo* em córtex cerebral de ratos de 30 dias de vida. Considerando que a superprodução de ER gera altos níveis de TBA-RS, por estimular a lipoperoxidação (Halliwell e Gutteridge, 2007), investigamos a influência de vários antioxidantes sobre os níveis aumentados de TBA-RS induzidos por NAA 80 mM *in vitro*. Observamos que a mistura de ácido ascórbico mais Trolox foi capaz de prevenir completamente o aumento dos níveis de TBA-RS promovido pelo NAA em córtex cerebral. Esses resultados podem sugerir a participação de $OH^{\bullet}$ nesse efeito, visto que essa ER é seqüestrada pelos antioxidantes usados na mistura (Bains e Shaw, 1997; Halliwell e Gutteridge, 2007). Por outro lado, H_2O_2 e $O_2^{\bullet-}$ provavelmente não estejam envolvidos, já que tanto CAT quanto SOD, que atuariam sobre esses substratos, não foram capazes de prevenir o aumento dos níveis de TBA-RS causados pelo NAA. Ainda, como o ditiotreitol

(protetor de grupos –SH) e a GSH foram capazes de prevenir apenas parcialmente o aumento dos níveis de TBA-RS induzido por NAA, não devemos excluir a possibilidade de que a oxidação de grupos –SH esteja envolvida nos efeitos neurotóxicos do NAA. No entanto, $NO^{\bullet}$ provavelmente não esteja envolvido nesse efeito, visto que o L-NAME (inibidor da óxido nítrico sintase) não foi capaz de reduzir o aumento dos níveis de TBA-RS promovido pelo NAA.

Investigamos também no presente estudo o efeito da administração intracerebroventricular de NAA sobre a atividade da G6PD em córtex cerebral de ratos de 30 dias de vida. Como previamente dito, a G6PD é a enzima marca-passo da via da pentose fosfato, sendo regulada por vários fatores, dentre os quais o estresse oxidativo (Kletzien *et al.*, 1994). A G6PD é a enzima limitante da detoxificação de H_2O_2 dependente de GSH e NADPH, o que mostra sua importância na função antioxidante cerebral (Adams *et al.*, 2001; Hashida *et al.*, 2002). Por esse motivo a medida da atividade dessa enzima regulatória pode ser considerada como um marcador precoce de estresse oxidativo (Kletzien *et al.*, 1994). Observamos em nosso trabalho que o NAA é capaz de reduzir significativamente a atividade da G6PD, o que pode comprometer a produção de NADPH e alterar o equilíbrio redox celular em córtex cerebral de ratos de 30 dias de vida.

Quanto às defesas antioxidantes enzimáticas, medimos primeiramente o efeito do NAA sobre a atividade da CAT, que catalisa diretamente a decomposição do H_2O_2 a oxigênio molecular (Aebi, 1984). Observamos que a atividade dessa enzima é fortemente inibida *in vitro* pelo NAA, com ou sem incubação prévia, e também *in vivo,* tanto através da administração aguda quanto intracerebroventricular de NAA a ratos de 14 e 30 dias de vida, corroborando nossos resultados *in vitro*. Além disso, resultados similares foram

obtidos com uma preparação comercial de CAT purificada, indicando uma possível interação direta do NAA com a CAT. Na tentativa de avaliar o mecanismo de inibição da CAT pelo NAA, realizamos estudos cinéticos sobre a interação do NAA com a CAT em homogeneizados de córtex cerebral de ratos de 14 dias de vida. Os valores de K_m e V_{max} obtidos foram similares aos relatados previamente em cérebro de ratos (Somani e Husain, 1996; Baud *et al.*, 2004). Nossos resultados mostraram também que o NAA inibe a atividade da CAT de forma acompetitiva, e que o K_i' (constante de inibição de inibidor acompetitivo) para o NAA foi menor que as concentrações encontradas no cérebro de pacientes afetados pela Doença de Canavan, sugerindo que a inibição da CAT pelo NAA pode ter relevância fisiopatológica.

Estudamos também o efeito do NAA sobre a atividade da GPx, que decompõe H_2O_2 através de sua redução à H_2O, com a concomitante oxidação da GSH; apesar de ser praticamente específica para GSH como doadora de hidrogênios, a GPx pode também atuar em outros peróxidos orgânicos, sendo os mesmos reduzidos a um álcool correspondente (Wendel, 1981). A atividade da GPx é inibida *in vitro* pela presença de NAA no meio de reação, sem incubação prévia, e também com 1 hora de pré-incubação. Além disso, o NAA também inibe a atividade da preparação comercial de GPx purificada, indicando uma possível interação direta com a enzima. Apesar de a administração intracerebroventricular de NAA a ratos de 30 dias de vida não alterar a atividade da GPx, a administração subcutânea de NAA a ratos de 14 dias de vida é capaz de reduzir significativamente a atividade cortical dessa enzima, corroborando nossos resultados *in vitro*. A inibição das atividades da CAT e GPx pode indicar um possível comprometimento da detoxificação de H_2O_2, substrato igualmente decomposto pelas duas enzimas. Observamos ainda no presente

trabalho que a exposição dos homogeneizados de córtex cerebral ao NAA 80 mM por 1 hora é capaz de aumentar significativamente *in vitro* o conteúdo de H_2O_2. *In vivo,* o conteúdo de H_2O_2 em córtex cerebral de ratos de 14 dias de vida também foi significativamente aumentado após administração aguda de NAA, o que está de acordo com nossos resultados *in vitro*. Esses resultados sugerem que o aumento no conteúdo de H_2O_2 está provavelmente envolvido na neurotoxicidade do NAA e pode ser secundário à redução das atividades da CAT e GPx promovidas pelo NAA. Por outro lado, o aumento no conteúdo de H_2O_2 é capaz de promover a auto-inativação da CAT (Aebi, 1984), inibindo irreversivelmente a atividade dessa enzima; a GPx, no entanto, é menos suscetível à inativação mediada por H_2O_2 (Baud *et al.*, 2004). Apesar de apresentarem diferentes afinidades pelo H_2O_2, ambas são requeridas para a detoxificação de H_2O_2, sendo a toxicidade do H_2O_2 enormemente potencializada quando CAT e/ou GPx são inibidas (Baud *et al.*, 2004). Cabe ressaltar que o H_2O_2 se mistura facilmente com a água e pode difundir para o meio intracelular, sendo tóxico a diversos tipos celulares na faixa de 10 a 100 μM, causando senescência e apoptose, e em concentraçãoes mais elevadas pode promover morte celular necrótica (Halliwell e Gutteridge, 2007). Além disso, o H_2O_2 é capaz de abrir canais catiônicos na membrana plasmática, permitindo a entrada de cálcio em certos neurônios e glia, entre outros tipos celulares, levando conseqüentemente ao aumento na concentração intracelular de cálcio (Halliwell e Gutteridge, 2007). Como um dos efeitos neurotóxicos apresentados pelo NAA é justamente o aumento na concentração intracelular de cálcio (Rubin *et al.*, 1995), e como foi observado que o NAA é capaz de aumentar o conteúdo de H_2O_2, é possível que o aumento da concentração de cálcio seja potencializado *in vivo* por um sinergismo desses efeitos. É interessante observar que o efeito de aumento da

concentração intracelular de cálcio foi observado em concentrações relativamente baixas de NAA (10 mM) (Rubin *et al.*, 1995), sendo então possível que concentrações de NAA maiores, como as observadas nos pacientes afetados pela Doença de Canavan, promovam uma exacerbação desse efeito. Aumentos transitórios da concentração de cálcio regulam diversos processos fisiológicos, como a proliferação celular e a liberação de neurotransmissores; no entanto, aumentos descontrolados da concentração de cálcio intracelular podem estimular a óxido nítrico sintase, a fosfolipase A2, calpaínas e proteases, promovendo alterações de citoesqueleto e podendo desencadear a transição de permeabilidade mitocondrial, que abre póros na membrana mitocondrial interna e causa um colapso no potencial de membrana (Halliwell e Gutteridge, 2007). Esse evento requer a presença de agentes indutores, que podem ser peróxidos orgânicos, agentes oxidantes de grupos –SH (como o H_2O_2, que é aumentado pelo NAA) e o $ONOO^-$. À medida que solutos escapam da matriz através do póro, ocorre um desequilíbrio osmótico e edema mitocondrial, que podem culminar com morte celular necrótica ou apoptótica (Halliwell e Gutteridge, 2007). De fato, Baud e colaboradores (2004) observaram em cultura de células a presença de edema celular em quase 100% das células expostas por 1 hora ao H_2O_2. Vale lembrar que dentre os achados neuropatológicos da Doença de Canavan há edema astrocitário e cerebral, com a presença de mitocôndrias alongadas e distorcidas (Adachi *et al.*, 1972), podendo tais alterações estarem relacionadas ao aumento da concentração intracelular de cálcio, que pode ser exacerbado pelo aumento do conteúdo de H_2O_2 promovido pelo NAA, caso esses efeitos também ocorram no cérebro dos pacientes afetados pela Doença de Canavan.

Avaliamos ainda o efeito do NAA sobre a atividade da SOD, enzima antioxidante

que dismuta o $O_2^{\bullet -}$, produzindo H_2O_2 e H_2O. O NAA não é capaz de alterar a atividade dessa enzima em córtex cerebral nem *in vitro* (com ou sem pré-incubação) nem *in vivo* pela administração subcutânea de NAA a ratos de 14 dias de vida. Por outro lado, a administração intracerebroventricular de NAA é capaz de aumentar significativamente a atividade da SOD medida 1 hora após a injeção em córtex cerebral de ratos de 30 dias de vida. Alguns trabalhos mostram que há um aumento na atividade da SOD em cérebro de ratos adultos, bem como um aumento gradual e progressivo na geração mitocondrial de $O_2^{\bullet -}$, em relação ao cérebro de ratos de 12 dias de vida (Schreiber *et al.*, 1995; Tsay *et al.*, 2000). Tem sido relatado também que ratos *zitter*, que são ratos Sprague-Dawley mutantes caracterizados por tremores, hipomielinização, edema astrocitário e degeneração espongiforme precoce, exibem também alterações no metabolismo cerebral de ER (Kondo *et al.*, 1995; Nakadate *et al.*, 2006). Gomi e colaboradores (1994) demonstraram um aumento significativo na atividade da SOD e uma redução significativa na atividade da CAT no cérebro de ratos *zitter* adultos, mas tais alterações não foram observadas no cérebro de ratos *zitter* neonatos. Ainda, Ueda e colaboradores (2002) observaram acúmulo de H_2O_2 no cérebro de ratos *zitter* adultos, sugerindo que a atividade aumentada da SOD e a atividade reduzida da CAT, também observadas no cérebro de ratos *zitter* adultos, podem levar ao acúmulo excessivo de H_2O_2 no tecido cerebral (Gomi *et al.*, 1994), potencializando o dano celular. Esses resultados observados em ratos *zitter*, que apresentam alterações neuropatológicas similares às observadas na Doença de Canavan, na qual o NAA está acumulado, corroboram nossos achados de um aumento da atividade da SOD e diminuição da atividade da CAT promovidos pelo NAA administrado por via intracerebroventricular no cérebro de ratos de 30 dias de vida. Além disso, o fato de que o

NAA é capaz de aumentar significativamente o conteúdo de H_2O_2 *in vitro* e *in vivo* em cérebro de ratos de 14 dias de vida sugere que o conteúdo de H_2O_2 possa estar também aumentado no cérebro de ratos de 30 dias de vida. Considerando que nesses animais a atividade da SOD está aumentada, a atividade da CAT está reduzida e a atividade da G6PD, que fornece o NADPH necessário para o funcionamento normal da GPx, está também reduzida, pode-se sugerir que a detoxificação de H_2O_2 esteja de fato comprometida no cérebro de ratos de 30 dias de vida submetidos à administração intracerebroventricular de NAA. Alguns trabalhos mostram que condições que alterem o metabolismo de ER são capazes de aumentar a atividade da SOD (Rister e Baehner, 1976; Lièvre *et al*., 2000). Rister e Baehner (1976) mostraram que cobaias expostos à condições hiperóxicas apresentam aumento de $O_2^{\bullet -}$ e indução da SOD, com redução simultânea da CAT e GPx; o fato não haver indução correspondente da CAT e GPx torna muito difícil a manutenção da concentração de H_2O_2 em níveis normais, e o excesso de H_2O_2 provavelmente resultará na formação de ER mais danosas, como o $OH^{\bullet}$, através da reação de Fenton. Além disso, Lièvre e colaboradores (2000) observaram que a atividade da SOD é significativamente aumentada em 1 hora após a hipóxia/reperfusão aplicada à cultura de neurônios, e sugeriram que isso possa significar a mobilização de enzimas pré-existentes ou o resultado de uma indução muito precoce do gen da SOD em resposta ao estresse oxidativo. A transcrição do gen da SOD é regulada em resposta a diversos estímulos, como citocinas pró-inflamatórias, fatores de crescimento e estresse oxidativo, que gera uma *up-regulation* da expressão da SOD (Zelko *et al*., 2002). A importância específica de ER ou de alterações no *status* redox celular na transdução de sinal e regulação de genes está se tornando cada dia mais evidente, estando atualmente bem estabelecido que o H_2O_2 é a principal ERO

mediadora de sinalização celular (Aslan e Özben, 2003). Diversos estudos mostram que o agrotóxico Paraquat, agente gerador de $O_2^{\bullet -}$, é capaz de induzir a SOD (Nicotera *et al.*, 1989; Yoo *et al.*, 1999). Além disso, o gen da SOD é particularmente regulado por NF-κB, fator de transcrição essencial a diversos processos celulares, como inflamação, imunidade, proliferação celular e apoptose (Warner *et al.*, 1996; Manna *et al.*, 1998). O NF-κB, por sua vez, além de ser controlado pelo *status* redox celular, é ativado rapidamente por diversas ER, principalmente por H_2O_2, mas também por $^1O_2$, HOCl e $ONOO^-$ (Wang *et al.*, 2002; Gloire *et al.*, 2006). De fato, o tratamento de diversas linhagens celulares com H_2O_2 é capaz de aumentar significativamente a expressão da SOD já a partir de 1 hora de exposição, indicando que o H_2O_2 está de fato diretamente envolvido na indução dessa enzima antioxidante (Warner *et al.*, 1996; Yoo *et al.*, 1999; Rojo *et al.*, 2004). Considerando que a expressão da SOD pode aumentar já a partir de 1 hora de exposição ao H_2O_2, pode-se sugerir que a indução do gen da SOD pode se dar rapidamente em resposta ao estresse oxidativo. É interessante observar que a expressão da SOD aumenta significativamente em córtex cerebral de ratos Wistar expostos a um estresse agudo, mas não crônico (Filipovic e Radojcic, 2005). O fato de que a expressão da SOD pode ser prontamente induzível por H_2O_2 faz com que essa enzima possa funcionar como uma resposta protetora eficiente contra o estresse oxidativo (Yoo *et al.*, 1999), e a ativação da via desencadeada pelo NF-κB, ativado por H_2O_2, pode reforçar a capacidade antioxidante celular e proporcionar proteção efetiva contra estresse oxidativo através da *up-regulation* de genes que codificam enzimas antioxidantes (Rojo *et al.*, 2004). A resposta ao estresse oxidativo, no entanto, nem sempre ocorre através de uma expressão coordenada de todas as enzimas antioxidantes, visto que a expressão gênica das mesmas é regulada por diferentes

mecanismos (Röhrdanz *et al*., 2000; Wilson e Johnson, 2000). Considerando que nossos resultados demonstraram um aumento na atividade da SOD em 1 hora após a administração intracerebroventricular de NAA em cérebro de ratos de 30 dias de vida, considerando que a redução da CAT e G6PD juntamente com o aumento da SOD observados nesses animais podem promover um comprometimento na detoxificação de H_2O_2, que provavelmente esteja acumulado no cérebro dos mesmos, e considerando ainda que a expressão da SOD pode ser prontamente e rapidamente induzível por H_2O_2, sugere-se que o aumento da SOD observado em nosso estudo possa ter havido por um aumento na expressão dessa enzima antioxidante, em resposta ao estresse oxidativo promovido pelo NAA.

Considerando que o ácido ascórbico é capaz de prevenir o dano celular induzido por H_2O_2 (Avshalumov *et al*., 2004), e que a mistura de ácido ascórbico mais Trolox, *scavengers* de $OH^{\bullet}$, (Halliwell e Gutteridge, 2007), foi capaz de prevenir completamente o aumento dos níveis de TBA-RS em homogeneizados de córtex cerebral e que observamos um aumento no conteúdo de H_2O_2 mediado pelo NAA, pode-se sugerir a participação efetiva de H_2O_2, e possivelmente $OH^{\bullet}$, que é produzido a partir do H_2O_2 através da reação de Fenton, na neurotoxicidade do NAA. Cabe lembrar que previamente a mistura de CAT mais SOD, que detoxificam H_2O_2 e $O_2^{\bullet-}$, respectivamente, não havia sido capaz de prevenir o aumento dos níveis de TBA-RS promovido pelo NAA *in vitro* em córtex cerebral de ratos de 14 dias de vida, e por isso se imaginou que o H_2O_2 e $O_2^{\bullet-}$ provavelmente não estivessem envolvidos nos efeitos do NAA. No entanto, sabe-se agora que o NAA é capaz de inibir fortemente a atividade da CAT, sendo possível que a CAT estivesse então inibida na medida dos níveis de TBA-RS, e por isso não tivesse sido capaz de prevenir o aumento dos níveis de TBA-RS induzido por NAA.

Ainda, considerando que o NAA é capaz de aumentar significativamente o conteúdo de H_2O_2, considerando que o H_2O_2 pode oxidar diretamente grupos –SH (Halliwell e Gutteridge, 2007), e considerando que tanto o ditiotreitol (protetor de grupo –SH) quanto a GSH foram capazes de prevenir apenas parcialmente o aumento dos níveis de TBA-RS induzido por NAA, não devemos excluir a possibilidade de que a oxidação de grupos –SH esteja também envolvida nos efeitos neurotóxicos do NAA.

Em conjunto, nossos resultados indicam que o NAA pode promover estresse oxidativo *in vitro* e *in vivo* em córtex cerebral de ratos de 14 dias de vida, através da diminuição das defesas antioxidantes enzimáticas e não-enzimáticas e da geração de dano oxidativo a lipídios e proteínas, provavelmente pelo aumento de ER no córtex cerebral. Além disso, a administração intracerebroventricular de NAA pode comprometer as defesas antioxidantes também em córtex cerebral de ratos de 30 dias de vida, por reduzir o TRAP e a atividade da CAT, comprometendo assim a eficiência da detoxificação de ER; pode promover dano oxidativo protéico; e também comprometer a produção de NADPH e alterar o equilíbrio redox celular, podendo-se sugerir que o NAA é também capaz de promover estresse oxidativo em córtex cerebral de ratos de 30 dias de vida. Especificamente a redução das defesas antioxidantes enzimáticas, através da inibição das atividades da CAT e GPx, pode de fato comprometer a eficiência da detoxificação de ER, o que pode levar por fim ao dano oxidativo às biomoléculas. Além disso, o NAA pode promover um aumento no conteúdo de H_2O_2 *in vitro* e *in vivo*, o que pode estar envolvido na neurotoxicidade do NAA e, possivelmente, na progressão e manutenção da neurodegeneração característica da Doença de Canavan. Nosso estudo introduz outro possível mecanismo patológico para o dano cerebral observado nos pacientes afetados, sugerindo-se que o estresse oxidativo pode

estar envolvido na neuropatologia da Doença de Canavan, cujo marcador bioquímico é o acúmulo de NAA.

De uma forma geral, observamos nesse trabalho a presença de alterações de parâmetros de estresse oxidativo promovidas pelo NAA muito mais proeminentes no cérebro de ratos de 14 dias de vida do que no cérebro de ratos de 30 dias de vida. Cabe ressaltar que aos 14 dias de vida o cérebro de ratos está ainda em pleno desenvolvimento, estando ainda imaturo, havendo um pico de mielinização, crescimento cerebral e neurogênese em torno dessa idade; já aos 30 dias de vida, há uma redução significativa de todos esses processos (Morgane *et al*., 2002). Sabe-se que mamíferos neonatos podem exibir uma capacidade menor de metabolizar diversas substâncias e xenobióticos, estando portanto mais suscetíveis à toxicidade aguda de certas toxinas (Lotti *et al*., 2002). Além disso, nossos resultados estão de acordo com diversas evidências existentes na literatura que mostram que o cérebro de ratos em desenvolvimento pode ser mais sensível ao estresse oxidativo do que o cérebro de ratos adultos. Khaing e colaboradores (2000) demonstraram que o cérebro imaturo de ratos neonatos responde à infusão cerebral de uma excitotoxina com fragmentação de DNA de forma muito mais proeminente e abundante do que no cérebro de ratos adultos, sugerindo que um insulto excitotóxico neonatal resulta em morte celular mais extensiva do que a provocada por semelhante insulto ao cérebro adulto. Vale lembrar que a excitotoxicidade já foi relacionada ao estresse oxidativo (Pellegrini-Giampietro *et al*., 1990; Lees, 1993). Di Toro e colaboradores (2007) demonstraram que a formação cerebral de ER aumenta significativamente com a idade dos ratos após o nascimento, culminando em aproximadamente 10 dias de vida, decaindo significativamente aos 30 dias de vida e estabilizando aos 90 dias de vida. Baud e colaboradores (2004)

observaram que oligodendrócitos em desenvolvimento são mais vulneráveis ao H_2O_2 e mais sensíveis ao estresse oxidativo do que oligodendrócitos maduros, e que a CAT de oligodendrócitos em desenvolvimento é inativada pela exposição a H_2O_2. Além disso, o H_2O_2 é degradado mais rapidamente e induz a formação de menos ER intracelulares nos oligodendrócitos maduros do que nos imaturos; o *clearance* de H_2O_2 nos oligodendrócitos maduros é expressivamente maior do que nos imaturos, e portanto apresentam uma capacidade antioxidante pelo menos 8 vezes maior do que as células imaturas (Baud *et al*., 2004). Driver e colaboradores (2000) observaram que a produção de ER estimulada por ferro no cérebro de ratos de 14 dias é significativamente maior do que no cérebro de ratos adultos, sugerindo que ratos em desenvolvimento apresentam uma maior sensibilidade à produção cerebral de ER após a exposição a toxinas do que ratos adultos. Essa maior sensibilidade pode estar na verdade relacionada ao *status* antioxidante cerebral. De fato, as enzimas antioxidantes SOD, CAT e GPx são encontradas em baixos níveis no cérebro de ratos neonatos e aumentam com a idade (Mavelli *et al*., 1982; Schreiber *et al*., 1995), sugerindo que ratos neonatos estariam menos protegidos contra um aumento patológico de ER e, por isso, estariam mais suscetíveis ao estresse oxidativo. Dessa forma, pode-se concluir que nossos achados demonstrando uma maior susceptibilidade de ratos nenonatos ao estresse oxidativo está de acordo com as evidências existentes na literatura.

Quanto aos efeitos do NAAG, metabólito do NAA também acumulado nos pacientes afetados pela Doença de Canavan, sobre parâmetros de estresse oxidativo avaliados em nosso estudo, foi possível observar que o NAAG não é capaz de alterar o TRAP nem *in vitro* nem *in vivo*. Observamos também que esse ácido orgânico não é capaz de causar dano oxidativo protéico, à medida que não altera o conteúdo de carbonilas nem *in*

vitro nem *in vivo*. Observamos que a quimiluminescência espontânea e o TBA-RS não são afetados pela administração intracerebroventricular de NAAG, e também não são afetados *in vitro* pelo NAAG presente no meio de incubação, sugerindo que o dano oxidativo aos lipídios não é estimulado por esse ácido orgânico em córtex cerebral de ratos de 30 dias de vida. Assim como já havia sido observado com os demais parâmetros de estresse oxidativo, novamente o NAAG não é capaz de alterar as atividades das enzimas antioxidantes CAT, SOD e GPx, nem a atividade da G6PD. Apesar de Pliss e colaboradores (2003) terem recentemente relatado que a administração intracerebroventricular de NAAG é capaz de induzir clivagem de DNA, pensando-se que esse efeito poderia ter sido mediado por estresse oxidativo, no presente trabalho constatamos que o NAAG não é capaz de induzir estresse oxidativo, já que não alterou nenhum dos parâmetros testados nem *in vitro* nem *in vivo*. No entanto, quebra de fitas também ocorre durante o reparo do DNA e não pode ser atribuída unicamente ao estresse oxidativo (Halliwell e Gutteridge, 2007). Parece possível que os efeitos neurotóxicos exercidos pelo NAAG, observados anteriormente por outros pesquisadores (Pliss *et al*., 2000; Pliss *et al*., 2002; Pliss *et al*., 2003; Bubeníková-Valesová *et al*., 2006), não tenham sido mediados por estresse oxidativo, já que em nosso estudo o NAAG não alterou o *status* antioxidante e não promoveu dano oxidativo a biomoléculas. Além disso, nesses estudos a possibilidade de efeitos sinérgicos entre NAA e NAAG, já que o NAAG pode ser convertido enzimaticamente a NAA, não foi investigada, e não deve ser descartada. Enquanto parece que o NAA pode estar realmente relacionado aos mecanismos responsáveis pelo dano cerebral observado nos pacientes com Doença de Canavan, o papel neuroquímico do NAAG na patogênese dessa doença deve ser melhor elucidado.

Apesar de as concentrações de NAA usadas em nosso trabalho serem similares às observadas no plasma, cérebro e LCR de pacientes afetados pela Doença de Canavan, nos quais se pode observar um aumento nos níveis de NAA de até 4 vezes (Tsai e Coyle, 1995; Blüml, 1999; Surendran *et al.*, 2003), é difícil extrapolar nossos achados para a condição humana. No entanto, esses resultados estão de acordo com Tsai e colaboradores (1998) que estudaram a associação entre estresse oxidativo e discinesia tardia. Foi observado que pacientes com discinesia tardia também apresentam concentrações fortemente aumentadas de NAA no LCR (até 100 mM), da mesma forma que ocorre na Doença de Canavan. Os sintomas da discinesia tardia mostraram correlação positiva com os marcadores de neurotransmissão excitatória e com conteúdo de grupos carbonila (Tsai *et al.*, 1998). Níveis elevados de dienos conjugados e TBA-RS no LCR de pacientes com discinesia tardia já foram também relatados (Pall *et al.*, 1987; Lohr *et al.*, 1990). A hipótese de dano oxidativo nos pacientes com discinesia tardia é reforçada por relatos de que a Vitamina E é capaz de reverter os sintomas dos pacientes afetados por essa condição (Adler *et al.*, 1993; Lohr e Caligiuri, 1996; Zhang *et al.*, 2004). O aumento do conteúdo de carbonilas e dos níveis de produtos de dano oxidativo a lipídios (MDA e dienos conjugados) no LCR indica a ocorrência de proteínas oxidadas e de lipoperoxidação aumentada em pacientes com discinesia tardia, que também apresentam elevações expressivas dos níveis de NAA, e estão de acordo com nossos achados de um aumento de oxidação protéica e de lipoperoxidação promovidos pelo NAA em córtex cerebral de ratos.

Concluindo, nossos resultados mostrando o comprometimento das defesas antioxidantes (enzimáticas e não-enzimáticas) e a geração de dano oxidativo tanto a lipídios quanto a proteínas, sugerem que o NAA é capaz de promover estresse oxidativo

tanto *in vitro* quanto *in vivo* em córtex cerebral de ratos de 14 e 30 dias de vida, apontando para um importante papel do H_2O_2, e provavelmente do $OH^{\bullet}$, na patogênese da Doença de Canavan. Em conjunto, pode-se sugerir que o comprometimento das defesas antioxidantes, o dano oxidativo aos lipídios e proteínas, o aumento do conteúdo de H_2O_2, a oxidação de grupos –SH, o comprometimento na produção de NADPH e alteração no *status* redox celular resultem em estresse oxidativo e estejam de fato envolvidos na neurotoxicidade do NAA. Se esses efeitos também ocorrerem no cérebro de pacientes afetados pela Doença de Canavan, é possível que possam contribuir, juntamente com outros mecanismos, para a disfunção neurológica característica dessa doença. Baseado nesses resultados, propõe-se que a administração de antioxidantes, especialmente das vitaminas E e C, seja considerada como adjuvante na terapêutica dos pacientes afetados pela Doença de Canavan.

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ANEXOS

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Figura 1. Ciclo γ-glutamil. Enzimas envolvidas: ① = γ-glutamilcisteína sintetase; ② = glutationa sintetase; ③ = γ-glutamil transpeptidase; ④ = γ-glutamil ciclotransferase; ⑤ = 5-oxoprolinase; ⑥ = dipeptidase. 5

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