UNIVERSIDADE FEDERAL DO RIO GRANDE DO SUL

INSTITUTO DE CIÊNCIAS BÁSICAS DA SAÚDE

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ESTRESSE OXIDATIVO RENAL EM RATOS SUBMETIDOS À HIPER-HOMOCISTEINEMIA

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Dissertação apresentada ao Programa de Pós-Graduação em Ciências Biológicas: Bioquímica da Universidade Federal do Rio Grande do Sul, como requisito para a obtenção do título de Mestre em Bioquímica.

Porto Alegre, 2013

Às pessoas mais importantes da minha vida,

Meus pais, Geraldo e Nara, pelo amor dedicado,
Minhas irmãs, Carla e Cristiane, grandes companheiras,
e
Rafael, meu namorado, pela compreensão e torcida por meu crescimento profissional.

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SUMÁRIO

RESUMO

O estresse oxidativo tem sido considerado parte importante na etiopatogenia das alterações cardiovasculares e cerebrais causadas pela hiper-homocisteinemia (HHcy), uma condição caracterizada por elevados níveis plasmáticos de homocisteína (Hcy), um aminoácido derivado da metionina. A HHcy leve ocorre quando os níveis plasmáticos de Hcy variam de 15 a 30 µmol/L e pode resultar da deficiência de ácido fólico, vitamina B_6 e/ou vitamina B_{12}. A HHcy severa é caracterizada por níveis plasmáticos de Hcy superiores a 100 µmol/L e é encontrada na homocistinúria (HCU) clássica, um erro inato do metabolismo caracterizado pela deficiência da enzima cistationina β-sinatse (CBS), que catalisa a via de transulfuração da Hcy. Visto que estudos mais recentes demonstram que a HHcy também está associada a um comprometimento da função renal, a hipótese deste trabalho é que o estresse oxidativo também esteja envolvido nas alterações renais causadas pela Hcy. Dessa forma, o objetivo deste estudo foi investigar o efeito da administração de Hcy sobre a formação de espécies reativas de oxigênio (ERO), lipoperoxidação, dano às proteínas e formação de óxido nítrico (NO), além de avaliar as defesas antioxidantes enzimáticas e não enzimáticas em rins de ratos. Visando testar a hipótese de que as possíveis mudanças no *status* oxidativo/nitrativo nos rins de ratos hiperhomocisteinêmicos poderiam levar à nefropatia, também se verificaram parâmetros séricos de função renal, tais como creatinina, uréia, ácido úrico e albumina. Ratos Wistar foram submetidos a modelos experimentais de HHcy leve e severa desenvolvidos em nosso grupo de pesquisa. No modelo experimental de HHcy leve crônica, ratos adultos receberam injeções subcutâneas diárias de Hcy do 30º ao 60º dia de vida. A dose utilizada foi de 0,03 µmol Hcy/g de peso corporal e induzia concentrações plasmáticas de Hcy de cerca de 30µM. O grupo controle recebeu salina e os animais foram decapitados 12 horas após a última injeção. Desenvolveu-se ainda um modelo de HHcy leve aguda, induzida através de uma única injeção subcutânea de Hcy em ratos de 29 dias, utilizando-se a mesma dose. Os ratos foram decapitados 1 ou 12 horas após a injeção. No modelo experimental de HHcy severa crônica, ratos neonatos receberam injeções subcutâneas diárias de Hcy do 6^o ao 28^o dia de vida. As doses utilizadas foram de 0,3 a 0,6 µmol Hcy/g de peso corporal e elevavam as concentrações plasmáticas de Hcy para cerca de 500µM. O grupo controle recebeu salina e os animais foram decapitados 1 ou 12 horas após a última injeção. Os resultados obtidos a partir do modelo de HHcy leve demonstram que a administração crônica de Hcy aumentou as atividades das enzimas antioxidantes superóxido dismutase (SOD) e catalase (CAT) e a produção de NO (medida através dos níveis de nitritos) nos rins, assim como reduziu os níveis de albumina no soro dos animais 12 horas após a última injeção. A indução de HHcy leve aguda aumentou os níveis de diclorofluoresceína (DCF) e de nitritos em rins de ratos 1 hora após a injeção. Os resultados do modelo de HHcy severa crônica mostram um aumento significativo dos níveis de DCF e de substância reativas ao ácido tiobarbitúrico (TBARS) 1 e 12 horas após a última injeção, sugerindo que a Hcy aumentou os níveis de ERO e a lipoperoxidação em rins de ratos, respectivamente. O aumento da atividade da SOD e a redução da atividade da CAT também foram observados em 1 e 12 horas. Por outro lado, a redução das defesas antioxidantes não enzimáticas e o aumento dos níveis de nitritos foram observados somente 1 hora após a última administração de Hcy. A administração crônica de altas doses do aminoácido ainda elevou os níveis de uréia e reduziu os níveis de albumina 12 horas após a última injeção. Tais achados demonstram uma ligação entre a HHcy leve e severa e o estresse oxidativo em rins, o que pode representar, pelo menos em parte, um dos importantes mecanismos que contribuem para o risco de dano renal durante a HHcy.

ABSTRACT

Oxidative stress has been considered an important part in the etiopathogenesis of cardiovascular and brain changes caused by hyperhomocysteinemia (HHcy), a condition characterized by elevated plasma levels of homocysteine (Hcy), an amino acid derived from methionine. Mild HHcy occurs when Hcy plasma levels range from 15 to 30 µmol/L and may result from deficiency of folic acid, vitamin B_6 and/or vitamin B_{12}. Severe HHcy is characterized by plasma Hcy levels above 100 µmol/L and is found in classical homocystinuria (HCU), an inborn error of metabolism characterized by a deficiency of the enzyme cystathionine β-sinatse (CBS), which catalyzes the Hcy transsulfuration pathway. Since recent studies demonstrate that HHcy is also associated with impaired renal function, the hypothesis of this study is that oxidative stress is also involved in the renal changes caused by Hcy. Thus, the aim of this study was to investigate the effect of Hcy administration on the formation of reactive oxygen species (ROS), lipid peroxidation, protein damage and formation of nitric oxide (NO), in addition to evaluating the enzymatic and nonenzymatic antioxidant defenses in rat kidneys. To test the hypothesis that possible changes in oxidative/nitrative status in kidneys of hyperhomocysteinemic rats could lead to renal failure, serum parameters of renal function (creatinine, urea, uric acid and albumin) were also tested. Rats were subjected to experimental models of mild and severe HHcy developed in our research group. In the experimental model of chronic mild HHcy, adult rats received daily subcutaneous injection of Hcy from the 30^{th} to the 60^{th} day-of-age. The dose was 0.03 µmol Hcy/g body weight and induced plasma Hcy levels of about 30µM. The control group received saline and the animals were decapitated 12 hours after the last injection. An experimental model of acute mild HHcy was also performed and was induced by a single subcutaneous injection of Hcy in rats of 29 days, using the same dose. The rats were decapitated 1 or 12 hours after injection. In the experimental model of chronic severe HHcy, neonatal rats received daily subcutaneous injection of Hcy from the 6^{th} to the 28^{th} day-of-age. The doses used were 0.3 to 0.6 µmol Hcy/g body weight and elevated plasma concentrations of Hcy to about 500µM. The control group received saline and the animals were decapitated 1 or 12 hours after the last injection. The results obtained from the model of mild HHcy demonstrate that chronic Hcy administration increased superoxide dismutase (SOD) and catalase (CAT) activities and NO production (measured as nitrite levels) in kidneys, as well as reduced serum albumin levels at 12 hours after the last injection. Induction of acute mild HHcy increased dichlorofluorescein (DCF) and nitrite levels in rat kidneys at 1 hour after injection. The results of the model of chronic severe HHcy show a significant increase in DCF and thiobarbituric acid reactive substances (TBARS) levels at 1 and 12 hours after the last injection, suggesting that Hcy increased ROS levels and lipid peroxidation in rat kidneys, respectively. The increase in SOD activity and the reduction in CAT activity were also observed at 1 and 12 hours. Moreover, the reduction of non-enzymatic antioxidant defenses and increase in nitrite levels were only observed at 1 hour after the last administration of Hcy. The chronic administration of high doses of the amino acid still increased urea levels and decreased albumin levels at 12 hours after the last injection. These findings demonstrate a link between severe and mild HHcy and oxidative stress in the kidneys, which may represent, at least in part, one of the important mechanisms that contribute to the risk of kidney damage during HHcy.

LISTA DE ABREVIATURAS

-SH – grupamento sulfidrila

5-MTHF – 5-metil tetrahidrofolato

AdoHcy – *S*-adenosil homocisteína

AdoMet – *S*-adenosil metionina

ATP – trifosfato de adenosina (adenosine triphosphate)

BCS – barreira cérebro-sangue

CAT – catalase

CBS – cistationina β-sintase

DCF – diclorofluoresceína

ERO – espécies reativas de oxigênio

GP_X – glutationa peroxidase

GR – glutationa redutase

GSH – glutationa (forma reduzida)

GSSG – dissulfeto de glutationa (forma oxidada)

H_2O_2 – peróxido de hidrogênio

HCU – homocistinúria

Hcy – homocisteína

HHcy – hiper-homocisteinemia

$OH^{\bullet}$ – radical hidroxila

MMP-9 – metaloproteinase de matriz 9

MS – metionina sintase

MTHR – 5, 10-metilenotetra-hidrofolato redutase

NADPH – nicotinamida adenina dinucleotídeo fosfato (forma reduzida)

$NADP^+$ – nicotinamida adenina dinucleotídeo fosfato (forma oxidada)

NF-κB – fator nuclear κB

NMDA – *N*-metil-D-aspartato

NO – óxido nítrico

$O_2^{\bullet-}$ – ânion superóxido

$OONO^-$ – peroxinitrito

SNC – sistema nervoso central

SOD – superóxido dismutase

TBARS – substâncias reativas ao ácido tiobarbitúrico

TRAP – potencial antioxidante total (total reactive antioxidant potential)

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I. INTRODUÇÃO

1. Estresse oxidativo

1.1. Conceito

Um radical livre é qualquer espécie de existência independente que contenha um ou mais elétrons desemparelhados (Halliwell, 1987), enquanto que as espécies reativas de oxigênio (ERO) são átomos, íons ou moléculas que contêm oxigênio com um elétron não pareado em sua órbita externa, sendo caracterizadas por grande instabilidade e elevada reatividade (Halliwell e Gutteridge, 2007). As ERO compreendem todos os radicais do oxigênio, como o ânion radical superóxido ($O_2^{\bullet -}$), radical hidroxila ($OH^{\bullet}$), radical alquila ($L^{\bullet}$), alcoxila ($LO^{\bullet}$) e peroxila ($LOO^{\bullet}$). O peróxido de hidrogênio (H_2O_2), o oxigênio singlete ($^1O_2$) e o ozônio (O_3) não são radicais livres, mas podem induzir reações radicalares no organismo, sendo também considerados espécies reativas (Porter, 1984; Benzie, 1996; Patel, 1999).

As ERO são produzidas normalmente por células aeróbicas como um produto da respiração mitocondrial, porém existe na célula um balanço com as defesas antioxidantes. Uma condição de estresse oxidativo ocorre quando esse balanço é rompido devido à depleção de antioxidantes, um excesso de acúmulo de ERO ou ambos. Tal estresse ocorre quando condições ambientais ou fisiológicas severamente adversas oprimem os sistemas biológicos. Assim, um rápido e claro indicador de estresse oxidativo é a indução de defesas antioxidantes e/ou aumento dos níveis de ERO, as quais podem causar danos celulares severos, levando à disfunção fisiológica e morte celular (Dalton et al., 1999).

1.2. Implicações biológicas

Os efeitos lesivos causados pelas ERO incluem danos às membranas ou outras estruturas lipídicas celulares, modificação nas proteínas e dano ao DNA (Halliwell e Gutteridge, 2007), sendo que os danos oxidativos induzidos nas células e tecidos têm sido relacionados com a etiologia de várias doenças, incluindo cardiopatias, aterosclerose, problemas pulmonares, câncer, doenças hepáticas e envelhecimento (Ames et al., 1993; Witzum, 1994; Roy e Kulkarni, 1996; Stahl e Sies, 1997; Esterbauer et al., 1992; Chirico et al., 1993; Moriel et al., 1999; Chisolm e Steinberg, 2000).

A peroxidação lipídica ou lipoperoxidação é um processo caracterizado pelo ataque de ERO aos lipídeos presentes nas membranas celulares, desintegrando-as e permitindo a entrada dessas espécies no interior da célula (Halliwell e Gutteridge, 2007). Dessa forma, a membrana plasmática é considerada uma das estruturas celulares mais suscetíveis às ERO, visto que as alterações na estrutura e na permeabilidade das membranas celulares levam à perda da seletividade na troca iônica e liberação do conteúdo de organelas, culminando com a morte celular (Ferreira e Matsubara, 1997).

Proteínas também são bastante suscetíveis ao ataque de ERO devido à sua abundância nas células e no plasma, e à sua rápida taxa de reação com muitos radicais e com outros oxidantes (Hawkins at al., 2009). As ERO podem levar à oxidação de resíduos de aminoácidos, formação de ligações cruzadas entre proteínas e fragmentação das mesmas, levando a uma perda funcional (Berlett e Stadtman, 1997; Poon et al., 2004). O H_2O_2, em particular, tem a capacidade de provocar a oxidação de alguns resíduos específicos de aminoácidos, como também a quebra da cadeia polipeptídica ao atacar resíduos de prolina, aspartato e glutamato (Stadtman, 1993; Levine et al., 1994). Além disso, o dano às proteínas pode ocorrer através de reações com aldeídos (4-hidroxi-2-nonenal, malondialdeído) produzidos durante a lipoperoxidação (Uchida e Stadtman,

1993; Kristal e Yu, 1992). Aminoácidos contendo grupamentos sulfidrila (–SH) são alvos suscetíveis para uma variedade de pró-oxidantes, sendo que marcadores detectáveis de oxidação protéica incluem mudanças no conteúdo de tióis (Aksenova e Markesbery, 2001).

Já os danos ao DNA ocasionados pelos radicais livres desempenham um importante papel nos processos de mutagênese e carcinogênese (Poulsen et al., 1998) e incluem a clivagem de ligações fosfodiéster, modificação dos açúcares, ligação cruzada entre fita dupla e simples e ainda oxidação e/ou modificação das bases do DNA, sendo que essas últimas lesões citadas são as mais envolvidas com formação e propagação do câncer (Ribeiro et al., 2006).

1.3. Defesas antioxidantes

Para impedir os danos gerados pelos radicais livres, o organismo desenvolveu vários mecanismos de defesa capazes de neutralizar os radicais livres. Entre os mecanismos antioxidantes não-enzimáticos presentes nas células destacam-se as vitaminas C e E, carotenóides, flavonóides, pigmentos biliares, urato e o tripeptídeo glutationa (GSH), sendo todos captadores de radicais. A GSH é o maior componente não-protéico contendo grupamento –SH e é o maior antioxidante intracelular hidrofílico (Melov, 2002). A GSH tem diversas funções biológicas, incluindo o metabolismo de nutrientes e a regulação de eventos celulares, tais como a expressão de genes, síntese de DNA e proteínas, proliferação celular e apoptose, transdução de sinal, produção de citocinas e resposta imune, e glutationilação protéica. A GSH também tem o papel de manter os grupamentos –SH das proteínas de membrana na sua forma reduzida, visto que sua oxidação pode alterar a estrutura e função celular (Wu et al., 2004).

Para proteger a célula contra o ataque de radicais livres, a glutationa peroxidase celular (GPx) utiliza a GSH como um cofator para reduzir o H_2O_2 e hidroperóxidos orgânicos. A reação leva à redução de um peróxido em duas moléculas de H_2O e une oxidativamente duas moléculas

de glutationa liberando o dissulfeto de glutationa (GSSG) (Heverly-Coulson e Boyd, 2010):

$$H_2O_2 + 2GSH \longrightarrow GSSG + 2H_2O$$

A glutationa redutase (GR) também tem um importante papel na manutenção do *status* oxidativo da célula, sendo responsável pela regeneração da glutationa à sua forma reduzida (Becker et al., 1995):

$$GSSG + NADPH + H \longrightarrow 2GSH + NADP^+$$

Além da GPx e da GR, outras enzimas têm a função de proteger as células aeróbicas dos danos causados pelas ERO. A superóxido dismutase (SOD) e a catalase (CAT) são umas das mais importantes enzimas antioxidantes (Halliwell e Gutteridge, 2007; Fisher-Wellman et al., 2009).

A molécula da SOD citoplasmática contém átomos de cobre e zinco (Cu/Zn-SOD), enquanto que a SOD mitocondrial contém manganês (Mn-SOD) (Scandalios, 2005), sendo que a função de ambas as formas é a dismutação do $O_2^{-\bullet}$ em H_2O_2, cuja reação é representada da seguinte forma (Fattman et al., 2003):

$$O_2^{-\bullet} + O_2^{-\bullet} + 2H^+ \longrightarrow O_2 + H_2O_2$$

Embora o H_2O_2 não seja um radical livre, pode ser rapidamente convertido no radical $OH^{\bullet}$, o qual é muito reativo (Fattman et al., 2003). Assim, outra importante enzima do sistema antioxidante é a CAT, a qual é abundantemente, mas não exclusivamente, localizada nos peroxissomas, onde se encontra uma alta quantidade de H_2O_2. A CAT é capaz de degradar rapidamente o H_2O_2 através da seguinte reação (Scandalios, 2005):

$$2\ H_2O_2 \longrightarrow 2\ H_2O + O_2$$

2. Homocisteína

2.1. Metabolismo

A homocisteína (Hcy) é um aminoácido não essencial, formado a partir do metabolismo da metionina (Finkelstein et al., 1971), sendo essa oriunda de fontes alimentares ou do catabolismo de proteínas endógenas.

A primeira etapa do metabolismo da metionina é caracterizada pela sua conversão em S-adenosilmetionina (AdoMet) pela ação da enzima metionina adenosil transferase (MAT) (Mudd, 1973). A AdoMet é o mais importante doador de grupamentos metil em diversas reações biológicas (DNA, proteínas, neurotransmissores, hormônios, fosfolipídeos) (Chiang, 1998), sendo que o produto de sua desmetilação é denominado S-adenosilhomocisteína (AdoHcy). A AdoHcy sofre então uma reação de hidrólise gerando adenosina e Hcy (Richards et al., 1978; Doskeland e Ueland, 1982; Fujioka e Takata, 1981), a qual pode ser metabolizada por duas vias diferentes: a via da remetilação ou a via da transsulfuração (Selhub, 1999).

A via metabólica de remetilação é catalisada pela metionina sintase (MS), uma enzima dependente de vitamina B_{12} (cobalamina) que transefere um grupamento metil para a Hcy, regenerando a metionina. O grupamento metil para a formação de metionina é proveniente do 5-metil tetrahidrofolato (5-MTHF), que por sua vez, é formando durante a metabolização do ácido fólico. A remetilação da Hcy também pode ocorrer através da ação da enzima betaína Hcy metiltransferase, que catalisa a transeferência do grupo metila da betaína para a Hcy, formando dimetil glicina e metionina (Klee et al., 1961; Finkelstein e Martin, 1984).

Alternativamente, a Hcy pode ingressar na via de transulfuração e se condensar com a serina para formar cistationina, através da ação da cistationina β-sintase (CBS, E.C. 4.2.1.22), uma enzima dependente de vitamina B_6 (piridoxal fosfato). A cistationina sofre então, a ação da

γ-cistationase (também dependente de vitamina B_6), formando α-cetobutirato e cisteína (Mudd et al., 2001). Esse processo ocorre principalmente no fígado, rim, intestino delgado e pâncreas.

A rota da transulfuração da Hcy é considerada um importante suprimento de gutationa para o fígado, visto que forma cisteína, precursor da glutationa, a qual consiste em um antioxidante não-enzimático muito importante (Beatty e Reed, 1980; Mosharov et al., 2000).

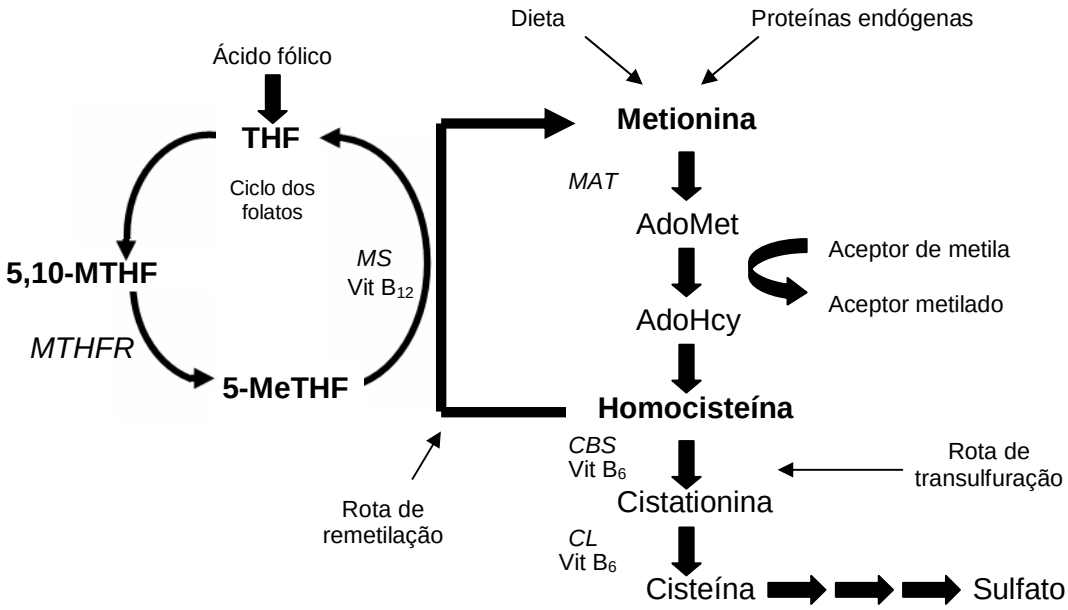

Figura 1. Metabolismo da homocisteína (adaptado de Mudd et al., 2001).

MAT – metionina adenosil transferase; CBS – cistationina β-sintase; CL – cistationina γ-liase; MS – metionina-sintase; MTHFR – metileno tetrahidrofolato redutase; AdoMet – *S*-adenosil metionina; AdoHcy – *S*-adenosil homocisteína; THF – tetrahidrofolato; 5,10-MTHF – 5,10-metileno-tetrahidrofolato.

2.2. Regulação do metabolismo

A CBS é regulada alostérica e positivamente pela AdoMet, o que contribui para a depleção de um excesso de Hcy, quando os níveis plasmáticos de metionina estão altos (Selhub e

Miller, 1992). Já a enzima 5,10-metilenotetra-hidrofolato redutase (MTHR), que cataliza a conversão de 5,10-metilenotetra-hidrofolato à 5-MTHF, é inibida alostericamente pela AdoMet e regulada positivamente pela AdoHcy (Selhub e Miller, 1992; Castro et al., 2006). Assim, níveis elevados de metionina previnem a formação de 5-MTHF e, indiretamente, a síntese de metionina a partir de Hcy (Selhub e Miller, 1992). Sabe-se também, que a Adomet é capaz de inibir e betaína Hcy metiltransferase, favorecendo a via de transulfuração (Fowler, 1997; Finkelstein, 1998).

Ainda, estudos demonstraram o envolvimento de estrógenos na ativação da via de transsulfuração da Hcy, o que justifica os menores níveis plasmáticos desse aminoácido em mulheres quando comparados aos de indivíduos do sexo masculino. Essa afirmação também justifica o aumento da concentração plasmática de Hcy após a menopausa, quando há uma redução dos níveis de estrógenos (Dimitrova et al., 2002).

Fatores nutricionais também estão envolvidos na regulação do metabolismo da Hcy, visto que deficiências de ácido fólico, vitamina B_{12} e vitamina B_6, e a conseqüente redução da atividade das enzimas envolvidas no metabolismo da Hcy, reduzem a eliminação da mesma, elevando seus níveis plasmáticos (Durand et al., 2001; Jacques et al., 1999; Selhub et al., 1993).

Por fim, condições oxidativas favorecem a via da transulfuração para a formação de cisteína e glutationa (Mosharov et al., 2000). O estresse oxidativo diminui a remetilação e aumenta a transulfuração, mantendo o *pool* intracelular de glutationa (auto-regulação) (Finkelstein, 1998).

2.3. Distribuição plasmática

A Hcy se distribui no plasma sob diversas formas: Hcy livre; associada à dissulfetos; formas oxidadas; e Hcy-tiolactona (Svardal et al., 1986; Jakubowski, 2002; 2008a). Sob

condições fisiológicas, menos de 1% da Hcy total está presente na forma livre. Cerca de 10–20% da Hcy total está presente em diferentes formas oxidadas, tais como o dissulfeto Hcy-Cys e homocistina (dímero de Hcy). A grande maioria da Hcy plasmática está ligada à albumina e γ-globulinas (Jakubowski, 2002).

2.4. Homocisteína e o estresse oxidativo/nitrativo

A Hcy é prontamente auto-oxidada quando adicionada ao plasma, levando à formação de homocistina, Hcy associada à dissulfetos e Hcy tiolactona. Durante a oxidação do grupamento –SH da Hcy, um $O_2^{\bullet-}$ e um H_2O_2 são gerados. O $OH^{\bullet}$ também pode ser gerado pela reação de Fenton, quando o H_2O_2 recebe outro elétron e um íon hidrogênio. Dessa forma, o $O_2^{\bullet-}$ e o $OH^{\bullet}$ podem iniciar um processo de peroxidação lipídica sobre as células (Loscalzo, 1996).

Além disso, há dados na literatura que apontam a produção de ERO induzida pela Hcy via ativação da NADPH oxidase (Pin-Lan et al., 2007), a qual é uma enzima associada à membrana que gera $O_2^{\bullet-}$ através da transferência de elétrons do NADPH para o O_2. O complexo enzimático é composto por duas subunidades citosólicas (p47phox e p67phox), duas subunidades ligadas à membrana (gp91phox ou Nox2 e p22phox) e uma GTPase (geralmente Rac1 ou Rac2) (Babior, 1999), sendo que a subunidade catalítica gp91phox, na qual o NADPH e o O_2 se ligam, pode ser substituída pelos homólogos Nox1 ou Nox4 dependendo do tecido.

Além disso, acredita-se que compostos contendo grupamento –SH, como a Hcy, diminuem os níveis de óxido nítrico (NO) bioativo através da reação do $O_2^{\bullet-}$ com o NO, resultando na geração do peroxinitrito ($OONO^-$). Assim, apesar da produção aumentada de NO após exposição à Hcy, menos NO bioativo está disponível devido à sua inativação pela produção de $O_2^{\bullet-}$ durante a oxidação da Hcy (Loscalzo, 1996).

3. Hiper-homocisteinemia

O termo hiper-homocisteinemia (HHcy) não define uma condição patológica, mas sim uma anormalidade bioquímica caracterizada pelo aumento dos níveis plasmáticos de Hcy, a qual sendo citotóxica, é exportada para fora da célula e se torna detectável no plasma (Lentz e Haynes, 2004). As concentrações plasmáticas normais de Hcy variam de 5 a 14 µmol/L, sendo que a HHcy pode ser classificada em leve (15-30 µmol/L), moderada (31-100 µmol/L) e severa (>100 µmol/L) (Hansrani et al., 2002).

3.1. Hiper-homocisteinemia leve

A HHcy leve pode resultar de diferentes fatores, incluindo dano renal, deficiências genéticas heterozigóticas da CBS ou MTHFR e deficiências de ácido fólico, vitamina B_{12} e/ou vitamina B_6, visto que são cofatores necessários para o metabolismo da Hcy (Zhang et al., 2009; Righetti et al., 2003; Zoungas et al., 2006).

Dados mostram que a HHcy leve é encontrada em mais de 40% dos pacientes com doenças cardiovasculares (Durand et al., 2001; Refsum et al., 1998; Selhub et al., 1993) e que uma redução dos níveis plasmáticos de Hcy pode prevenir em mais de 25% esses eventos (Nallamothu et al., 2002; Ueland et al., 2000; Wald et al., 2002). Além disso, evidências de estudos epidemiológicos sugerem um aumento no risco de desenvolver trombose venosa diante de níveis elevados de Hcy plasmática (Den Heijer et al., 1998; Langman et al., 2000). Dessa forma, a HHcy leve tem sido descrita como um importante fator de risco para doenças cardiovasculares (Diaz-Arrastia, 2000; Mattson et al., 2002; Sachdev, 2004), sendo que a importância da Hcy como um fator de risco é aproximadamente equivalente ao fumo ou à hiperlipemia (Boushey et al., 1995; Graham et al., 1997). Concentrações plasmáticas levemente

elevadas de Hcy também são consideradas um fator de risco tanto para doença de Alzheimer quanto para demência vascular, já que muitos estudos têm mostrado associações entre o declínio cognitivo e a HHcy (Morris et al., 2007; Hin et al., 2006). Dessa forma, tais evidências sugerem que a Hcy pode ser um marcador que predispõe ou está relacionada ao prognóstico de doenças neurodegenerativas.

3.2. Hiper-homocisteinemia severa

A HHcy severa ocorre na homocistinúria (HCU), que consiste em uma elevação anormal do dímero homocistina na urina e é causada por desordens genéticas autossômicas recessivas. A HCU clássica é causada por uma deficiência genética homozigótica na enzima CBS, tem incidência em neonatos de um para 344.000 no mundo e eleva os níveis séricos de Hcy para mais de 100 μmol/L (McKusick, 1992). Já a deficiência na enzima MTHFR é uma causa genética mais comum de HCU (McKusick, 1992) e envolve uma variação na posição 677 no gene da MTHFR, no qual a citosina é trocada por uma timina (assim denominada C677T ou 677C>T) (Wald et al., 2002).

A HHcy severa encontrada em pacientes afetados pela HCU clássica é acompanhada por diversas anormalidades em diferentes sistemas e órgãos, incluindo os sistemas nervoso e vascular, ossos e olhos. Outros tecidos, tais como fígado, cabelo, pele e rins, também podem ser comprometidos (Malloy et al., 1981; Sen et al., 2010).

Os achados mais consistentes na HCU clássica compreendem a disfunção neurológica, que pode levar ao retardo mental e convulsões, e as alterações cardiovasculares, sendo que a maior causa de morbidade nessa condição é o tromboembolismo (Mudd et al., 2001).

Alterações freqüentes também incluem o descolamento da retina, que leva ao desenvolvimento de miopia e subluxação ocular, e alterações ósseas, tais como osteoporose e

desenvolvimento de ossos longos. Escoliose também é freqüente, mas como uma conseqüência da osteoporose na coluna (Mudd et al., 2001).

3.3. Ações tóxicas da homocisteína

Muitas teorias para explicar a toxicidade da Hcy têm sido elaboradas ao longo dos últimos anos, mas apesar dos esforços nenhuma está completamente compreendida. Uma das primeiras hipóteses sugere que as potentes ERO, como o H_2O_2, o $O_2^{\bullet-}$ e o $OH^{\bullet}$, formadas como produto das reações redox envolvendo o grupo tiol da Hcy, fossem responsáveis pela toxicidade desse composto. Porém, verificou-se que a produção de ERO induzida pela Hcy também pode ocorrer através da ativação da NADPH oxidase e da xantina oxidase, duas importantes enzimas pró-oxidantes que geram $O_2^{\bullet-}$ nas células endoteliais (Edirimanne et al., 2007).

Além disso, existem estudos indicando que a Hcy reduz a transcrição do gene que codifica a GPx1 (Lubos et al., 2007) e reduz a SOD ligada à membrana extracelular presente na face luminal do endotélio, levando a uma maior exposição aos efeitos prejudiciais do $O_2^{\bullet-}$ (Yamamoto et al., 2000). Dentre esses efeitos, destaca-se a interação do $O_2^{\bullet-}$ com o NO, produzindo o ânion $ONOO^-$. O consumo de NO através dessa reação explicaria a alteração do tônus vascular observado durante a HHcy. Além disso, o $ONOO^-$ é responsável pela indução da tromboxano A2 sintase, tanto em células endoteliais como em plaquetas, levando à vasconstrição (Ungvari et al., 2000; Bagi et al., 2002).

Ainda, a alta produção de $O_2^{\bullet-}$ mediada pela Hcy, parece ser responsável pela ativação do fator nuclear κB (NF-κB) (Au-Yeung et al., 2004), o qual induz a expressão de genes que codificam proteínas pró-inflamatórias como a proteína 1 quimioatraente de monócitos, molécula-1 de adesão celular vascular, molécula-1 de adesão intracelular, E-selectina e receptor para produtos finais de glicação avançada em células endoteliais (Poddar et al., 2001; Hofmann et al.,

2001; Carluccio et al., 2007; Hwang et al., 2008). Assim, a Hcy aumenta a ligação de monócitos ao endotélio vascular, um evento chave na formação de placa aterosclerótica (Dalal et al., 2003; Zeng et al., 2003).

Estudos *in vitro* sugerem que um mecanismo adicional para a toxicidade exercida pela Hcy seria através da promoção da oxidação de partículas de LDL, as quais são altamente pró-aterogênicas quando modificadas, visto que se ligam a receptores *scavenger* expressos por monócitos, formando células espumosas (Exner et al., 2002; Griffiths et al., 2006). Além disso, a Hcy parece estimular o crescimento da camada muscular vascular e reduzir o crescimento das células endoteliais (Austin et al., 2004).

Uma teoria recente também sugere que a toxicidade da Hcy é uma conseqüência de uma ligação covalente desse composto a proteínas, modificando assim, suas funções. Esse processo é chamado de homocisteinilação e pode ser S-homocisteinilação, quando a Hcy se liga através de uma ligação dissulfídica a proteínas livres, ou N-homocisteinilação, quando a Hcy se liga através de uma ligação amida a um grupo ε amino de um resíduo de lisina de uma proteína (Barbato et al., 2007).

A Hcy se liga através de ligações dissulfídicas à fibronectina, anexina II e metalotioneína intracelular, entre outras proteínas (Majors et al., 2002; Hajjar et al., 1998; Barbato et al., 2007). Já a N-homocisteinilação é uma conseqüência de uma alta reatividade da Hcy-tiolactona a aminoácidos livres, especialmente ao grupo ε amino de resíduos de lisina de proteínas (Jakubowski, 1997; 1999). *In vivo*, a Hcy-tiolactona modifica a albumina sanguínea, hemoglobina e imunoglobulinas, lipoproteínas de baixa (LDL) e alta (HDL) densidade, transferrina, antitripsina e fibrinogênio (Jakubowski, 2002; 2008b). Nesse contexto, tem sido demonstrado que proteínas modificadas através de N-homocisteinilação podem atuar como neo-antígenos, levando à ativação de uma resposta inflamatória, um componente chave na

aterogênese, aterotrombose e na etiologia do acidente vascular cerebral (Undas et al., 2004).

Quanto aos seus efeitos neurotóxicos, estudos *in vitro* e *in vivo* têm demonstrado que a Hcy induz dano neural através de excitotoxicidade e apoptose. Esses eventos poderiam ser, ao menos parcialmente, uma conseqüência da inabilidade do tecido cerebral em metabolizar a Hcy, favorecendo a acúmulo desse aminoácido no sistema nervoso central (SNC) (Finkelstein, 1998).

A Hcy pode levar ao rompimento da barreira cérebro-sangue (BCS) (Kamath et al., 2006), já que é capaz de aumentar a atividade da metaloproteinase 9 de matriz (MMP-9) e diminuir os níveis de inibidor de metaloproteinase 4 de matriz (Moshal et al., 2006; Tyagi et al., 2010). Como conseqüência, a MMP-9 atua sobre diferentes componentes da BCS, levando ao rompimento de sua estrutura. Uma segunda possibilidade seria o fato de a Hcy atuar como um neurotransmissor excitatório, levando a um aumento da permeabilidade vascular (Tyagi et al., 2005; 2007).

A Hcy atua como um agonista tanto de receptores metabotrópicos de glutamato dos grupos I e III, quanto de receptores para N-metil-d-aspartato (NMDA) (Boldyrev e Johnson, 2007; Bleich et al., 2004; Ho et al., 2002). A superestimulação desses receptores leva a pulsos de cálcio citoplasmático, alta produção de radicais livres e ativação de caspases, levando à apoptose. Outra conseqüência da exposição do SNC à Hcy é a ativação da poli-ADP-ribose polimerase em neurônios, levando a um consumo de ATP e NAD^+, causando prejuízo ao metabolismo energético, seguido da produção de radicais livres (Kruman et al., 2000).

Neste contexto, nosso grupo tem mostrado que a HHcy severa altera a captação de glutamato, induz estresse oxidativo e altera o metabolismo energético em cérebro de ratos (Machado et al., 2011; Streck et al., 2003a; 2003b).

3.5. Hiper-homocisteinemia e a doença renal

Visto que os rins possuem a habilidade de excretar metabólitos e que estudos prévios

identificaram os rins como o maior sítio para a depuração da Hcy plasmática, esse órgão pode se tornar suscetível aos efeitos tóxicos causados por esse aminoácido (Bostom et al., 1995).

Sustentando essa hipótese, estudos experimentais demonstraram que a exposição a altos níveis de Hcy causa danos glomerulares e intersticiais, os quais são marcadamente proporcionais à concentração sérica desse aminoácido (Arnadottir et al., 1996). Embora o efeito direto da Hcy sobre os rins ainda não esteja bem elucidado (Ninomiya et al., 2004), alguns mecanismos têm sido sugeridos. O principal deles é o aumento na produção de $O_2^{\bullet-}$ nos rins diante de altos níveis de Hcy, o que pode ocorrer via ativação da NADPH oxidase (Yi et al., 2006).

O envolvimento da NADPH oxidase e consequente estresse oxidativo nos rins foram demonstrados em vários estudos. Ratos alimentados com uma dieta deficiente em ácido fólico para desenvolver HHcy, por exemplo, exibiram um aumento da expressão de receptores NMDA associado a um aumento de $O_2^{\bullet-}$ oriundo da atividade da NADPH oxidase no glomérulo, o que foi acompanhado por glomeruloesclerose notável. O tratamento com um antagonista de receptores NMDA inibiu significativamente a produção de $O_2^{\bullet-}$ induzida pela HHcy, reduzindo o dano glomerular. Assim, suporta-se a ideia de que os receptores NMDA podem mediar a ativação da NADPH oxidase nos rins durante a HHcy e ter um papel importante no desenvolvimento de glomeruloesclerose (Zhang et al., 2010).

Há uma forte evidência de que o aumento da produção de $O_2^{\bullet-}$ esteja envolvido na redução da disponibilidade de NO bioativo (Tolins e Shultz, 1994). Sabe-se que a associação de uma alta taxa de síntese de NO a uma alta produção de $O_2^{\bullet-}$, induz uma rápida formação de $OONO^-$ (Ishii et al., 2001), que é responsável pela nitração de resíduos de tirosina proteicos, consistente com o aumento de proteínas renais nitrotirosinadas observadas em ratos com HHcy (Baylis et al., 1996).

Estudos *in vitro* em células mesangiais evidenciaram que a HHcy crônica, além de induzir um aumento da subunidade Gp91 da NADPH oxidase, eleva os níveis de RNAm do inibidor de

metaloproteinase 1, levando a um acúmulo de colágeno I acompanhado por um aumento da proliferação celular. Esse efeito poderia ter um papel importante na deposição de colágeno ou de componentes da matriz extracelular nos glomérulos durante a HHcy (Yang e Zou, 2003).

Com relação à avaliação da função renal, estudos demonstraram que ratos Sprague-Dawley uninefrectomizados tratados com metionina para gerar HHcy, apresentaram uma alteração na excreção de albumina urinária na segunda semana de tratamento e uma redução no *clearance* de creatinina após seis semanas de tratamento (Yi et al., 2007).

Ratos heterozigotos para CBS (CBS+/–) também apresentaram redução da filtração glomerular. Houve ainda aumento da expressão de moléculas inflamatórias e da molécula 1 de adesão de célula vascular, assim como o aumento da infiltração de macrófagos, sugerindo que o estresse oxidativo induzido pela HHcy causa remodelação da matriz extracelular e inflamação, resultando em glomeruloesclerose e redução da função renal (Sen et al., 2010).

3.6. Medidas de prevenção da hiper-homocisteinemia leve e tratamento da hiper-homocisteinemia severa

O estado nutricional deficiente em ácido fólico e vitaminas B_{12} e B_6 é a causa mais comum da HHcy leve. Dessa forma, o consumo de alimentos fontes dessas vitaminas constitui um método simples, eficaz e econômico para prevenir a HHcy (Vannucchi e Melo, 2009). Além disso, demonstrou-se que indivíduos que praticam exercício físico regularmente, parecem ter seus níveis plasmáticos de Hcy significativamente reduzidos (Ali et al., 1998).

No que se refere à HHcy severa que ocorre na HCU clássica, o principal tratamento inclui a restrição de metionina na dieta para reduzir o acúmulo de metionina, Hcy e seus metabólitos, além da suplementação com L-cistina para elevar os níveis de cisteína (Svardal et al., 1986).

Uma suplementação com ácido fólico pode ajudar a reduzir os níveis de Hcy por ativar

sua remetilação (De Bree et al., 1997). Uma suplementação adicional com vitamina B_{12} é recomendada para evitar a deficiência de ácido fólico e dar o suporte para a utilização de folato (visto que o folato fica "preso" na forma de 5-MTHF na deficiência de vitamina B_{12}) (Shane e Stokstad, 1985). A suplementação com vitamina B_{12} também é recomendada para prevenir o dano neurodegenerativo.

Em 1967, Barber e Spaeth reportaram que três pacientes com deficiência na CBS responderam a altas doses de piridoxina (250 a 500 mg por dia), visto que é um importante cofator na via de transulfuração (Tada et al., 1967). Porém, verificou-se que nem todos os pacientes com deficiência na CBS respondem ao tratamento com vitamina B_6. Estudos sustentam a hipótese de que a resposta ao tratamento com piridoxina pode ser determinada por propriedades específicas da enzima mutante, sendo que existe uma forte correlação entre a presença de atividade residual da CBS e uma resposta clínica à vitamina B_6, e entre a ausência de atividade residual da enzima e a falha na resposta ao tratamento em questão (Mudd et al., 1995). Demonstrou-se também que o tratamento com betaína pode ser associado à terapêutica de pacientes que respondem ao tratamento com vitamina B_6.

Certas drogas (análogos de adenosina, d-penicilamina N-acetilcisteína, estrógenos e tamoxifeno) também podem ser utilizadas, pois reduzem a concentração de Hcy plasmática através de diferentes mecanismos (por exemplo, remetilação e inibição da atividade da hidrolase de AdoHcy) (Pezzini et al., 2007).

II. OBJETIVOS

Objetivos gerais

No sentido de ampliar o conhecimento sobre as alterações bioquímicas envolvidas na patogênese da disfunção renal causada pela HHcy, este trabalho teve como objetivos gerais investigar o efeito da administração de Hcy sobre alguns parâmetros de estresse oxidativo e nitrativo, bem como verificar o efeito da administração de Hcy sobre parâmetros séricos de função renal em ratos.

Objetivos específicos

Capítulo I

Avaliar os efeitos da HHcy leve crônica e aguda sobre parâmetros de estresse oxidativo/nitrativo denominados diclorofluoresceína (DCF), substâncias reativas ao ácido tiobarbitúrico (TBARS), conteúdo de grupamentos –SH e níveis de nitritos, bem como enzimas antioxidantes (SOD e CAT), em rins de ratos. Para testar a hipótese de que a HHcy leve causa disfunção renal, também foram avaliados os níveis de creatinina, uréia, ácido úrico e albumina no soro.

Capítulo II

Investigar os efeitos da HHcy severa crônica sobre parâmetros de estresse oxidativo denominados DCF, TBARS, conteúdo de grupamentos –SH, potencial antioxidante total (TRAP) e níveis de nitritos, bem como enzimas antioxidantes (SOD e CAT), em rins de ratos. Os níveis de uréia e albumina no soro também foram avaliados.

III. METODOLOGIA E RESULTADOS

MODELOS EXPERIMENTAIS

Os capítulos I e II serão apresentados na forma de manuscritos científicos, os quais apresentam os modelos experimentais de HHcy leve e HHcy severa utilizados nesta dissertação.

Hiper-homocisteinemia leve crônica: ratos Wistar adultos receberam injeções subcutâneas diárias de Hcy do 30º ao 60º dia de vida. A dose utilizada foi de 0,03 μmol Hcy/g de peso corporal e induzia concentrações plasmáticas de Hcy de cerca de 30μM. O grupo controle recebeu salina e os animais foram decapitados 12 horas após a última injeção.

Hiper-homocisteinemia leve aguda: a HHcy leve aguda foi induzida através de uma única injeção subcutânea de Hcy em ratos Wistar de 29 dias, utilizando-se a dose de 0,03 μmol Hcy/g de peso corporal. O grupo controle recebeu salina e os ratos foram decapitados 1 ou 12 horas após a injeção.

Hiper-homocisteinemia severa crônica: ratos Wistar neonatos receberam injeções subcutâneas diárias de Hcy do 6º ao 28º dia de vida. As doses utilizadas foram de 0,3 a 0,6 μmol Hcy/g de peso corporal e elevavam as concentrações plasmáticas de Hcy para cerca de 500μM. O grupo controle recebeu salina e os animais foram decapitados 1 ou 12 horas após a última injeção.

Os demais itens da metodologia estão inseridos nos manuscritos científicos.

Capítulo I

MANUSCRITO 1

Homocysteine levels considered a risk factor to neurodegenerative disorders alter oxidative/nitrative status in kidneys and reduce serum albumin levels in rats.

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Homocysteine levels considered a risk factor to neurodegenerative disorders alter oxidative/nitrative status in kidneys and reduce serum albumin levels in rats

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Hyperhomocysteinemia levels considered a risk factor to neurodegenerative disorders alters oxidative/nitrative status in kidneys and reduces serum albumin levels in rats

Abstract

Objective: Mild hyperhomocysteinemia has been considered a risk factor for cardiovascular and neurodegenerative diseases. However, it has been also reported that this condition may cause renal injury, whose mechanisms are still not well elucidated. Thus, in the present study we evaluated the effect of chronic and acute mild hyperhomocysteinemia on the activities of the antioxidants enzymes superoxide dismutase (SOD) and catalase (CAT), as well as on other parameters of oxidative/nitrative damage, namely 2′7′ dichlorofluorescein fluorescence assay (DCF), thiobarbituric acid-reactive substances (TBARS), sulfhydryl content and nitrite levels in kidneys of rats. To test the hypothesis that mild hyperhomocysteinemia causes renal dysfunction we also tested creatinine, urea, uric acid and albumin in serum.

Design: For chronic treatment, Wistar rats received daily subcutaneous injections of homocysteine (0.03 μmol/g body weight) from the 29^{th} to the 60^{th} days-of-age and control group received saline. Rats were sacrificed at 12 h after last injection. For acute treatment, rats of 29 days-of-age received a single subcutaneous injection of homocysteine (0.03 μmol/g body weight) or saline (control). Rats were sacrificed at 1 or 12 h after injection.

Results: Chronic mild hyperhomocysteinemia increased activities of antioxidant enzymes and nitrite levels while acute mild hyperhomocysteinemia increased only DCF and nitrite levels in kidneys at 1 h. Chronic Hcy treatment also decreased serum albumin levels, but did not alter other renal function parameters.

Conclusion: Our findings demonstrate that mild hyperhomocysteinemia induces oxidative/nitrative stress in kidney, which may represent one of the mechanisms that contribute to renal injury during mild hyperhomocysteinemia.

Keywords: Kidney; mild hyperhomocysteinemia; nitrative stress; oxidative stress; renal function parameters.

1. Introduction

Hyperhomocysteinemia, a condition of elevated plasma homocysteine (Hcy) levels, is often classified as mild (15-30 μM), moderate (31-100 μM) and severe (>100 μM)[1]. During the last years, mild hyperhomocysteinemia has received a great deal of attention as a risk factor for atherosclerosis, cardiovascular, cerebral vascular and neurodegenerative diseases[2-7]. However, it has been also reported that mild hyperhomocysteinemia is a common finding among patients with renal failure[8-9]. Although renal dysfunction or failure is known to be an important factor causing hyperhomocysteinemia, the effects of Hcy at abnormally high concentrations on the kidney are not clear.

Preclinical studies show that Hcy, at slightly elevated levels, induces renal function alterations in male adult rats[10]. The ability of the kidney in performing the excretion of metabolites could predispose this organ to changes caused by toxic effects of Hcy. Previous works identified the kidney as a major site for the disappearance and metabolism of plasma Hcy; approximately 20% of the arterial plasma Hcy is removed on passage through the kidney [11].

The underlying mechanism by which Hcy exerts its toxic effects on different organs remains unexplained; however, some studies have suggested the role of oxidative damage, which is defined as a serious imbalance between production of reactive species and antioxidant defenses, and could result from diminished levels of antioxidants and/or increased production of reactive species[12-14]. Studies show that hyperhomocysteinemia is associated with oxidative stress in tissues such as brain, heart and lung [15-20].

Since experimental evidence supports the role of oxidants not only in inducing tubulointerstitial damage which accompanies progression, but also in the early stages of

glomerular disease, our hypothesis is that oxidative stress is also involved in the renal injury during mild hyperhomocysteinemia[21].

Therefore, in the present study we evaluated the effect of chronic and acute mild hyperhomocysteinemia on the activities of the antioxidants enzymes superoxide dismutase (SOD) and catalase (CAT), as well as on other parameters of oxidative and nitrative damage, namely 2′7′ dichlorofluorescein fluorescence assay (DCF), thiobarbituric acid-reactive substances (TBARS), sulfhydryl content and nitrite levels in kidneys of rats. To test the hypothesis that mild hyperhomocysteinemia leads to renal dysfunction, we also tested the levels of creatinine, urea, uric acid and albumin in serum.

2. Materials and Methods

2.1 Animals and reagents

Thirty Wistar rats were obtained from the Central Animal House of the Departamento de Bioquímica, Instituto de Ciências Básicas da Saúde, Universidade Federal do Rio Grande do Sul, Porto Alegre, RS, Brazil. Animals were maintained on a 12/12 h light/dark cycle in an air-conditioned constant temperature (22±1^{o}C) colony room. Rats had free access to a 20% (w/w) protein commercial chow and water. The NIH "Guide for the Care and Use of Laboratory Animals" (NIH publication No. 80-23, revised 1996) and the official governmental guidelines in compliance with the Federação das Sociedades Brasileiras de Biologia Experimental were followed in all experiments. All chemicals were obtained from Sigma Chemical Co., St. Louis, MO, USA.

2.2 Chronic homocysteine treatment

D,L-Hcy were dissolved in 0.9% NaCl solution and buffered to pH 7.4. Hcy solution was administered subcutaneously twice a day from their 29th to their 60th day-of-age. During the treatment, animals received 0.03 μmol Hcy/g body weight. Plasma Hcy concentration in rats subjected to this treatment achieved levels similar to those considered as a risk factor for cardiovascular and neurodegenerative diseases (15-30 μmol/L)[22]. Control animals received saline solution in the same volumes as those applied to Hcy-treated rats. The rats were sacrificed by decapitation without anesthesia 12 h after the last injection. Kidneys and blood were quickly removed.

2.3 Acute homocysteine treatment

D,L-Hcy were dissolved in 0.9% NaCl solution and buffered to pH 7.4. Rats at the age of 29 days received a single subcutaneous injection of Hcy solution (0.03 μmol Hcy/g body weight) or saline (control). The plasma Hcy concentration of rats subjected to this treatment reached levels similar to those considered as a risk factor for cardiovascular and neurodegenerative diseases. The rats were sacrificed by decapitation without anesthesia 1 or 12 h after injection. Kidneys and blood were quickly removed.

2.4 Tissue preparation

Kidneys were homogenized in 10 volumes (1:10, w/v) of 20 mM sodium phosphate buffer, pH 7.4, containing 140 mM KCl. The homogenate was centrifuged at 750 *g* for 10 min at 4^{o}C. The pellet was discarded and the supernatant was immediately separated and used for the measurements.

2.5 Serum samples

Blood was sampled for determination of serum creatinine, urea, uric acid and albumin levels. After standing for 30 min at 4°C, blood was centrifuged at 500 *g* for 15 min to obtain serum.

2.6 Superoxide dismutase assay

SOD activity assay is based on the capacity of pyrogallol to autoxidize, a process highly dependent on superoxide, which is the substrate for SOD. The inhibition of the autoxidation of this compound occurs in the presence of SOD, whose activity can be then indirectly assayed at 420 nm using the SpectraMax M5/M5 Microplate Reader (Molecular Devices, MDS Analytical Technologies, Sunnyvale, California, USA)[23]. A calibration curve was performed with purified SOD as standard, in order to calculate the activity of SOD present in the samples. The results are reported as units/mg protein.

2.7 Catalase assay

CAT activity was assayed using SpectraMax M5/M5 Microplate Reader (Molecular Devices, MDS Analytical Technologies, Sunnyvale, California, USA). The method used is based on the disappearance of hydrogen peroxide (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[24]. One CAT unit is defined as one μmol of H_2O_2 consumed per minute and the specific activity is calculated as pmol/mg protein.

2.8 2′7′ dichlorofluorescein fluorescence assay

Reactive species production was measured following a method described by Lebel and collaborators[25] based on the oxidation of 2′7′-dichlorofluorescein (H_2DCF). Tissue supernatant (60 µL) was incubated for 30 min at 37°C in the dark with 240 µL of 100 µM 2′7′-dichlorofluorescein diacetate (H_2DCF-DA) solution in a 96-well plate. H_2DCF-DA is cleaved by cellular esterases and the resultant H_2DCF is eventually oxidized by the Reactive Oxygen Species (ROS) present in samples. The last reaction produces the fluorescent compound, dichlorofluorescein (DCF), which was quantified following 488 nm excitation and 525 nm emission, where results are represented by nmol DCF/mg protein.

2.9 Thiobarbituric acid-reactive substances

TBARS were measured according to Ohkawa and collaborators[26]. Briefly, the following reagents were added (in this order) to glass tubes: 200 µL of tissue supernatant; 20 µL of SDS 8.1%; 600 µL of 20% acetic acid in aqueous solution (v/v) pH 3.5; 600 µL of 0.8 % thiobarbituric acid. The mixture was vortexed and the reaction was carried out in a boiling water bath for 1 h. The tube was then allowed to cool on water for 5 min, and was centrifuged at 1000 *g* for 10 min. The resulting pink stained TBARS were determined spectrophotometrically at 535 nm in a Beckman DU® 800 (Beckman Coulter, Inc., Fullerton, CA, USA). A calibration curve was generated using 1,1,3,3-tetramethoxypropane as a standard. TBARS were calculated as nmol TBARS/mg protein.

2.10 Sulfhydryl content

This assay is based on the reduction of 5,5'-dithiobis-(2-nitrobenzoic acid) (DTNB) by thiols, which in turn become oxidized (disulfide), generating the yellow derivative

thionitrobenzoic acid (TNB) whose absorption is measured spectrophotometrically at 412 nm[27]. Briefly, 50 mL of homogenate were added to 1 mL of PBS buffer pH 7.4 containing 1 mM EDTA. Then 30 mL 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 DU1 640 spectrophotometer. The sulfhydryl content is inversely correlated to oxidative damage to proteins. Results were reported as nmol TNB/mg protein.

2.11 Nitrite assay

Nitrite levels were measured using the Griess reaction; 100 µL of supernatant of kidney was mixed with 100 µL Griess reagent (1:1 mixture of 1% sulfanilamide in 5% phosphoric acid and 0.1% naphthylethylenediamine dihydrochloride in water) and incubated in 96-well plates for 10 min at room temperature. The absorbance was measured on a microplate reader (SpectraMax M5/M5 Microplate Reader - Molecular Devices, MDS Analytical Technologies, Sunnyvale, California, USA) at a wavelength of 543 nm. Nitrite concentration was calculated using sodium nitrite standards[28].

2.12 Protein determination

Protein concentration was measured by the method of Lowry and collaborators[29] using bovine serum albumin as standard.

2.13 Biochemical parameters assays

Creatinine, urea, uric acid and albumin levels were analyzed using Labtest kits (Labtest Diagnóstica SA, Lagoa Santa, MG, Brazil). The biochemical parameters were run on a Labmax 240.

2.14 Statistical determination

Data were analyzed by the Student`s *t* test for unpaired samples and One-way ANOVA followed by the Tukey test, when F-test was significant. All analyses were performed using the Statistical Package for the Social Sciences (SPSS) software in a PC-compatible computer. Differences were considered statistically significant if $p<0.05$.

3.0 Results

Firstly, we evaluated the effect of chronic mild hyperhomocysteinemia on parameters of oxidative/nitrative stress in kidneys of rats. Figure 1 shows that this condition significantly increased the activity of the antioxidant enzymes SOD [A: $t(6)=3.406$; $p<0.05$] and CAT [B: $t(6) = 5.087$; $p<0.01$]. On the other hand, chronic mild hyperhomocysteinemia did not alter reactive species production (as measured by DCF) [control: 56.09 ± 1.84; Hcy-treated: 55.65 ± 2.67; $t(6) = 0.329$; $p>0.05$], oxidative lipid damage (as measured by TBARS) [control: 3.04 ± 0.62; Hcy-treated: 3.00 ± 0.61; $t(6) = 0.092$; $p>0.05$] as well as sulfhydryl content [control: 42.44 ± 2.85; Hcy-treated: 39.78 ± 2.99; $t(6) = 1.694$; $p>0.05$]. The next step was investigating the effect of chronic mild Hcy administration on kidneys nitrite levels and, as can be observed in figure 2, this parameter was significantly increased by Hcy administration [$t(6) = 4.909$; $p<0.01$].

It has been previously described that in rats subjected to mild hyperhomocysteinemia, Hcy is present in the plasma until 1h, returning to baseline levels 12 h after the last injection of

this amino acid[22]. On this basis, we also investigated the effect of acute mild Hcy treatment at 1 and 12 h after Hcy administration with the objective to evaluate if the effects caused by Hcy on the kidneys depend on the presence or not of this amino acid.

Differently of chronic treatment, acute mild hyperhomocysteinemia did not alter SOD [control: 2.97 ± 0.27; Hcy-treated 1h: 2.69 ± 0.29; Hcy-treated 12 h: 2.88 ± 0.24; $F(2,15) = 1.940$; $p>0.05$] and CAT activity [control: 3.18 ± 1.00; Hcy-treated 1h: 2.63 ± 0.91; Hcy-treated 12 h: 2.70 ± 0.70; $F(2,15) = 0.551$; $p>0.05$] in kidneys of rats, while the evaluation of reactive species production (figure 3) demonstrates that acute Hcy treatment significantly increased DCF levels at 1 h [$F(2,15) = 5.05$; $p<0.05$], but not at 12 h [$F(2,15) = 5.05$; $p>0.05$]. On the other hand, slightly elevated levels of Hcy did not lead to lipoperoxidation process [control: 1.51 ± 0.29; Hcy-treated 1h: 1.41 ± 0.27; Hcy-treated 12 h: 1.58 ± 0.20; $F(2,15) = 0.698$; $p>0.05$] and did not alter sulfhydryl content [control: 41.95 ± 5.18; Hcy-treated 1h: 43.76 ± 4.96; Hcy-treated 12 h: 45.74 ± 4.07; $F(2,15) = 1.694$; $p>0.05$] in acute administration. Lastly, Figure 4 shows that mild hyperhomocysteinemia significantly increased nitrite levels in kidneys at 1 h after acute Hcy treatment [$F(2,15) = 7.87$; $p<0.01$], but not at 12 h [$F(2,15) = 7.87$; $p>0.05$].

In order to investigate if the changes on the oxidative/nitrative status observed in kidneys could lead to a renal dysfunction, we also tested some biochemical parameters in serum of rats and observed that chronic Hcy treatment was not able to alter serum creatinine [control: 0.37 ± 0.04; Hcy-treated: 0.39 ± 0.02; $t(6) = 0.586$; $p>0.05$], urea [control: 45,67 ± 6.71; Hcy-treated: 41.17 ± 9.26; $t(6) = 0.964$; $p>0.05$] and uric acid levels [control: 0.89 ± 0.17; Hcy-treated: 0.92 ± 0.23; $t(6) = 0.317$; $p>0.05$], but figure 5 shows a decrease in serum albumin levels [$t(6) = 2.255$; $p<0.05$].

4.0 Discussion

Several factors are known to lead to increased plasma concentrations of Hcy, including genetic, hormonal and dietetic deficiencies. Slight enhance in plasma Hcy levels may be a consequence of deficiency of folate, cobalamin and/or vitamin B_6, which are cofactors for Hcy metabolism[30]. Mild hyperhomocysteinemia has been described as prevalent in the general population and is considered an independent risk factor for atherosclerosis in the coronary, cerebral, and peripheral circulations[31-33]. Nevertheless, results from animal studies have also suggested that hyperhomocysteinemia may cause renal injury[34-36], but mechanisms are still not well elucidated.

Some previous works show that mild hyperhomocysteinemia developed evidence of oxidative stress in rabbit liver, in rat brain and serum and in cultured endothelial cells[37-40]. On this basis, the aim of this study was evaluating the oxidative effect of mild hyperhomocysteinemia in kidneys of rats.

Using an experimental model developed in our laboratory, we firstly evaluated the effect of chronic mild hyperhomocysteinemia on the activities of the antioxidant enzymes SOD and CAT. SOD is one of the primary antioxidant in a network of detoxification enzymes that neutralizes the highly reactive superoxide ions to less reactive H_2O_2 followed by its immediate conversion to water by CAT and other peroxidases[41]. In our study we found an increase in the activities of these enzymes in kidneys of rats submitted to chronic Hcy treatment. In agreement, a previous study showed that patients with inherited defects of Hcy metabolism had an increase in the activities of antioxidant enzymes in the circulation, when total plasma Hcy levels were higher than 20 μM[42]. In addition, animals exposed to chronic mild hyperhomocysteinemia were found to present an imbalance between SOD and CAT activities, expressed as an increased SOD/CAT ratio in erythrocytes and cerebral cortex[22].

Since antioxidants are defined as any substance that significantly delays or prevents the oxidation of that substrate[43]; we propose that oxidative stress and the necessity of protection against oxidative damage may be responsible, at least partially, for the elevation in the activity of these enzymes induced by Hcy. However, we believe that, in the present study, the response of the antioxidant detoxification system was enough to reduce ROS levels in kidneys of rats submitted to chronic hyperhomocysteinemia, since DCF assay was no altered.

In the other side, we observed that acute Hcy treatment enhanced DCF levels at 1 h after the injection of this amino acid, but did not alter the activities of antioxidant enzymes neither at 1 and 12 h. We believe that SOD and CAT were only changed in the chronic treatment because theses modifications may represent a cell adaptation in face of a constant physiological stress, in this case, by production of reactive species.

The increase in ROS levels may be a consequence of the free or reduced sulfhydryl group of Hcy to be highly reactive at physiological pH, and in the presence of molecular oxygen, Hcy undergoes thiol oxidation reactions. ROS such as superoxide anion and H_2O_2 are formed during auto-oxidation of Hcy[44]. It has been suggested that auto-oxidation of Hcy represents one of the important mechanisms contributing to Hcy-induced cell damage.

Another hypothesis for this ROS production is the NADPH oxidase activation, which is a membrane-associated enzyme that catalyzes the production of superoxide[48]. The kidney expresses all components of the phagocyte NADPH oxidase (gp91phox or Nox-2) as well as NADPH oxidase homologues Nox-1 and Nox-4[49]. Previous works have demonstrated that increased plasma Hcy levels induce NADPH oxidase activity, accompanied by marked glomerular injury[50].

Since lipid peroxidation is an indicative of ROS generation, the next step of this study was investigating the effect of chronic and acute Hcy administration on TBARS levels. It has been

previously reported that Hcy, at severely elevated levels, contributes to ischemiareperfusion-induced lipid peroxidation in the kidney[51]. Other works have also shown that severe hyperhomocysteinemia induces lipid peroxidation in extrarenal tissues, such as lung[20], heart[19], liver[52] and hippocampus[16]. Besides, chronic mild hyperhomocysteinemia increased TBARS levels in plasma and cerebral cortex[22]. However, our results showed that this parameter was not altered by mild Hcy administration neither at the chronic and acute treatment in kidneys of rats. It is probably that acute treatment was not able to cause excessive oxidation necessary to lipoperoxidation. In this context, in the chronic treatment, an increased activity of antioxidant enzymes, which scavenge ROS, could prevent this process.

In order to verify if sulfhydryl containing amino acid residues in renal proteins of rats submitted to mild hyperhomocysteinemia were affected by pro-oxidants effects, we evaluated the protein-bound sulfhydryl status. Although it was observed indication of oxidative stress generation, our results showed no alterations in sulfhydryl content in kidneys of rats submitted to this model, neither at chronic and acute treatment.

The activity of nitric oxide (NO) was reflected in the measurement of its oxidation end product nitrite. In the present study, there was a significant increase in the levels of nitrites in kidneys of rats during chronic mild hyperhomocysteinemia and 1 h after acute treatment. In agreement, data from literature showed that acute severe hyperhomocysteinemia increases nitrite levels in the hippocampus, cerebral cortex and serum of rats[53]. On the other hand, chronic severe hyperhomocysteneimeia was not able to alter nitrite levels in lung of rats[20]. Further analysis reveals that the activation of a transcription factor called nuclear factor-κB (NF-κB) is involved in Hcy-induced inducible nitric oxide synthase expression in the kidney[36] and activation of NF-κB has been shown to be involved in the induction of glomerulosclerosis[54-55]. In addition, this finding allows us to conclude that the rise in nitrite levels could also be involved in the increased

CAT activity seen during chronic mild hyperhomocysteinemia, since another study demonstrated that NO can enhance the activity of this antioxidant enzyme[56].

In order to test the hypothesis that these changes on the oxidative/nitrative status observed in kidneys of hyperhomocysteinemic rats could lead to a nephropathy process, we assessed some biochemical parameters that are usually altered during renal dysfunction. Creatinine and urea have been described since 1904 and 1952, respectively[57-58], and are still the "gold standard" for renal function avaliation. This study demonstrated no alterations in these parameters, not showing an apparent loss of renal function by mild hyperhomocysteinemia. Uric acid, on the other hand, is well known to be an independent predisposing factor of renal dysfunction[59]. However it has been also reported that renal dysfunction promotes increased serum concentration of uric acid[60]. In accordance with the above results, we demonstrated here that mild hyperhomocysteinemia was not able to alter this parameter in rats.

Lastly, we evaluated serum albumin levels in rats with mild hyperhomocysteinemia and observed that this parameter was reduced under this condition. One explanation for this finding may be the increase in permeability of the renal glomerulus to proteins, which can lead to proteinuria and hypoalbuminemia[61]. Works described in literature which show that total Hcy is associated with microalbuminuria in population-based studies hold this hypothesis[62-64]. Besides, this study showed that mild hyperhomocysteinemia altered redox status in kidneys of rats and it is known that ROS play a role in a variety of renal diseases which can contribute to the loss of albumin in the urine[65-66]. However, further studies are necessary to confirm this supposition.

Although in the present work, rats subjected to treatment did not present an apparent alteration in renal clearance, the small changes in ROS production and increased antioxidant activity may represent only an initial response to hyperhomocysteinemia. Therefore, the persistence of chronic hyperhomocysteinemia could result in a pathological condition in which

the antioxidant enzymes would no longer be able to prevent oxidative stress. Thus, a prolonged chronic treatment with Hcy may be necessary to observe changes in the levels of creatinine, urea and uric acid.

5.0 Practical Application

In summary, chronic mild hyperhomocysteinemia increased CAT and SOD activity and nitrite levels, while acute mild hyperhomocysteinemia increased reactive species (measured by DCF) and nitrite levels. Chronic Hcy treatment also decreased serum albumin levels in rats. Therefore, the present study demonstrates a link between mild hyperhomocysteinemia and oxidative/nitrative stress in the kidney, which may contributes to expand the knowledge about the biochemical changes involved in the risk of renal dysfunction during mild hyperhomocysteinemia.

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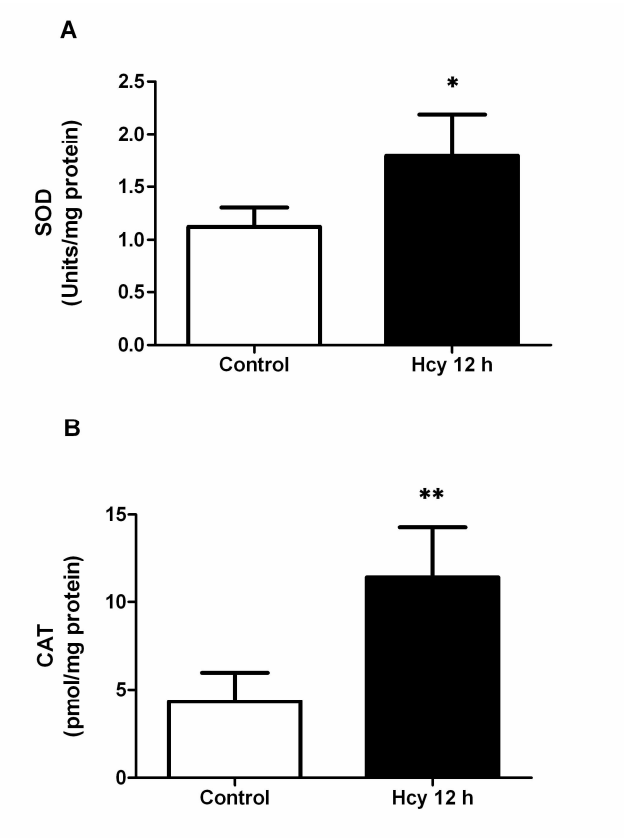

Figure 1. Effect of chronic mild Hcy administration on superoxide dismutase (A) and catalase (B) activity in the kidneys of rats. Results are expressed as means ± SD for six animals in each group. Different from control, $^{*}p<0.05$; $^{**}p<0.01$ (Student's *t*-test). Hcy: homocysteine.

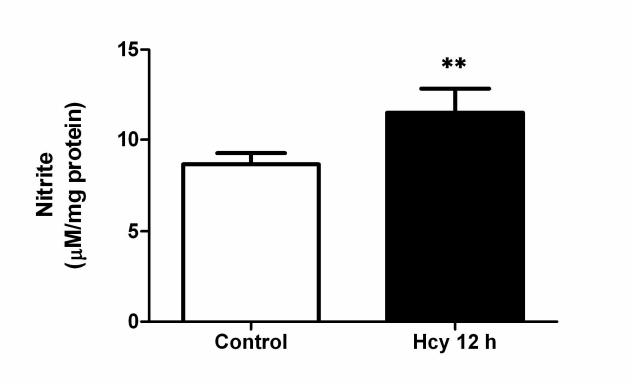

Figure 2. Effect of chronic mild Hcy administration on nitrite levels in the kidneys of rats. Results are expressed as means ± SD for six animals in each group. Different from control, $^{**}p<0.01$ (Student's *t*-test). Hcy: homocysteine.

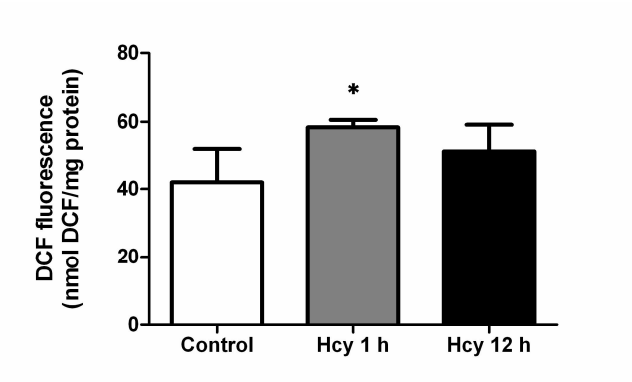

Figure 3. Effect of acute mild Hcy administration on ROS production in the kidneys of rats. Results are expressed as means ± SD for six animals in each group. Different from control, $^{*}p<0.05$ (One-way ANOVA and Tukey test). Hcy: homocysteine.

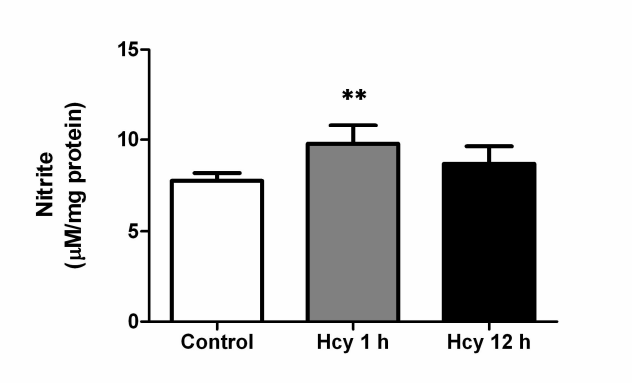

Figure 4. Effect of acute mild Hcy administration on nitrite levels in the kidneys of rats. Results are expressed as means ± SD for six animals in each group. Different from control, $^{**}p<0.01$ (One-way ANOVA and Tukey test). Hcy: homocysteine.

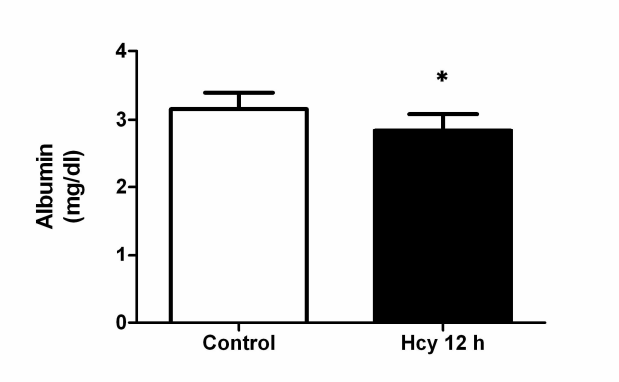

Figure 5. Effect of chronic mild Hcy administration on albumin levels in serum of rats. Results are expressed as means ± SD for six animals per group. Different from control, $^{*}p<0.05$ (Student's *t*-test). Hcy: homocysteine.

Capítulo II

MANUSCRITO 2

Induction of oxidative/nitrative stress in kidney and changes in albumin and urea levels in serum of rats subjected to severe hyperhomocysteinemia.

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INDUCTION OF OXIDATIVE/NITRATIVE STRESS IN KIDNEY AND CHANGES IN ALBUMIN AND UREA LEVELS IN SERUM OF RATS SUBJECTED TO SEVERE HYPERHOMOCYSTEINEMIA

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Abstract

Purpose: Since severe hyperhomocysteinemia can induce renal injury and oxidative stress is associated with renal dysfunction, in the present study we investigated the effects of chronic homocysteine administration on some parameters of oxidative stress namely 2′7′ dichlorofluorescein fluorescence assay (DCF), thiobarbituric acid-reactive substances (TBARS), sulfhydryl content, total radical-trapping antioxidant potential (TRAP) and antioxidant enzymes (superoxide dismutase and catalase), as well as nitrite levels in kidneys of rats. The levels of urea and albumin in serum were also evaluated.

Methods: Wistar rats received daily subcutaneous injections of homocysteine (0.3-0.6 µmol/g body weight) from the 6^{th} to the 28^{th} days-of-age and control group received saline. Rats were sacrificed at 1 or 12 h after the last injection; kidneys and blood were collected.

Results: Results showed that severe hyperhomocysteinemia significantly increased DCF and TBARS at 1 and 12 h after last injection, suggesting that homocysteine increased reactive species production and lipid peroxidation in kidneys of rats. Increase in superoxide dismutase activity and decrease in catalase activity were also observed at 1 and 12 h after the last injection. Decrease in non-enzymatic antioxidant defenses (TRAP) and increase in nitrite levels were observed only at 1 h after the last administration of homocysteine. Homocysteine administration also increased urea and decreased albumin levels at 12 h after injection.

Conclusions: Our findings showed a profile of oxidative stress in kidneys of rats, elicited by homocysteine, which could explain, at least partly, the mechanisms involved in the renal damage present in some homocystinuric patients.

K**eywords:** Severe hyperhomocysteinemia; albumin; urea; serum; kidney; oxidative/nitrative stress.

1. Introduction

Homocysteine (Hcy), a sulfur-containing amino acid, is derived from the intracellular metabolism from the indispensable amino acid methionine[1]. Factors that are responsible for the elevation of plasma Hcy levels include genetic deficiencies such as classical homocystinuria, an inborn error of metabolism characterized biochemically by cystathionine β-synthase (EC 4.2.1.22) deficiency[2]. Affected patients by this disease can present mental retardation, seizures and vascular complications[2-4]. It has also been reported that severe hyperhomocysteinemia causes remodeling of extracellular matrix and inflammation in the kidneys[5], which has been shown to be the major site for the removal of plasma Hcy and possesses the enzymes for both remethylation and transulphuration pathway[1, 6]. In addition, a recent epidemiological study revealed a positive association between plasma Hcy levels and the development of chronic renal disease in the general population[7]. The loss of the kidney function may signify a role for this tissue in the metabolic clearance of plasma Hcy, leading to hyperhomocysteinemia[8] that in turn, could compromise renal function[9]. However these mechanisms are still not well elucidated.

Oxidative stress is generated by an imbalance between antioxidant and oxidants, leading to reactive oxygen species (ROS) production and accumulation, which can lead to severe cellular damage, such as physiological dysfunction and cell death. When oxidative stress occurs, cells work to neutralyze the oxidant effects and to restore redox balance by resetting critical homeostatic parameters. Such cellular activity leads to activation or silencing of genes encoding antioxidant enzymes, transcription factors and structural proteins[10]. Previous results showed that Hcy induces oxidative stress in brain of rats, reducing antioxidant defenses and increasing lipid peroxidation[11-14]. In addition, chronic hyperhomocysteinemia decreases antioxidant defenses, increases lipid peroxidation and/or decreases nitrite levels in liver and heart of rats[15-

16]. It is possible that alterations in oxidative/nitrative status could be also involved in the renal injury during hyperhomocysteinemia.

On this basis, in the present study we investigated the effects of chronic Hcy administration on some parameters of oxidative stress namely 2′7′ dichlorofluorescein fluorescence assay (DCF), thiobarbituric acid-reactive substances (TBARS), sulfhydryl content, total radical-trapping antioxidant potential (TRAP) and antioxidant enzymes (superoxide dismutase and catalase), as well as on nitrite levels in kidneys of rats. The levels of urea and albumin in serum were also evaluated. Our hypothesis is that severe hyperhomocysteinemia may alter oxidative/nitrative status in kidney, leading to renal dysfunction.

2. Materials and Methods

2.1 Animals and reagents

Eighteen Wistar rats were obtained from the Central Animal House of the Departamento de Bioquímica, Instituto de Ciências Básicas da Saúde, Universidade Federal do Rio Grande do Sul, Porto Alegre, RS, Brazil. Animals were maintained on a 12/12 h light/dark cycle in an air-conditioned constant temperature ($22^{o}C \pm 1^{o}C$) colony room. Rats had free access to a 20% (w/w) protein commercial chow and water. The NIH "Guide for the Care and Use of Laboratory Animals" (NIH publication No. 80-23, revised 1996) and the official governmental guidelines in compliance with the Federação das Sociedades Brasileiras de Biologia Experimental were followed in all experiments. All chemicals were obtained from Sigma Chemical Co., St. Louis, MO, USA.

2.2 Chronic homocysteine treatment

D,L-Hcy were dissolved in 0.9% NaCl solution and buffered to pH 7.4. Hcy solution was administered subcutaneously twice a day from their 6^{th} to their 28^{th} day-of-age. Hcy doses were calculated from pharmacokinetic parameters previously determined in our laboratory[17-18]. During the first week of treatment, animals received 0.3 μmol Hcy/g body weight. In 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. Plasma Hcy concentration in rats subjected to this treatment achieved levels similar to those found in homocystinuric patients[2]. Control animals received saline solution in the same volumes as those applied to Hcy-treated rats. The rats were sacrificed by decapitation without anesthesia at 1 or 12 h after the last injection. The kidneys and blood were quickly removed.

2.3 Tissue preparation

Kidneys were homogenized in 10 volumes (1:10, w/v) of 20 mM sodium phosphate buffer, pH 7.4, containing 140 mM KCl. The homogenate was centrifuged at 750 *g* for 10 min at 4^{o}C. The pellet was discarded and the supernatant was immediately separated and used for the measurements.

2.4 Serum samples

Blood was sampled for determination of serum urea and albumin levels. After standing for 30 min at 4°C, blood was centrifuged at 500 *g* for 15 min to obtain serum.

2.5 2′7′ dichlorofluorescein fluorescence assay

ROS production was measured following a method described by Lebel and collaborators[19] based on the oxidation of 2′7′-dichlorofluorescein. Tissue supernatant (60 μL) was incubated for 30 min at 37°C in the dark with 240 μL of 100 μM 2′7′-dichlorofluorescein diacetate solution in a 96-well plate. 2′7′-dichlorofluorescein diacetate is cleaved by cellular esterases and the resultant 2′7′-dichlorofluorescein is eventually oxidized by the ROS present in samples. The last reaction produces the fluorescent compound, dichlorofluorescein, which was quantified following 488 nm excitation and 525 nm emission, where results are represented by nmol dichlorofluorescein/mg protein.

2.6 Thiobarbituric acid-reactive substances

Lipid peroxidation was estimated by measuring TBARS in Kidney samples according to a modified method of Jentzsch and collaborators[20]. Briefly, 0.2 mL of tissue supernatant was added to the reaction mixture containing 1 ml of 1% ortho-phosphoric acid and 0.25 ml alkaline solution of thiobarbituric acid (final volume 2.0 ml), followed by 45 min heating at 95^{o}C. After cooling, samples and standards of malondialdehyde were read at 532 nm against the blank of the standard curve. The results were expressed as nmol malondialdehyde/mg protein.

2.7 Sulfhydryl content

This assay is based on the reduction of 5,5'-dithiobis-(2-nitrobenzoic acid) by thiols, which in turn become oxidized (disulfide), generating a yellow derivative namely nitro-5-thiobenzoic acid whose absorption is measured spectrophotometrically at 412 nm[21]. Briefly, 50 mL of homogenate were added to 1 mL of phosphate buffered saline pH 7.4 containing 1 mM ethylenediaminetetraacetic acid. Then 30 mL of 10 mM 5,5'-dithiobis-(2-nitrobenzoic acid),

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 DU1 640 spectrophotometer. The sulfhydryl content is inversely correlated to oxidative damage to proteins. Results were reported as nmol nitro-5-thiobenzoic acid/mg protein.

2.8 Total radical-trapping antioxidant potential

TRAP was determined by measuring the chemiluminescence intensity of luminol induced by 2,2'-azo-bis-(2-amidinopropane) thermolysis in a Perkin-Elmer Microbeta Microplate Scintillation Analyzer (PerkinElmer Life and Analytical Sciences, Waltham, MA, USA)[22]. Two-hundred and forty microliters of a system containing 2,2'-azo-bis-(2-amidinopropane) (10 mM), dissolved in 50 mM sodium phosphate buffer pH 8.6, and luminol (5.6 mM), was added to a microplate, and the initial chemiluminescence was measured. Ten microliters of 300 μM Trolox (water-soluble α-tocopherol analogue, used as a standard) or 10 μl of tissue supernatant was then added to each plate well, producing a decrease in the initial chemiluminescence value. This value is kept low, until the antioxidants present in the sample are depleted, chemiluminescence then returns to its initial value. The time taken by the sample to maintain chemiluminescence low is directly proportional to the antioxidant capacity of the tissue. Results are represented as nmol Trolox/mg protein.

2.9 Superoxide dismutase assay

Superoxide dismutase (SOD) activity measurement is based on the inhibition of the radical superoxide reaction with adrenalin as described by McCord and Fridovich[23]. In this method, SOD present in the sample competes with the detection system for superoxide anion. A

unit of SOD is defined as the amount of enzyme that inhibits the rate of adrenalin oxidation by 50%. Adrenalin oxidation leads to the formation of the colored product, adrenochrome, which is detected by spectrophotometer. SOD activity is determined by measuring the rate of adrenochrome formation, observed at 480 nm, in a reaction medium containing glycine-NaOH (50 mM, pH 10) and adrenalin (1 mM).

2.10 Catalase assay

The determination of catalase (CAT) activity was carried out in accordance with a modified method of Nelson and Kiesow[24]. This assay involves the change in absorbance at 240 nm due to CAT-dependent decomposition of hydrogen peroxide (H_2O_2). The spectrophotometric determination was initiated by the addition of 0.07 ml in an aqueous solution of 0.3 mol/l H_2O_2. The change in absorbance at 240 nm was measured for 2 min. The results were expressed as pmol/mg protein.

2.11 Nitrite assay

Nitrite levels were measured using the Griess reaction; 100 µL of supernatant of kidney was mixed with 100 µL Griess reagent (1:1 mixture of 1% sulfanilamide in 5% phosphoric acid and 0.1% naphthylethylenediamine dihydrochloride in water) and incubated in 96-well plates for 10 min at room temperature. The absorbance was measured on a microplate reader (SpectraMax M5/M5 Microplate Reader - Molecular Devices, MDS Analytical Technologies, Sunnyvale, California, USA) at a wavelength of 543 nm. Nitrite concentration was calculated using sodium nitrite standards[25]. The results were expressed as µM/mg protein.

2.12 Protein determination

Protein concentration was measured by the method of Lowry[26] using bovine serum albumin as standard.

2.13 Urea assay

Urea levels were analyzed by a method using a Labtest-Diagnostic kit. In this method, urea is hydrolyzed by urease to ammonium ions and CO_2. The ammonium ions react in alkaline pH, with sodium hypochlorite and salicylate under the catalytic action of sodium nitroprusside to form indophenol blue. The color formation is proportional to the amount of urea in the sample [kit label]. Urea assay was run on a COBAS MIRA PLUS.

2.14 Albumin assay

Albumin levels were analyzed by a method using a Labtest-Diagnostic kit. In this assay, albumin interacts with buffered bromocresol green and due to the protein error of indicators, there is formation of green color, proportional to the concentration of albumin in the sample [kit label]. Albumin assay was run on a COBAS MIRA PLUS.

2.15 Statistical determination

Data were analyzed by the Student's *t*-test for unpaired samples and One-way ANOVA followed by Tukey test, when the F-test was significant. All analyses were performed using the Statistical Package for the Social Sciences software in a PC-compatible computer. Differences were considered statistically significant if $p<0.05$.

3. Results

ROS production was measured by DCF and renal lipid damage was measured by TBARS. Figure 1 shows that chronic hyperhomocysteinemia significantly increased the levels of reactive species in kidney, as indicated by dichlorofluorescein formed from the oxidation of 2′7′-dichlorofluorescein, at 1 h [$F(2,15) = 15.62$; $p<0.01$] and 12 h [$F(2,15) = 15.62$; $p<0.001$] after the last administration of Hcy. As can be observed in figure 2, Hcy significantly also increased TBARS at 1 h [$F(2,15) = 42.17$; $p<0.001$] and 12 h [$F(2,15) = 42.17$; $p<0.001$] after chronic hyperhomocysteinemia. The effect of chronic hyperhomocysteinemia on sulfhydryl content was also tested; results showed that this parameter was not altered by Hcy administration neither at 1 h [control: 2.47 ± 0.12; Hcy-treated: 2.39 ± 0.18; $F(2,15) = 1.30$; $p>0.05$] and 12 h [control: 2.47 ± 0.12; Hcy-treated: 2.52 ± 0.08; $F(2,15) = 1.30$; $p>0.05$] after the last injection, as compared to the control group.

Next, we evaluated the status of kidney non-enzymatic and enzymatic antioxidant. Figure 3 shows that Hcy administration significantly reduced the total antioxidant potential (TRAP) at 1 h [$F(2,15) = 18.54$; $p<0.001$], but did not alter this parameter at 12 h [$F(2,15) = 18.54$; $p>0.05$] after the last injection. As can be observed in figures 4A and 4B, Hcy significantly increased SOD activity at 1 h [$F(2,15) = 15.88$; $p <0.001$] and 12 h [$F(2,15) = 15.88$; $p<0.05$] and decreased CAT activity at 1 h [$F(2,15) = 30.77$; $p<0.001$] and 12 h [$F(2,15) = 30.77$; $p<0.01$] after the last Hcy administration, respectively.

We also investigated the effect of chronic Hcy administration on kidney nitrite levels. Figure 5 shows that chronic Hcy administration significantly increased nitrite levels at 1 h [$F(2,15) = 16.93$; $p<0.001$], but did not alter this parameter at 12 h [$F(2,15) = 16.93$; $p>0.05$] after the last administration of Hcy.

In order to evaluate the renal dysfunction, we investigated the effect of hyperhomocysteinemia on some renal function parameters of rats sacrificed at 12 h after the last injection of Hcy. Figure 6A shows an increase in serum urea [$t(6) = 2.62$; $p<0.05$], whereas figure 6B shows a reduction in serum albumin levels after chronic Hcy administration [$t(6) = 3.73$; $p<0.01$].

4.0 Discussion

Hyperhomocysteinemia is a consequence of a wide range of determinants: genetic, such as classical homocystinuria; nutritional, such as diet rich in methionine and/or poor in B vitamins; pathological, such as renal disease; or medications, such as antifolate drugs[27-28]. Moreover, increased levels of Hcy also appear to be associated with neurodegenerative and vascular disorders and more recently, with renal injury[9, 28-35].

Since recent studies indicate that elevated plasma total Hcy is independently associated with increased kidney allograft loss in humans[36] and that Hcy induces oxidative stress in other tissues such as brain, liver, heart and endothelium[11, 13-16, 37-38], in the present study we extend our investigation, evaluating the effect of chronic hyperhomocysteinemia on the kidneys of developing rats.

We used an experimental model of chronic hyperhomocysteinemia developed in our group, in which the Hcy levels present a peak in plasma at 15 min up to 1h after injection of this amino acid and returns to baseline levels after 12 h[17-18]. Based on this, we firstly investigated some oxidative stress parameters and observed that Hcy increased DCF and TBARS levels in kidneys of rats at 1 and 12 h after the last injection of this amino acid. These results respectively demonstrate that Hcy promoted ROS production and lipoperoxidation, whilst highlight the endurance of its effects, since these parameters remained altered even after Hcy levels have

returned to normal (12 h after the interruption of treatment). In agreement, data from literature showed that Hcy, at elevated levels, is associated with oxidative stress in the kidney[39]. These changes observed in the present work may be a consequence of the auto-oxidation of Hcy, which leads to a generation of ROS such as anion superoxide radical and H_2O_2[40]. The hydroxyl radical can also be generated by the Fenton reaction, when H_2O_2 receives another electron and a hydrogen ion. Hydroxyl radical is the most reactive radical and may lead to lipid peroxidation process[41], which may cause damage to renal membranes. The ROS production may also occur via NAD(P)H oxidase activation and probably contributes to the Hcy effect on extracellular matrix homeostasis and consequent sclerosis in glomeruli[42].

Since sulfhydryl containing amino acid residues in proteins are susceptible targets for a variety of pro-oxidants, we also evaluated the protein-bound sulfhydryl status. Although sulfhydryl content was decreased in liver of rats submitted to hyperhomocysteinemia model[15], no alterations in the sulfhydryl levels were observed in the kidneys of rats sacrificed at 1 and 12 h after chronic hyperhomocysteinemia. It could be explained by the fact that mammalian cells from different tissues possess a system that regulates redox status of cellular thiols and protects sulfhydryl-containing proteins from excessive oxidation[43]. In addition, there are other detectable markers, including changes in the content of protein carbonyls, nitrotyrosines and advanced glycation end-products that could be explored in the future, to better evaluate protein damage.

The next step was investigating the effect of chronic Hcy administration on TRAP, an index of antioxidant capacity. The results showed a reduction of non-enzymatic defense in kidney of hyperhomocysteinemic rats at 1 h after the last Hcy injection. It shows consistency with the increased renal oxidative stress observed, whereas the tissue becomes more susceptible to attack by ROS. Furthermore, Streck and collaborators have already demonstrated that Hcy *in vitro*

significantly decreases TRAP in rat hippocampus[11] and other works have previously shown that Hcy administration reduces the antioxidant potential in the parietal cortex of rats[13].

We also evaluated the effect of chronic hyperhomocysteinemia on the activities of the antioxidant enzymes SOD and CAT, which compose an efficient system responsible for removing ROS[44-45]. In our study we found an increase in SOD activity in kidney of rats at 1 and 12 h after the last injection of Hcy. Probably it represents an antioxidant response against the possible increase in superoxide radical production, since it is well known that this enzyme catalyzes superoxide radical dismutation[44]. These results are in agreement with a report that suggests an existence of a positive correlation between the dosage of SOD and total Hcy[46].

On the other hand, CAT activity was decreased in this study. It has been previously shown that acute Hcy administration decreases CAT activity in the rat hippocampus and that vitamins E and C completely prevented this effect, indicating that the participation of oxidative stress is probably involved in the actions of Hcy[12]. Whereas it is known that CAT is able to convert H_2O_2 to water and molecular oxygen[45], one explanation for these results is the possibility of early depletion of renal antioxidant reserves, in face the initial stimulus characterized by the increased in SOD activity, which probably elevated H_2O_2 levels. Additionally, the decrease in CAT activity may be explained by the fact that antioxidant enzymes are inhibited by specific ROS[47-48], which are probably formed from Hcy[49].

Nevertheless, it has been reported that Hcy can regulate protein turnover and gene expression, including antioxidant enzymes[50-51]. Therefore, we cannot discard the possibility of these changes in SOD and CAT activities may be caused by these mechanisms.

The reduction in the CAT activity is an important result since it may explain why the levels of TRAP increased back to the control levels at 12 h after injection of Hcy, but ROS and TBARS levels are still significantly higher at 12 h compared to the control group. Although the

levels of TRAP returned to the control levels at 12 h, CAT activity remained reduced at this time point. A possible mechanism to explain these results may be due to the fact that the cellular environment was made more susceptible to the formation of H_2O_2 and oxidative stress generation, increasing DCF levels. Besides, hydroxyl radical can be generated from H_2O_2, leading to lipid peroxidation process and increasing TBARS levels[41].

The activity of nitric oxide (NO) in this study was reflected in the measurement of nitrite, its oxidation end product, which was significantly increased at 1 h after the last administration of Hcy. It has been reported that oxidative stress causes an increase in the production of NO, a molecule which is envolved in both oxidative stress and cell death responses[52]. Previous studies showed that hyperhomocysteinemia activates nuclear factor kappa B in rat kidney by increasing phosphorylation of IκBα. Activation of nuclear factor kappa B causes up-regulation of inducible NO synthase expression leading to an incresead NO production[53]. Thus, since NO stimulates production of tumor necrosis factor-alpha, what could result in inflammatory injury[52], it may play an important role in the renal injury observed during severe hyperhomocysteinemia. In this context, more studies will be necessary to elucidate such mechanisms.

In order to test the hypothesis that these changes on the oxidative/nitrative status observed in kidneys of hyperhomocysteinemic rats could lead to a nephropathy process, we assessed urea and albumin levels in serum of rats. Urea is the main product of protein metabolism, circulates in the blood and is freely filtered at the glomerulus, mostly excreted in the urine; thus renal insufficiency can lead to hyperuremia[54]. Determination of serum urea is not specific to general changes in renal function, but it is more sensitive to changes of the primary kidney conditions, so it is an important and widely used marker in cases involving this condition. Although the kidney glomeruli filter the plasma at a high rate, it produces a filtrate that is nearly protein-free. Lund

and collaborators[55] provided data in rats demonstrating that the albumin glomerular sieving coefficient rises as the glomerular filtration rate falls, thus lowering serum albumin levels. In the present study, we verified that Hcy increased urea levels and decreased albumin levels, which may represent an impaired kidney function. These results corroborate other works described in literature, which show that total Hcy is associated with microalbuminuria in population-based studies[56-58].

In conclusion, chronic hyperhomocysteinemia altered renal function parameters and increased reactive species production, lipid peroxidation and nitrite levels, as well as caused changes in the antioxidant defenses (enzymatic and non-enzymatic). Our findings suggest a consistent profile of oxidative stress in the kidneys of rats, elicited by Hcy, which could contribute to explain, at least in part, the mechanisms involved in the pathogenesis of kidney damage in homocystinuric patients.

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Conflict of Interest statement

The authors declare that there are no conflicts of interest.

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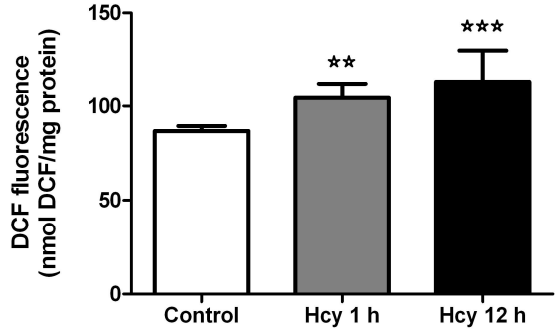

Figure 1. Effect of chronic administration of Hcy on 2',7'-dichlorofluorescein fluorescence assay in the kidneys of rats. Results are expressed as means ± SD for six animals per group. Different from control, $^{**}p<0.01$; $^{***}p<0.001$ (One-way ANOVA and Tukey test). Hcy: homocysteine; DCF: dichlorofluorescein. GraphPad Prism 5 was used to create the artwork.

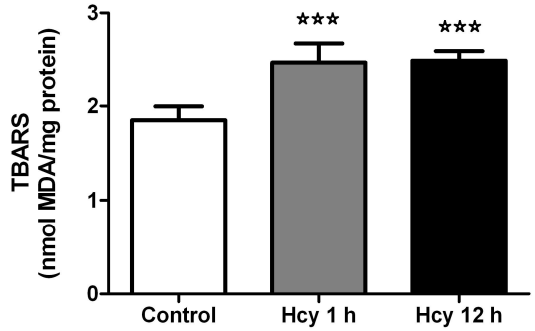

Figure 2. Effect of chronic administration of Hcy on thiobarbituric acid-reactive substances in the kidneys of rats. Results are expressed as means ± SD for six animals per group. Different from control, $^{***}p<0.001$ (One-way ANOVA and Tukey test). Hcy: homocysteine; MDA: malondialdehyde. GraphPad Prism 5 was used to create the artwork.

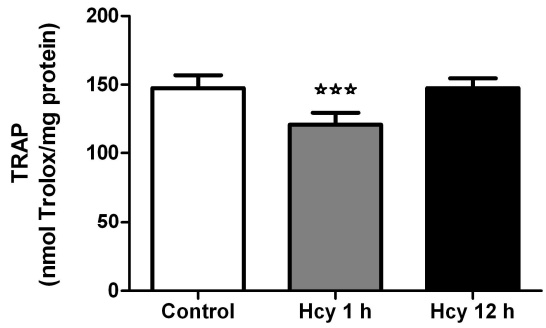

Figure 3. Effect of chronic administration of Hcy on total radical-trapping antioxidant potential in the kidneys of rats. Results are expressed as means ± SD for six animals in per group. Different from control, $^{***}p<0.001$ (One-way ANOVA and Tukey test). Hcy: homocysteine. GraphPad Prism 5 was used to create the artwork.

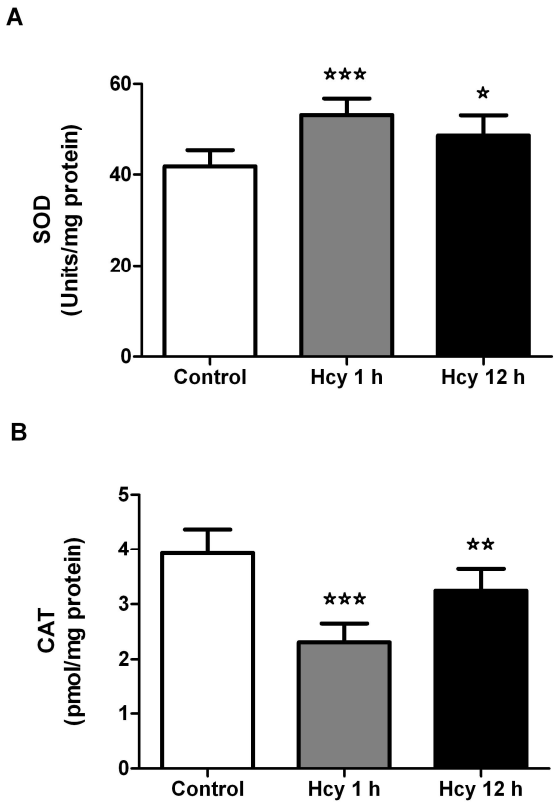

Figure 4. Effect of chronic administration of Hcy on superoxide dismutase (A) and catalase (B) in the kidneys of rats. Results are expressed as means ± SD for six animals per group. Different from control, $^{*}p<0.05$; $^{**}p<0.01$; $^{***}p<0.001$ (One-way ANOVA and Tukey test). Hcy: homocysteine. GraphPad Prism 5 was used to create the artwork.

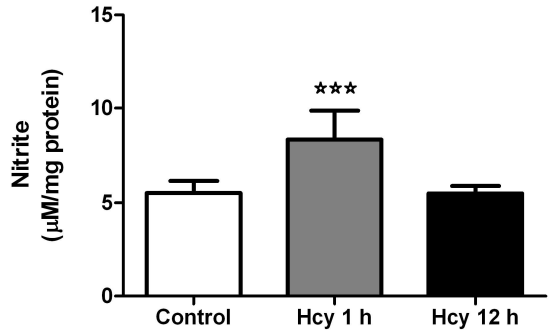

Figure 5. Effect of chronic administration of Hcy on nitrite levels in the kidneys of rats. Results are expressed as means ± SD for six animals per group. Different from control, $^{***}p<0.001$ (One-way ANOVA and Tukey test). Hcy: homocysteine. GraphPad Prism 5 was used to create the artwork.

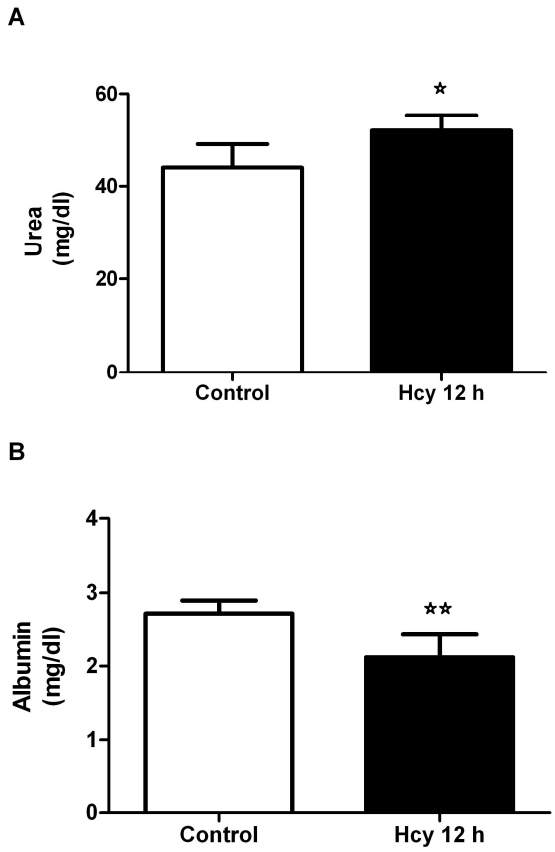

Figure 6. Effect of chronic administration of Hcy on urea (A) and albumin (B) levels in serum of rats. Results are expressed as means ± SD for six animals per group. Different from control, $^{*}p<0.05$; $^{**}p<0.01$ (Student's *t*-test). Hcy: homocysteine. GraphPad Prism 5 was used to create the artwork.

IV. DISCUSSÃO

A HHcy consiste em uma condição clínica caracterizada por elevados níveis plasmáticos de Hcy, sendo freqüentemente classificada em leve (15-30 μM), moderada (31-100 μM) e severa (>100 μM) (Hansrani et al., 2002). Essa condição pode ser uma conseqüência de diversos fatores, como por exemplo: genéticos, tais como a HCU clássica; fisiológicos, tais como dietas ricas em metionina e/ou pobre em vitaminas B; patológicos, tais como doença renal; ou medicamentosos, tais como drogas anti-folato (Perry, 1999; Eikelboom et al., 1999).

A HHcy leve e severa estão associadas ao desenvolvimento de desordens neurodegenerativas e vasculares (Refsum et al., 1998; De Bree et al., 2002; Boers, 2000; Eikelboom et al., 1999; Welch e Loscalzo, 1998; Diaz-Arrastia, 2000; Mattson et al., 2002; Mattson e Haberman, 2003; Veeranna et al., 2011; Sachdev, 2004; Ullegaddi et al., 2006). Entretanto, estudos em animais têm sugerido que a HHcy também pode causar dano renal, mas os mecanismos desse processo ainda necessitam ser elucidados (Kumagai et al., 2002; Li et al., 2002; Zhang et al., 2004).

O objetivo do presente estudo foi investigar o efeito da HHcy leve e severa sobre o *status* redox em rins de ratos, visto que há evidências de que o estresse oxidativo participa da toxicidade exercida pela Hcy em órgãos como o cérebro, fígado, coração e endotélio (Streck et al., 2003a; Matté et al., 2004; 2007; 2009; Kolling et al., 2011; Zhu et al., 2009; Edirimanne et al., 2007; Scherer et al., 2011).

Nosso laboratório desenvolveu modelos experimetais de HHcy leve e severa, sendo que no modelo de HHcy leve utilizaram-se ratos adultos de 29 dias, visto que este modelo mimetiza uma condição em que a HHcy se desenvolve ao longo da vida. No modelo de HHcy severa, utilizaram-se ratos neonatos de 6 dias, já que tinha o objetivo de mimetizar a HCU, a qual sendo

uma deficiência genética, acomete os indivíduos afetados desde o nascimento.

Com base em trabalhos anteriores que mostram que a Hcy está presente no plasma em altos níveis durate 1 hora e retorna aos níveis basais 12 horas após a injeção subcutânea desse aminoácido (Streck et al., 2002a; 2002b; Scherer et al., 2011), desenvolveram-se diferentes modelos experimentais de HHcy nos quais os animais foram decapitados 1 ou 12 horas após a administração de Hcy. A escolha dos pontos de tempo justifica-se pela importância de se avaliar se os efeitos causados pelo tratamento dependem ou não da presença de Hcy no sangue periférico. Dessa forma, no modelo de HHcy leve os animais foram sacrificados 12 horas após a administração crônica de Hcy e 1 ou 12 horas após a administração aguda. Já no modelo experimental de HHcy severa, avaliou-se somente o efeito do tratamento crônico 1 ou 12 horas após a última injeção de Hcy.

Utilizando-se o modelo experimental de HHcy leve, primeiramente se avaliou o efeito do tratamento crônico sobre as atividades das enzimas antioxidantes SOD e CAT. Neste estudo, encontrou-se um aumento da atividade dessas enzimas em rins de ratos submetidos ao tratamento. Em concordância, estudos prévios mostram que pacientes com erros inatos no metabolismo da Hcy possuem um aumento das atividades de enzimas antioxidantes na circulação quando os níveis plasmáticos de Hcy ultrapassam 20 μM (Moat et al., 2000). Além disso, animais expostos à HHcy leve crônica apresentaram um desequilíbrio entre as atividades da SOD e da CAT, expresso pelo aumento da razão SOD/CAT no córtex cerebral e em eritrócitos (Scherer et al., 2011).

Visto que os antioxidantes são definidos como quaisquer substâncias que significativamente inibem ou previnem a oxidação excessiva (Halliwell et al., 1995), propõe-se aqui que o estresse oxidativo e a necessidade de proteção contra o dano oxidativo possam ser responsáveis, pelo menos parcialmente, pela elevação da atividade dessas enzimas. Entretanto, é

possível que, no presente trabalho, o balanço entre o sistema antioxidante de detoxificação e as ERO foi suficiente para reduzir os níveis de ERO em rins de ratos submetidos à HHcy leve crônica, já que os níveis de DCF não foram alterados.

Por outro lado, observou-se que o tratamento agudo com Hcy elevou os níveis de ERO 1 hora após a injeção desse aminoácido, mas não alterou as atividades das enzimas antioxidantes nem 1 e nem 12 horas após a injeção. O aumento de ERO observado pode ser uma conseqüência da auto-oxidação da Hcy, que leva à geração de $O_2^{\bullet-}$ e H_2O_2 (Starkebaum e Harlan, 1986). Essa produção de ERO também pode ocorrer via ativação da NADPH oxidase e provavelmente contribui para o efeito da Hcy na homeostase da matriz extracelular e conseqüente esclerose no glomérulo (Yi et al., 2006). No que se refere às enzimas antioxidantes, é provável que as atividades da SOD e da CAT foram alteradas apenas no tratamento crônico porque essas modificações representam uma adaptação celular frente a um estresse fisiológico constante, neste caso, a produção de espécies reativas.

Visto que a peroxidação lipídica também é um indicativo de geração de ERO, o próximo passo foi investigar o efeito da HHcy leve sobre os níveis de TBARS. Embora trabalhos anteriores mostrem que a HHcy leve crônica eleva os níveis de TBARS no plasma e no córtex cerebral (Scherer et al., 2011), no presente estudo não houve alteração nesse parâmetro em rins de ratos. É provável que o tratamento agudo não tenha causado oxidação excessiva o suficiente para levar à lipoperoxidação. No tratamento crônico, por sua vez, o aumento das enzimas antioxidantes possivelmente preveniu esse processo.

Do mesmo modo, resíduos de aminoácidos contendo grupamentos –SH em proteínas são alvos suscetíveis para uma variedade de pró-oxidantes. Porém, a avaliação do *status* de grupamentos -SH não demonstrou alterações induzidas pela HHcy leve. Isto pode ser explicado pelo fato de que células de mamíferos de diferentes tecidos possuem um sistema que protege

proteínas contendo –SH da oxidação excessiva (Rahman et al., 1999). Além disso, existem outros marcadores detectáveis, incluindo mudanças no conteúdo de proteínas carboniladas, nitrotirosinas e produtos finais de glicação avançada que podem ser explorados no futuro para melhor avaliar danos a proteínas.

A atividade do NO, por sua vez, foi medida através dos níveis de nitritos (produto final de sua oxidação), os quais estavam significativamente elevados nos rins de ratos com HHcy leve crônica e 1 hora após o tratamento agudo. Tem sido descrito que o estresse oxidativo causa um aumento na produção de NO, uma molécula que possui um complexo papel tanto no estresse oxidativo quanto na resposta à morte celular (Sass et al., 2001). Estudos anteriores mostraram ainda, que a HHcy ativa o FN-κB em rins de ratos via fosforilação de IκBα. A ativação do FN-κB causa aumento da expressão da NO sintase induzível, levando ao aumento da produção de NO. Assim, visto que o NO estimula a produção do fator de necrose tumoral alfa, que por sua vez pode causar dano inflamatório (Sass et al., 2001), esse pode ser um processo importante envolvido no dano renal observado durante a HHcy. Porém, mais estudos serão necessários para elucidar tais mecanismos. Além disso, esse resultado permite concluir que o aumento nos níveis de NO possa estar envolvido com a elevação da atividade da CAT observada durante a HHcy leve crônica, visto que outro estudo demonstrou que o NO pode elevar a atividade dessa enzima antioxidante (Yoshioka et al., 2006).

Com o objetivo de testar a hipótese de que as mudanças no *status* oxidativo/nitrativo poderiam causar danos funcionais aos rins de ratos com HHcy leve crônica, avaliaram-se alguns parâmetros bioquímicos que geralmente se encontram alterados durante a disfunção renal. A creatinina e a uréia foram descritas em 1904 e 1952, respectivamente (Narayanan e Appleton, 1980; Bateman et al., 1952) e ainda são consideradas “padrão ouro” para a avaliação da função renal. Neste estudo, não se observaram alterações nesses parâmetros e, portanto, não houve

evidências de uma perda da capacidade de depuração renal pela HHcy leve. O ácido úrico, por outro lado, é bem conhecido por ser um fator predisponente independente para disfunção renal (Weiner et al., 2008); entretanto, tem sido descrito que a disfunção renal também promove o aumento das concentrações séricas desse parâmetro (Zapolski et al., 2011). Corroborando os resultados acima, demonstrou-se aqui que a HHcy leve não foi capaz de alterar esse parâmetro em ratos.

Por fim, avaliaram-se os níveis de albumina sérica em ratos com HHcy leve crônica e observou-se que esse parâmetro foi reduzido sob essa condição. Visto que a literatura mostra uma associação entre a HHcy e a microalbuminúria (Francis et al., 2004; Hoogeveen et al., 1998; Jager et al., 2001), esse resultado pode indicar um aumento da permeabilidade dos glomérulos. Lund e colaboradores (2003) obtiveram dados a partir de trabalhos realizados com ratos, demonstrando que o coeficiente de permeabilidade glomerular à albumina aumenta à medida que a taxa de filtração glomerular cai, reduzindo assim, os níveis séricos de albumina. Além disso, este estudo demonstrou uma alteração no *status* redox nos rins dos animais e sabe-se que ERO participam de uma variedade de doenças renais, podendo contribuir para a perda de albumina na urina (Halliwell, 1994; Southorn e Powis, 1988). Entretanto, mais estudos são necessários para confirmar essa hipótese.

Embora, no presente trabalho, os ratos submetidos à HHcy leve não apresentaram indícios de perda da capacidade de depuração renal, sabe-se que a persistência do aumento da permeabilidade glomerular a proteínas pode progredir para falência renal em alguns casos. Além disso, as pequenas alterações vistas na produção de ERO e o aumento da atividade antioxidante podem ser apenas uma resposta inicial à HHcy, sendo que a persistência da HHcy leve crônica poderia resultar em uma condição patológica em que as enzimas antioxidantes não seriam mais capazes de evitar o estresse oxidativo. Dessa forma, um tratamento crônico prolongado com Hcy

pode ser necessário para se observar mudanças nos níveis de creatinina, uréia e ácido úrico.

Na etapa seguinte deste estudo, desenvolveu-se um modelo experimental de HHcy severa crônica. Inicialmente, investigaram-se alguns parâmetros de estresse oxidativo e se observou que a Hcy promoveu um aumento dos níveis de DCF e TBARS em rins de ratos 1 e 12 horas após a última injeção do aminoácido, demonstrando um aumento da produção de ERO e da peroxidação lipídica, respectivamente. Em concordância com esses resultados, dados da literatura apontam que a Hcy está associada com estresse oxidativo nos rins (Diez et al., 2005). Além disso, foi previamente descrito que a Hcy, em níveis altamente elevados, contribui para a lipoperoxidação induzida por insquemia-reperfusão em rins (Prathapasinghe et al., 2007). Outros trabalhos também demonstraram que a HHcy severa induz peroxidação lipídica em tecidos extrarenais, tais como pulmão (da Cunha et al, 2011), coração (Kolling et al., 2011), fígado (Matté et al., 2009) e hipocampo (Streck et al., 2003a).

Como descrito anteriormente, a produção de ERO provavelmente ocorra devido à auto-oxidação de Hcy (Misra, 1974) e/ou ativação da NADPH oxidase (Pin-Lan et al., 2007). Além de $O_2^{\bullet -}$ e H_2O_2, o $OH^{\bullet}$ também pode ser gerado pela reação de Fenton, quando o H_2O_2 recebe outro elétron e um íon hidrogênio. O $OH^{\bullet}$ é o radical mais reativo e pode levar ao processo de lipoperoxidação observado pelo aumento dos níveis de TBARS (Jenkins, 1988). Em contrapartida, o aumento do estresse oxidativo local não foi capaz de alterar o *status* de grupamentos –SH ligados a proteínas em rins de ratos após indução de HHcy severa, embora esse parâmetro tenha sido reduzido no fígado de ratos submetidos ao mesmo modelo (Matté et al., 2009).

O próximo passo foi investigar o efeito da administração crônica de Hcy sobre o TRAP, um índice de capacidade antioxidante. Os resultados mostraram uma redução dessa defesa não-enzimática nos rins de ratos hiperhomocisteinêmicos 1 hora após a última injeção de Hcy. Esse

resultado mostra consistência com o aumento do estresse oxidativo observado, visto que o tecido se torna mais suscetível à formação de ERO. Além disso, Streck e colaboradores (2003a) já haviam demonstrado que a Hcy, *in vitro*, diminui significativamente o TRAP em hipocampo de ratos e outros trabalhos mostraram previamente que a administração de Hcy reduz o potencial antioxidante no córtex parietal de ratos (Matté et al., 2004).

Foi avaliado, também, o efeito da HHcy crônica sobre as atividades das enzimas antioxidantes SOD e CAT, as quais compõem um eficiente sistema responsável pela remoção de ERO (Halliwell, 2001; Halliwell e Gutteridge, 2007). No presente estudo, observou-se um aumento da atividade da SOD em rins de ratos 1 e 12 horas após a última injeção de Hcy. Provavelmente esse resultado represente uma resposta antioxidante contra o possível aumento na produção de $O_2^{\bullet-}$, visto que essa enzima catalisa a dismutação desse radical (Halliwell e Gutteridge, 2007). Esses resultados estão em concordância com um estudo que sugere a existência de uma correlação positiva entre a dosagem de SOD e a Hcy total (Wilcken et al., 2000).

Por outro lado, houve uma redução da atividade da CAT neste estudo. Previamente, foi demonstrado que a administração aguda de Hcy diminui a atividade da CAT em hipocampo de rato e que as vitaminas E e C previnem completamente esse efeito, indicando uma provável participação do estresse oxidativo nas ações da Hcy (Wyse et al., 2002). Visto que a CAT é capaz de converter o H_2O_2 em água e oxigênio molecular (Halliwell e Gutteridge, 2007), uma explicação para esse resultado é a possibilidade de uma depleção precoce das reservas renais de CAT diante de um estímulo inicial caracterizado pelo aumento da atividade da SOD, que provavelmente elevou os níveis de H_2O_2. Adicionalmente, a redução da atividade da CAT pode ser explicada pelo fato de que enzimas antioxidantes são inibidas por ERO específicas (McCord e Fridovich, 1969; Blum e Fridorich, 1985; Vessey e Lee, 1993), que poderiam estar sendo

formadas a partir da Hcy. Como a Hcy pode regular o *turnover* protéico e a expressão de genes, incluindo enzimas antioxidantes (Stern et al., 2004; Sharma et al., 2006), não se pode descartar a possibilidade de que as mudanças nas atividades da SOD e da CAT sejam causadas por esses mecanismos.

É necessário ressaltar que a redução da atividade da CAT é um resultado importante visto que pode explicar por que os níveis de TRAP retornaram ao normal 12 horas após a injeção de Hcy, mas as ERO e os níveis de TBARS permaneceram significativamente altos. Embora os níveis de TRAP tenham retornado aos níveis do controle em 12 horas, a atividade da CAT permaneceu reduzida nesse ponto de tempo. Dessa forma, o ambiente celular se tornou mais suscetível à formação de H_2O_2 e à geração de estresse oxidativo.

Com relação à atividade de NO, os resultados observados neste estudo mostram um aumento desse parâmetro 1 hora após a última injeção, estando de acordo com outros dados da literatura que demonstraram que a HHcy severa aguda eleva os níveis de nitritos em hipocampo, córtex cerebral e soro de ratos (da Cunha et al., 2010). Em contraste, a HHcy severa crônica não foi capaz de alterar os níveis de nitritos em pulmões de ratos (Da Cunha et al., 2011).

Considerando que mudanças no *status* oxidativo/nitrativo observado em rins de ratos hiperhomocisteinêmicos poderiam levar a um processo nefropático, verificaram-se os níveis de uréia e albumina em soro de ratos submetidos à HHcy severa crônica. Constatou-se que a Hcy elevou os níveis séricos de uréia, a qual sendo livremente filtrada pelo glomérulo, pode indicar insuficiência renal quando em excesso (Berl e Schrier, 2002). A determinação da uréia sérica não é específica para alterações da função renal, mas é mais sensível a danos recentes aos rins, sendo um importante marcador para injúria renal. Já os níveis séricos de albumina encontravam-se reduzidos após HHcy severa, da mesma forma como foi observado durante a HHcy leve.

Em conjuto, nossos achados demonstram uma associação entre a HHcy (leve e severa) e o

estresse oxidativo/nitrativo em rins, o que pode representar, pelo menos em parte, um dos importantes mecanismos que contribuem para o risco de dano renal observado em indivíduos e/ou pacientes com HHcy.

V. CONCLUSÕES

A HHcy leve crônica aumentou as atividades das enzimas antioxidantes SOD e CAT e os níveis de nitritos, bem como reduziu os níveis séricos de albumina em ratos. A HHcy leve aguda elevou os níveis de ERO e de nitritos nos rins.

A HHcy severa crônica aumentou a produção de ERO, peroxidação lipídica e os níveis de nitritos, assim como alterou as defesas antioxidantes (enzimáticas e não-enzimáticas) nos rins. Albumina e uréia séricas também foram alteradas pela HHcy severa.

Em conclusão, o presente estudo demonstra uma relação entre a HHcy (leve e severa) e o estresse oxidativo em rins de ratos, o que pode indicar, pelo menos em parte, que as alterações no estado oxidativo podem ser um dos importantes mecanismos que contribuem para o risco de dano renal observado durante a HHcy.

VI. PERSPECTIVAS

Diante dos resultados verificados neste estudo, faz-se necessário:

- Verificar o possível papel protetor do ácido fólico e/ou outros antioxidantes, tais como vitaminas C e E, sobre os efeitos da HHcy leve e severa em rins de ratos.

- Avaliação morfológica dos rins de ratos submetidos aos modelos de HHcy leve e severa.

- Realizar o teste de microalbuminúria na urina dos ratos submetidos aos tratamentos com o objetivo de comprovar a hipótese de que a hipoalbuminemia observada durante a HHcy é decorrente da perda urinária.

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