UNIVERSIDADE FEDRERAL DO RIO GRANDE DO SUL

EFEITO *IN VIVO* E *IN VITRO* DA HOMOCISTEÍNA SOBRE FATIAS DE HIPOCAMPO DE RATOS SUBMETIDAS À PRIVAÇÃO DE OXIGÊNIO E GLICOSE

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À minha mãe e ao meu pai

Que perderam noites de sono para que eu dormisse tranqüila

Que deixaram seus sonhos para que eu sonhasse

“Se eu vi mais longe, foi por estar de pé sobre ombros de gigantes”.

Isaac Newton

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RESUMO

A homocistinúria é um erro inato do metabolismo de aminoácidos causado pela deficiência severa na atividade da enzima cistationina β-sintase, o que resulta no acúmulo tecidual de homocisteína e metionina. Pacientes afetados geralmente apresentam aterosclerose, retardo mental, convulsões e isquemia cerebral. Entretanto, os mecanismos fisiopatológicos responsáveis por estas manifestações são pouco conhecidos.

A isquemia cerebral é caracterizada por uma redução grave ou pelo bloqueio completo do fluxo sanguíneo normal em alguma região do cérebro, geralmente causada por um trombo ou uma hemorragia. O estresse oxidativo parece ser um dos principais mecanismos envolvidos no dano celular induzido por isquemia e a administração de antioxidantes, em alguns casos, pode prevenir alguns desses danos.

Considerando que: a) pacientes com homocistinúria apresentam alterações neurológicas e que são mais suscetíveis à isquemia, b) o estresse oxidativo parece estar envolvido na fisiopatogenia tanto da homocistinúria quanto da isquemia cerebral c) o ácido fólico reduz os níveis plasmáticos de homocisteína e pode ter efeitos antioxidantes, e) o pré-tratamento com vitaminas E e C previne os efeitos da Hcy sobre a Na^+, K^+-ATPase e sobre a memória, neste trabalho nós verificamos os efeitos *in vivo* e *in vitro* da homocisteína sobre fatias de hipocampo de ratos submetidas à privação de oxigênio e glicose, um modelo *in vitro* de isquemia cerebral, e também os efeitos do pré-tratamento com antioxidantes, vitamina E mais C e ácido fólico sobre o dano celular causado pela homocisteína.

Os resultados mostraram que a homocisteína *in vitro* (100 e 500 μM), aumentou a liberação de lactato desidrogenase (LDH) para o meio de incubação, sugerindo um aumento no dano celular causado pela isquemia. Além disso, tanto o modelo agudo quanto o crônico de hiperhomocisteinemia aumentou a morte celular quando os animais foram sacrificados 1 hora após a administração de homocisteína.

Nossos resultados também mostraram que o pré-tratamento com ácido fólico foi capaz de prevenir completamente o dano causado pela administração aguda de homocisteína, enquanto que a vitamina E preveniu apenas parte deste efeito. Estes achados podem ser relevantes para explicar, pelo menos em parte, a maior susceptibilidade dos pacientes hiperhomocisteinêmicos de apresentar eventos issquêmicos e apontam uma possível estratégia de prevenção.

ABSTRACT

Homocystinuria is an inherited metabolic disorder caused by severe deficiency of cystationine β-synthase activity, resulting in the tissue accumulation of homocysteine and methionine. Affected patients usually present atherosclerosis, mental retardation, seizures, and stroke. However, the physiopatological mechanisms are not yet fully established.

Cerebral ischemia is defined as a severe reduction or blockage of normal blood flow in the brain, usually caused by thrombosis or hemorrhage. Oxidative stress seem to be one of major mechanisms involved on cellular damage induced by ischemia, and antioxidants administration, sometimes, can prevent this damage.

Considering that: a) homocystinuric patients present neurological alterations and are more susceptible to ischemia, b) oxidative stress is involved on pathogenia such of homocystinuria as of cerebral ischemia, c) folic acid reduces the serum levels of homocisteine and could have antioxidant effects, c) pretreatment with vitamins E and C prevent the effects of homocysteine on Na^+, K^+-ATPase activity and on memory, in this work we verified the *in vivo* and *in vitro* effects of homocysteine on rat hippocampal slices exposed to oxygen and glucose deprivation, an *in vitro* model of cerebral ischemia, we also investigated the effects of pretreatment with antioxidants, vitamin E plus C and folic acid on cellular damage caused by homocysteine.

Results showed that homocysteine *in vitro* (100 and 500μM), both chronic (1 h after homocysteine administration) and acute hyperhomocysteinemia increased the LDH release to the incubation medium, suggesting an increase of tissue damage caused by ischemia. Furthermore, results showed that both chronic (1 h after homocysteine administration) and acute hyperhomocysteinemia increased the cellular death.

Our results also demonstrated that pretreatment with folic acid completely prevented the damage caused by acute homocysteine administration, whereas vitamin E just partially prevented such effect. These findings may be relevant to explain, at least in part, the higher susceptibility of hyperhomocysteinemic patients to bear ischemic events and point to a possible preventive treatment.

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LISTA DE ABREVIATURAS

CBS – Cistationina β-sintase

EIM – Erros Inatos do Metabolismo

Hcy – Homocisteína

HCU – Homocistinúria

LDH – Lactato desidrogenase

LDL – Lipoproteína de baixa densidade

NMDA – N-metil-D-aspartato

POG – Privação de oxigênio e glicose

SAM – S-adenosil-metionina

SNC – Sistema nervoso central

1. INTRODUÇÃO

1.1 Erros inatos do metabolismo

O termo erros inatos do metabolismo (EIM) foi introduzido na literatura médica em 1902 pelo inglês Sir Archibald E. Garrod, baseado em suas investigações sobre consangüinidade e distribuição de casos de alcaptonúria em famílias (Garrod, 1902). Hoje, o termo é usado para designar cerca de 500 defeitos genéticos, a maioria deles envolvendo processos de síntese, degradação, transporte e armazenamento de moléculas no organismo (Scriver et al., 2002).

Dessa forma, os EIM são alterações genéticas que se manifestam pela síntese de uma proteína anômala, geralmente uma enzima, ou por uma diminuição ou mesmo ausência de sua síntese. Como conseqüência pode ocorrer o acúmulo de substâncias que estão presentes em pequena quantidade em indivíduos normais, a deficiência de produtos intermediários críticos e a deficiência de produtos finais específicos ou ainda o excesso nocivo de produtos de vias metabólicas acessórias (Bickel, 1987).

Embora individualmente raras, essas doenças afetam entre 1% e 2% da população, constituindo-se um importante problema de saúde pública (Baric et al., 2001).

1.2 Homocistinúria

A homocistinúria (HCU) é um erro inato do metabolismo de aminoácidos causado pela deficiência severa na atividade da enzima cistationina β-sintase (CBS), o que resulta no acúmulo tecidual principalmente de homocisteína (Hcy). A herança é autossômica recessiva e a freqüência varia de população para população, sendo que a incidência mundial é estimada em 1 caso para 200.000 nascimentos vivos (Mudd et al., 2001).

Nos pacientes afetados, os níveis plasmáticos de Hcy podem chegar a 500μmol/L, enquanto que em indivíduos normais estes níveis estão entre 5 e 10 μmol/L (Mudd et al., 2001). No líquor os níveis de Hcy podem aumentar cerca de 10 vezes (Fowler e Jakobs, 1998).

As manifestações clínicas da homocistinúria incluem trombose e doença aterosclerótica vascular prematuras, lesões oculares, malformações do esqueleto e desordens do sistema nervoso central (SNC), como retardo mental, distúrbios psiquiátricos e cognitivos, convulsões e isquemia cerebral e cardíaca (Mudd et al., 2001). Embora as alterações vasculares apresentadas pelos pacientes sejam bem caracterizadas, os demais mecanismos fisiopatológicos da homocistinúria ainda são pouco conhecidos (De Franchis et al., 1998).

O primeiro achado clínico encontrado nos pacientes homocistinúricos é geralmente, o retardo mental. Miopia e deslocamento da lente ocular, além de alterações vasculares e anormalidades esqueléticas também são observados nesses pacientes (De Franchis et al., 1998). Entretanto, o diagnóstico definitivo é

baseado na presença das alterações bioquímicas características (Mudd et al., 2001).

Os achados laboratoriais consistem na presença de elevadas concentrações de Hcy na urina e no plasma dos pacientes. Também pode ocorrer uma diminuição de cisteína e um aumento de metionina no plasma de pacientes homocistinúricos (Fowler e Jakobs, 1998).

Para confirmação do diagnóstico, utiliza-se o ensaio enzimático direto da atividade da enzima CBS que pode ser realizado em biópsia de fígado, cultura de fibroblastos e/ou linfócitos (Uhlendorf e Mudd, 1968; Goldstein et al., 1972; Mudd et al., 2001).

Embora ainda não exista um tratamento eficaz para grande parte dos EIM, a maioria busca aumentar a atividade residual da enzima deficiente, diminuir o fluxo ao longo da via afetada, suplementar produtos deficientes e utilizar vias alternativas para remover substratos tóxicos ou metabólitos acumulados (Walter et al., 1998). No caso da HCU, além da dieta com restrição de metionina, a administração de vitamina B_{12} e ácido fólico tem sido estudada como forma de estimular a via alternativa de remetilação da Hcy e diminuir os níveis plasmáticos desse aminoácido (Homocysteine Studies Collaboration, 2002).

Modelos farmacológicos e genéticos de hiper-homocisteinemia têm sido desenvolvidos com o objetivo de elucidar os mecanismos pelos quais a Hcy promove neurotoxicidade e as disfunções cerebrovasculares observadas nos pacientes afetados, abrindo perspectivas de tratamento (Troen, 2005). Neste contexto, nosso laboratório desenvolveu um modelo químico experimental de

HCU, onde os animais apresentam níveis plasmáticos de Hcy similares àqueles dos pacientes homocistinúricos (Streck et al., 2002).

1.3 Homocisteína

A homocisteína é um aminoácido sulfurado, formado durante o metabolismo da metionina, que é proveniente da dieta ou da degradação de proteínas endógenas. O aminoácido está na intersecção de duas vias metabólicas: remetilação e transsulfuração. Na via de remetilação, a Hcy adquire um grupo metil do 5-metil-tetrahidrofolato, para formar metionina em uma reação catalisada pela enzima metionina sintase, a qual é dependente de vitamina B_{12} e folato (Finkelstein, 1998; Troen, 2005).

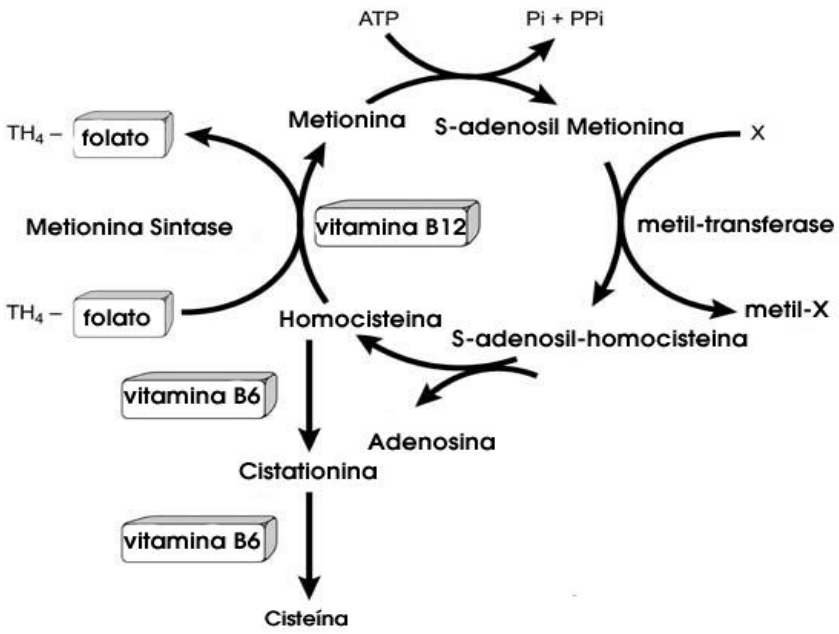

Figura 1. Metabolismo da homocisteína.

Na via de transsulfuração, Hcy se condensa com a serina para formar cistationina, em uma reação irreversível catalisada pela enzima cistationina β-sintase (CBS), que utiliza vitamina B6 como cofator. A cistationina é subseqüentemente hidrolizada para cisteína, a qual pode voltar a ser incorporada em glutationa ou adiante ser metabolizada a sulfato e excretada na urina. Esta via normalmente cataboliza o excesso de Hcy que não é requerido para as reações de metilação (Finkelstein, 1998; Troen, 2005).

Em indivíduos normais a concentração de Hcy no plasma fica entre 5 e 10 μmol/L, sendo que mais de 97% está na forma oxidada. Define-se como hiper-homocisteinemia leve níveis inferiores a 16 μmol/L, como moderada, entre 16 e 30 μmol/L, níveis intermediários estão entre 31 e 100 μmol/L e severa quando as concentrações plasmáticas são superiores a 100 μmol/L (Perna et al., 2003).

Diversos estudos têm demonstrado que a hiper-homocisteinemia é um fator de risco independente para doenças cardiovasculares e acidente vascular cerebral (Perna et al., 2004). Recentemente demonstrou-se que uma diminuição de 25% nos níveis de Hcy diminuiu em 11% e 19% o risco de indivíduos desenvolverem isquemia cardíaca e cerebral, respectivamente (Homocysteine Studies Collaboration, 2002). Além disso, elevada concentração de Hcy está relacionada ao déficit cognitivo, demência, doença de Alzheimer, depressão e esquizofrenia (Korczyn, 2002; McCaddon, 2002; Reynolds, 2002; Mattson e Shea, 2003).

Níveis elevados de Hcy produzem modificações na estrutura e função dos vasos sanguíneos. Ela tem ação tóxica diretamente sobre as células endoteliais ou através da produção de óxido nítrico (ON), sendo que tais efeitos podem

prejudicar a vasodilatação (Faraci e Lentz, 2004). Além disso, a Hcy induz um estado pró-coagulante, devido ao aumento na produção de tromboxano, da agregação plaquetária, ativação de fator XII e inibição do ativador de proteína C (Perna et al., 2003; Thambyrajah e Townend, 2000). A formação de moléculas adesivas e a estimulação da proliferação de células musculares lisas na parede vascular também têm sido relatadas (Hassan et al., 2004).

No SNC, a Hcy produz excitotoxicidade através da estimulação de receptores NMDA e do conseqüente influxo de cálcio (Kim e Pae, 1996; Lipton et al., 1997; Kruman et al., 2000). Estudos também têm demonstrado que a Hcy potencializa os efeitos excitotóxicos do glutamato (Kruman et al., 2000) e do peptídeo β-amilóide (Ho et al., 2001; White et al., 2001). Além disso, a Hcy parece provocar dano ao DNA, induzindo apoptose (Kruman et al., 2000). Ho e colaboradores demonstraram que a Hcy, em cultura de células, aumenta a concentração citosólica de Ca^{2+}, a produção de radicais livres e induz apoptose.

Inúmeros estudos têm correlacionado níveis elevados de Hcy com a indução de estresse oxidativo. Neste contexto, trabalhos mostram que concentrações elevadas de Hcy aumentam a produção do ânion superóxido que pode reagir com ON e formar peroxinitrito (Perna et al., 2003; Faraci e Lentz, 2004). Pacientes com homocistinúria geralmente apresentam um aumento no nível dos produtos finais da lipoperoxidação, como malondialdeído e F_2 isoprostanos (Ullegaddi et al., 2004, Racek et al., 2005) e têm maior susceptibilidade à oxidação de LDL (Racek et al., 2005). Além disso, a Hcy induz o estresse oxidativo *in vivo* como resultado da diminuição da concentração

plasmática de cisteína, que leva a uma redução dos nos níveis de glutationa (Mudd et al., 1985). Recentes trabalhos publicados mostram que a Hcy *in vitro* aumenta a peroxidação de lipídeos e diminui a capacidade antioxidante tecidual em hipocampo e córtex parietal de ratos (Streck et al., 2003b; Matté et al., 2004).

1.4 Ácido Fólico

O ácido fólico é uma vitamina hidrossolúvel que promove a remetilação da Hcy, ele doa o grupo metil para a conversão de metionina em S-adenosilmetionina (SAM), que é o principal doador de grupamentos metil para muitas reações de transmetilação (Mattson e Shea, 2003).

A deficiência de folato leva à depleção dos níveis de SAM e à diminuição das reações de metilação de citosina no DNA. Dessa forma, há um prejuízo da transcrição do DNA resultando em mutações ou causando apoptose (Mattson et al., 2002). Níveis baixos de folato também podem causar efeitos deletérios para a célula por permitir a acumulação de Hcy, já que os níveis plasmáticos desse aminoácido e de ácido fólico são inversamente proporcionais. Estudos epidemiológicos recentes têm relacionado a deficiência de folato e a resultante elevação nos níveis plasmáticos de Hcy com aumento no risco de doenças cardiovasculares, isquemia cerebral (Hankey e Eikelboom, 2001; Faraci e Lentz, 2004; He et al., 2004) e desordens neurodegenerativas e neuropsiquiátricas, incluindo a doença de Alzheimer, depressão e esquizofrenia (Korczyn, 2002; McCaddon, 2002; Reynolds, 2002; Mattson e Shea, 2003). Estudos também têm demonstrado que a suplementação com ácido fólico é eficiente em reduzir os

níveis plasmáticos de Hcy, e conseqüentemente pode reduzir o risco dessas doenças (Mattson e Shea, 2003; Assanelli et al., 2004; Ullegaddi et al., 2004).

Evidências mostram que o ácido fólico apresenta propriedades antioxidantes, independente de sua ação sobre o metabolismo da Hcy. Por outro lado, a administração crônica de folato aumenta os níveis plasmáticos do antioxidante glutationa e diminui os níveis de malondialdeído em pacientes com hiperhomocisteinemia (Racek et al., 2005). Além disso, o folato parece ter um efeito antioxidante direto sobre a oxidação de LDL *in vitro* (Nakano et al., 2001) e estudos mostram que essa vitamina melhora a função endotelial de pacientes com doença arterial coronária mesmo sem diminuir a concentração de Hcy (Doshi et al., 2002).

1.5 Vitamina E

A vitamina E ou α-tocoferol é uma molécula lipossolúvel, que tende a se concentrar no interior das membranas biológicas, nas lipoproteínas do sangue e nas glândulas adrenais (Wang e Quinn, 1999). Sua principal função *in vivo* é bloquear a reação em cadeia da lipoperoxidação, reagindo com os radicais peroxil e alcoxil. O radical tocoferoxil formado não é suficientemente reativo para retirar o H° dos lipídeos de membrana porque o elétron desemparelhado no átomo de oxigênio pode sofrer ressonância dentro do anel aromático do α-tocoferol (Wang e Quinn, 1999). A molécula de α-tocoferol pode ser regenerada através de redução por vitamina C ou ubiquinol-10 (Tucker e Townsend, 2005). Esta regeneração

aumenta a capacidade antioxidante da vitamina E, e dessa forma, vários estudos clínicos têm utilizado as duas vitaminas concomitantemente.

A deficiência de vitamina E em humanos, causada pela má absorção de lipídeos ou por anormalidades genéticas, resulta em neuropatia periférica e ataxia (Wang e Quinn, 1999). Baixos níveis desse antioxidante estão relacionados a distúrbios de lipoperoxidação em ratos e podem influenciar as atividades das enzimas superóxido dismutase, catalase e glutationa peroxidase (Gilgun-Sherki, 2002).

A formação de radicais livres e o estresse oxidativo têm sido relacionados à fisiopatogenia de muitas doenças crônicas incluindo câncer, aterosclerose, diabetes, doença de Alzheimer, e outras doenças neurodegenerativas (Tucker e Townsend, 2005). Além disso, o estresse oxidativo também parece exercer um importante papel no dano cerebral causado por outras doenças como, por exemplo, isquemia cerebral. Estudos mostram que a capacidade antioxidante medida no plasma de pacientes isquêmicos é inversamente proporcional ao prejuízo neurológico posterior ao acidente vascular cerebral (Cherubini et al., 2000). Dessa forma, o emprego de antioxidantes, entre eles as vitaminas E e C, tem sido amplamente estudado com o objetivo de prevenir ou atenuar os danos decorrentes da formação de radicais livres em várias doenças que afetam o SNC.

Neste contexto, pesquisadores têm demonstrado que a administração de vitamina E exerce efeitos protetores contra eventos isquêmicos em ratos (Van der Worp et al., 1998; Chaudhary et al., 2003; Zhang et al., 2004). Em modelos experimentais de doença de Alzheimer a vitamina E reduz a neurotoxicidade do peptídeo β-amilóide (Bell e Moosmann, 2002). Além disso, o pré-tratamento com

as vitaminas E e C previne a inibição das atividades da Na^+, K^+, ATPase (Wyse et al., 2002) e butirilcolinesterase (Stefanello et al., 2005) e o déficit de memória causados pela hiper-homocisteinemia em ratos (Reis et al., 2002).

1.6 Isquemia Cerebral

A isquemia cerebral é caracterizada por uma redução grave ou pelo bloqueio completo do fluxo sanguíneo normal em alguma região do cérebro, geralmente causada por um trombo ou uma hemorragia (Lipton, 1999). Em roedores, assim como em humanos, esse bloqueio causa morte celular principalmente em neurônios localizados na região CA_1 do hipocampo (Lipton, 1999). A isquemia constitui uma das principais causas de mortalidade e invalidez entre adultos e idosos ocidentais (Cherubini et al., 2000).

Durante o bloqueio do fluxo sanguíneo normal, o cérebro é capaz de manter os níveis de ATP por apenas 1 minuto (White et al., 2000). Os exatos mecanismos responsáveis pelo dano celular decorrente da isquemia ainda são pouco conhecidos, porém vários eventos bioquímicos parecem estar envolvidos, tais como depleção de energia, acidose celular, quebra dos gradientes iônicos, influxo de Ca^{2+} e ativação de fosfolipases e proteases (White et al., 2000). Muitos destes fenômenos são acompanhados pela formação de espécies reativas de oxigênio e nitrogênio. Assim, o estresse oxidativo parece estar diretamente envolvido com o dano celular induzido por isquemia. O aumento dos produtos da lipoperoxidação e a diminuição nos níveis teciduais de antioxidantes têm sido reportados como

evidências indiretas da indução de estresse oxidativo durante os eventos de isquemia e reperfusão (El Kossi e Zakhary, 2000; Warner et al., 2004).

Vários modelos animais têm sido desenvolvidos e aplicados no intuído de compreender os mecanismos responsáveis pelo dano neurológico causado pela isquemia cerebral e testar possíveis estratégias de neuroproteção. Um desses modelos, que têm sido utilizado com sucesso é o modelo de privação de oxigênio e glicose (POG) *in vitro* (Schurr et al., 1995; Taylor et al., 1995; Siqueira et al., 2004; Fontella et al., 2005). Tal modelo oferece importantes vantagens, já que a composição celular, assim como os neurônios funcionais, as células inflamatórias competentes, os efetores localmente liberados e as conexões intercelulares são preservados (Taylor et al., 1995).

2. OBJETIVOS

Considerando que: a) pacientes com homocistinúria apresentam alterações neurológicas e que são mais suscetíveis à isquemia, b) o estresse oxidativo parece estar envolvido nos danos neurológicos decorrentes da isquemia/reperfusão, c) a homocisteína *in vivo e in vitro* aumenta o estresse oxidativo, d) a suplementação com ácido fólico reduz os níveis plasmáticos de Hcy e pode ter efeitos antioxidantes, e) o pré-tratamento com antioxidantes (vitaminas E e C) previne os efeitos da Hcy sobre as atividades das enzimas Na^+, K^+-ATPase e butirilcolinesterase, bem como o déficit de memória em ratos, f) os mecanismos pelos quais a Hcy exerce seus efeitos neurotóxicos ainda não foram completamente estabelecidos, os objetivos do presente trabalho foram:

1. Avaliar o efeito *in vitro* da homocisteína sobre a POG em fatias de hipocampo de ratos.

2. Verificar o efeito da administração aguda e crônica de Hcy sobre a POG em fatias de hipocampo de ratos.

3. Avaliar o efeito do pré-tratamento com o ácido fólico ou com as vitaminas E mais C sobre o dano celular causado pela homocisteína.

3. ARTIGOS

Homocysteine increases neuronal damage in hippocampal slices receiving oxygen and glucose deprivation

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Metabolic Brain Disease (Submetido)

Homocysteine increases the tecidual damage in hippocampal slices: studies in model of *in vitro* ischemia.

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Abstract

Homocystinuria is an inherited metabolic disorder caused by severe deficiency of cystationine β-synthase activity, resulting in the tissue accumulation of homocysteine (Hcy). Affected patients usually present many signs and symptoms such as seizures, mental retardation, atherosclerosis and stroke. The aim of this study is to evaluate the *in vivo* and *in vitro* effects of Hcy using hippocampal slices from Wistar rats exposed to oxygen and glucose deprivation (OGD, followed by reoxygenation), an *in vitro* model of hypoxic–ischemic events. Neural cell injury was quantified by the measurement of lactate dehydrogenase (LDH) released from damaged cells into the extracellular fluid. The results showed that Hcy *in vivo* and *in vitro* increased the LDH released to de incubation medium, suggesting an increase of tissue damage caused by OGD. This fact can be related with the high incidence of stroke in homocystinuric patients.

Key words: Homocysteine; Homocystinuria; Metabolic Disease; Cerebral Ischemia.

Introduction

Ischemia is defined as a severe reduction or blockage of blood flow and is a pathophysiological event that causes cerebral damage (Fontella *et al.,* 2005). Global brain ischemia in rodents, as well as in humans, causes delayed cell death in neurons mainly located in the CA_1 region of the hippocampus. The pathogenesis of cerebral ischemia/reperfusion has been associated with depletion of cellular energy sources, release of excitatory amino acids, mitochondrial dysfunction and excessive generation of free radicals (White *et al.,* 2000).

Tissue accumulation of homocysteine (Hcy) occurs in homocystinuria, an inherited metabolic disorder caused by severe deficiency of cystationine β-synthase (CBS, EC 4.2.1.22) activity. Affected patients usually present mental retardation, seizures and stroke (Kraus, 1998; Mudd *et al.,* 2001). Hyperhomocysteinemia is a well-known risk factor for cerebrovascular lesions in adult (Faraci and Lentz, 2004) and children (Van Beynum *et al.,* 1999). It has been known that high concentration of Hcy produces changes in the structure and function of cerebral blood vessels, increases oxidation of low-density lipoprotein, stimulates smooth muscle cell proliferation and promotes prothrombotic effects and thrombolysis (Faraci and Lentz, 2004; Schwammenthal and Tanne, 2004). Furthermore, we have recently showed that Hcy induces oxidative stress (Streck *et al.,* 2003), reduces Na^+, K^+, ATPase activity (Streck *et al.,* 2002) and energy metabolism in rat hippocampus (Siqueira *et al.,* 2004) and impairs memory in rats (Streck *et al.,* 2004).

The oxygen and glucose deprivation (OGD) in slices is an *in vitro* model of ischemia that has been used to investigate mechanisms of cell death and neuroprotection (Fontella *et al.,* 2005; Siqueira *et al.,* 2004). It offers important advantages because cell composition, such as functional neurons, inflammatory competent cells, locally released effectors and intercellular connections are preserved (Taylor *et al.,* 1995).

Considering that homocystinuric patients are susceptible to cerebral ischemia and that the mechanisms responsible for such effects are poorly known, in the present study we investigated the *in vivo* and *in vitro* effects of Hcy using

hippocampal slices from Wistar rats exposed to oxygen and glucose deprivation (OGD, followed by reoxygenation), an *in vitro* model of hypoxic–ischemic events (Cárdenas *et al.,* 2000). Our hypothesis is that Hcy will possibly increase susceptibility to ischemic injury.

Material and Methods

Animals and Reagents

Male Wistar rats aged 60 days obtained from the Central Animal House of the Department of Biochemistry, ICBS, Federal University of Rio Grande do Sul, Porto Alegre, RS, Brazil, were housed in groups of eight with their mothers on the day of birth. Animals were maintained on a 12:12 h light/dark cycle (lights on 7:00–19:00 h) in an air-conditioned constant-temperature (22 ± 1ºC) colony room, with free access to water and a 20% (wt.) protein commercial. Animal care followed the official governmental guidelines in compliance with the Federation of Brazilian Societies for Experimental Biology and was approved by the Ethics Committee of the Federal University of Rio Grande do Sul, Brazil. All chemicals were purchased from Sigma Chemical Co., St Louis, MO, USA.

In vitro *studies*

For the *in vitro* studies rats were decapitated and the hippocampi were quickly dissected out and transverse sections (400 μm) were prepared using a

McIlwain tissue chopper. Hippocampal slices were divided into two equal sets (control and OGD), placed into separate 24-well culture plates, and preincubated for 30 min in a modified Krebs-Henseleit solution (preincubation solution): 120 mM NaCl, 2 mM KCl, 0.5 mM $CaCl_2$, 26 mM $NaHCO_3$, 10 mM $MgSO_4$, 1.18 mM KH_2PO_4, 11 mM glucose, in a tissue culture incubator at 37º C with 95% O_2 /5% CO_2 (Cimarosti *et al.,* 2001) in the presence or absence of Hcy (final concentration 10 to 500 μM) (Streck *et al.,* 2003).

Acute treatment

For acute treatment, rats received one single subcutaneous injection of Hcy (0.6 μmol/ g body weight) (Streck *et al.,* 2002) and control animals received an equivalent volume of saline. The rats were killed 1 h after injection and the hippocampi were immediately isolated. The slices were preincubated for 15 min in a modified Krebs-Henseleit solution as described above. After preincubation, the medium in the control plate was replaced with another modified Krebs-Henseleit solution (KHS incubation solution, pH 7.4): 120 mM NaCl, 2 mM KCl, 2 mM $CaCl_2$, 2.6 mM $NaHCO_3$, 1.19 mM $MgSO_4$, 1.18 mM KH_2PO_4, 11 mM glucose, and incubated for 45 min (OGD period) in a tissue culture incubator at 37ºC with 95% O_2 /5% CO_2. After 45 minutes, the control medium was replaced by a fresh one and slices incubated for another 3 hours (recovery period) in the same conditions.

Oxygen and glucose deprivation

To model ischemic conditions, after preincubation OGD slices were washed twice with a KHS medium without glucose saturated with N_2 and incubated for 45 min (OGD period) at 37 ºC in an anaerobic chamber saturated with nitrogen, as fully detailed elsewhere (Cárdenas *et al.,* 2000; Cimarosti *et al.,* 2001). After 45 min of incubation, the medium from both control and OGD slices was removed and the two groups received KHS with glucose. Then, the slices were incubated for 3 h (recovery period) in the culture incubator at 37ºC with 95% O_2 /5% CO_2. Control and OGD experiments were run concomitantly using four slices of the same animal in each plate.

Assessment of neural injury-LDH assay

Cellular damage was quantified by measuring lactate dehydrogenase (LDH) released into the medium (Koh and Choi, 1987). After the recovery period, LDH activity was determined using a kit (Doles Reagents, Goiânia, Brazil). Each experiment was normalized by subtracting the background levels of LDH produced from the non-treatment slices (Almli *et al.,* 2001).

Statistical analysis

Data were analyzed by one-way ANOVA followed by the Duncan Multiple range test when the *F* test was significant. All analyses were performed using the

Statistical Package for the social Sciences (SPSS) software. Differences were considered statistically significant if $p<0.05$.

Results

Figure 1 shows the effects of preincubation with Hcy on the model of ischemia *in vitro*. The OGD exposure and reoxygenation caused an increase of LDH release into the incubation media, a marker of tissue damage, in all groups studied, as compared to control slices (F (7,40)=9.55, $p<0.05$). Post hoc analysis showed that Hcy 500 μM provokes an increase in the cell death on the non-OGD group, but the highest level of LDH released was found in the ischemic-Hcy (500 μM) group.

In the figure 2 we can see that acute administration of Hcy followed by the exposition of the tissue to OGD condition lead to an increase of cell loss. Oxygen glucose deprivation exposure and reoxygenation caused an increase of LDH released into the incubation media and acute administration of Hcy significantly increased such release in OGD-group (F (3,12)=32.38, $p<0.05$).

Discussion

ATP produced by aerobic metabolism is the major source of energy in the brain, and it may be compromised by the interruption of oxygen and substrate delivery and disturbances in cerebral metabolism, such as the condition resulting

from ischemia (White *et al.,* 2000). The exposure of hippocampal slices to an *in vitro* model of hypoxia–ischemia, followed by reoxygenation, resulted in increased LDH in the medium, which is a consequence of cell damage or death.

In the present work, the measurement of LDH released into the medium indicates that Hcy *in vivo* and *in vitro* increase the effect of OGD on this parameter. This result may be interpreted as an increased vulnerability of these cells to ischemia, induced by Hcy.

Our results are in agreement with others studies suggesting that Hcy may sensitize the brain to a variety of insults, like ischemic brain injury *in vivo* (Endres *et al.,* 2005) and neurodegenerative disorders (Mattson and Shea, 2003; Duan *et al.,* 2002).

The model of oxygen and glucose deprivation in slices offers important advantages because cell composition, such as functional neurons, inflammatory competent cells, locally releases effectors and intercellular connections are preserved (Taylor *et al.,* 1995).

Faraci and Lentz (2004) have been demonstrated that in an animal model of homocistinúria, Hcy exert multiple effects within blood vessels, include increase formation of reactive oxygen species, reduced bioavailability of NO, inflammation, hypertrophy of vascular muscle and changes in DNA methylation. On the other hand, Hcy can induce a procoagulative state due to action on the coagulation cascade and fibrinolysis, thus directly inducing or acting in a synergistic manner with other factors in determining the appearance of atherosclerosis (Perna et al., 2003; Thambyrajah and Townend, 2000). However, in this model the vascular

epithelium is absent, so the effects of Hcy observed in our experiments are not consequence of vascular damage and thrombosis.

On the other hand, the same mechanisms suggested to be involved in neuronal damage caused by Hcy, e.g. inhibition of Na^+, K^+, ATPase (Streck *et al.,* 2002), impairment of energy metabolism (Streck *et al.,* 2003a) and reactive oxygen species production (Streck *et al.,* 2003b) participate in the neuronal damage and death that is caused in pyramidal neurons by ischemia (Lipton, 1999; Warner and Sheng, 2004; Wyse *et al.,* 2000). This can be one explanation for the fact that Hcy increase ischemic damage, in our study, since a combination of two insults is usually more devastating than either of them alone.

In spite that in this model of ischemic injury we cannot access long-term alterations in response to OGD insult, it has been used to investigate the effect on vulnerability of brain tissue to ischemic injury of many drugs and agents. As we demonstrate in our experiments, it was possible to establish differences in brain tissue response to OGD condition between Hcy-treated and control animals. Studies of the effects of Hcy using *in vivo* ischemic models would bring interesting information concerning long-term effects, as well as the investigation of the mechanism involved in the effect of Hcy on cell signaling pathway associated with cell death or survival. Anyway, this finding may be relevant for the ischemia-induced pathophysiological squeals *in vivo*, and the effects of the high level of homocysteine to which the patient was possible submitted previously to the ischemic event should be considered.

Acknowledgments

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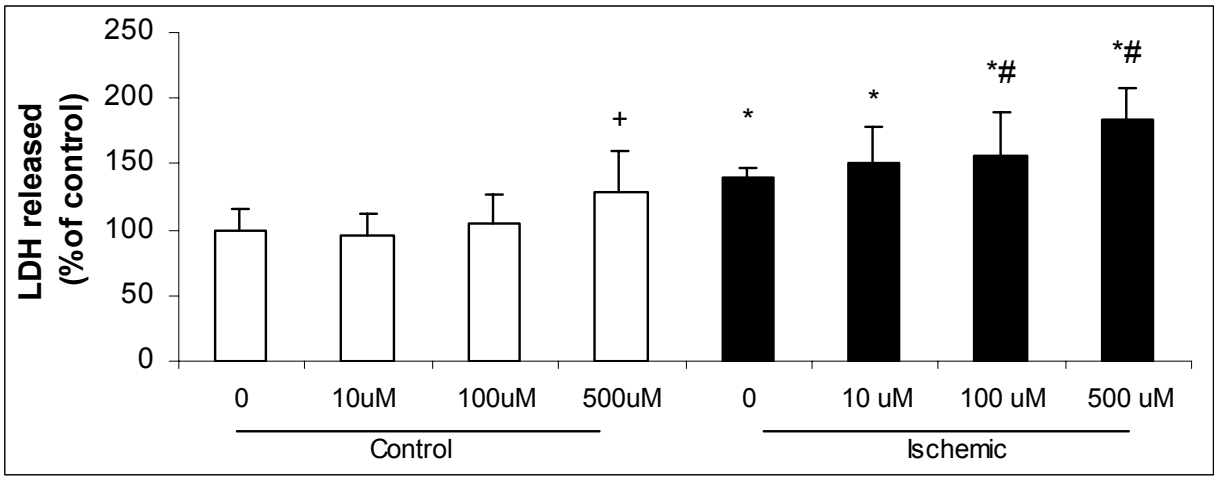
Fig. 1 Effects of preincubation with Hcy (10-500 μM) on cell death, as assessed by LDH released to the medium, of rat hippocampal slices exposed or not - control group to oxygen and glucose deprivation (OGD) for 45 min and reoxygenation for 3h - the ischemic groups. Results are expressed as percentage of the saline non-OGD group. Columns represent mean ± S.D. for 4 experiments performed in triplicate. + Value significantly different from significantly different from Hcy 0 non-OGD, * Values significantly different from those of non-OGD groups, # values significantly different from those Hcy 0 and Hcy 10 μM OGD, as determined by one-way ANOVA followed by Duncan's test ($p < 0.05$).

Fig. 2 In vivo effects of Hcy (acute s.c. injection of 0.6 μmol/g body weight) on cell death, as assessed by LDH released to the medium, of rat hippocampal slices exposed or not - control group to oxygen and glucose deprivation (OGD) for 45 min and reoxygenation for 3h - the ischemic groups. Results are expressed as percentage of the saline non-OGD group. Columns represent mean ± S.D. for 4 experiments performed in triplicate * Values significantly different from those of non-OGD groups, # value significantly different from those saline OGD, as determined by one-way ANOVA followed by Duncan's test ($p < 0.05$).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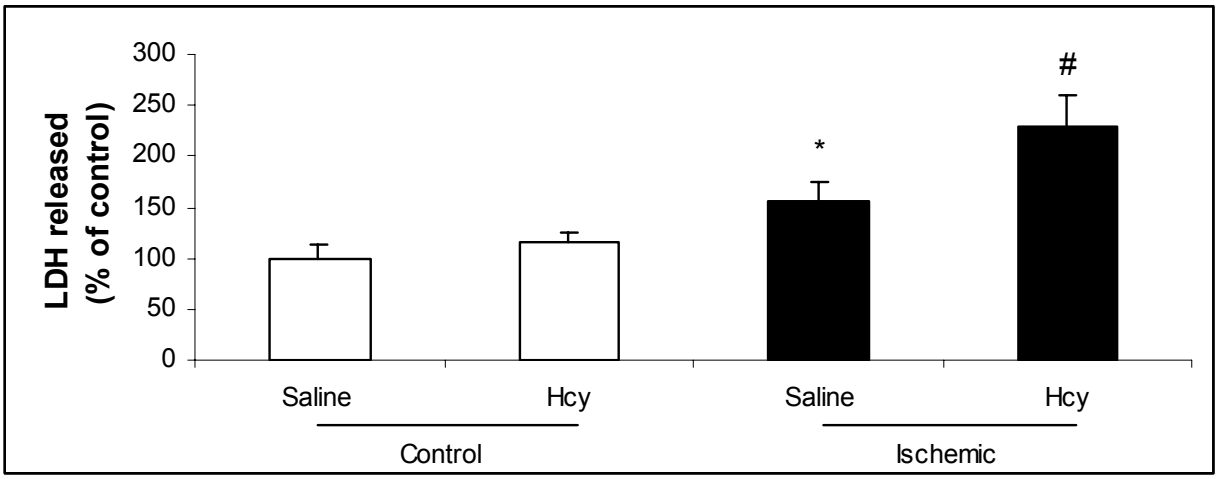
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Hyperhomocysteinemia increases damage on brain slices exposed to *in vitro* model of oxygen and glucose deprivation: prevention by folic acid

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Hyperhomocisteinemia provokes damage on hippocampal slices exposed to *in vitro* model of oxygen and glucose deprivation: prevention by folic acid.

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Abstract

In the present study we evaluate the effects of homocysteine on cellular damage using hippocampal slices from Wistar rats exposed to oxygen and glucose deprivation (OGD, followed by reoxygenation), an *in vitro* model of hypoxic–ischemic events. For chronic treatment, we induced elevated levels of homocysteine in blood (500 μM), comparable to those of human homocystinuria, and in brain (60 nmol/g wet tissue) of young rats by injecting subcutaneously homocysteine (0.3-0.6 μmol/g of body weight) twice a day at 8 h intervals from the 6^{th} to the 28^{th} postpartum day and controls received saline. Rats were sacrificed 1, 3 or 12 h after the last injection. For acute treatment, 29-days old rats received one single injection of homocysteine (0.6 μmol homocysteine/g body weight) or saline and were sacrificed 1 h later. In other set of experiments rats were pretreated with vitamins E (40 mg/Kg) and C (100mg/Kg) or folic acid (5mg/Kg) during one week; 12 h after the last administration they received a single injection of homocysteine or saline and were sacrificed 1 h later. Results showed that chronic (1 h after homocysteine administration) and acute hyperhomocysteinemia increased the cellular damage measured by LDH released to de incubation medium, suggesting an increase of tissue damage caused by OGD. Pretreatment with folic acid completely prevented the damage caused by acute hyperhomocysteinemia, whereas vitamin E just partially prevented such effect. These findings may be relevant to characterization of the sequels presented in patients subject to hyperhomocysteinemia and can suggest a possible prevention strategy.

Key words: Inborn Errors of Metabolism; Homocysteine; Cerebral Ischemia; Vitamin E; Folic Acid; Rat

1. Introduction

Ischemia is defined as a severe reduction or blockage of blood flow and is a pathophysiological event that causes cerebral damage (Lipton, 1999). Global brain ischemia in rodents, as well as in humans, causes delayed cell death in neurons mainly located in the CA_1 region of the hippocampus. Although the precise mechanism responsible for ischemic brain damage is still unclear, a number of interconnecting biochemical events appear to be involved, such as energy depletion, cellular acidosis, ion homeostasis breakdown, influx of Ca^{2+} and activation of phospholipases and proteases (Lipton, 1999). Most of these events are accompanied by generation of oxygen free radicals, thus oxidative stress is an important mechanism of brain injury (Warner et al., 2004; El Kossi and Zakhary, 2000).

Tissue accumulation of homocysteine (Hcy) is found in homocystinuria, an inherited metabolic disorder caused by severe deficiency of cystationine β-synthase (CBS, EC 4.2.1.22) activity. Affected patients usually present mental retardation, seizures and stroke (Mudd et al., 2001), whose pathogenia is not well known. However, it has been known that elevated Hcy levels produces changes in the structure and function of cerebral blood vessels, increases oxidation of low-density lipoprotein, stimulates smooth muscle cell proliferation and promotes prothrombotic effects and thrombolysis (Faraci and Lentz, 2004; Hassan et al., 2004; Van Beynum et al., 1999). Furthermore, by using the chemically induced model of homocystinuria developed in our laboratory, we showed that hyperhomocisteinemia reduces Na^+, K^+, ATPase activity (Streck et al., 2002) and energy metabolism in hippocampus (Streck et al., 2003a) and impairs memory in rats (Streck et al., 2004). On the other hand, Hcy induces oxidative stress *in vitro* (Streck et al., 2003b).

Folate (5'-methyltetrahydrofolate) is a water-soluble vitamin that promotes the remethylation of homocysteine. It provides the methyl group for the conversion

of methionine to *S*-adenosylmethionine (SAM), the major methyl donor for most methyltranferase reactions (Mattson and Shea, 2003). A growing number of epidemiological studies have linked folate deficiency and resultant elevated plasma total Hcy levels with an increased risk of vascular disease, cerebral ischemia (Hankey and Eikelboom, 2001; Faraci and Lentz, 2004; He et al., 2004) and neurodegenerative and neuropsychiatric disease, including Alzheimer disease, depression, and schizophrenia (Mattson and Shea, 2003; Korczyn, 2002; McCaddon, 2002; Reynolds, 2002). It has been also shown that folate supplementation can reduce the risk of these diseases (Mattson and Shea, 2003; Assanelli et al., 2004; Ullegaddi et al., 2004).

Vitamin E (α-tocopherol) functions as the most potent naturally occurring scavenger of reactive oxygen and nitrogen species (Tucker and Townsend, 2005). It breaks the propagation of the free radical chain reaction in the lipids of biological membranes (Gilgun-Sherki et al., 2002). The water-soluble antioxidant vitamin C can reduce tocopheroxyl radicals directly or indirectly and thus support the antioxidant activity of vitamin E (Sies et al., 1995). In addition, it has been reported that vitamin E administration exerts protective effects against ischemic events (Zhang et al., 2004; Chaudhary et al., 2003; Van der Worp et al., 1998). On the other hand, we have shown that pretreatment with vitamins E and C prevents the inhibition of activities of Na^+, K^+, ATPase and butyrylcholinesterase (Stefanello et al., 2005; Wyse et al., 2002) and the impairment of memory caused by Hcy (Reis et al., 2002).

In the present study we investigated the effect of acute and chronic administration of Hcy on brain slices exposed to oxygen and glucose deprivation (OGD), an *in vitro* model of hypoxic-ischemic events (Cárdenas et al., 2000). We also studied the protective effect of vitamin E and C and folate on the ischemic damage caused by homocysteine. Our hypothesis is that hyperhomocysteinemia provokes cellular damage and that folate and vitamins E and C could prevent such effects.

2. Experimental

2.1 Animal and reagents

Male Wistar rats obtained from the Central Animal House of the Department of Biochemistry, ICBS, Federal University of Rio Grande do Sul, Porto Alegre, RS, Brazil, were housed in groups of eight with their mothers on the day of birth. Animals were maintained on a 12:12 h light/dark cycle (lights on 7:00–19:00 h) in an air-conditioned constant-temperature (22 ± 1ºC) colony room, with free access to water and a 20 wt.% protein commercial. Animal care followed the official governmental guidelines in compliance with the Federation of Brazilian Societies for Experimental Biology and was approved by the Ethics Committee of the Federal University of Rio Grande do Sul, Brazil. All chemicals were purchased from Sigma Chemical Co., St Louis, MO, USA.

2.2 Acute administration of Hcy

Hcy was dissolved in 0.9% NaCl solution and buffered to pH 7.4. Rats of 29 days old received one single s.c. injection of Hcy (0.6 μmol/ g body weight) (Streck et al., 2002) and control animals received an equivalent volume of saline. The rats were killed 1 h after injection.

2.3 Chronic administration of Hcy

Homocysteine solution was administered subcutaneously twice a day at 8-h intervals from 6 to 28 days of age. Control animals received saline solution in the same volumes as those applied to Hcy-treated rats. Hcy doses were calculated from pharmacokinetic parameters previously determined in our laboratory (Streck et al., 2002). During the first week of treatment, animals received 0.3 μmol Hcy/g body weight. On the second week, 0.4 μmol Hcy/g body weight was administered to the animals, and in the last week rats received 0.6 μmol Hcy/g body weight. Rats subjected to this treatment achieved plasma Hcy levels similar to those found in homocystinuric patients. Maximal brain levels were achieved 15 min after Hcy administration (0.04–0.06 mmol/kg wet weight tissue). Twelve hours after treatment, plasma Hcy concentrations returned to normal levels (0.01 mmol/l) and brain Hcy levels were not detected. The animals were decapitated 1h, 3h or 12h after the last injection and the hippocampi were quickly dissected out.

2.4 Vitamins E and C pretreatment

Animals were treated for 7 days with a daily i.p. administration of vitamins E (40 mg/Kg) and C (100 mg/Kg) (Wyse et al., 2002) or with saline (0.9 % NaCl). Twelve hours after the last injection, rats received a single s.c. administration of Hcy as describe above and were decapitated 1h later.

2.5 Folate pretreatment

Animals were treated for 7 days with a daily i.p. administration of folic acid (5 mg/Kg) or saline (0.9 % NaCl) (Lalonde et al., 1993). After this pretreatment, rats received a single s.c. injection of Hcy as describe above and were decapitated 1h later.

2.6 Preparation and incubation of slices

After decapitation the hippocampi were immediately isolated and transverse sections (400 μm) were prepared using a McIlwain tissue chopper. Hippocampal slices were divided into two equal sets (control and OGD), placed into separate 24-well culture plates, and preincubated for 30 min in a modified Krebs-Henseleit solution (pre-incubation solution): 120 mM NaCl, 2 mM KCl, 0.5 mM $CaCl_2$, 26 mM $NaHCO_3$, 10 mM $MgSO_4$, 1.18 mM KH_2PO_4, 11 mM glucose, in a tissue culture incubator at 37º C with 95% air/5% CO_2 (Cárdenas et al., 2000).

2.7 Oxygen and glucose deprivation (OGD)

After pre-incubation, the medium in the control plate was replaced with another modified Krebs-Henseleit solution (KHS incubation solution, pH 7.4): 120 mM NaCl, 2 mM KCl, 2 mM $CaCl_2$, 2.6 mM $NaHCO_3$, 1.19 mM $MgSO_4$, 1.18 mM KH_2PO_4, 11 mM glucose, and incubated for 45 min in a tissue culture incubator at

37ºC with 95% air/5% CO_2. After 45 minutes, the control medium was replaced by a fresh one and slices incubated for another 3 hours in the same conditions.

In the ischemic plate, after pre-incubation OGD slices were washed twice with a KHS medium without glucose saturated with N_2 and incubated for 45 min (OGD period) at 37 ºC in an anaerobic chamber saturated with nitrogen, as fully detailed elsewhere (Cárdenas et al., 2000; Cimarosti et al., 2001). After 45 min of incubation, the medium from both control and OGD slices was removed and the two groups received KHS with glucose. Then, the slices were incubated for 3 h (recovery period) in the culture incubator. Control and OGD experiments were run concomitantly using four slices of the same animal in each plate.

2.8 Assessment of neural injury-LDH assay

Cellular damage was quantified by measuring lactate dehydrogenase (LDH) released into the medium (Koh and Choi, 1987). After the recovery period, LDH activity was determined using a kit (Doles Reagents, Goiânia, Brazil). Each experiment was normalized by subtracting the background levels of LDH produced from the no-treatment wells (Almli et al., 2001).

2.9 Statistical analysis

Data were analyzed by one-way ANOVA followed by the Duncan Multiple range test when the *F* test was significant. All analyses were performed using the Statistical Package for the social Sciences (SPSS) software. Differences were considered statistically significant if $p<0.05$.

3. Results

In order to evaluate whether exposure to Hcy affects cellular susceptibility to OGD we assessed LDH activity in the incubation medium of hippocampal slices of rats submitted to acute administration of Hcy. Figure 1 shows that OGD exposure and 3h of reoxygenation caused an increase of LDH release into the incubation media, a marker of cellular death, in all groups studied, as compared to control slices *(F* (3,12)=72.64, *p*<0.001). Post hoc analysis showed that acute Hcy administration provoked an increase in the cellular damage on the non-OGD group, but the highest level of LDH released was found in the Hcy-ischemic group.

Figure 2 shows the effect of Hcy chronic administration on cellular damage. This experiment aimed to evaluate whether high concentrations of Hcy during on development similar to those found in homocystinuric patients interfere with the neuronal damage induced by OGD. Results showed that OGD caused an increase of cell damage 1, 3 and 12h after the last injection of Hcy, however, chronic Hcy administration increased LDH release into the incubation media when the animals were killed only 1h, but not 3 or 12 h (*F* (3,12)=63.97, *p*<0.001), indicating that the presence of Hcy is necessary to cause cellular damage, since 3 or 12 h after injection it was not present in plasma and brain of rats.

Next, we investigated the effect of possible neuroprotectors agents against the damage caused by Hcy and ischemia. Figures 3 and 4 show that pretreatment with vitamin E and C partially prevented the damage caused by Hcy and OGD in hippocampal slices (*F*(7,24)=29.55, *p*<0.001) and that folic acid pretreatment

completely prevented the effects of Hcy administration (fig. 4) ($F(7,24)=58.42$, $p<0.001$). None treatment *per se* altered LDH release.

4. Discussion

Hyperhomocysteinemia has been associated with homocystinuria (Mudd et al., 2001) and many diseases involving the CNS, such as stroke (Yoo et al., 1998; Clarke et al., 1998; Eikelboom et al., 2000; Mudd et al., 2001; Loscalzo, 2002; Morris, 2003; Dwyer et al., 2004). On the other hand, energy metabolism and oxidative stress are important events that have been related to the pathogenesis of these diseases (White et al., 2000; Halliwell and Gutteridge, 1999). In this context, we have shown that chronic homocysteinemia decreases energy metabolism in rat hippocampus (Streck et al., 2003a) and the presence of Hcy in the incubation medium induces oxidative stress (increased TBA-RS and decreased TRAP) in hippocampus and parietal cortex of rats (Streck et al., 2003b; Matté et al., 2004).

Energy demands of central nervous system are fulfilled almost entirely with continuous supplies of oxygen and glucose from the blood flow. Consequently, cerebral ischemia drastically reduces ATP available for neurons and causes severe neuronal injury (Fujimoto et al., 2004). On the other hand, experimental *in vitro* model of ischemia in hippocampal slices is a model amply used in the literarture (Schurr et al., 1995; Cárdenas et al., 2000; Siqueira et al., 2004) and offers important advantages because cell composition, such as functional neurons, inflammatory competent cells, locally releases effectors and intercellular connections are preserved (Taylor et al., 1995). However, in this model the

vascular epithelium is absent, so the effects of Hcy observed in our experiments are not consequence of vascular damage and thrombosis.

In the present study we first investigated the effect of acute and chronic Hcy administration on hippocampal slices exposed to this *in vitro* model of hypoxia–ischemia. Results showed that the exposure of slices to ischemia followed by reoxygenation resulted in increased LDH activity in the medium, which is a consequence of cell damage and acute hyperhomocysteinemia increased the effect of OGD on this parameter in young rats. We also verified that the chronic Hcy administration, an experimental model of homocystinuria, also increased de LDH release when the animals were sacrificed 1h, but not 3 or 12 h, after the last injection of Hcy. These result may be interpreted as an increased vulnerability of these cells to ischemia in presence of Hcy, since our previous studies showed that 1 h after Hcy injection the levels of this amino acid are elevated in tissues. However, these results indicate that to produce deleterious effects its necessary that Hcy concentration in brain remain high. According to the pharmacokinetics parameters determined by Streck and colleages (Streck et al., 2002), the Hcy concentration begins lower 90 min after the administration, this way, after 3h the Hcy levels would be normal.

Numberless studies have been demonstrated that hyperhomocysteinemia produces endothelial damage and dysfunction increasing the risk of atherogenesis and thrombogenesis through oxidative mechanisms (Faraci and Lentz, 2004; Assanelli et al., 2004; Racek et al., 2005). On the other hand, many researchers have reported that administration of vitamin E exerts protective effects against ischemic events (Van der Worp et al., 1998; Chaudhary et al., 2003; Zhang et al.,

2004). This way, we tested the effect of pretreatment with antioxidants (vitamins E and C) on the damage caused by high concentrations of Hcy associated with OGD. Results showed that the pretreatment of vitamin E and C partially prevented the effect of acute administration of Hcy, indicating that probably oxidative stress is not the only mechanism involved in cellular dead provoked by Hcy, since that the administration of these vitamins (in the same dose and duration of treatment used in the present study) prevented some biochemistry and behavioral parameters in rats caused by Hcy and proline administration (Reis et al., 2002; Wyse et al., 2002; Franzon et al., 2003; Wyse et al., 2004; Delwing, et al., 2005). This result is in agreement with other studies showing that there is a lack of effect on neuronal death, endothelial function and plasma antioxidant capacity when vitamin E is administered during short periods (Zhang et al., 2004; Assanelli et al., 2004).

It has been shown that folic acid deficiency increases the risk of stroke, Parkinson's disease and Alzheimer's disease (Mattson et al., 2002) and that supplementation of this vitamin can significantly attenuate the clinical features of homocystinuria, stroke and of various neurodegenerative disorders (Botez et al., 1982; Daly et al., 1995; Mattson et al., 2002; Verhaar et al., 2002; Mattson and Shea, 2003; Bottiglieri, 2005; Moore et al., 2005; Walter et al., 1998). In this regard, some reports have shown that folic acid administration can reduce blood Hcy concentrations in humans and rats (Homocysteine Lowering Trialists' Collaboration, 1998; Rydlewicz et al., 2002; Lamers et al., 2004). In addition, there is also strong evidence suggesting an acute antioxidant effect of this vitamin, which is independent of its effect on Hcy metabolism (Ullegaddi et al., 2004; Racek et al., 2005). We observed in the present study that the pretreatment with folic acid

during 7 days before OGD completely prevented the effects of acute administration of Hcy, indicating that Hcy probably cause damage in hippocampal slices by way of two mechanisms. One is producing direct neurotoxicity, this effect is avoid lowering the Hcy concentration. The second mechanism is increasing the free radical production, thus folic acid acts as antioxidant. **Since vitamins E and C were not able in prevent completely the effects of Hcy, it seems that oxidative stress is not the only mechanism involved in the observed cellular damage.**

We demonstrated in our study that it was possible to establish differences in brain tissue response to OGD condition between Hcy-treated and control animals and that folic acid was effective in prevent the increase of the susceptibility caused by Hcy. Studies of the effects of Hcy using *in vivo* ischemic models would bring interesting additional information concerning the relationship between Hcy, ischemic risk and possible prevention strategies. Anyway, this finding may be relevant for the ischemia-induced pathophysiological squeals *in vivo*, and the effects of the high level of homocysteine to which the patient was possible submitted previously to the ischemic event should be considered. Moreover, we demonstrate that if our results will be confirmed in *in vivo* experiments, supplementation with folic acid may be used as adjuvant therapy in patients with elevate risk of ischemic events.

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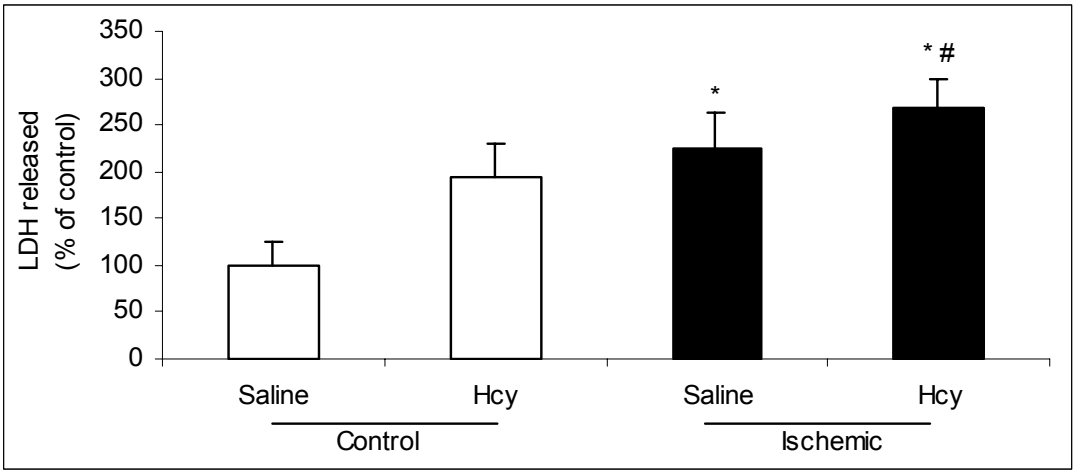
Fig. 1 - Effects of acute administration of homocysteine on cell injury, as assessed by LDH release to the medium, of hippocampal slices from rats exposed to oxygen and glucose deprivation (OGD) for 45 min and reoxygenation for 3h - the ischemic groups. Results are expressed as percentage of the saline non-OGD group. Columns represent mean ± S.D. for 4 experiments performed in triplicate. * Values significantly different from those of non-OGD groups, *# value significantly different from those saline OGD, as determined by ANOVA followed by Duncan's test ($p < 0.001$). Hcy- homocysteine.

Fig. 2 - Effects of chronic administration of homocysteine on cell injury, as assessed by LDH release to the medium, of hippocampal slices from rats exposed to oxygen and glucose deprivation (OGD) for 45 min and reoxygenation for 3h - the ischemic groups. A) Animals killed 1h after the last injection. B) Animals killed 3h after the last injection. C) Animals killed 12h after the last injection. Results are expressed as percentage of the saline non-OGD group. Columns represent mean ± S.D. for 4 experiments performed in triplicate. * Values significantly different from those of non-OGD groups, *# value significantly different from those saline OGD,

as determined by ANOVA followed by Duncan's test (p < 0.001). Hcy - hmocysteine.

Fig. 3 - Effects of pretreatment with vitamins E and C before acute administration of homocysteine on cell injury, as assessed by LDH release to the medium, of hippocampal slices from rats exposed to oxygen and glucose deprivation (OGD) for 45 min and reoxygenation for 3h - the ischemic groups. Results are expressed as percentage of the saline non-OGD group. Columns represent mean ± S.D. for 4 experiments performed in triplicate. * Values significantly different from those of non-OGD groups, *# value significantly different from those saline OGD, *+ value significantly different from other groups, as determined by ANOVA followed by Duncan's test (p< 0.001). Sal - saline; Hcy – homocysteine; Vit - vitamins C and E.

Fig. 4 - Effects of pretreatment with folic acid before acute administration of homocysteine on cell injury, as assessed by LDH release to the medium, of hippocampal slices from rats exposed to oxygen and glucose deprivation (OGD) for 45 min and reoxygenation for 3h - the ischemic groups. Results are expressed as percentage of the saline non-OGD group. Columns represent mean ± S.D. for 4 experiments performed in triplicate. * Values significantly different from those of non-OGD groups, *# value significantly different from those saline OGD, as determined by ANOVA followed by Duncan's test (p < 0.001). Sal – saline; Hcy – homocysteine.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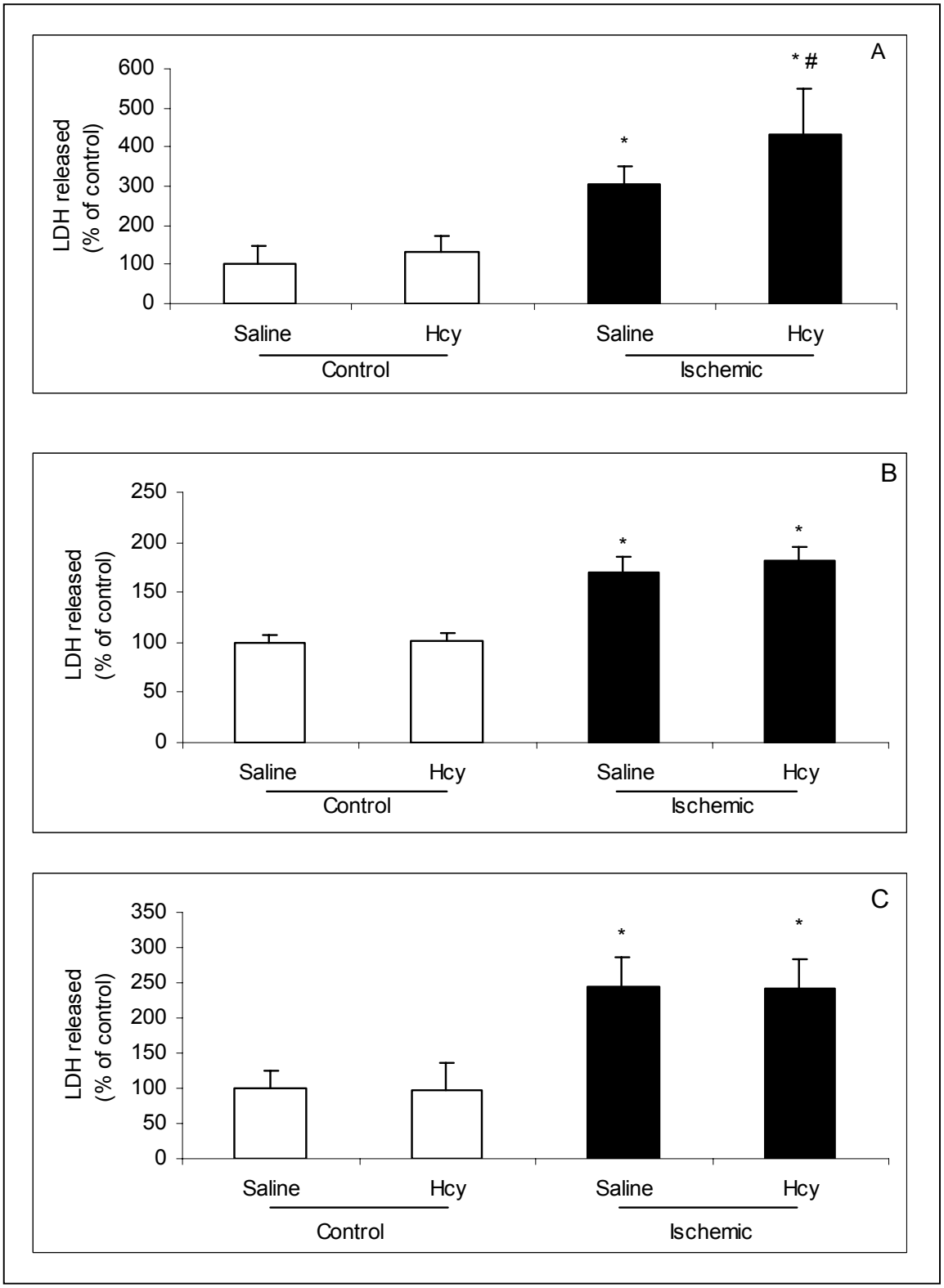
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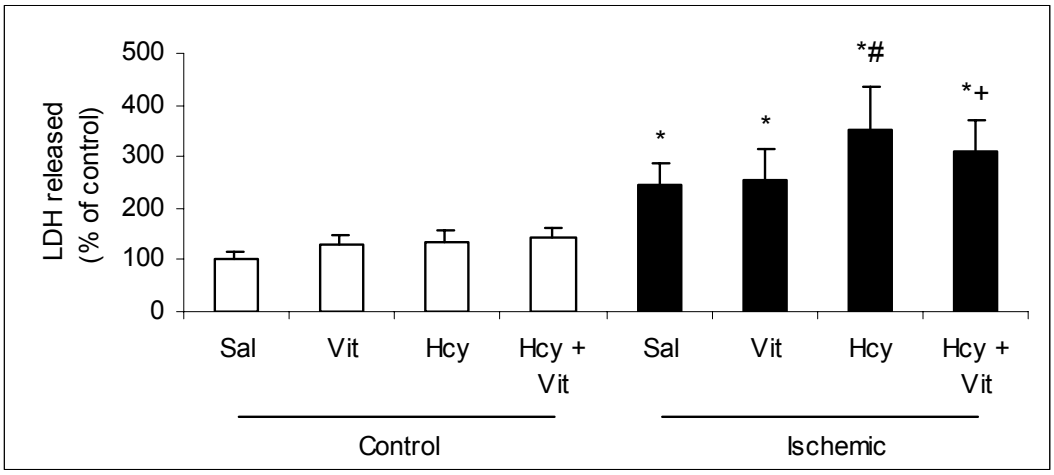
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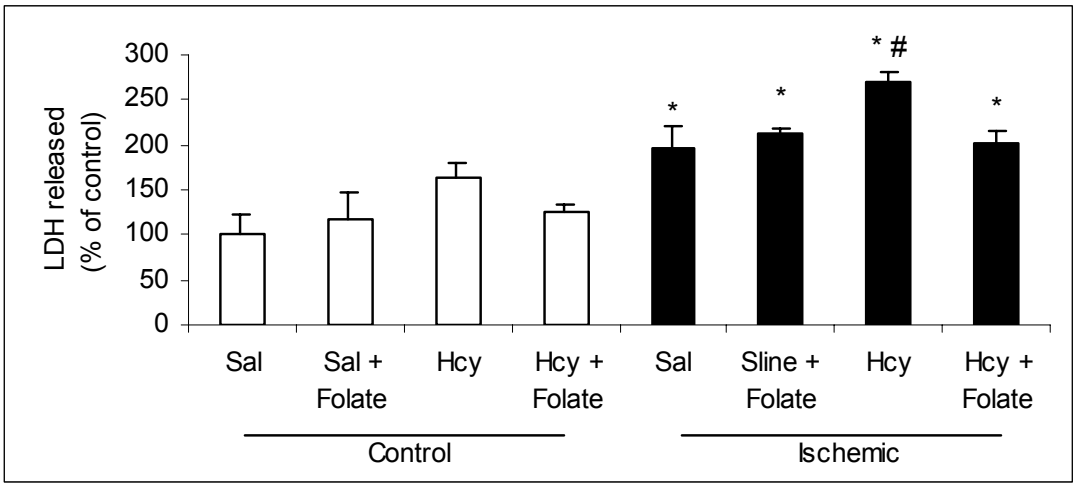
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4. DISCUSSÃO

A homocistinúria é um erro inato do metabolismo que ocorre devido à deficiência parcial ou total da atividade da enzima cistationina β-sintetase (CBS), resultando no acúmulo tecidual principalmente de Hcy. Pacientes afetados apresentam alterações em vários órgãos e sistemas, incluindo os sistemas nervoso central e vascular (Mudd et al., 2001).

Embora a exata etiopatogenia da HCU ainda não seja completamente conhecida, sabe-se que níveis plasmáticos elevados de Hcy produzem modificações na estrutura e função dos vasos sanguíneos, aumentam a oxidação de LDL, estimulam a proliferação das células musculares lisas e promovem efeitos pró-trombóticos (Van Beynum et al., 1999; Faraci e Lentz, 2004; Hassan et al., 2004). Além disso, a Hcy apresenta efeitos neurotóxicos como, por exemplo, o aumento na formação de radicais livres, a elevação do influxo de cálcio via receptores NMDA, o que pode levar a excitotoxicidade pelo glutamato. A Hcy também aumenta a imunoreatividade da fosfo-tau, estimula apoptose em cultura de neurônios de ratos (Ho et al., 2002) e pode potencializar o efeito de outros agentes neurotóxicos e promotores de estresse oxidativo, como a proteína β-amilóide (Kruman et al., 2002; White et al., 2001), cobre e ferro (Pfanzagl, 2005).

Neste contexto, estudos realizados no nosso laboratório, utilizando um modelo de homocistinúria quimicamente induzida desenvolvido por Streck e colaboradores (2002), demonstraram que a hiper-homocisteinemia prejudica a memória (Streck et al., 2004) e reduz a atividade da enzima Na^+, K^+, ATPase

(Streck et al., 2002) e o metabolismo energético em hipocampo de ratos (Streck et al., 2003a). Por outro lado, a Hcy *in vitro* induz estresse oxidativo em hipocampo e córtex parietal de ratos (Streck et al., 2003b; Matté et al., 2004).

O estresse oxidativo parece estar intimamente envolvido na patogênese de várias doenças que afetam o SNC, incluindo as doenças de Parkinson e de Alzheimer, esquizofrenia, isquemia cerebral e HCU. Baseado nesses estudos, outros trabalhos têm sido realizados no intuito de testar o potencial terapêutico de antioxidantes nessas patologias. Alguns desses trabalhos demonstram que a administração de vitamina E exerce efeitos protetores contra a isquemia cerebral em ratos (Van der Worp et al., 1998; Chaudhary et al., 2003; Zhang et al., 2004). Por outro lado, recentes estudos do nosso grupo mostraram que o pré-tratamento com vitaminas E e C previne a inibição das atividades das enzimas Na^+, K^+, ATPase e butirilcolinesterase (Wyse et al., 2002; Stefanello et al., 2005) e o prejuízo da memória causados pela Hcy (Reis et al., 2002).

Dados da literatura mostram que a deficiência de ácido fólico aumenta o risco de derrame e das doenças de Parkinson e Alzheimer (Mattson et al., 2002) e que a suplementação com essa vitamina pode atenuar algumas das manifestações clínicas da homocistinúria e das doenças acima citadas (Botez et al., 1982; Daly et al., 1995; Walter et al., 1998; Mattson et al., 2002; Verhaar et al., 2002; Mattson e Shea, 2003; Bottiglieri, 2005; Moore et al., 2005). Neste contexto, alguns autores têm mostrado que a administração de ácido fólico pode reduzir os níveis plasmáticos de Hcy em humanos e ratos (Homocysteine Lowering Trialists' Collaboration, 1998; Rydlewicz et al., 2002; Lamers et al., 2004). Além disso, evidências também sugerem uma ação antioxidante do ácido fólico, independente

de sua atuação no metabolismo da Hcy (Ullegaddi et al., 2004; Racek et al., 2005).

O ATP produzido pelo metabolismo aeróbico é a principal fonte de energia para o cérebro, dessa forma, o prejuízo do suprimento de oxigênio e glicose causado pela interrupção do fluxo sanguíneo durante a isquemia reduz o ATP disponível para os neurônios e causa severo dano celular (Fujimoto et al., 2004). Além da diminuição do ATP, outros eventos que contribuem para o prejuízo neuronal decorrente da privação de oxigênio e glicose incluem o aumento do influxo de Ca^{2+}, liberação de glutamato, diminuição do pH, alteração de permeabilidade celular, ativação de fosfolipases e proteases e formação de radicais livres e óxido nítrico (Lipton, 1999).

Estudos em modelos de isquemia/hipóxia cerebral em fatias de hipocampo têm sido utilizados como método para avaliação dos danos neuronais, seus mecanismos e possíveis medidas neuroprotetoras (Schurr et al., 1995; Taylor et al., 1995; Siqueira et al., 2004; Fontella et al., 2005). No modelo utilizado neste trabalho, não há a presença de epitélio vascular, assim ele pode ser considerado uma boa ferramenta para o estudo de isquemia de origem não aterotrombótica.

No presente trabalho nós utilizamos o modelo de privação de oxigênio e glicose (POG) em fatias de hipocampo de ratos para avaliar os efeitos *in vivo* e *in vitro* da Hcy sobre o dano celular causado pela isquemia. A liberação de LDH para o meio de incubação foi o parâmetro de avaliação do dano tecidual utilizado em todo o nosso estudo.

Inicialmente, testamos o efeito da adição de elevadas concentrações de Hcy (10, 100 e 500 μM) ao meio de incubação das fatias de hipocampo de ratos

de 60 dias. As fatias foram incubadas por 30 min e posteriormente submetidas a 45 min de POG e 3h de reoxigenação. Nossos resultados mostraram que a exposição à POG aumentou a liberação de LDH e que nos grupos Hcy 100 μM e Hcy 500 μM houve uma liberação significativamente maior do que nos grupos controle e 10 μM. Em vista disso, podemos aferir que concentrações de Hcy similares àquelas encontradas nos pacientes homocistinúricos aumentam a vulnerabilidade do tecido cerebral ao dano celular causado pela isquemia.

Embora os modelos animais de EIM não possam mimetizar completamente uma doença humana, eles dão uma idéia do quadro clínico apresentado durante a instalação e desenvolvimento da mesma. Em nosso laboratório desenvolvemos modelos animais experimentais de vários EIM, como a fenilcetonúria (Wyse et al., 1995), a hiperprolinemia tipo II (Moreira et al., 1989), a homocistinúria (Streck et al., 2002) e as acidemias metilmalônica (Dutra et al., 1991) e propiônica (Brusque et al., 1999), com o objetivo de melhor compreender os mecanismos fisiopatológicos da doença.

Neste trabalho utilizamos o modelo químico experimental de homocistinúria desenvolvido por Streck e colaboradores (2002), onde os animais apresentam níveis plasmáticos de Hcy similares aos dos pacientes homocistinúricos (entre 0,4 e 0,5 mM). No modelo agudo, os ratos receberam uma única injeção de Hcy (0,6 μmol/g de peso corporal) ou de solução salina (NaCl 0,9%). Os animais foram sacrificados 1h após a injeção. O modelo crônico consiste na administração subcutânea de Hcy, duas vezes ao dia, entre o 6º e o 28º dias de vida do animal. As doses de Hcy variaram de acordo com o peso e a idade dos ratos e foram

determinadas conforme os parâmetros farmacocinéticos desse aminoácido (de 0,3 a 0,6 μmol/g de peso corporal). Animais controle receberam igual volume de solução salina.

Em relação aos modelos de hiperhomocisteinemia, primeiramente avaliamos o efeito da administração aguda de Hcy em animais adultos (60 dias), sobre modelo de POG. Da mesma forma que *in vitro*, a Hcy provocou um aumento no dano celular causado pela POG de aproximadamente 45% em relação ao grupo tratado com salina. Em todos os experimentos, os grupos isquêmicos foram significativamente diferentes dos respectivos grupos não-isquêmicos.

Em outra série de experimentos, utilizamos o mesmo modelo agudo, mas em animais jovens (29 dias). A administração de Hcy aumentou (20%) a liberação de LDH quando comparada com o grupo controle. Diferentemente dos animais de 60 dias, a administração de Hcy a ratos jovens provocou um aumento na liberação de LDH das fatias do grupo não submetido à POG. Esses achados poderiam ser resultantes de uma maior permeabilidade barreira hemato-encefálica à Hcy nos animais jovens (29 dias). Como as concentrações de Hcy no cérebro de ratos adultos ainda não foram medidas, não podemos afirmar se o aminoácido alcança níveis tão elevados quanto os observados por nosso grupo (Streck et al., 2002) em animais mais jovens (0,04-0,06 mmol/kg de tecido). Dessa forma, novos estudos serão necessários para elucidar esses resultados.

O aumento da liberação de LDH para o meio de incubação após a administração aguda de Hcy pode ser interpretado como um aumento da vulnerabilidade das células neuronais à isquemia. Tais resultados corroboram com

outros trabalhos que mostram que a Hcy sensibiliza o cérebro a uma variedade de insultos, como isquemia cerebral (Endres et al., 2005) e desordens neurodegenerativas (Duan et al., 2002; Mattson e Shea, 2003).

Por outro lado, os mesmos mecanismos envolvidos no dano neuronal causado pela Hcy, isto é, a inibição da Na^+, K^+, ATPase (Streck et al., 2002), o prejuízo do metabolismo energético cerebral (Streck et al., 2003a) e a produção de radicais livres (Streck et al., 2003b) também participam na morte celular observada nos neurônios piramidais após eventos isquêmicos (Lipton, 1999; Wyse et al., 2000; Warner et al., 2004). Isto pode explicar em parte, o fato da Hcy aumentar o dano provocado pela isquemia, já que a combinação de dois insultos costuma ser mais devastadora que um ou outro ocorrendo independentemente.

Posteriormente, nós testamos o efeito da administração crônica de Hcy, um modelo experimental de HCU, sobre o dano celular. Três grupos de animais receberam Hcy do 6º ao 28º dia de vida e foram sacrificados em diferentes tempos (1, 3 ou 12h) após a última injeção de Hcy, com o objetivo de avaliar a necessidade da presença de elevadas concentrações de Hcy no cérebro dos animais no momento do sacrifício. O tratamento crônico provocou um aumento no dano celular apenas quando os animais foram mortos 1h após a última injeção. Nos demais grupos não houve diferença significativa entre os animais tratados e controles.

Esses resultados sugerem um aumento da vulnerabilidade dessas células à isquemia na presença de Hcy, já que os estudos farmacocinéticos (Streck et al., 2002) demonstraram que 1h após a injeção, os níveis desse aminoácido estão elevados nos tecidos (incluindo o cérebro) dos ratos, e que esses níveis começam

a diminuir 90 min após a administração de Hcy, retornando aos níveis normais em 3h. Assim, podemos afirmar que elevados níveis teciduais de Hcy são necessários para produzir o dano celular, uma vez que este dano não ocorreu quando os animais foram sacrificados 3h ou 12 h após a última injeção.

Sabendo que a presença de Hcy aumenta o dano tecidual de fatias de hipocampo submetidas à POG, e que o aumento do estresse oxidativo pode ser um dos mecanismos responsáveis por esse efeito, nós decidimos testar o possível papel neuroprotetor do ácido fólico e das vitaminas E e C.

Nossos resultados mostraram que o pré-tratamento com as vitaminas E e C durante uma semana, preveniu parcialmente o efeito da administração aguda de Hcy, indicando que o estresse oxidativo não deve ser o único mecanismo envolvido no dano celular causado por esse aminoácido. Essa interpretação é baseada em estudos que mostram que a administração dessas vitaminas (nas mesmas doses e tempo de tratamento) foi capaz de prevenir alguns efeitos bioquímicos e comportamentais em ratos tratados com Hcy e prolina (Reis et al., 2002; Wyse et al., 2002; Franzon et al., 2003; Wyse et al., 2004; Delwing, et al., 2005). Por outro lado, os dados do presente trabalho estão de acordo com outros estudos que mostraram que a administração de vitamina E por curtos períodos, não preveniu a morte neuronal e a função endotelial em ratos (Zhang et al., 2004) e não aumentou a capacidade antioxidante do plasma de pacientes após infarto do miocárdio (Assanelli et al., 2004). Além disso, também tem sido demonstrado que α-tocoferol, que é uma molécula lipossolúvel, pode apenas parar o ataque oxidativo inicial à membrana; uma vez que a cascata de radicais livres entra no

citoplasma, a vitamina E é pouco eficiente como antioxidante (Wang e Quinn, 1999), o que poderia explicar, pelo menos em parte, nossos resultados.

Nós mostramos também que o pré-tratamento com ácido fólico durante 7 dias antes da POG preveniu completamente os efeitos da administração aguda de Hcy sobre a susceptibilidade celular. Nossa hipótese é que o ácido fólico poderia estar atuando através de dois mecanismos distintos: o primeiro seria protegendo contra os efeitos neurotóxicos da Hcy, já que ele pode diminuir os níveis desse aminoácido (Ho et al., 2002; Mattson e Shea, 2003); o segundo seria bloqueando a produção de radicais livres, pois essa vitamina também tem ação antioxidante (Ullegaddi et al., 2004; Racek et al., 2005). Até o momento não sabemos se o ácido fólico altera os níveis de Hcy ou atua como antioxidante. Portanto, novos estudos serão necessários para elucidar esses mecanismos.

Neste estudo foi possível estabelecer diferenças entre as respostas do tecido cerebral à condição de POG entre animais tratados com Hcy e controles. O ácido fólico foi efetivo em prevenir totalmente o aumento da susceptibilidade celular causado pela Hcy, enquanto que as vitaminas E e C preveniram parcialmente. Estes achados podem contribuir para entender a maior suscetibilidade dos pacientes hiperhomocisteinêmicos de ter eventos isquêmicos e sugerem que, desde que confirmados em experimentos *in vivo*, a suplementação com ácido fólico ou possivelmente com antioxidantes possa ser usada como terapia adjuvante nestes pacientes.

5. CONCLUSÕES

1- A Hcy *in vitro*, nas concentrações similares àquelas encontradas em pacientes com HCU, aumentou significativamente a morte celular causada pela POG em fatias de hipocampo de ratos de 60 dias de vida.

2- A administração aguda de Hcy aumentou significativamente a suscetibilidade das fatias à morte celular causada pela POG em ratos de 29 e 60 dias de vida.

3- A administração crônica de Hcy aumentou significativamente a suscetibilidade das fatias à morte celular causada pela POG quando os animais foram mortos 1h após a última injeção desse aminoácido.

4- O pré-tratamento com as vitaminas E e C, durante 7 dias antes da POG, preveniu significativamente e parcialmente os efeitos da Hcy sobre a morte celular.

5- O pré-tratamento com o ácido fólico, durante 7 dias antes da POG, preveniu significativamente e completamente o aumento do dano neuronal causado pela Hcy em fatias de hipocampo de ratos submetidas à POG.

6. PERSPECTIVAS

1- Medir alguns parâmetros de estresse oxidativo nas fatias de hipocampo de ratos com hiperhomocisteinemia submetidas à POG.

2- Avaliar os efeitos do tratamento com ácido fólico e com as vitaminas E e C durante a indução do modelo crônico experimental de HCU.

3- Medir os níveis plasmáticos e teciduais de Hcy após a administração de ácido fólico e das vitaminas E e C.

4- Verificar os efeitos da Hcy, do ácido fólico e das vitaminas E e C em modelos animais de isquemia *in vivo.*

5- Avaliar os efeitos da Hcy em culturas organotípicas de hipocampo de ratos.

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