Universidade Federal do Rio Grande do Sul

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Papel do estresse oxidativo na fisiopatologia da fenilcetonúria

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Orientadora: Profª Drª Carmen Regla Vargas

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“A mente que se abre a uma nova ideia jamais voltará ao seu tamanho original.”

Albert Einstein

ÍNDICE

RESUMO

A fenilcetonúria é um erro inato do metabolismo de aminoácidos, causada pela deficiência severa ou ausência na atividade da fenilalanina hidroxilase, enzima que catalisa a hidroxilação da fenilalanina em tirosina na presença do cofator tetra-hidrobiopterina. Como consequência, ocorre o acúmulo da fenilalanina e seus metabólitos nos tecidos e nos líquidos biológicos dos pacientes afetados. O tratamento para a fenilcetonúria consiste em uma dieta restrita em fenilalanina e proteínas, suplementada com uma fórmula especial, contendo aminoácidos (exceto a fenilalanina) e micronutrientes. A principal característica clínica dos pacientes fenilcetonúricos não tratados é o retardo mental e outras alterações neurológicas, cuja base bioquímica é ainda pouco compreendida. Entretanto, nos últimos anos evidências indicam que o estresse oxidativo está envolvido na fisiopatologia da doença. Em estudos prévios, demonstramos que pacientes fenilcetonúricos diagnosticados tardiamente apresentavam aumento na peroxidação lipídica e redução de antioxidantes no momento do diagnóstico e também durante o tratamento, e que esses parâmetros não estavam diretamente relacionados com os níveis sanguíneos de fenilalanina. O objetivo deste trabalho foi o de investigar o papel do dano oxidativo e também das defesas antioxidantes na patogênese da fenilcetonúria. Foi demonstrado que pacientes fenilcetonúricos tratados apresentaram maior dano ao DNA, medido através do ensaio cometa, em comparação aos controles, e que este dano estava relacionado aos níveis sanguíneos elevados de fenilalanina. Neste particular, testes *in vitro* revelaram um efeito dose-dependente da fenilalanina sobre o dano ao DNA, reforçando os achados *in vivo* e indicando que a fenilalanina foi responsável por esse dano. Também verificamos que os pacientes fenilcetonúricos com diagnóstico tardio apresentaram maior oxidação a lipídios (determinado através da técnica das espécies reativas ao ácido tiobarbitúrico) e a proteínas (medido através do conteúdo de sulfidrilas e carbonilas) em comparação aos pacientes diagnosticados no período neonatal e aos controles. Portanto, o diagnóstico precoce, além de prevenir o retardo mental, como já descrito na literatura científica, também previne o dano oxidativo a biomoléculas. Por outro lado, foi observada uma redução nas

concentrações de antioxidantes não enzimáticos (níveis de glutationa e reatividade antioxidante total) e na atividade da enzima antioxidante glutationa peroxidase em ambos os grupos de pacientes. A diminuição nos antioxidantes é comum em pacientes fenilcetonúricos, sendo atribuída principalmente à dieta restrita. Neste trabalho também verificamos que os pacientes que aderiam estritamente à dieta recomendada apresentavam redução nos níveis sanguíneos de L-carnitina, um composto com ação antioxidante. Além disso, os níveis de L-carnitina nesses pacientes mostraram uma correlação negativa significativa com a lipoperoxidação (medida pelas espécies reativas ao ácido tiobarbitúrico) e uma correlação positiva significativa com a reatividade antioxidante total. Os dados sugerem que a deficiência em L-carnitina está relacionada com o estresse oxidativo em pacientes fenilcetonúricos e, portanto, sua suplementação deva ser considerada como uma terapia adjuvante. De fato, a suplementação com L-carnitina e selênio (outro composto antioxidante deficiente em pacientes fenilcetonúricos) foi capaz de corrigir a oxidação a lipídios e proteínas (medida pelas espécies reativas ao ácido tiobarbitúrico e pelo conteúdo de sulfidrilas, respectivamente), além de normalizar a atividade da enzima glutationa peroxidase. Adicionalmente, foi verificada uma correlação negativa significativa entre a peroxidação lipídica e os níveis sanguíneos de L-carnitina, assim como uma correlação positiva significativa entre a atividade da glutationa peroxidase e a concentração sanguínea de selênio. Em conjunto, nossos resultados sugerem que o estresse oxidativo está envolvido na patogênese da fenilcetonúria. Considerando que nossos resultados possam ser extrapolados para o cérebro, que possui menos defesas antioxidantes e vários fatores que aumentam a produção de radicais livres, pode ser proposto que o dano oxidativo contribui, pelo menos em parte, com a disfunção neurológica na fenilcetonúria, e, portanto, que a administração dos antioxidantes deficientes nesta patologia deva ser considerada na terapia da doença.

ABSTRACT

Phenylketonuria is an inborn error of amino acid metabolism, caused by severe deficiency or absence of phenylalanine hydroxylase activity, enzyme that catalyzes the hydroxylation of phenylalanine to tyrosine in the presence of the cofactor tetrahydrobiopterin. As consequence, the accumulation of phenylalanine and its metabolites in tissues and biologic fluids of affected patients occurs. The treatment for phenylketonuria consists in a phenylalanine and protein-restricted diet, supplemented with a special formula containing amino acids (except phenylalanine) and micronutrients. The main clinical characterization of untreated phenylketonuric patients is mental retardation and other neurological features, whose biochemical basis is poorly understood. However, in recent years evidences indicate that oxidative stress is involved in the pathophysiology of the disease. In previous studies it was demonstrated that phenylketonuric patients late diagnosed presented increased lipid peroxidation and reduced antioxidants at the moment of diagnosis and also during the treatment, and that these parameters were not directly related to the phenylalanine blood levels. The objective of this work was to investigate the role of the oxidative damage and of antioxidant defenses on pathogenesis of phenylketonuria. It was demonstrated that phenylketonuric patients under treatment presented increased DNA damage, measured by the comet assay, compared to controls, which was related to phenylalanine blood levels. In this particular, *in vitro* tests revealed a dose-dependent effect of phenylalanine on DNA damage, reinforcing *in vivo* findings indicating that the phenylalanine was responsible for this damage. We also verified that phenylketonuric patients late diagnosed presented increased lipid (determined by thiobarbituric acid-reactive species) and protein oxidation (measured by sulphydryl and carbonyl groups) when compared to patients diagnosed in the neonatal period and to controls. Therefore, early diagnosis besides to prevent mental retardation, as described in the scientific literature, also prevents oxidative damage to biomolecules. On the other hand, it was observed a reduction in the concentration of non-enzymatic antioxidants (glutathione levels and total antioxidant reactivity) as well as in the activity of glutathione peroxidase enzyme in both groups of patients. The reduction in antioxidants is common in phenylketonuric patients being mainly

attributed to the restricted diet. In this work, we also verified that patients who strictly adhered to the recommended diet present reduction in blood L-carnitine levels, a compound with an antioxidant action. Also, the levels of L-carnitine in these patients showed a significant negative correlation with lipid peroxidation (measured by thiobarbituric acid-reactive species) and a significant positive correlation with the total antioxidant reactivity. This suggests that L-carnitine deficiency is related to oxidative stress in phenylketonuric patients and therefore the supplementation should be considered as an adjuvant therapy. In fact, the supplementation with L-carnitine and selenium (other antioxidant compound deficient in phenylketonuric patients) was capable to correct the lipid and protein oxidation (measured by thiobarbituric acid-reactive species and sulphydryl content, respectively) besides to normalize the glutathione peroxidase activity. In addiction, it was verified a significant inverse correlation between lipid peroxidation and L-carnitine blood levels as well as a significant positive correlation between glutathione peroxidase activity and blood selenium concentration. Taken these results together, our results suggest that oxidative stress is involved in the pathogenesis of phenylketonuria. Considering that our results may be extrapolated to the brain, which has less antioxidant defenses and several other factors that increase the production of free radicals, it may be propose that the oxidative damage contributes, at least in part, to the neurological dysfunction in phenylketonuria and, therefore, the administration of deficient antioxidants in this pathology should be considered in the therapy of the disease.

LISTA DE ABREVIATURAS

BH_2 – di-hidrobiopterina

BH_4 – tetra-hidrobiopterina

BHE – barreira hematoencefálica

CAT – catalase

DHPR – di-hidropteridina redutase

EIM – erros inatos do metabolismo

ERO – espécies reativas de oxigênio

GR – glutationa redutase

GSH – glutationa reduzida

GSH-Px – glutationa peroxidase

GSSG – glutationa oxidada

HPA – hiperfenilalaninemia

LC – L-carnitina

PA - fenilacetato

PAH – fenilalanina hidroxilase

Phe – fenilalanina

PKU – fenilcetonúria

PLA – fenil-lactato

PLP – piridoxal fosfato

PPA – fenilpiruvato

Q10 – ubiquinona-10

QI – quociente de inteligência

RL – radical livre

SNC – sistema nervoso central

SOD – superóxido dismutase

TBARS – espécies reativas ao ácido tiobarbitúrico

Tyr – tirosina

INTRODUÇÃO

1. Erros Inatos do Metabolismo

A primeira menção aos erros inatos do metabolismo (EIM) data de 1908, quando Sir Archibald Garrod usou este termo para designar doenças como a alcaptonúria, patologia na qual os pacientes afetados excretam grandes quantidades do ácido homogentísico na urina. Garrod observou uma maior frequência desta alteração em indivíduos de uma mesma família e maior incidência de consanguinidade entre os pais dos pacientes afetados. Sabendo disso e baseado nas leis de Mendel, Garrod propôs um modelo de herança autossômica recessiva para este distúrbio. Através da observação de que o ácido homogentísico, excretado em altas concentrações na urina dos pacientes com alcaptonúria, era um metabólito normal da degradação protéica, ele relacionou este distúrbio com um bloqueio na rota do catabolismo das proteínas (Scriver et al., 2001).

Desde os estudos de Garrod, muitos pesquisadores têm detectado novas doenças metabólicas hereditárias e os EIM já foram descritos em todas as áreas do metabolismo humano normal (aminoácidos, ácidos orgânicos, lipídios, carboidratos, etc.), compreendendo hoje mais de 500 defeitos genéticos (Scriver et al., 2001). Embora individualmente raras, estas doenças em seu conjunto afetam, aproximadamente, 1 a cada 500/2.000 nascimentos (Baric, Furnic e Hoffmann, 2001).

Os EIM são caracterizados pela síntese alterada de uma proteína, geralmente uma enzima, com atividade parcial ou totalmente reduzida. Essa

alteração resulta no bloqueio da via metabólica com consequente acúmulo de seus substratos e outros derivados deles, bem como diminuição da síntese dos produtos. Tal bloqueio, dependendo da via afetada, repercute clinicamente de maneira bastante variável no indivíduo, sendo geralmente de sintomatologia grave e muitas vezes letal (Scriver et al., 2001).

Os EIM podem ser classificados de diversas maneiras. Saudubray e Charpentier (2001) catalogaram os EIM em três grandes grupos: distúrbios de síntese ou degradação de moléculas complexas, que incluem as doenças lisossômicas de depósito e as doenças peroxissomais; doenças com déficit de energia, que incluem as doenças de depósito de glicogênio, defeitos de gliconeogênese e defeitos de oxidação de ácidos graxos; e erros inatos do metabolismo intermediário, que incluem as aminoacidopatias, as acidemias orgânicas, os defeitos do ciclo da ureia e as intolerâncias aos açúcares.

Neste último grupo de doenças pode ocorrer intoxicação aguda ou crônica, causada pelo acúmulo dos metabólitos tóxicos devido a um bloqueio em suas vias metabólicas normais. Estudos revelam que aproximadamente um terço dos EIM corresponde a aminoacidopatias, outro terço a acidemias orgânicas e o terço final a todos os outros EIM (Hoffmann, 1994).

1.1 Hiperfenilalaninemia e Fenilcetonúria

Hiperfenilalaninemia (HPA) é o termo genérico que se designa a um fenótipo bioquímico no qual existe um aumento persistente da concentração plasmática do aminoácido essencial fenilalanina (“phenylalanine” - Phe), causado pela deficiência da hidroxilação hepática deste aminoácido. A

hidroxilação da Phe à tirosina (“tyrosine” - Tyr) é catalisada pela enzima fenilalanina hidroxilase (Phe-4-monooxigenase – PAH – EC 1.14.16.1) com a participação da coenzima tetra-hidrobiopterina (BH4), que se reduz na reação de hidroxilação e deve ser regenerada por outra enzima, a di-hidropteridina redutase (DHPR – EC 1.6.99.7) (Scriver e Kaufman, 2001), conforme ilustrado na figura 1.

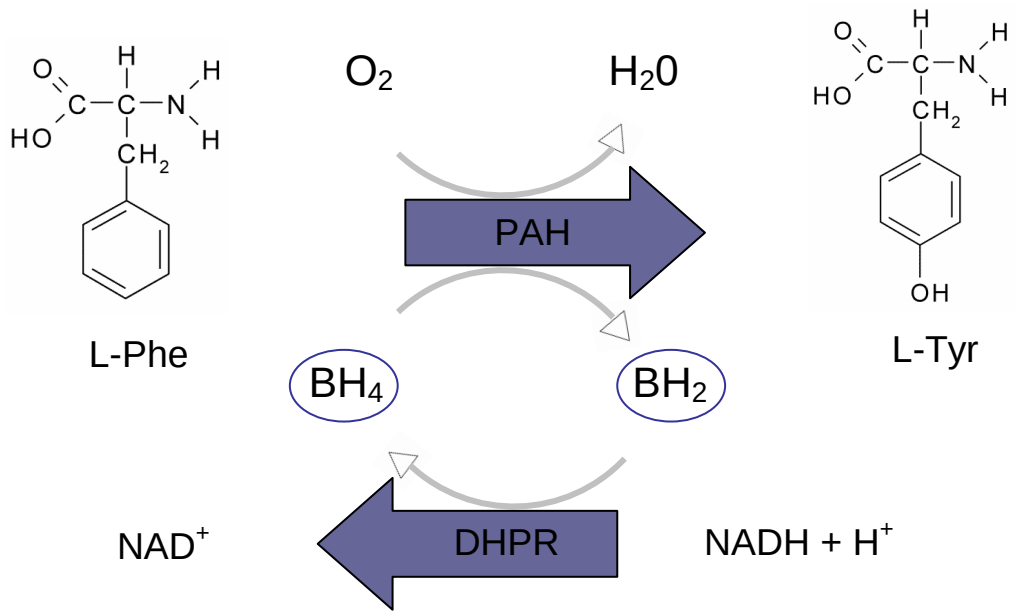

Figura 1. Sistema de hidroxilação da L-Phe em L-Tyr em seres humanos. São mostradas as enzimas, os substratos e cofatores. A PAH é capaz de hidroxilar a L-Phe utilizando oxigênio molecular e o cofator BH4 como doador de elétrons. Em cada reação, o cofator é oxidado a di-hidrobiopterina (BH2). Os níveis de BH4 são mantidos no fígado mediante a redução de BH2 a BH4 pela enzima DHPR, utilizando como cofator o NADH.

Qualquer defeito que interfira no sistema de hidroxilação (deficiência da PAH, da DHPR ou da biossíntese de BH4) causará uma HPA persistente, já que este sistema é o principal determinante do metabolismo da Phe em seres humanos. Não obstante, a principal causa de HPA é o defeito na atividade da PAH, que constitui 98% dos casos diagnosticados de HPA e é conhecida como

fenilcetonúria – (“phenylketonuria” – PKU) – (OMIM 261600) (Scriver e Kaufman, 2001).

Com o bloqueio na rota de hidroxilação da Phe, além dela, outros metabólitos anormais como o fenilpiruvato (PPA), o fenilacetato (PA) e o fenil-lactato (PLA) também se acumulam no sangue e tecidos dos pacientes fenilcetonúricos e são excretados em níveis elevados na urina desses pacientes (figura 2). Cabe salientar que o nome da doença deriva dos altos níveis de fenilpiruvato, uma fenilcetona, encontrada na urina de crianças afetadas (Cooper, 2000).

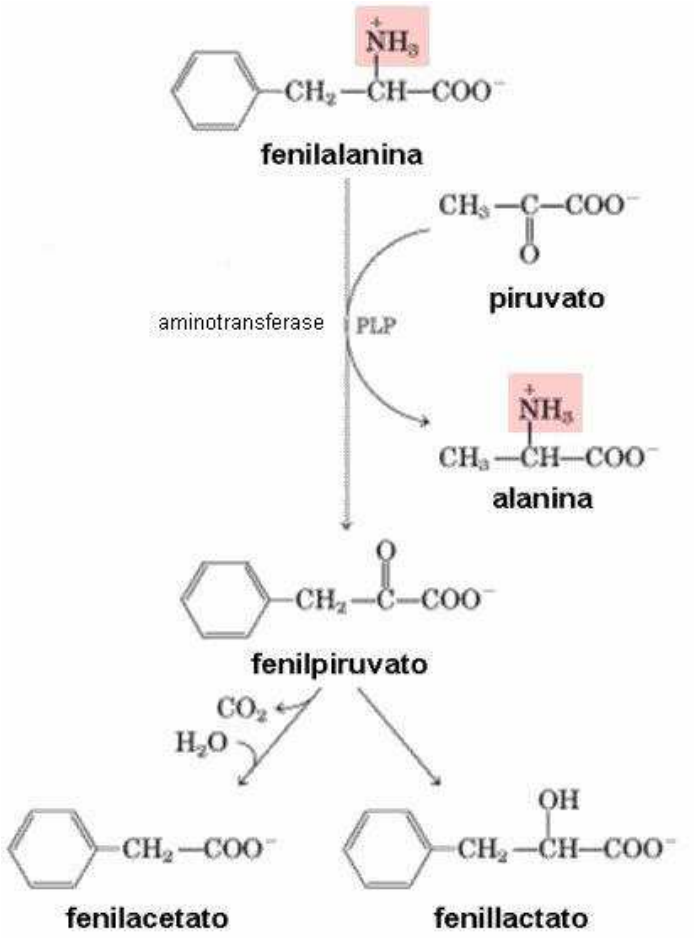

Figura 2. Rotas alternativas para o catabolismo da Phe na PKU (adaptado de Lehninger, 2008).

A PKU apresenta herança autossômica recessiva, possuindo uma incidência global de 1:10.000 nascimentos, sendo, portanto, o mais frequente erro inato do metabolismo dos aminoácidos, e, possivelmente, sendo também o mais estudado (Scriver e Kaufman, 2001; Berg, Tymoczko e Stryer, 2010). A incidência de portadores é estimada em 2% da população em geral. O gene que codifica a atividade da PAH está localizado no cromossomo 12 (Woo et al., 1983), sendo uma enfermidade muito heterogênea, com mais de 500 mutações já descritas (Phenylalanine Hydroxylase Locus Knowledgebase).

1.1.1 Classificação

A PKU apresenta um amplo espectro de fenótipos bioquímicos, possivelmente devido à combinação múltipla de um grande número de mutações alélicas. Nesse particular, as concentrações plasmáticas de Phe ao diagnóstico variam consideravelmente, havendo também diferenças importantes na resposta à dieta restritiva (tolerância) que mantém as concentrações plasmáticas do aminoácido dentro de uma faixa recomendada (Scriver e Kaufman, 2001; Guttler e Lou, 1990).

A forma grave, também conhecida como PKU clássica, é caracterizada por concentrações plasmáticas de Phe ao diagnóstico superiores a 1.200 µM e a tolerância à Phe inferior a 350 mg/dia. A atividade residual da PAH é praticamente indetectável.

A variante moderada da PKU apresenta concentrações plasmáticas de Phe entre 600 e 1.200 µM e os pacientes apresentam uma tolerância de 350 a 400 mg/dia de Phe na dieta. A variante leve mostra uma Phe de 360 a 600 µM e

uma tolerância entre 400 e 600 mg/dia. A atividade enzimática residual é inferior a 10% nessas duas variantes e por isso também requerem tratamento.

Ainda existe uma forma benigna de HPA, caracterizada bioquimicamente por concentrações plasmáticas de Phe menores que 360 µM. É causada por mutações leves de pouca repercussão na atividade da PAH (atividade residual de 10 a 35% do normal), o que determina que não necessite de tratamento (Campistol et al., 2006).

1.1.2. Manifestações clínicas

Quando não tratada, a forma grave, ou PKU clássica, causa retardo mental e motor graves, manifestando-se já nos primeiros meses de vida. Essa é a razão pela qual em um grande número de países existem programas de detecção precoce, no período neonatal, o que permite o tratamento precoce da doença, indispensável para evitar o aparecimento dos sintomas. A PKU clássica não tratada causa, portanto, retardo mental grave, bem como microcefalia, epilepsia, eczema e hiperatividade (Scriver e Kaufman, 2001).

As crianças tratadas precocemente apresentam uma boa evolução, com um quociente de inteligência (QI) dentro dos limites da normalidade, ainda que, no geral, inferior ao de seus irmãos não afetados, apresentando pequenas dificuldades de aprendizagem (Weglage et al., 1995).

1.1.3. Diagnóstico

O diagnóstico da PKU dá-se pela detecção de elevadas concentrações de Phe no sangue dos pacientes afetados, bem como por concentrações elevadas de Phe e seus derivados (PPA, PA e PLA) na urina. Inicialmente, nos anos 60 o diagnóstico dessa doença era realizado através dos métodos semi-quantitativos como a cromatografia de aminoácidos em papel ou camada delgada. No entanto, a quantificação exata da Phe no sangue é importante não somente para diagnosticar, mas também para monitorar o tratamento dos pacientes fenilcetonúricos.

Em 1963, Guthrie publicou um método de análise da Phe em sangue, baseado no fato de a Phe em altas concentrações, em sangue impregnado em papel filtro (Guthrie card), bloquear a ação inibidora do crescimento bacteriano de um composto adicionado ao meio de cultura, produzindo-se um halo de crescimento ao redor do sangue dos pacientes afetados (Guthrie e Susi, 1963).

Os procedimentos atuais de detecção precoce da PKU baseiam-se no método de Guthrie (ainda utilizado em alguns países), em métodos cromatográficos, fluorimétricos ou espectrofotométricos, este último baseado na reação específica da PAH sobre a Phe. Já a espectrometria de massas em tandem tem sido o método de escolha para o diagnóstico neonatal. A confirmação do defeito da atividade enzimática da PAH só é possível na biópsia hepática, já que a enzima não se expressa em fibroblastos (Campistol et al., 2006).

1.1.4. Tratamento

Nos anos 50, foi estabelecido o tratamento para a PKU, baseado na restrição de Phe e proteína na dieta, que continua sendo base para o tratamento atual (Bickel, Gerrard e Hickmans, 1953). Com a detecção precoce da enfermidade e o início da dieta foi possível prevenir o retardo mental e os outros sintomas associados à doença, resultando em uma geração de pacientes fenilcetonúricos com boa qualidade de vida.

O tratamento da PKU é para toda a vida e baseia-se na redução do aporte de alimentos que contêm Phe, combinado com a administração de uma fórmula especial que contém os demais aminoácidos e micronutrientes, necessários ao desenvolvimento normal do paciente (Burgard et al., 1999, Wappner et al., 1999).

É sabido que o manejo dos pacientes é complexo, mas também que quando seguem uma dieta estrita o prognóstico é muito melhor. Neste contexto, deve-se fazer uma restrição de Phe que mantenha concentrações plasmáticas inferiores a 360 µM para crianças e 600 µM para o adulto, apesar de estes valores não serem totalmente consensuais. A dieta do paciente fenilcetonúrico deve ser individualizada, usualmente devendo conter entre 250 e 500 mg de Phe/dia, quando a de um paciente não-fenilcetonúrico deve ser de cerca de 2.500mg de Phe/dia. A quantidade de proteínas totais na dieta também deve ser individualizada e calculada de acordo com a faixa etária do paciente, ficando entre 2,5 e 3,0 g/kg/dia para crianças com menos de um ano de idade (Levy, 1999).

1.1.5. Fisiopatologia

Ainda que a PAH seja uma enzima hepática, o principal efeito clínico associado às HPAs é uma alteração do desenvolvimento e funções cerebrais, sendo que há várias hipóteses sobre os mecanismos causadores da neurotoxicidade na PKU (Weglage et al., 1996).

Tem-se postulado que a neurotoxicidade da PKU poderia dever-se, em parte, à elevada concentração de Phe, que utiliza o mesmo sistema de transporte que outros aminoácidos neutros de cadeia longa ou ramificada, o que dificulta a passagem dos mesmos através da barreira hematoencefálica (BHE), resultando em menor concentração no cérebro. Como resultado, ocorre a diminuição na síntese de proteínas, causando proliferação dendrítica e mielinização defeituosas (Scriver e Kaufman, 2001 Hoeksma et al., 2009).

Por outro lado, tem-se especulado muito sobre a deficiência relativa de Tyr, que se torna um aminoácido essencial nos pacientes, já que sua síntese está bloqueada na doença (Van Spronsen et al., 1996). Além disso, a Tyr e o triptofano são aminoácidos neutros cujo transporte através da BHE é prejudicado pelas altas concentrações de Phe. Como a Tyr e o triptofano são precursores de neurotransmissores, a deficiência relativa destes aminoácidos traduz-se em uma redução na síntese de serotonina, dopamina e norepinefrina (Tam e Roth, 1997). Existe também a hipótese de que a neurotoxicidade nesta doença possa ser ocasionada pelo acúmulo dos metabólitos da Phe. Neste sentido, o componente mais tóxico parece ser o PA (Kaufman, 1989).

Ainda, também foi demonstrado que a atividade da Na^+, K^+-ATPase está reduzida na membrana sináptica em um modelo animal quimicamente induzido de PKU (Wyse et al, 1994), podendo contribuir com o dano neurológico

encontrado na doença. Adicionalmente, vários estudos têm sugerido que o metabolismo energético em cérebro de ratos é prejudicado pela Phe *in vivo* e *in vitro*. A Phe, em concentrações usualmente encontradas no plasma de pacientes com PKU, reduz as atividades dos complexos I-III da cadeia respiratória e diminui a atividade da succinato desidrogenase em cérebro de ratos submetidos à HPA quimicamente induzida (Rech et al., 2002). Também foi verificado que a Phe inibe as atividades das enzimas piruvato quinase e creatina quinase em córtex cerebral de ratos (Costabeber et al., 2003; Feksa et al., 2002).

Nos últimos anos tem sido também demonstrada a participação do estresse oxidativo no dano neuronal na PKU, que seria provocado pelo acúmulo de metabólitos, por uma alteração do sistema antioxidante nos pacientes ou pela combinação de ambos os fatores (Sierra et al., 1998; Artuch et al., 2004; Sirtori et al., 2005; Sitta et al., 2006).

Estes estudos, em seu conjunto, sugerem que o dano neuronal na PKU é multifatorial, podendo estar associado com as concentrações plasmáticas e cerebrais elevadas de Phe, com a redução nas concentrações plasmáticas e cerebrais de Tyr e de neurotransmissores. Vários mecanismos de neurotoxicidade foram propostos para esta doença incluindo o acúmulo de radicais livres formados, associado à diminuição do sistema de defesa antioxidante (Ormazábal et al., 2004). O presente estudo objetiva investigar esse mecanismo em pacientes fenilcetonúricos tratados.

2. Radicais livres e estresse oxidativo

Um radical livre (RL) é definido como uma estrutura química com um ou mais elétrons desemparelhados no seu último orbital, ou seja, ocupando um orbital atômico ou molecular sozinho. Este elétron desemparelhado confere uma reatividade extremamente alta a esta molécula, uma vez que ela terá uma grande tendência em adquirir um segundo elétron para emparelhar o orbital (Halliwell e Gutteridge, 2007).

Nos mamíferos, são produzidos radicais livres de oxigênio, carbono, nitrogênio e enxofre, mas os de maior destaque são os de oxigênio, como os radicais superóxido ($O_2^{\bullet -}$) e hidroxila ($OH^{\bullet}$), devido à sua grande reatividade e, portanto, aos danos que podem causar. Entretanto, há compostos tão reativos como os RL, mas que não possuem elétrons desemparelhados na sua última camada, sendo estes classificados como espécies reativas. O termo espécies reativas de oxigênio (ERO) é frequentemente usado para incluir compostos derivados do oxigênio que não são propriamente radicalares, mas que produzem RL, como o peróxido de hidrogênio e o oxigênio *singlet* (Dröge, 2002).

Diversas são as fontes geradoras de RL e ERO. Os sistemas biológicos são expostos a estas moléculas continuamente, já que elas são produzidas endogenamente, por exemplo, na cadeia transportadora de elétrons mitocondrial, na degradação de ácidos graxos e nos processos de fagocitose (Dröge, 2002). Além disso, essas moléculas podem ser formadas por fatores exógenos, como a radiação ionizante e a exposição a solventes orgânicos (Halliwell e Gutteridge, 2007).

Em condições fisiológicas do metabolismo celular aeróbio, o oxigênio molecular (O_2) sofre redução tetravalente, com a incorporação de quatro elétrons, resultando na formação de água (H_2O). No entanto, aproximadamente 5% do oxigênio utilizado na cadeia respiratória mitocondrial não é completamente reduzido à água, podendo ser convertido a intermediários reativos, como os radicais superóxido ($O_2^{\cdot -}$) e hidroxila ($OH^{\cdot}$) e também o peróxido de hidrogênio (H_2O_2), processo que pode ser exacerbado em condições patológicas (Boveris e Chance, 1973).

A reatividade química dos RL é determinada pela molécula que carrega o elétron desemparelhado e, por isso, a reatividade varia muito entre um radical e outro. Entretanto, em quantidades excessivas, os RL irão causar dano oxidativo aos componentes celulares como as proteínas, os lipídios e mesmo os ácidos nucléicos (Halliwell e Gutteridge, 2007).

No entanto, as células possuem defesas contra os efeitos danosos produzidos pelos RL, as chamadas defesas antioxidantes que convertem as espécies reativas em compostos inativos. Os antioxidantes podem ser definidos, portanto, como qualquer substância que, quando presente em baixas concentrações com relação ao substrato oxidável, significativamente retarda ou previne a oxidação deste substrato. As defesas antioxidantes podem ser enzimáticas ou não enzimáticas (Halliwell e Gutteridge, 2007, Halliwell, 1994).

As enzimas antioxidantes agem impedindo a geração de espécies reativas, bem como as removendo. Dentre os principais antioxidantes enzimáticos, estão a enzima superóxido dismutase (SOD), que catalisa a dismutação de dois radicais superóxidos, formando H_2O_2 e O_2, a enzima catalase, que é responsável pela degradação do H_2O_2, formando água (H_2O) e

O_2 e a enzima glutationa peroxidase (GSH-Px), que catalisa a decomposição de hidroperóxidos, utilizando glutationa reduzida (GSH) como substrato para formar glutationa oxidada (GSSG) e o produto de redução do hidroperóxido. Fisiologicamente, a GSH-Px atua acoplada à enzima glutationa redutase (GR) que, por sua vez, catalisa a redução de GSSG, usando NADPH como coenzima (Halliwell, 2001; Bonnefoy, Drai e Kostka, 2002; Salvador e Henriques, 2004). Na figura 3 é possível observar-se a redução do O_2 à água, os sítios de formação de RLs e a ação das enzimas antioxidantes.

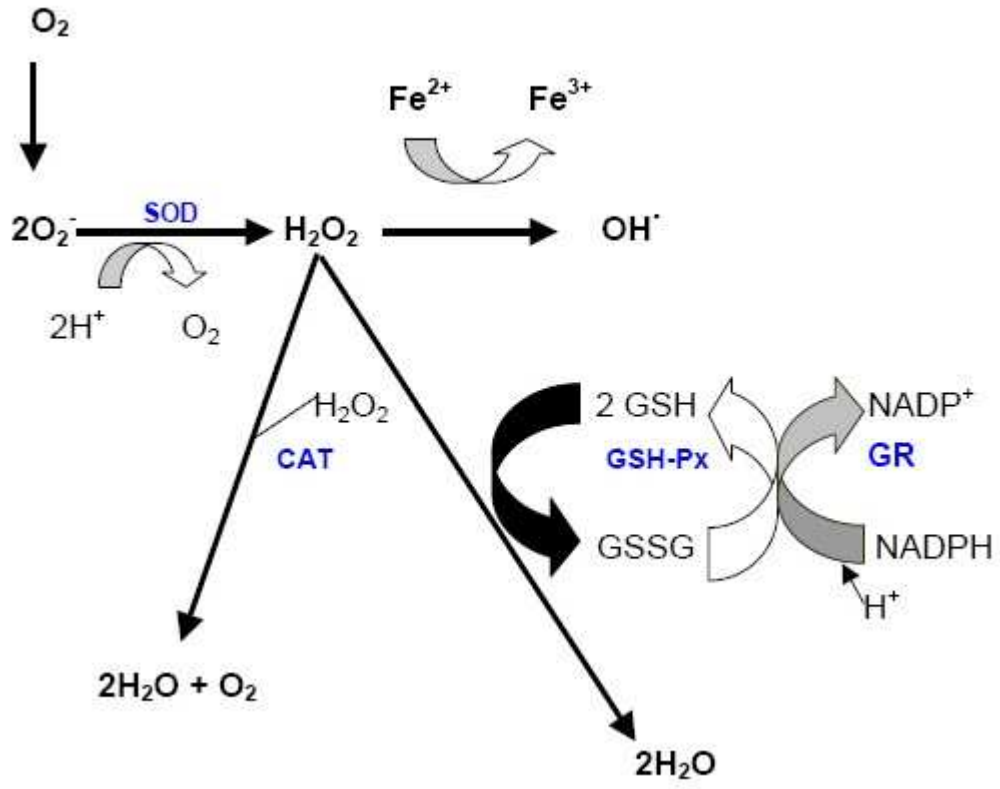

Figura 3: Redução do oxigênio à água (Adaptado de Lieberman e Marks, 2009).

Além das enzimas antioxidantes, o organismo tem a capacidade de sintetizar compostos não-enzimáticos que apresentam, direta ou indiretamente, capacidade eficaz de defesa antioxidante, atuando a fim de manter o estado de

equilíbrio celular. São exemplos deles a bilirrubina, o ácido úrico, a melatonina, o estrógeno e a glutationa (Halliwell e Gutteridge, 2007). Alguns antioxidantes não podem ser sintetizados pelo organismo, devendo ser ingeridos na dieta. Dentre estes antioxidantes não-enzimáticos, podem-se citar vitaminas, como as vitaminas A, C, E, a riboflavina e a tiamina, o selênio, a L-carnitina e os polifenóis (Salvador e Henriques, 2004). O modo de ação dos antioxidantes não-enzimáticos é, de fato, bastante diverso, podendo abranger a remoção do oxigênio presente no meio, o sequestro das ERO ou de seus precursores, bem como a inibição da formação das espécies reativas (Halliwell, 1994).

A L-carnitina (LC) é uma amina quaternária e altamente polar que pode ser sintetizada em seres humanos, ao contrário de outros organismos. Entretanto, a maior parte da LC encontrada no homem é de origem exógena, devendo ser adquirida da dieta (Gülçin, 2006). Embora a sua principal função no organismo seja garantir o transporte de ácidos graxos de cadeia longa para dentro da mitocôndria para que possam ser oxidados, diversos estudos têm demonstrado que a LC desempenha um papel de proteção contra as ERO. Esse papel é resultado principalmente do sequestro de radicais hidroxila e da inibição da formação destes radicais na reação de Fenton (Derin et al., 2004). O selênio, por sua vez, é um elemento traço que deve ser adquirido na alimentação, principalmente a partir de alimentos com alto conteúdo protéico. Ele é considerado uma substância antioxidante principalmente por estar incorporado em enzimas que possuem atividade catalítica que impede a formação de espécies reativas, sendo o exemplo mais comum a GSH-Px (Steinbrenner e Sies, 2009).

O organismo está normalmente em equilíbrio entre a produção e a degradação de RL e ERO, que existem em baixas concentrações em todos os tecidos (Dröge, 2002). Entretanto, quando um desequilíbrio ocorre entre a capacidade antioxidante e as espécies reativas formadas, em favor das pró-oxidantes, cria-se um estado denominado de estresse oxidativo (Halliwell e Gutteridge, 2007). O estresse oxidativo pode resultar de uma situação em que há uma diminuição nos níveis das defesas antioxidantes, uma elevada velocidade de produção de espécies reativas ou uma combinação de ambos (Salvador e Henriques, 2004).

Na vigência do estresse oxidativo, o organismo pode reagir de duas formas: adaptando-se ou sofrendo dano celular. Em caso de estresse oxidativo brando, as células suportam e respondem a essa situação com o aumento na produção de defesas antioxidantes, tentando com essa adaptação restabelecer o equilíbrio pró-oxidante/antioxidante. Entretanto, um estresse oxidativo muito severo leva a danos irreversíveis podendo causar até mesmo a morte celular (Halliwell e Gutteridge, 2007).

O desequilíbrio pró-oxidante/antioxidante pode causar danos potencialmente a todos os tipos de moléculas, como os lipídios, as proteínas e o DNA. Assim, as principais consequências secundárias ao estresse oxidativo nos organismos biológicos são a lipoperoxidação das membranas celulares, podendo alterar sua fluidez e permeabilidade; a oxidação de proteínas, que leva à alteração da atividade enzimática e mesmo à desnaturação; e lesão ao DNA/RNA celular, podendo causar mutações, que, por sua vez, levam ao aparecimento de doenças como câncer, doença de Parkinson, entre outras (Halliwell e Gutteridge, 2007).

2.1 Estresse oxidativo em alterações do sistema nervoso central

Está bem descrito que o cérebro é um órgão extremamente suscetível à ação dos RL e, portanto, ao estresse oxidativo. Isto é devido, entre outras causas, ao seu baixo conteúdo de defesas antioxidantes, ao alto conteúdo lipídico, ao alto consumo de oxigênio por unidade de massa de tecido e ao alto conteúdo de ferro em algumas áreas particulares (Reznick e Packer, 1993; Halliwell e Gutteridge, 2007). Desta forma, um crescente número de trabalhos descrevem a participação do estresse oxidativo na fisiopatologia de várias doenças neurológicas, incluindo a doença de Parkinson, a doença de Alzheimer, e esclerose lateral amiotrófica e a esclerose múltipla, por exemplo (Reznick e Packer, 1993; Przedborski et al., 1996; Ben-Menachem, Killerman e Markleind, 2000).

Cabe salientar que estas doenças podem ser tanto resultantes como geradoras de um desequilíbrio redox na produção e consumo de ERO ou de espécies reativas de nitrogênio. Esta alteração redox pode ser detectada em sangue total ou em um de seus constituintes, como eritrócitos, leucócitos, plasma ou soro. Estas amostras biológicas são fontes de marcadores *in vivo* de estresse oxidativo, uma vez que através do sangue circulam antioxidantes, substâncias que sofreram a ação de espécies reativas e mesmo produtos de reações entre biomoléculas e os agentes oxidantes (Dalle-Donne et al., 2006; Halliwell e Gutteridge, 2007).

2.2 Estresse oxidativo nos erros inatos do metabolismo

Nos últimos anos, diversos estudos em modelos animais e em pacientes vêm demonstrando a participação do estresse oxidativo na fisiopatologia de alguns EIM intermediário, incluindo as acidemias orgânicas, as doenças peroxissomais, os defeitos de oxidação mitocondrial de ácidos graxos e as aminoacidopatias (Barschak et al., 2006; Vargas et al., 2004; Ribas et al., 2010; Wajner et al., 2004; Tonin et al., 2010, Schuck et al., 2009; Sgaravatti et al., 2009). Acredita-se que o acúmulo dos metabólitos tóxicos e/ou alterações no *status* antioxidante nestas desordens possa levar a um desequilíbrio redox, contribuindo para o desenvolvimento dos sintomas, principalmente os neurológicos, apesar de o mecanismo responsável pelo estresse oxidativo nos EIM ainda não estar completamente esclarecido.

Com relação à PKU, em modelos animais demonstrou-se o aumento de marcadores de estresse oxidativo em cérebro e cerebelo de ratos fenilcetonúricos, sendo que a melatonina e outros antioxidantes foram capazes de reverter esse processo (Martinez-Cruz et al., 2002). Além disso, demonstrou-se que a Phe induz lipoperoxidação e reduz o potencial antioxidante total, além de reduzir a atividade da GSH-Px *in vivo* em ratos (Hagen et al., 2002). Um estudo recente demonstrou que a Phe é capaz de induzir oxidação a lipídios e a proteínas em hipocampo e córtex cerebral de ratos, sendo que esses efeitos foram revertidos pela melatonina e pelo α-tocoferol (Fernandes et al., 2010).

Estudos também têm demonstrado que pacientes fenilcetonúricos submetidos a dietas restritivas acabam por apresentar baixos níveis sanguíneos de defesas antioxidantes, entre elas o selênio e a ubiquinona-10 (Q10) (Van Bakel et al., 2000; Lombeck, Jochum e Terwolbeck, 1996; Artuch et al., 2004;

Hargreaves, 2007). Além disso, é frequente verificar-se nestes pacientes a diminuição da atividade das enzimas antioxidantes, entre elas a GSH-Px, dependente de selênio (Sierra et al., 1998: Reilly et al., 1990; Wilke et al., 1992).

Finalmente, em estudos recentes realizados por nosso grupo de pesquisa, foi demonstrado que pacientes fenilcetonúricos, seja no momento do diagnóstico, seja em vigência do tratamento dietético, apresentam índices elevados de peroxidação lipídica, diminuição da reatividade antioxidante total e redução na atividade da enzima GSH-Px (Sirtori et al., 2005; Sitta et al., 2006).

Este trabalho visa ampliar os conhecimentos no que se refere à participação do estresse oxidativo na fisiopatologia da PKU, investigando para tanto amostras de sangue de pacientes tratados.

OBJETIVOS

Objetivo geral

Investigar a participação de alterações no equilíbrio redox na fisiopatologia da fenilcetonúria, avaliando dano oxidativo e alterações no sistema antioxidante em sangue de pacientes portadores da doença.

Objetivos específicos

Capítulo 1: Investigar em sangue total os efeitos in *vivo* e *in vitro* da fenilalanina sobre o dano ao DNA, determinado pelo ensaio cometa.

Capítulo 2: Avaliar e comparar parâmetros de estresse oxidativo em sangue obtido de dois grupos de pacientes fenilcetonúricos, um diagnosticado no período neonatal e outro com diagnóstico tardio da doença.

Capítulo 3: Investigar o papel da deficiência de L-carnitina sobre parâmetros de estresse oxidativo em plasma de pacientes fenilcetonúricos.

Capítulo 4: Avaliar o efeito da suplementação com L-carnitina e selênio sobre parâmetros de estresse oxidativo em sangue de pacientes fenilcetonúricos.

RESULTADOS

Os resultados deste trabalho estão apresentados na forma de artigos científicos.

Capítulo 1 – Artigo 1: *Evidence that DNA damage is associated to phenylalanine blood levels in leukocytes from phenylketonuric patients*

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Evidence that DNA damage is associated to phenylalanine blood levels in leukocytes from phenylketonuric patients

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ABSTRACT

Phenylketonuria (PKU) is an inborn error of phenylalanine (Phe) metabolism, biochemically characterized by the accumulation of Phe and its metabolites in blood and tissues of affected patients. Treatment for PKU consists of a protein restricted diet supplemented with a mixture containing essential amino acids (other than Phe) and micronutrients. In recent years several authors have studied the pathomechanisms of the disease and demonstrated the existence of lipid and protein oxidative damage in PKU patients. In this work we investigated the *in vivo* and *in vitro* effects of Phe on DNA damage determined by the alkaline comet assay using silver staining and visual scoring. We found a dose-dependent effect of Phe on DNA damage in leukocytes from normal individuals incubated with different concentrations of Phe. Additionally, by analyzing blood leukocytes from two groups of treated PKU patients based on their blood Phe levels, we verified that the DNA damage index was significantly higher in PKU patients with high Phe blood levels (DI = 68.2 ± 12.3), compared to well-treated patients and the control group (healthy individuals). Furthermore, well-treated PKU patients had greater DNA damage (DI = 44.9 ± 7.6) relatively to controls (DI = 12.7 ± 4.1). Our present *in vitro* and *in vivo* findings indicate that DNA damage occurs in peripheral blood from PKU patients and is associated to Phe blood levels.

1. Introduction

Phenylketonuria (PKU) is an autosomal recessive disorder of amino acid metabolism, which results from primary dysfunction of phenylalanine hydroxylase, the hepatic enzyme responsible for catalyzing the conversion of phenylalanine (Phe) to tyrosine. Untreated PKU patients present elevated levels of Phe and its metabolites phenylpyruvate, phenylacetate and phenyllactate in body fluids. Retarded development and intellectual impairment are the most important clinical features presented by untreated PKU patients [1].

Treatment for patients with PKU consists of restriction of Phe intake, achieved with natural-protein-restricted diets supplemented with a Phe-free amino acid mixture enriched with essential micronutrients, such as vitamins, minerals, and trace elements [2]. Untreated PKU patients can develop severe neurological symptoms, but despite numerous studies the pathogenetic mechanisms involved in brain injury remain to be fully elucidated [3].

In this context, it was demonstrated that high Phe levels interfere with the production of the neurotransmitters dopamine and noradrenaline, decreases the availability of tryptophan and tyrosine, and causes serotonin and catecholamine depletion thus influencing brain function [4,5]. Additionally, high Phe concentrations were found to influence several mechanisms such as neural excitability, axonal conduction and synaptic transmission velocity [1,6,7].

In recent years, several works have revealed a role for oxidative stress in the pathophysiology of PKU, so that free radicals elicited by Phe could attack vital molecules, such as DNA, protein and lipids causing cell damage and malfunction [8–10]. More recently, we demonstrated lipid and protein oxidation in blood from treated and non-treated PKU patients [11–13].

In the present work, we extended these investigations analyzing DNA damage in leukocytes from treated PKU patients using the comet assay. We also evaluated the *in vitro* effect of different concentrations of Phe on DNA oxidative damage in white blood cells from normal individuals.

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2. Materials and methods

2.1. Blood sample

Venous blood was collected from PKU patients and controls under sterile conditions in heparinized vials. Whole blood was frozen at −80 °C until analysis. The present study was approved by the Ethical Committee of Hospital de Clínicas de Porto Alegre, RS, Brazil. All parents of the patients included in the present study gave informed consent.

2.2. In vitro studies

Venous blood sample was collected from 3 healthy volunteers. Leukocytes from each control subject were pre-treated with various concentrations of phenylalanine (100, 250, 500, 1000, and 2500 μmol/L) for 6 h at 37 °C. These phenylalanine concentrations are similar to those found in blood from PKU patients that can vary from 100 μmol/L in well-treated patients to more than 2500 μmol/L at diagnosis.

2.3. In vivo studies

A total of 18 treated PKU patients admitted at the Medical Genetic Service of Hospital de Clínicas de Porto Alegre, aged between 3 and 25 years were studied. The patients were diagnosed by selective screening for PKU (age at diagnosis 25.4 ± 23.1 months). Treatment consisted of natural-protein-restricted diet supplemented with a Phe-free amino acid mixture enriched with vitamins and minerals, without L-carnitine and selenium (length of treatment 11.7 ± 4.7 years). Patients were divided into two groups according to their annual average Phe blood levels. The first group consisted of 8 patients, who had good compliance with the special diet (mean Phe 396.4 ± 151.6 μmol/L). The second group included 10 PKU patients, who did not strictly adhere to the diet resulting in high Phe blood levels (mean Phe 848.8 ± 150.8 μmol/L). Regarding to the optimal levels of blood Phe for PKU patients, the most commonly reported blood Phe recommendations are 120–360 μmol/L, although acceptable concentrations are considered below 600 μmol/L (normal range 60–240 μmol/L). Age-matched healthy individuals (n = 17) were used as the control group (mean Phe 108.7 ± 38.1 μmol/L).

2.4. Determination of phenylalanine levels

Plasma Phe levels were measured spectrofluorometrically based on the method of McCaman and Robins [14]. Phe reacts with ninhydrin in the presence of copper ions to form a highly fluorescent complex, which is proportional to Phe plasma concentration. The results were represented in μmol/L.

2.5. Single cell gel electrophoresis (comet assay)

The alkaline comet assay was performed as described by Singh et al. [15], in accordance with general guidelines for use of the comet assay [16,17]. Isolated human leukocytes were suspended in agarose and spread into a glass microscope slide pre-coated with agarose. Agarose was allowed to set at 4 °C for 5 min. Slides were incubated in ice-cold lysis solution to remove cell proteins, leaving DNA as "nucleoids". After the lysis procedure, slides were placed on a horizontal electrophoresis unit, covered with fresh buffer (300 mM NaOH and 1 mM EDTA, pH >13) for 20 min at 4 °C to allow DNA unwinding and the expression of alkali-labile-sites. Electrophoresis was performed for 20 min (25 V; 300 mA; 0.9 V/cm). Slides were then neutralized, washed in bi-distilled water and stained using a silver staining protocol [18]. After drying at room temperature overnight, gels were analyzed using an optical microscope. One hundred cells (50 cells from each of the two replicate slides) were selected, and analyzed. Cells were visually scored according to tail length and receive scores from 0 (no migration) to 4 (maximal migration) according to tail intensity. Therefore, the damage index (DI) for cells ranged from 0 (all cells with no migration) to 400 (all cells with maximal migration). The slides were analyzed under blind conditions at least by two different individuals.

2.6. Statistical analysis

Data were analyzed using nonparametric Kruskal–Wallis test followed by Mann–Whitney U-test. A p value lower than 0.05 was considered significant. The values were presented as median ± S.E. All analyses were performed using the Statistical Package for the Social Sciences (SPSS) software in a PC-compatible computer.

3. Results

Tables 1 and 2 show individual DI values and the number of cells found in each damage class for the treated PKU patients and controls, respectively. Fig. 1 shows the *in vitro* effect of Phe on DNA damage in white blood cells. We verified a concentration-dependent effect of Phe and DI until the Phe concentration of 1000 μmol/L ($p < 0.01$). No significantly difference was found on

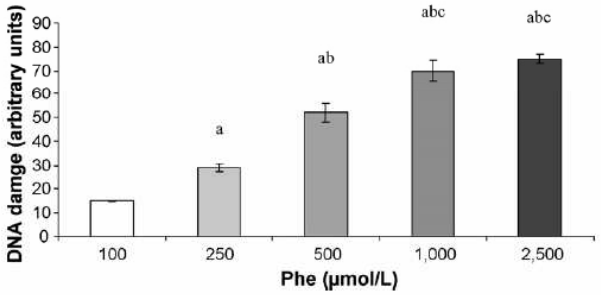

Fig. 1. *In vitro* effect of phenylalanine on DNA damage (comet assay) in leukocytes from whole blood. Data represent median ± S.E. of 3 independent experiments (individuals). (a) $p < 0.01$ compared to the Phe 100 μmol/L group; (b) $p < 0.01$ compared to the 250 μmol/L group; (c) $p < 0.01$ compared to the Phe 500 μmol/L group (Kruskal–Wallis test followed by Mann–Whitney U-test).

Table 1
Age, Phe levels and individual DI values and number of cells found in each damage class in the PKU group.

PKU patient	Average Phe levels (μmol/L)	Age (years)	DI	Damage class				
				0	1	2	3	4
1	574.0	10	41	67	26	6	1	0
2	441.1	10	50	59	34	5	2	0
3	410.9	12	48	61	30	9	0	0
4	1003.0	15	68	65	10	18	6	1
5	507.6	15	56	57	33	7	3	0
6	712.3	18	69	50	36	9	5	0
7	664.5	7	92	43	32	17	7	1
8	942.6	11	65	57	27	10	6	0
9	1087.6	9	48	70	17	8	5	0
10	688.8	15	62	67	12	14	6	1
11	199.4	9	51	68	20	6	5	1
12	864.0	25	57	65	19	11	4	1
13	277.9	13	42	74	16	5	4	1
14	555.9	10	38	75	15	7	3	0
15	749.2	24	65	62	20	11	5	2
16	779.2	25	80	43	40	11	6	0
17	997.0	18	76	48	35	11	5	1
18	205.4	3	33	79	13	5	2	1
$\sum$				1110	435	170	75	10

DI: damage index.

Table 2
Age and individual DI values and number of cells found in each damage class in the control group.

Control subject	Age (years)	DI	Damage class				
			0	1	2	3	4
1	15	13	90	7	3	0	0
2	20	9	93	5	2	0	0
3	21	11	92	5	3	0	0
4	11	8	93	6	1	0	0
5	14	7	93	7	0	0	0
6	8	18	87	8	5	0	0
7	16	17	88	7	5	0	0
8	12	18	84	14	2	0	0
9	12	10	90	10	0	0	0
10	18	13	89	9	2	0	0
11	11	17	83	17	0	0	0
12	8	8	93	6	1	0	0
13	5	17	83	17	0	0	0
14	6	13	91	5	4	0	0
15	7	18	88	6	6	0	0
16	4	7	93	7	0	0	0
17	5	12	89	10	1	0	0
$\sum$			1519	146	35	0	0

DI: damage index.

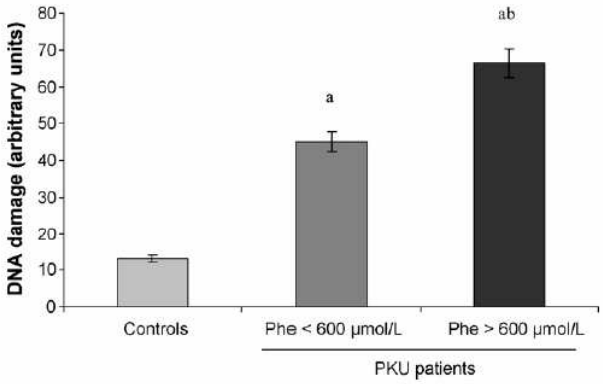

Fig. 2. DNA damage (comet assay) of peripheral blood leukocytes from two groups of PKU patients, one with Phe <600 μmol/L ($n = 8$) and the other with Phe >600 μmol/L ($n = 10$) and controls ($n = 17$). Data represent median ± S.E. (a) $p < 0.0001$ compared to the control; (b) $p < 0.001$ compared to the Phe <600 μmol/L group (Kruskal–Wallis test followed by Mann–Whitney *U*-test).

DNA damage between 1000 and 2500 μmol/L, both considered extremely high Phe levels for PKU patients.

Fig. 2 shows that greater DNA migration was found in the well-controlled group of PKU patients (DI = 44.9 ± 7.6) when compared to the control group (DI = 12.7 ± 4.1). Additionally, patients who did not adhere to the recommended diet and have high Phe levels presented significantly increased damage index (DI = 68.2 ± 12.3) when compared to the well-treated patients and controls.

4. Discussion

Oxidative stress has been demonstrated in PKU patients and in experimental PKU animal models [8–10,19]. In this context, our laboratory has shown that lipid and protein oxidative damage occurs in PKU patients [11–13]. Considering that oxidative stress can provoke DNA damage [20], our goal in this work was to evaluate DNA damage in PKU patients, which are exposed for long periods to high levels of Phe and its metabolites.

It should be emphasized that DNA is a particularly important target for oxidation generating several classes of products (single- and double-strand breaks), inter/intra-strand cross-links, DNA-proteins cross-links, and sugar fragmentation products. These products may elicit mutations, microsatellite instability, loss of heterozygosity, chromosomal aberrations, cytotoxicity, and neoplasic growth [21].

We determined DNA damage in peripheral leukocytes by using the alkaline comet assay that measures DNA strand breaks in single cells. In the alkaline (pH> 13) version of the comet assay (single cell gel electrophoresis) developed by Singh et al. [15], increased DNA migration can be associated with incomplete excision repair sites [16], which are generated as an intermediate step during the action of different DNA-repair systems [22]. This assay possesses a number of advantages as compared to other tests, being extremely sensitive for detecting low levels of DNA damage, besides its low cost and short time necessary to perform the assay [23].

We verified significantly greater levels of DNA migration, and thus DNA damage, even in leukocytes from well-treated phenylketonuric patients (Phe <600 μmol/L) when compared to the control group. Additionally, the damage index was even greater in patients who did not comply adequately with the diet (Phe >600 μmol/L), when compared to the well-controlled PKU group. When comparing the distribution of damage class in the PKU groups, it is possible to observe that the differences were primarily caused by an increased number of cells in damage class 1 and 2, reflecting a homogeneously distributed increase in the number of slightly damage cells rather than a low number of highly damage cells. On the other hand, a significant number of cells presented damage class 3 and 4 in PKU patients, which did not occur in the control group.

These results are in agreement with our *in vitro* findings showing that the DNA damage index increased in parallel with the amount of Phe added to the medium and that Phe effect was maximal at 1000 μmol/L. It should be stressed that concentrations of Phe of 250 and 500 μmol/L already provoked a significant increase of DNA damage.

Taken these data together, we presume that DNA damage in PKU is probably related to blood Phe levels. Furthermore, considering that lipid and protein oxidative damage has been previously shown in PKU patients, it may be hypothesized that the present results of DNA damage may have occurred due to an excessive production of free radicals generating oxidative damage in cells [20]. Free radicals may lead to DNA damage by direct attack on the purine/pyrimidine bases and/or on the deoxyribose sugar, resulting in strand breaks and formation of DNA adducts and oxidized bases [24]. It is important to emphasize that we cannot rule out an oxidative DNA damage due to Phe metabolites (phenylpyru-

vate, phenyllactate and phenylacetate) that also accumulate in PKU patients.

In conclusion, the present work provides experimental evidence that DNA damage occurs in PKU patients, possibly secondarily to high tissue Phe levels. A previous study reported augmented levels of 8-hydroxy-2-deoxyguanosine, a product of DNA oxidative damage, in urine from PKU patients, which reinforces our present findings [25]. Our present results should be however interpreted with caution. In case DNA damage is also encountered in brain from animal models of hyperphenylalaninemia, DNA alterations may represent a further means to explain neurological dysfunction in this inherited metabolic disorder. In this context, it should be emphasized that the brain has low DNA-repair capacity and high vulnerability to oxidative damage [26,27], making this tissue extremely vulnerable to oxidative DNA damage.

Conflict of interest

The authors declare that there are no conflicts of interest.

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Capítulo 2 – Artigo 2: *Effect of short- and long-term exposition to high phenylalanine blood levels on oxidative damage in phenylketonuric patients*

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Effect of short- and long-term exposition to high phenylalanine blood levels on oxidative damage in phenylketonuric patients

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ABSTRACT

Phenylketonuria is the most frequent disturbance of amino acid metabolism. Treatment for phenylketonuric patients consists of phenylalanine intake restriction. However, there are patients who do not adhere to treatment and/or are not submitted to neonatal screening. These individuals are more prone to develop brain damage due to long-lasting toxic effects of high levels of phenylalanine and/or its metabolites. Oxidative stress occurs in late-diagnosed phenylketonuric patients, probably contributing to the neurological damage in this disorder. In this work, we aimed to compare the influence of time exposition to high phenylalanine levels on oxidative stress parameters in phenylketonuric patients who did not adhere to protein restricted diet. We evaluated a large spectrum of oxidative stress parameters in plasma and erythrocytes from phenylketonuric patients with early and late diagnosis and of age-matched healthy controls. Erythrocyte glutathione peroxidase activity and glutathione levels, as well as plasma total antioxidant reactivity were significantly reduced in both groups of patients when compared to the control group. Furthermore, protein oxidative damage, measured by carbonyl formation and sulfhydryl oxidation, and lipid peroxidation, determined by malondialdehyde levels, were significantly increased only in patients exposed for a long time to high phenylalanine concentrations, compared to early diagnosed patients and controls. In conclusion, exposition to high phenylalanine concentrations for a short or long time results in a reduction of non-enzymatic and enzymatic antioxidant defenses, whereas protein and lipid oxidative damage only occurs in patients with late diagnosis.

Phenylketonuria (PKU) is an autosomal recessive disorder involving mutations in the phenylalanine hydroxylase gene leading to a blockage in the conversion of phenylalanine (Phe) to tyrosine (Tyr) (Scriver and Kaufman, 2001). As a result, Phe accumulates in the blood and other tissues of the affected patients. Although severe brain damage is the hallmark on PKU untreated patients the precise mechanisms of brain damage in this disorder are still unclear (Van Spronsen et al., 2001). However, Phe seems to be the major neurotoxin in PKU (Scriver and Kaufman, 2001).

The only treatment currently available for PKU mainly involves a lifelong Phe-restricted diet. The diet consists of restriction of protein-rich foods, such as meat, fish, eggs and milk products, and a supplementation with an amino acid formula that contains micronutrients and the essential amino acids, except Phe (Start, 1998). The appropriate treatment to avoid mental retardation depends on early detection, which can only be done by newborn screening. Worldwide neonatal screening programs have expanded over the years, aiming to detect metabolic or endocrine diseases that are severe, frequent and treatable, and all programs include PKU (Dhondt, 2007). However, there is still a considerable number of PKU patients, especially in developing countries who are not diagnosed in the newborn period and start treatment late (Trefz et al., 2000). Furthermore, there are PKU patients that do not adhere strictly to treatment.

Oxidative stress is commonly observed in some inborn errors of intermediary metabolism, participating of their pathophysiology (Colome et al., 2000; Wajner et al., 2004). Although the

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Abbreviations: ANOVA, analysis of variance; GSH-Px, glutathione peroxidase; GSH, reduced glutathione; Phe, phenylalanine; PKU, phenylketonuria; ROS, reactive oxygen species; Se, selenium; TAR, total antioxidant reactivity; TBARS, thiobarbituric acid-reactive species; Tyr, tyrosine.

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mechanisms of increased oxidative stress in these diseases are not completely understood, it may be due to the accumulation of toxic metabolites that lead to excessive production of free radicals. Furthermore, restricted diets may also alter the antioxidant status (Artuch et al., 2004; Van Bakel et al., 2000).

In a recent study, we verified that oxidative stress is induced in treated phenylketonuric patients with late diagnosis (Sitta et al., 2006). In this work, we extended this investigation by analyzing two groups of PKU patients who did not adhere to the diet, one diagnosed late and other diagnosed in the neonatal period, in order to evaluate whether time exposition to high Phe levels could affect oxidative stress parameters in PKU.

1. Materials and methods

1.1. Patients and controls

Twenty patients with classical PKU under poor treatment control were studied. They were classified into two groups, according to their ages at diagnosis. Group A consisted of 10 patients diagnosed at the neonatal period (age at diagnosis 17 ± 6 days) and group B consisted of 10 patients diagnosed late (age at diagnosis 9.8 ± 7.4 months). Patients from the two groups had comparable age (group A: 8.9 ± 2.1 years; group B: 9.2 ± 1.7 years). The average blood Phe levels calculated from the various measurements obtained at every 2 months from the two groups of patients were also comparable (group A: 551 ± 245 μmol/L; group B: 518 ± 253). The patients' blood Phe levels at the moment of the tests were 874 ± 84.4 μmol/L for group A and 835 ± 78.9 μmol/L for group B. Age-matched healthy children ($n = 10$) were used as the control group.

The dietary treatment consisted of a restricted protein diet supplemented with an essential amino acid mixture. The diet contained 220–450 mg/(kg day) Phe and 2.55–4.00 g/(kg day) Tyr according to patients' age. There is no consensus concerning optimal levels of blood Phe for PKU patients. However, the most commonly reported blood Phe recommendations are 120–360 μmol/L. Therefore, our sample consisted of patients who did not strictly adhere to the dietary recommendation, presenting high blood Phe levels.

The study was approved by the Ethics Committee of the Hospital de Clínicas de Porto Alegre.

1.2. Erythrocyte and plasma preparation

Erythrocytes and plasma were prepared from whole blood samples obtained until 10:00 a.m. from fasting individuals (controls and PKU patients) by venous puncture with heparinized vials. Whole blood was centrifuged at 1000 × g, plasma was removed by aspiration and frozen at −80 °C until biochemical determinations. Erythrocytes were washed three times with cold saline solution (0.153 mol/L sodium chloride). Lysates were prepared by the addition of 1 mL of distilled water to 100 μL of washed erythrocytes and frozen at −80 °C until analysis.

1.3. Oxidative stress parameters

1.3.1. Thiobarbituric acid-reactive species (TBARS)

Thiobarbituric acid-reactive species (TBARS) were determined according to the method described by Esterbauer and Cheeseman (1990). Briefly, 300 μL of 10% trichloroacetic acid was added to 150 μL of plasma and centrifuged at 1000 × g for 10 min at 4 °C. Three hundred microlitre of the supernatant was transferred to a test tube and incubated with 300 μL 0.67% thiobarbituric acid (7.1% sodium sulfate) at 100 °C for 1 h. The resulting pink stained TBARS was determined at 535 nm wavelength in a spectrophotometer. Calibration curve was performed using 1,1,3,3-tetramethoxypropane subjected to the same treatment as that for the supernatants. TBARS were calculated as nmol TBARS/mg protein.

1.3.2. Total antioxidant reactivity (TAR)

Total antioxidant reactivity (TAR) was determined by measuring the luminol chemiluminescence intensity induced by 2,2′-azo-bis-(2-amidinopropane) (ABAP) according to the method of Lissi et al. (1995). The background chemiluminescence was measured by adding 4 mL of 2 mM ABAP (0.1 mol/L glycine buffer, pH 8.6) into a glass scintillation vial. Fifteen microlitre of luminol (4 mmol/L) was added to each vial and the chemiluminescence was measured. This was considered to be the basal value. Ten microlitre of 10 μmol/L Trolox or plasma were added and the chemiluminescence was measured during 60 s. The addition of Trolox or supernatant reduces the chemiluminescence. The rapid reduction in luminol intensity is considered the measure of the TAR capacity. TAR measurement was calculated as nmol Trolox/mg protein.

1.3.3. Glutathione peroxidase (GSH-Px)

GSH-Px was measured using the RANSEL kit (Randox Laboratories, UK). The method is based on Paglia and Valentine (1967). Glutathione peroxidase catalyses the oxidation of glutathione by cumene hydroperoxide. In the presence of glutathione reductase and NADPH, oxidized glutathione is immediately converted to its reduced form with a concomitant oxidation of NADPH to $NADP^+$. The decrease in absorbance at 340 nm is measured. One GSH-Px unit is defined as 1 μmol of NADPH consumed per minute and the specific activity is represented as units per mg protein.

1.3.4. Determination of protein carbonyl content

Protein carbonyl formation was measured spectrophotometrically according to Reznick and Packer (1994). One hundred microlitre of plasma was treated with 1 mL of 10 mM 2,4-dinitrophenylhidrazine (DNPH) dissolved in 2.5N HCl or with 2.5N HCl (blank) and left in the dark for 90 min. Samples were then precipitated with 500 μL 20% TCA and centrifuged for 5 min at 10,000 × g. The pellet was then washed with 1 mL ethanol:ethyl acetate (1:1, v/v) and dissolved in 200 μL 6 M guanidine prepared in 2.5N HCl at 37 °C for 5 min. The difference between the DNPH-treated and HCl-treated samples (blank) was used to calculate the carbonyl content determined at 370 nm. The results were calculated as nmol of carbonyl groups/mg of protein.

1.3.5. Reduced glutathione (GSH) levels

GSH concentrations were measured according to Browne and Armstrong (1998). One hundred microlitre of erythrocyte was incubated with an equal volume of o-phthaldialdehyde (1 mg/mL methanol) at room temperature for 15 min. Fluorescence was measured using excitation and emission wavelengths of 350 nm and 420 nm, respectively. Calibration curve was prepared with standard GSH (1–100 μM) and the concentrations were calculated as nmol/mg protein.

1.3.6. Sulfhydryl content

This assay is based on the reduction of 5,5′-dithio-bis (2-nitrobenzoic acid) (DTNB) by thiols, generating a yellow derivative (TNB) whose absorption is measured spectrophotometrically at 412 nm (Aksenov and Markesbery, 2001). Thirty microlitre of plasma was incubated with an equal volume of DTNB at room temperature for 30 min in a dark room. The sulfhydryl content is inversely correlated to oxidative damage to proteins. Results were reported as nmol TNB/mg protein.

1.3.7. Protein determination

Erythrocyte protein concentrations were determined by the method of Lowry et al. (1951), using bovine serum albumin as standard. Plasmatic protein concentrations were determined by the Biuret method using a diagnostic kit (Labtest Diagnóstica, MG, Brazil).

1.4. Statistical analysis

Data were expressed as mean ± standard deviation. Comparisons between means were carried out by one-way ANOVA followed by the Duncan multiple range test when the F value was significant. A P value lower than 0.05 was considered significant. All analyses were performed using the Statistical Package for Social Sciences (SPSS) software in a PC-compatible computer.

2. Results

Fig. 1 shows the enzymatic (GSH-Px activity) and non-enzymatic (GSH and TAR) antioxidant defenses in poor adherent PKU patients, with early (group A) and late (group B) diagnosis. It can be seen that erythrocyte GSH-Px activity (A) [$F(2, 27) = 3.369$, $P < 0.05$] and GSH levels (B) [$F(2, 27) = 8.465$, $P < 0.01$] were significantly reduced in both groups of patients, compared to the controls. Furthermore, plasma TAR measurement (C) [$F(2, 27) = 40.516$, $P < 0.01$] was similarly reduced in both groups of patients relatively to the controls.

Next, we evaluated lipid (TBARS) and protein (sulfhydryl content and carbonyl formation) oxidative damage in PKU patients diagnosed early and late (Fig. 2). TBARS, that reflects the amount of malondialdehyde formation, an end product of membrane fatty acid peroxidation was significantly increased only in the group of patients with late diagnosis (group B) [$F(2, 27) = 46.684$, $P < 0.01$], in comparison to patients with neonatal diagnosis (group A) and controls (A). In addition, plasma protein sulfhydryl groups was significantly reduced (B) [$F(2, 27) = 45.707$, $P < 0.01$], while plasma carbonyl formation (C) was significantly increased [$F(2, 27) = 9.163$, $P < 0.01$] only in patients with late diagnosis.

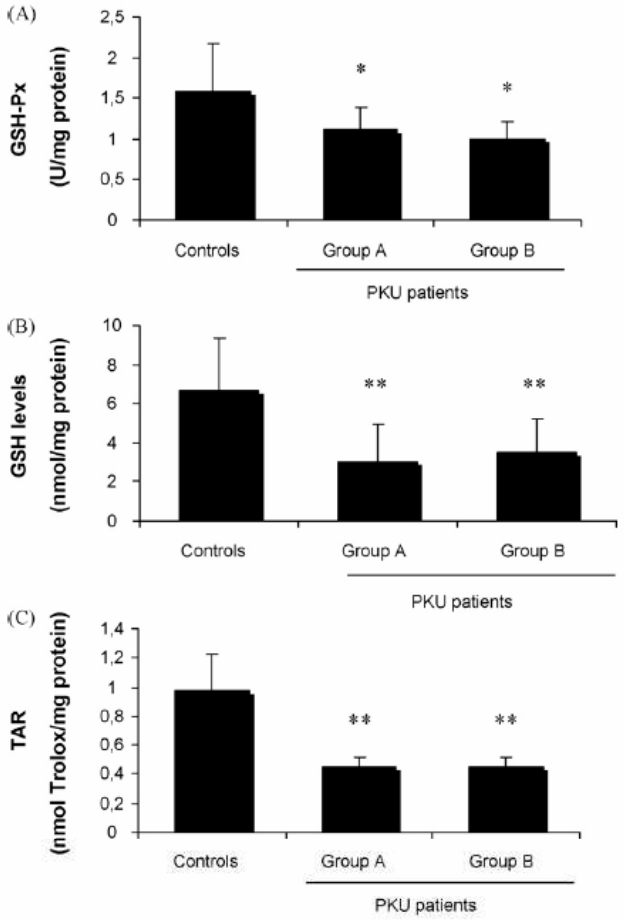

Fig. 1. Antioxidant measurements in two groups of poor adherent PKU patients and controls ($n = 10$). Group A represents patients early diagnosed (17 ± 6 days); group B represents patients with late diagnosis (9.8 ± 7.4 months). (A) Erythrocyte glutathione peroxidase activity (GSH-Px). (B) Erythrocyte glutathione levels (GSH). (C) Plasma total antioxidant reactivity (TAR). Data represent mean ± S.D. $^{*}P < 0.05$ and $^{**}P < 0.01$, different from controls (Duncan multiple range test).

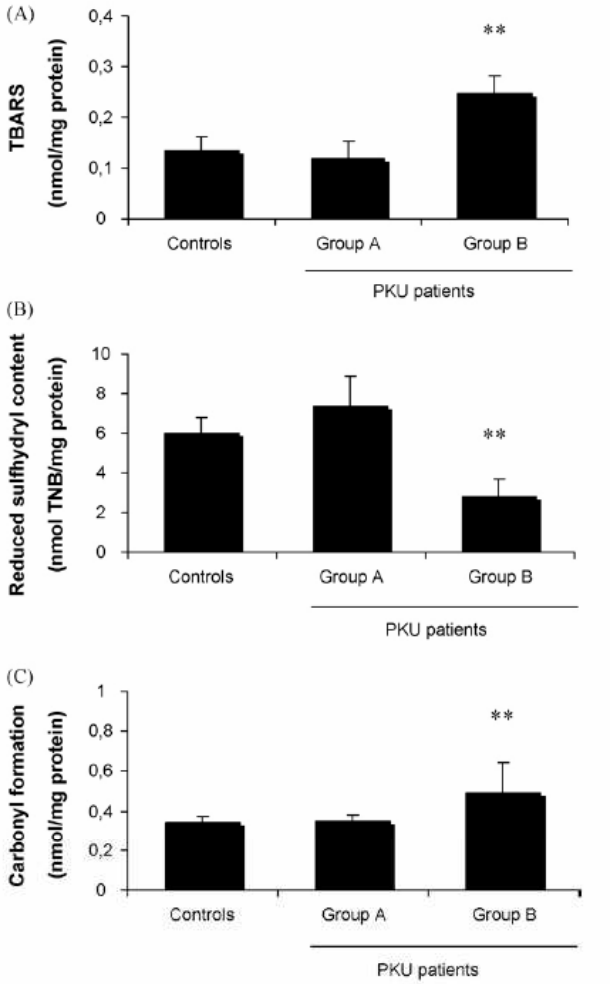

Fig. 2. Lipid and protein oxidative damage parameters in plasma from two groups of poor adherent PKU patients and controls ($n = 10$). Group A represents patients early diagnosed (17 ± 6 days) and group B indicates patients with late diagnosis (9.8 ± 7.4 months). (A) Thiobarbituric acid-reactive species (TBARS). (B) Sulfhydryl content. (C) Carbonyl content. Data represent mean ± S.D. $^{**}P < 0.01$, different from controls and from group A (Duncan multiple range test).

3. Discussion

PKU treatment consists of a low-phenylalanine diet that must be initiated in the neonatal period in order to prevent brain damage and the appearance of neurological symptoms in the affected patients (Scriver and Kaufman, 2001). However, some patients are missed in the newborn period and start treatment late, whereas other do not adhere to the diet.

Recently, it has been demonstrated that oxidative stress may contribute, at least in part, to the severe neurological dysfunction found in PKU (Sierra et al., 1998; Van Bakel et al., 2000; Colome et al., 2003; Artuch et al., 2004; Schulpis et al., 2005; Sirtori et al., 2005; Sitta et al., 2006). Our goal in the present study was to compare oxidative stress parameters in PKU patients who did not adhere to the recommended diet and were diagnosed in the neonatal period with those with late diagnosis. Both groups of patients were biochemically characterized by high Phe levels, implying that they did not follow the dietary therapy.

We found that the antioxidant status, measured by GSH levels and GSH-Px activity in erythrocytes and TAR in plasma, was significantly and similarly reduced in both groups of PKU patients. The reduction of blood antioxidant defenses could be tentatively attributed to dietary treatment, since the long-term administration of a protein restricted diet may result in deficiency of micronutrient, such as minerals, antioxidants and vitamins, which are necessary for tissue antioxidant defenses (Longhi et al., 1987). It is important to emphasize that patients included in this study were supplemented with a synthetic amino acids formula that did not contain L-carnitine and selenium. However, this is only a presumption that should be tested by including another group of untreated PKU patients receiving a normal protein diet.

In this context, some authors have already reported impaired of the antioxidant status in PKU as a consequence of the restricted diet. Thus, selenium (Se), an essential cofactor for GSH-Px activity, was found to be deficient in PKU patients and Se supplementation to these patients normalized GSH-Px activity (Wilke et al., 1992). Also, ubiquinone-10 (Q10) deficiency, another natural antioxidant, has been associated with a low protein dietary intake in human PKU (Artuch et al., 1999; Artuch et al., 2001). Furthermore, L-carnitine, a scavenger of free radicals, was also decreased in PKU patients submitted to a protein restricted diet (Schulpis et al., 1990; Sitta et al., in press). Alternatively, the low antioxidant status in both groups of PKU patients could be tentatively attributed to a

high production of reactive species, depleting therefore the major antioxidant defenses that potentially scavenge these species (Wajner et al., 2004).

We also observed that protein (carbonyl formation and sulfhydryl oxidation) and lipid (TBARS) oxidative damage were significantly increased only in the group of PKU patients with late diagnosis, as compared to early diagnosed patients and controls. Taken together, it is conceivable that tissue exposition to high levels of Phe and/or its metabolites for relatively short periods of time is not sufficient *per se* to cause oxidative damage, although the antioxidant defenses are already low. Otherwise, the data indicate that long time exposure to high Phe and/or its metabolites is capable to provoke tissue oxidative damage, besides reducing the antioxidant defenses, probably due to the excessive production of free radicals. In this context, it is possible that the antioxidant defenses can overcome free radical generation up to a certain extend (short reactive species exposition in early diagnosed) after which they may become insufficient and are not capable anymore to normalize these highly reactive radicals increased in blood (late diagnosed) leading to lipid peroxidation and protein oxidative damage. It should be also emphasized that patients with late diagnosis probably were submitted for a long time (since birth) to high concentrations of Phe before being diagnosed and start treatment, and this should also be taken into consideration in order to explain the damage observed in the measured parameters.

On the other hand, it is widely established that various reactive oxygen species (ROS) react with membrane lipids resulting in altered cell membrane fluidity and in the formation of end products which attack proteins and DNA bases, causing therefore mutagenic lesions (Halliwell and Gutteridge, 2001; Andersen et al., 2006). On the other hand, proteins can also be modified by direct attack of ROS, given rise to carbonyl group formation into side-chains or reduction of sulfhydryl groups of susceptible amino acids (Draper and Hadley, 1990; Ischiropoulos, 1998; Halliwell and Gutteridge, 2001; Levine, 2001).

In case the present findings showing a pro-oxidant status (decreased antioxidant defenses allied to increased oxidative damage) in blood of late diagnosed and poorly controlled PKU patients also occur in the brain, it may be presumed that irreversible cell oxidative injury may contribute, at least in part, to the neurological damage and symptoms presented by PKU patients who poorly adhere to treatment. At this point it should be emphasized that the central nervous system is highly sensitive to oxidative stress due to its high oxygen consumption, its high iron and lipid content, especially polyunsaturated fatty acids, and the low activity of antioxidant defenses (Halliwell, 1996).

Although the mechanisms underlying the increased oxidative damage in PKU are not completely understood, it may be well due to the accumulation of toxic metabolites such as phenylalanine, considered the major neurotoxic agent in this disorder, and/or its metabolites (Hagen et al., 2002; Ercal et al., 2002; Martinez-Cruz et al., 2002) that directly or indirectly induce excessive production of free radicals and/or deplete the tissue antioxidant capacity (Wajner et al., 2004).

In conclusion, to the best of our knowledge this is the first report demonstrating that long exposition to high Phe concentrations is determinant of tissue oxidative damage and reinforces previous *in vitro* and *in vivo* findings showing that oxidative stress probably is a pathomechanism of tissue damage in PKU.

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ORIGINAL PAPER

L-Carnitine Blood Levels and Oxidative Stress in Treated Phenylketonuric Patients

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Abstract *Aims* L-Carnitine exerts an important role by facilitating the mitochondrial transport of fatty acids, but is also a scavenger of free radicals, protecting cells from oxidative damage. Phenylketonuria (PKU), an inborn error of phenylalanine (Phe) metabolism, is currently treated with a special diet consisting of severe restriction of protein-enriched foods, therefore potentially leading to L-carnitine depletion. The aim of this study was to determine L-carnitine levels and oxidative stress parameters in blood of two groups of PKU patients, with good and poor adherence to treatment. *Methods* Treatment of patients consisted of a low protein diet supplemented with a synthetic amino acids formula not containing Phe, L-carnitine, and selenium. L-Carnitine concentrations and the oxidative stress parameters thiobarbituric acid reactive species (TBARS) and total antioxidant reactivity (TAR) were measured in blood of the two groups of treated PKU patients and controls. *Results* We verified a significant decrease of serum L-carnitine levels in patients who strictly adhered to the diet, as compared to controls and patients who did not comply with the diet. Furthermore, TBARS measurement was significantly increased and TAR was significantly reduced in both groups of phenylketonuric patients relatively to controls. We also found a significant negative correlation between TBARS and L-carnitine levels and a significant positive correlation between TAR and L-carnitine levels in well-treated PKU patients. *Conclusions* Our results suggest that L-carnitine should be measured in plasma of treated PKU patients, and when a decrease of this endogenous component is detected in plasma, supplementation should be considered as an adjuvant therapy.

Keywords Phenylketonuria · Oxidative stress · L-carnitine levels

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Introduction

Phenylketonuria (PKU) is an autosomal recessive disorder involving mutations in the phenylalanine

(Phe) hydroxylase gene. As a result, Phe cannot be converted to tyrosine and accumulates in the blood and other tissues of affected patients. Untreated PKU is associated with severe mental retardation, seizures, severe behavioral difficulties, and other symptoms (Scriver and Kaufman 2001). The currently available treatment consists of a lifelong Phe-restricted diet with low consumption of meat, fish, dairy products, nuts, beans, and other protein-containing foods (Start 1998).

L-Carnitine (4-*N*-trimethylammonium-3-hydroxybutyric acid) is a naturally endogenous compound that plays an important physiological role shuttling long-chain fatty acids from the cytoplasm across the inner mitochondrial membrane for β-oxidation and ATP production in peripheral tissues (Bahl and Bressler 1987). In addition, L-carnitine participates in the control of the mitochondrial acyl-CoA/CoA ratio, peroxisomal oxidation of fatty acids, and the production of ketone bodies. Due to its intrinsic interaction with the bioenergetic processes, it plays an important role in diseases associated with metabolic compromise and a deficiency of L-carnitine is known to have major deleterious effects on the CNS (Virmani and Binienda 2004).

In humans, L-carnitine is derived from dietary sources (75%) and endogenous biosynthesis (25%), which occurs in the liver, brain, and kidney (Bremer 1983). Primary and secondary L-carnitine deficiencies have been described, the latter being caused by genetically determined metabolic errors, acquired diseases, and iatrogenic factors (Pons and De Vivo 1995). Since the main dietary sources of L-carnitine are milk and red meat, secondary L-carnitine deficiency has been described in phenylketonuric patients, who are submitted to a protein-restricted diet (Schulpis et al. 1990), and inadequate iron availability has been suggested as its possible cause (Böhles et al. 1991).

In the last years, many studies have demonstrated antioxidant and antiradical activities for L-carnitine. Thus, L-carnitine was shown to have an antiperoxidative effect in several tissues by different mechanisms (Ferrari et al. 1988; Bertelli et al. 1994; Koudelova et al. 1994; Di Giacomo et al. 1993; Izgut-Uysal et al. 2001; Gülçin 2006). It was also found that L-carnitine has a scavenger effect on reactive oxygen species (ROS) and a stabilizing effect on damaged cell membranes (Fritz and Arrigoni-Martelli 1993).

In a recent study, we verified that oxidative stress is induced in treated phenylketonuric patients and that this process is not directly related to Phe blood levels (Sitta et al. 2006). In order to investigate the possible relation between L-carnitine levels and oxidative stress in treated phenylketonuric patients, in this study we determined total L-carnitine levels and oxidative stress parameters in plasma from two groups of patients with PKU under dietary treatment, one group that strictly adhered to the diet and the other group that did not comply with the recommended diet. We also correlated plasma L-carnitine concentrations with the oxidative stress parameters examined.

Methods

Patients and Controls

Twenty patients with classical PKU under dietary treatment were studied (treatment duration of 7.07 ± 2.85 years). Treatment consisted of a low protein diet supplemented with a synthetic amino acids formula that did not contain Phe, L-carnitine, and selenium. All patients were diagnosed after the neonatal period with high Phe blood levels and were classified into two groups according to their blood Phe levels. Group A consisted of 10 patients (mean age 8.28 ± 2.87 years), who had good compliance with the special diet (mean Phe blood levels 396.9 ± 46.8 μmol/l). Group B included 10 patients (mean age 9.59 ± 3.96 years), who did not strictly adhere to the diet (mean Phe blood levels 1,096.7 ± 78.3 μmol/l). Healthy children ($N = 10$) of comparable age (mean age 9.41 ± 3.50 years) and sex were used as control group (mean blood Phe levels 135.5 ± 21.4 μmol/l).

The study was approved by the Ethics Committee of the Hospital de Clínicas de Porto Alegre.

Plasma Preparation

Plasma was separated from whole blood samples obtained from fasting individuals (controls and PKU patients) by venous puncture with heparinized vials. Whole blood was centrifuged at 1,000*g*, and plasma was removed by aspiration and frozen at −80°C until analysis.

Determination of Phenylalanine Levels

Plasma Phe levels were measured spectrofluorometrically based on the method of McCaman and Robins (1962). Phe reacts with ninhydrin in the presence of copper ions to form a highly fluorescent complex, which is proportional to Phe plasma concentration. The results were represented as μmol/l.

Determination of Total L-carnitine Levels

Total plasma L-carnitine levels were determined based on the method of De Sousa et al. (1990). Initially, 100 μl of potassium hydroxide 1 mol/l was added to 50 μl of plasma and the mixture incubated at 37°C for 30 min. The solution to be assayed was added to 50 μl of HEPES 0.5 mol/l, 20 μl of *N*-ethylmaleimide 40 mmol/l, and 100 μl of [1–14C] acetyl coenzyme A 10 μmol/l. Then, 30 μl of L-carnitine acyltransferase (0.5 mg/ml) was added and the mixture was passed down a column of Dowex. The total column effluent was collected, the scintillation fluid was added, and the isotope content was determined using a liquid scintillation counter. The results were expressed as μmol/l.

Thiobarbituric Acid Reactive Species (TBARS)

Thiobarbituric acid reactive species (TBARS) values were determined according to the method described by Esterbauer and Cheeseman (1990). Briefly, 300 μl of 10% trichloroacetic acid was added to 150 μl of plasma and centrifuged at 1,000*g* for 10 min at 4°C. Then, 300 μl of the supernatant were transferred to a test tube and incubated with 300 μl 0.67% thiobarbituric acid (7.1% sodium sulfate) at 100°C for 1 h. The mixture was allowed to cool in water for 5 min. The resulting pink-stained TBARS was determined at 535 nm wavelength in a spectrophotometer. Calibration curve was performed using 1,1,3,3-tetramethoxypropane subjected to the same treatment as that for the supernatants. TBARS measurement was calculated as nmol TBARS/mg protein.

Total Antioxidant Reactivity (TAR)

Total antioxidant reactivity (TAR), which represents the quality of the tissue antioxidants, was determined by measuring the luminol chemiluminescence intensity induced by 2,2′-azo-bis-(2-amidinopropane) (ABAP) according to the method of Lissi et al. (1995). The background chemiluminescence was measured by adding 4 ml of 2 mM ABAP (0.1 mol/l glycine buffer, pH 8.6) into a glass scintillation vial. About 15 μl of luminol (4 mmol/l) was added to each vial and the chemiluminescence was measured. This was considered to be the basal value. Then, 10 μl of 10 μmol/l Trolox or plasma was added, and the chemiluminescence was measured for 60 sec. The addition of Trolox or supernatant reduces the chemiluminescence. The rapid reduction in luminol intensity is considered the measure of the TAR capacity. TAR measurement was calculated as nmol Trolox/mg protein.

Protein Determination

Plasma protein concentrations were determined by the Biuret method using the commercial kit of Labtest (Labtest Diagnóstica, MG, Brazil).

Statistical Analysis

Data were expressed as mean ± standard deviation. Comparisons between means were analyzed by one-way ANOVA followed by the Duncan multiple range test when the F value was significant. Correlations between variables were calculated using the Pearson correlation coefficient. A P value lower than 0.05 was considered significant. All analyses were performed using the Statistical Package for the Social Sciences (SPSS) software in a PC-compatible computer.

Results

Figure 1a shows Phe concentrations in plasma from the PKU patients studied and controls. We initially determined the total L-carnitine levels in plasma from two groups of treated phenylketonuric patients, one with a good response to dietary treatment (Group A) and the other with high plasma Phe levels (Group B) (Fig. 1b). We verified a significant decrease of plasma total L-carnitine levels in group A (mean L-carnitine 42.7 ± 3.2 μmol/l; mean Phe 396.9 ± 46.8 μmol/l), when compared to controls (mean L-carnitine 56.6 ± 6.8 μmol/l; mean Phe 135.5 ± 21.4 μmol/l) and to group B (mean L-carnitine 59.3 ± 2.2 μmol/l;

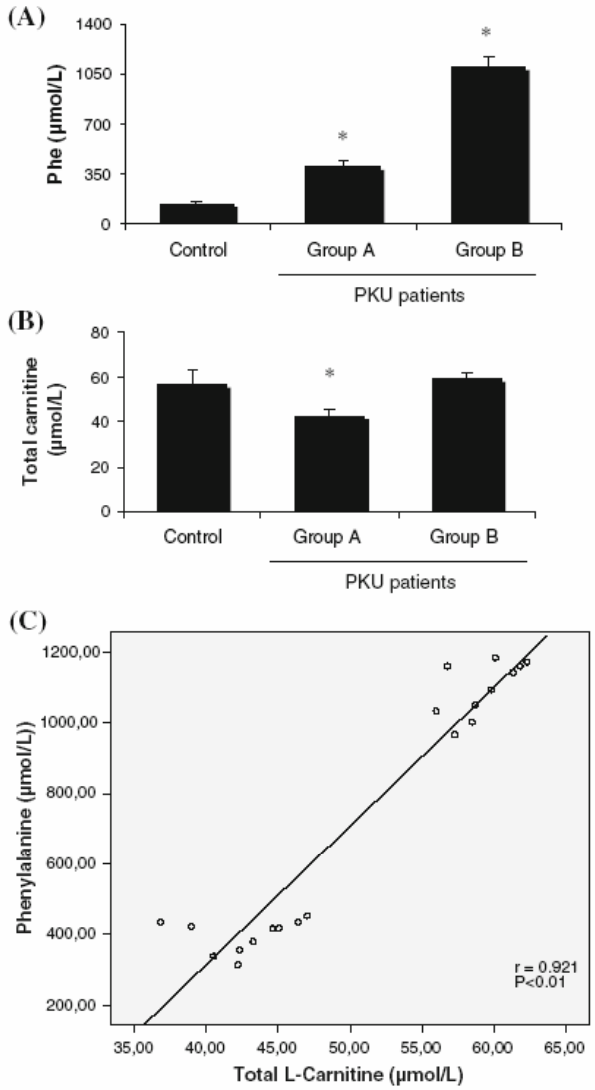

Fig. 1 Phenylalanine concentrations (**a**) and total L-carnitine levels (**b**) in plasma from treated PKU patients and controls. Data are mean ± SD. Group A represents patients who adhered to the diet, whereas patients from group B did no comply with the low Phe diet ($n = 10$). $^{*}P < 0.01$, different from control (Duncan multiple range test). Correlation (Pearson correlation coefficient) between L-carnitine levels and Phe concentrations (**c**)

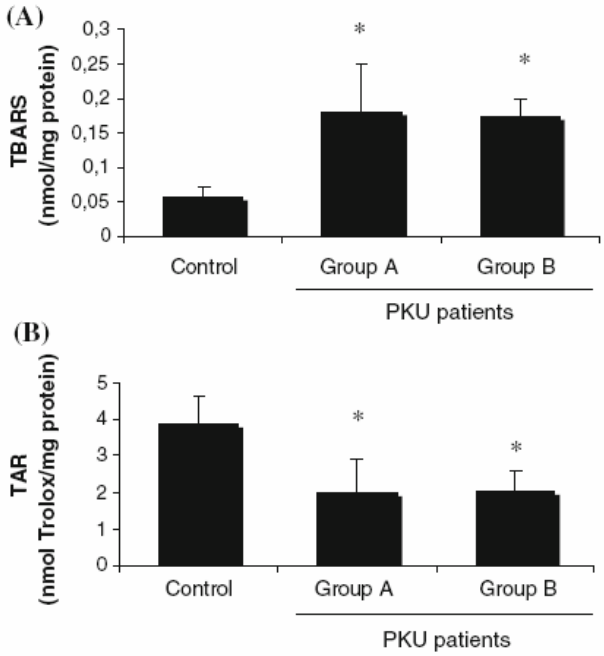

Fig. 2 Thiobarbituric acid reactive species (TBARS) measurement (**a**) and total antioxidant reactivity (TAR) (**b**) in plasma from treated PKU patients and controls. Data are mean ± SD. Group A represents patients with blood Phe levels of 396.9 ± 46.8 µmol/l. Group B represents patients with blood Phe levels of 1,096.7 ± 78.3 µmol/l. ($n = 10$). $^{*}P < 0.01$, different from control (Duncan multiple range test)

mean Phe 1,096.7 ± 78.3 µmol/l) [$F(2,27) = 38.174$, $P < 0.01$]. We also found a significant positive correlation between serum L-carnitine and Phe levels (Fig. 1c) [$r = 0.921$, $P < 0.01$].

Next, we measured the oxidative stress parameters TBARS and TAR in the same two groups of phenylketonuric patients and in controls. Figure 2a shows the results of TBARS measurement, a lipid peroxidation parameter, which was similarly increased in both groups of treated patients in respect to the controls (group A: mean TBARS 0.185 ± 0.068 nmol/mg protein; group B: mean TBARS 0.175 ± 0.024 nmol/mg protein; controls: mean TBARS 0.056 ± 0.015 nmol/mg protein) [$F(2,27) = 27.330$, $P < 0.01$]. As shown in Fig. 2b, TAR values, a measure of the tissue capacity to react with free radicals, were significantly reduced in both groups of PKU patients, when compared to the control group (group A: mean TAR 1.985 ± 0.941 nmol Trolox/mg protein; group B: mean TAR 2.044 ± 0.573 nmol Trolox/mg protein; controls: mean TAR 3.858 ± 0.777 nmol Trolox/mg protein) [$F(2,27) = 10.751$, $P < 0.01$].

In order to investigate whether blood L-carnitine levels were associated to oxidative stress in PKU patients, we correlated TBARS and TAR with total L-carnitine levels in treated PKU patients. We verified a significant negative correlation between TBARS values and L-carnitine concentrations ($r = -0.577$, $P < 0.05$) (Fig. 3a) and a significant positive correlation between TAR measurement and total L-carnitine levels ($r = 0.606$, $P < 0.05$) (Fig. 3b) in the group of patients presenting low blood L-carnitine

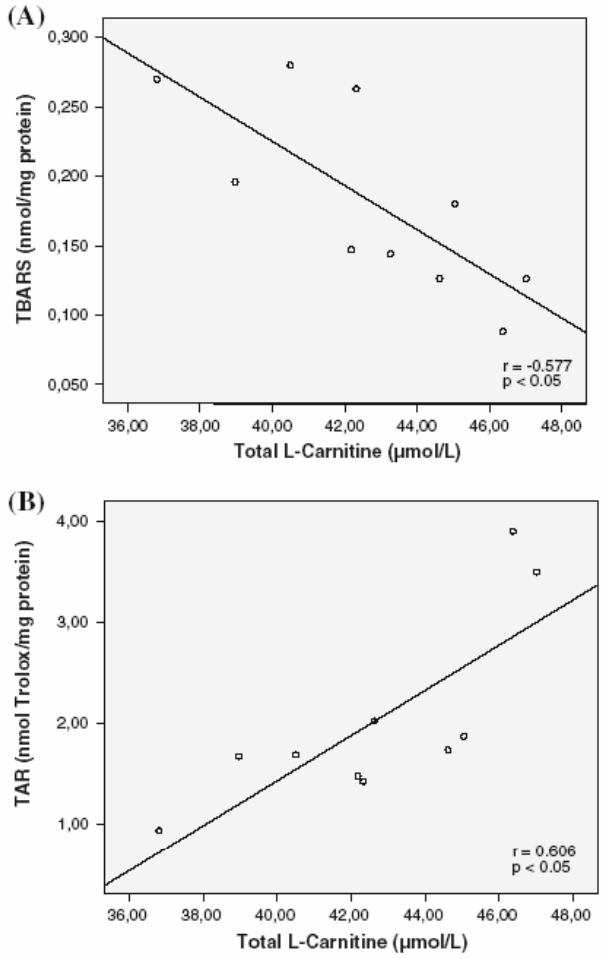

Fig. 3 Correlation between oxidative stress parameters and total L-carnitine levels in plasma from well-treated phenylketonuric patients (mean blood Phe 396.9 ± 46.8 µmol/l). Correlation between TBARS and L-carnitine levels (**a**). Correlation between TAR and L-carnitine levels (**b**). Graphs show the Pearson correlation coefficient and probabilities

levels (group A). In contrast, no correlation was found between oxidative stress parameters (TBARS and TAR) and L-carnitine concentrations in the group of patients with high blood Phe levels and normal plasma L-carnitine levels (group B).

Discussion

PKU is a common inborn error of metabolism with an approximate incidence of 1:10,000 newborns (Scriver and Kaufman 2001). Fortunately, it is a disorder in which developments in science and medicine have resulted in many advances in the last decades. Currently, the phenotype of the untreated disorder (severe mental retardation and epilepsy) can be almost completely replaced by normal cognitive and physical development by the use of a low-Phe dietary treatment (Hendriksz and Walter 2004). On the other hand, many studies have searched for the mechanisms underlying mental retardation from non-well-treated PKU, but no single factor has been identified as being responsible for the central nervous system related-problems in these patients (Ercal et al. 2002).

The importance of ROS and free radicals has attracted increasing attention over the past decade, since these molecules are exacerbating factors in cellular injury and aging processes (Halliwell and Gutteridge 2001; Gülçin 2006). In this context, oxidative stress has been observed in some inborn errors of intermediary metabolism and attributed to the accumulation of toxic metabolites eliciting excessive free radical production (Colome et al. 2000). Recent studies have shown that oxidative stress occurs in treated and non-treated PKU patients and that this pathologic process is not directly related to blood Phe levels, implying that other causes should be searched for (Sierra et al. 1998; Sirtori et al. 2005; Sitta et al. 2006).

A semi-synthetic formula low in Phe and protein sometimes associated with a mixture of essential amino acids (Matalon et al. 2006) is currently used to treat PKU patients. This diet is mainly made up essentially of vegetables and fruits (poor in Phe) and provides the minimal amounts of animal products appropriate for normal development growth, while keeping plasma Phe levels within normal (Giovanini et al. 2007). Since approximately 75% of L-carnitine content in human body is derived from the diet mainly from products of animal origin, PKU patients under dietary treatment are potentially prone to develop L-carnitine deficiency (Schulpis et al. 1990; Vilaseca et al. 1993). Moreover, some studies have demonstrated that special restricted diets may lead to a decrease of the antioxidant status in some inborn errors of metabolism (Artuch et al. 2004; van Backel et al. 2000). In this context, it should be stressed that various antioxidant properties have been described for L-carnitine (Ferrari et al. 1988; Bertelli et al. 1994; Koudelova et al. 1994; Di Giacomo et al. 1993; Izgut-Uysal et al. 2001; Gülçin 2006). Therefore, in this work we first measured various oxidative stress parameters and L-carnitine in blood of treated

phenylketonuric patients, and thereafter investigated the relationship between serum L-carnitine deficiency and the oxidative stress process observed.

We first observed that serum L-carnitine levels were significantly reduced in well-treated PKU patients with normal plasma Phe levels, but not in patients with poor compliance to the diet (with high Phe levels), reinforcing previous findings of other investigators (Schulpis et al. 1990; Vilaseca et al. 1993). Also, a positive correlation between serum Phe levels and serum L-carnitine levels was observed. Thus, it may be presumed that L-carnitine deficiency may be due to a very restricted diet in animal products.

We also observed a similar increase of lipid peroxidation (TBARS measurement) and a decrease of antioxidant defenses (TAR measurement) in plasma of both groups of PKU patients, i.e., those who strictly adhered to the diet and those who did not comply with the diet. We also evaluated the association between the decreased L-carnitine levels and the increased oxidative stress parameters found in plasma of treated PKU patients, and verified a significant negative correlation between TBARS measurement and total L-carnitine levels and a significant positive correlation between TAR and blood total L-carnitine levels in well-controlled patients, but not in those who did not adhere to the diet.

Some works have shown antiperoxidative effects of L-carnitine (blocks the accumulation of end products of lipid peroxidation) (Fariello and Calabrese 1988; Bertelli et al. 1994; Lowitt et al. 1995) and that L-carnitine prevents the formation of ROS, and regulates nitric oxide, cellular respiration, and the activity of enzymes involved in defense against oxidative damage (Brown 1999; Kremser et al. 1995). Therefore, it might be presumed that the lipid peroxidation process and the reduction of TAR reflecting oxidative stress in well-treated PKU patients can be, at least in part, due to lower levels of L-carnitine, at least in those who strictly adhered to the recommended diet. On the other hand, since lipid peroxidation and diminution of plasma antioxidant defenses also occurred to a similar extent in PKU patients not complying with the diet, other factors than L-carnitine and Phe levels (perhaps other metabolites accumulating in this disorder, such as phenyllactate, phenylpyruvate, and phenylacetate) may be acting to explain the oxidative stress demonstrated in these patients.

It is also important to emphasize that other antioxidants, such as selenium and coenzyme Q10 (Reilly et al. 1990; Artuch et al. 2004) have been found to be deficient in treated PKU patients. We did not measure coenzyme Q10 in our patients, but they had lower serum selenium levels (data not shown). However, we did not observe a correlation between serum selenium levels and the oxidative stress parameters studied, except for erythrocyte glutathione peroxidase activity, since selenium is necessary for this enzyme activity. Therefore, although decreased L-carnitine levels were observed in well-treated PKU patients, we cannot at present establish that this decrease is mainly responsible for the oxidative stress found in these patients since other antioxidants are also decreased due to the restricted diet. This conclusion is also based on the fact that oxidative stress parameters were altered in all patients, whereas L-carnitine levels were reduced only in well-controlled patients.

Thus, the increased oxidative damage in our sample of PKU patients is complex and probably multifactorial, implying that more investigation is needed to unravel the distinct cooperative mechanisms responsible for this process. On the other hand, L-carnitine supplementation to these patients followed by determination of L-carnitine and oxidative stress parameters may respond to the question of whether L-carnitine deficiency is a key feature in this whole process.

Oxidative stress process can cause damage in crucial cellular macromolecules (DNA, proteins and lipids), while the low L-carnitine levels might cause, in the long-term, energy metabolism impairment (Halliwell and Gutteridge 2001; Stanley 2004). Recent findings demonstrated that L-carnitine exerts antioxidant and neuroprotective effects in quinolinic acid-induced prooxidant and 3-nitropropionic-induced deficient metabolic models (Silva-Adaya et al. 2008). Its neuroprotective effects can be due to its antioxidant properties or to the facilitation of long-chain fatty acid transport into the mitochondria to produce energy through β-oxidation. Since this latter process is not used in the brain, which depends nearly exclusively on glucose (glycolysis) for its energetic needs, rather than on fatty acids, it may be hypothesized that the antioxidant activities or L-carnitine are more important to explain its neuroprotection. Thus, considering the evidence provided

in the present work that oxidative stress in well-treated PKU patients is possibly associated to decreased L-carnitine concentrations, it might be appropriate to supplement the diet of these patients with L-carnitine.

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Capítulo 4 – Artigo 4: *Evidence that L-carnitine and selenium supplementation reduces oxidative stress in phenylketonuric patients*

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ORIGINAL RESEARCH

Evidence that L-Carnitine and Selenium Supplementation Reduces Oxidative Stress in Phenylketonuric Patients

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Abstract It is well established that the involvement of reactive species in the pathophysiology of several neurological diseases, including phenylketonuria (PKU), a metabolic genetic disorder biochemically characterized by elevated levels of phenylalanine (Phe). In previous studies, we verified that PKU patients (treated with a protein-restricted diet supplemented with a special formula not containing L-carnitine and selenium) presented high lipid and protein oxidative damage as well as a reduction of antioxidants when compared to the healthy individuals. Our goal in the present study was to evaluate the effect of Phe-restricted diet supplemented with L-carnitine and selenium, two well-known antioxidant compounds, on oxidative damage in PKU patients. We investigated various oxidative stress parameters in blood of 18 treated PKU patients before and after 6 months of supplementation with a special formula containing L-carnitine and selenium. It was verified that treatment with L-carnitine and selenium was capable of reverting the lipid peroxidation, measured by thiobarbituric acid-reactive species, and the protein oxidative damage, measured by sulfhydryl oxidation, to the levels of controls. Additionally, the reduced activity of glutathione peroxidase was normalized by the antioxidant supplementation. It was also verified a significant inverse correlation between lipid peroxidation and L-carnitine blood levels as well as a significant positive correlation between glutathione peroxidase activity and blood selenium concentration. In conclusion, our results suggest that supplementation of L-carnitine and selenium is important for PKU patients since it could help to correct the oxidative stress process which possibly contributes, at least in part, to the neurological symptoms found in phenylketonuric patients.

Keywords Phenylketonuria · Oxidative stress · L-Carnitine · Selenium

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Introduction

The participation of reactive oxygen species (ROS) on the pathophysiology of a crescent number of pathologies, including cancer, neurodegenerative disorders, and inherited metabolic diseases is well established (Halliwell and Gutteridge 2007).

ROS are continuously produced during normal physiologic events and are capable of easily initiating deleterious

cascades inducing peroxidation of membrane lipids. In order to handle ROS production, organisms possess an efficient antioxidant system which includes non-enzymatic antioxidant defenses and antioxidant enzymes such as superoxide dismutase (SOD), catalase (CAT), and glutathione peroxidase (GSH-Px). However, when the balance between the generation of ROS and the inactivation of ROS by the antioxidant system is lost, oxidative stress occurs, leading to oxidative damage to cellular membrane or intracellular molecules, and causing pathological conditions (Halliwell and Gutteridge 2007; Chaudière 1994; German 1999).

Phenylketonuria (PKU) is an inborn error of phenylalanine (Phe) metabolism, caused by deficiency of the enzyme phenylalanine hydroxylase activity, which converts Phe to tyrosine (Tyr). Although Phe is thought to be neurotoxic in PKU, no single mechanism has been identified as being responsible for the central nervous system-related problems, and the pathophysiology of the disease still remains unclear (Scriver and Kaufman 2001; Van Spronsen et al. 2001).

In this context, there is strong evidence that oxidative stress contributes for the neurological damage in PKU, especially in untreated patients or in patients who do not comply with the low-Phe diet. It is presumed that Phe and/or its metabolites induce excessive production of free radicals and/or deplete the tissue antioxidant capacity (Colome et al. 2003; Schulpis et al. 2005; Sierra et al. 1998, 2006, 2009a; Sirtori et al. 2005). In addition, protein-restricted diet for PKU patients may lead to a decrease of the antioxidants intake, contributing to the oxidative damage verified in the disease (Artuch et al. 2004; van Backel et al. 2000).

We previously demonstrated that PKU patients submitted to a protein-restricted diet presented lipid and protein oxidative damage and a decrease in the antioxidant status (Sitta et al. 2006, 2009a). Additionally, we verified in PKU patients a positive correlation between the plasma antioxidant reactivity and L-carnitine (LC) concentrations and a negative correlation between malondialdehyde levels and LC levels, a substance with a potential antioxidant effect that is found to be reduced in PKU patients under strict diet (Sitta et al. 2009b). Therefore, in the present work we evaluated the effect of a long-term supplementation with LC and selenium (Se) on various oxidative stress parameters in PKU patients in order to test for the efficacy of this treatment on the antioxidant status of phenylketonuric patients.

Methods

Patients and Controls

Eighteen patients (mean age 17.2 ± 2.6 years; range 15–22 years-old) with classical PKU under treatment were studied. The average blood Phe levels calculated from the various measurements obtained at every 2 months was 686 ± 315 μmol/l. The dietary treatment consisted of a restricted protein diet supplemented with a special formula not containing LC and Se (PKU 3—Support®). The diet contained 220–450 mg/(kg day) Phe and 2.55–4.00 g/(kg day) Tyr according to patients' age. Oxidative stress parameters were analyzed in blood of PKU patients before and after at least 6 months of supplementation with Se and LC (PKU 3 Advanta—Support®—Se: 31.5 mcg/day; LC: 98 mg/day). Eighteen healthy children (mean age 19.4 ± 3.7 years; range 18–23 years-old) were used as the control group.

The study was approved by the Ethics Committee of the Hospital de Clínicas de Porto Alegre.

Erythrocyte and Plasma Preparation

Erythrocytes and plasma were prepared from whole blood samples obtained from fasting individuals (controls and PKU patients) by venous puncture with heparinized vials. Fifty microliters of the whole blood was spotted onto specialized paper cards for posterior analysis of free L-carnitine. The additional whole blood was centrifuged at 1,000×*g*, plasma was removed by aspiration and frozen at −80°C until determinations. Erythrocytes were washed three times with cold saline solution (0.153 mol/l sodium chloride). Lysates were prepared by the addition of 1 ml of distilled water to 100 μl of washed erythrocytes and frozen at −80°C until analysis.

Oxidative Stress Parameters

Determination of Thiobarbituric Acid-Reactive Species (TBARS)

Thiobarbituric acid-reactive species were determined according to the method described by Esterbauer and Cheeseman (1990). Briefly, 300 μl of 10% trichloroacetic acid was added to 150 μl of plasma and centrifuged at 1,000×*g* for 10 min at 4°C. Three hundred microliters of the supernatant was transferred to a test tube and incubated with 300 μl 0.67% thiobarbituric acid (7.1% sodium sulfate) at 100°C for 1 h. The resulting pink stained TBARS were determined at 535 nm wavelength in a spectrophotometer. Calibration curve was performed using 1,1,3,3-tetramethoxypropane subjected to the same treatment as that for the supernatants. TBARS were calculated as nanomoles per milligram protein.

Glutathione Peroxidase (GSH-Px)

GSH-Px activity was measured using the RANSEL kit (Randox Laboratories, UK). The method is based on Paglia

and Valentine (1967). Glutathione peroxidase catalyses the oxidation of glutathione by cumene hydroperoxide. In the presence of glutathione reductase and NADPH, oxidized glutathione is immediately converted to its reduced form with a concomitant oxidation of NADPH to $NADP^+$. The decrease in absorbance at 340 nm is measured. One GSH-Px unit is defined as 1 µmol of NADPH consumed per minute and the specific activity was represented as units per milligram protein.

Determination of Protein Carbonyl Content

Protein carbonyl formation was measured spectrophotometrically according to Reznick and Packer (1994). One hundred microliters of plasma was treated with 1 ml of 10 mM 2,4-dinitrophenylhidrazine (DNPH) dissolved in 2.5 N HCl or with 2.5 N HCl (blank) and left in the dark for 90 min. Samples were then precipitated with 500 µl 20% TCA and centrifuged for 5 min at 10,000×*g*. The pellet was then washed with 1 ml ethanol:ethyl acetate (1:1, V/V) and dissolved in 200 µl 6 M guanidine prepared in 2.5 N HCl at 37°C for 5 min. The difference between the DNPH-treated and HCl-treated samples (blank) was used to calculate the carbonyl content determined at 370 nm. The results were calculated as nanomoles of carbonyl goups per milligram protein.

Determination of Sulfhydryl Content

This assay is based on the reduction of 5,5′-dithio-bis (2-nitrobenzoic acid) (DTNB) by thiols, generating a yellow derivative (TNB) whose absorption is measured spectrophotometrically at 412 nm (Aksenov and Markesbery 2001). Thirty microliters of plasma was incubated with an equal volume of DTNB at room temperature for 30 min in a dark room. The sulfhydryl content is inversely correlated to oxidative damage to proteins. Results were reported in nanomoles of TNB per milligrams of protein.

Catalase Assay (CAT)

CAT activity was assayed by the method of Aebi (1984) measuring the absorbance decrease at 240 nm in a reaction medium containing 20 mM H_2O_2, 10 mM potassium phosphate buffer, pH 7.0, and 0.1–0.3 mg protein/ml. One unit of the enzyme is defined as 1 µmol of H_2O_2 consumed per minute and the specific activity was reported in units per milligram of protein.

Superoxide Dismutase (SOD)

SOD activity was determined using the RANSOD kit (Randox, United Kingdom). The method is based on the formation of red formazan from the reaction of 2-(4-iodophenyl)-3-(4-nitrophenol)-5-phenyltetrazolium chloride and superoxide radical (produced in incubation medium from the xanthine–xanthine oxidase reaction system), which is assayed spectrophotometrically at 505 nm. The inhibition of the produced chromogen is proportional to the activity of the SOD present in the sample. A 50% inhibition is defined as one unit of SOD and the specific activity was represented in units per milligram protein.

Free L-Carnitine Determination

Free LC levels were determined in blood spots by liquid chromatography electrospray tandem mass spectrometry (LC–MS/MS), using the multiple reaction monitoring (MRM) mode (Chace et al. 1997). Results were reported in micromoles/liter.

Selenium Determination

Atomic absorption spectrophotometry with hydride generation was used for plasma Se determination. Results were reported in micrograms/liter.

Protein Determination

Erythrocyte protein concentrations were determined by the method of Lowry et al. (1951), using bovine serum albumin as standard. Plasmatic protein concentrations were determined by the Biuret method using a diagnostic kit (Labtest Diagnóstica, MG, Brazil).

Statistical Analysis

Data were expressed as mean ± standard deviation, and were analyzed using repeated measures analysis of variance. Correlations between variables were calculated using the Spearman correlation coefficient. A P value lower than 0.05 was considered significant. All analyses were performed using the Statistical Package for Social Sciences (SPSS) software in a PC-compatible computer.

Results

Table 1 shows blood Phe, free LC, and Se concentrations in PKU patients before and after supplementation with LC and Se and also in controls. Both LC and selenium levels were significantly reduced in PKU patients before supplementation when compared to controls. It can also be seen in the table that the antioxidant treatment was capable of reverting this deficiency and that Phe values were higher than expected.

Table 1 Blood phenylalanine, free L-carnitine, and selenium concentrations in controls and in PKU patients before and after supplementation with L-carnitine and selenium

	Controls ($n = 18$)	PKU patients before supplementation ($n = 18$)	PKU patients after supplementation ($n = 18$)
Phe (μmol/l)	34.2 ± 12.1	751.6 ± 239.3**	598.4 ± 298.2**
Se (μg/l)	33.9 ± 7.2	21.2 ± 5.5*	30.4 ± 3.8
Free L-carnitine (μmol/l)	44.4 ± 11.8	21.9 ± 5.5*	38.0 ± 9.8

* $P < 0.05$, ** $P < 0.01$, different from controls

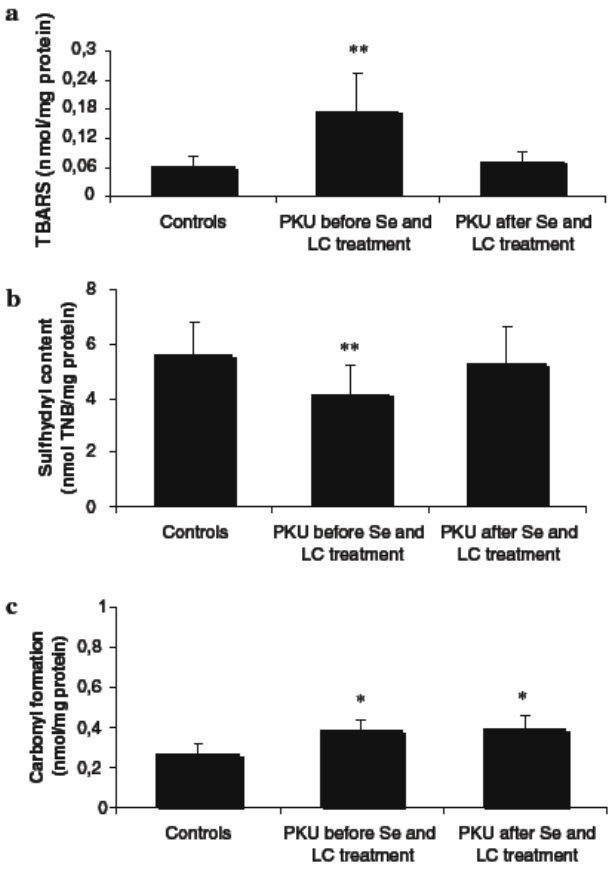

Fig. 1 Lipid and protein oxidative damage in plasma from PKU patients ($n = 18$). **a** Thiobarbituric acid-reactive species (TBARS). **b** Sulfhydryl content. **c** Carbonyl formation. Data represent mean ± S.D. * $P < 0.05$, ** $P < 0.01$, different from controls (Analysis of variance for repeated measures)

The evaluation of oxidative damage to lipids (TBARS) and proteins (sulfhydryl content and carbonyl formation) in PKU patients and controls is displayed in Fig. 1. TBARS were significantly increased in PKU patients before antioxidant supplementation, reflecting an elevated amount of malondialdehyde, an end product of membrane fatty acid

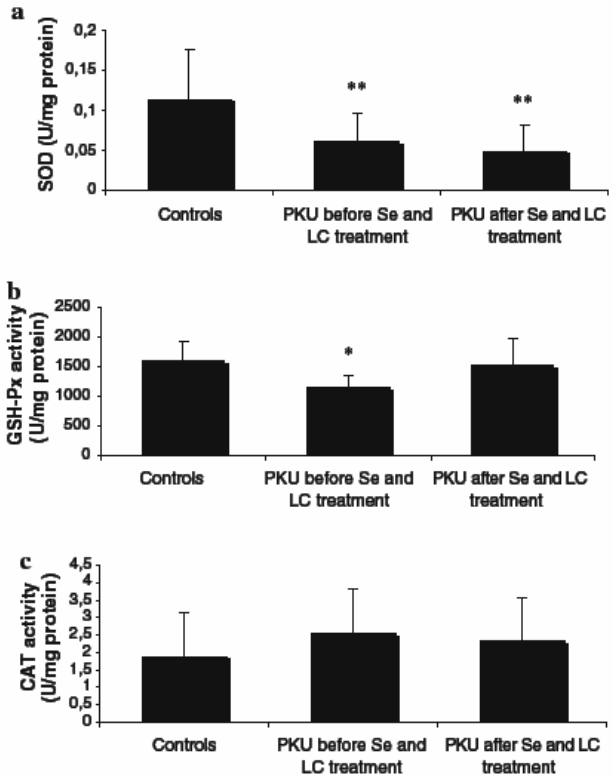

Fig. 2 Enzymatic antioxidant activities in erythrocytes from PKU patients and controls ($n = 10$). **a** Superoxide dismutase activity (SOD). **b** Glutathione peroxidase activity (GSH-Px) **c** Catalase activity (CAT). Data represent mean ± S.D. * $P < 0.05$, ** $P < 0.01$, different from controls (Analysis of variance for repeated measures)

peroxidation. The supplementation with Se and LC was capable of reverting this process. In addition, plasma protein sulfhydryl groups were significantly reduced while plasma carbonyl formation was significantly increased in PKU patients without supplementation of antioxidants when compared to controls. The administration of Se and LC corrected the oxidation of sulfhydryl groups but no effect was observed upon carbonyl formation.

Figure 2 shows the enzymatic antioxidant defenses (SOD, GSH-Px, and CAT) in erythrocytes from PKU patients and controls. It can be seen that GSH-Px and SOD activities were significantly reduced in PKU patients before antioxidant supplementation when compared to controls. The Se and LC treatment increased GSH-Px activity to the levels of controls, but did not alter SOD activity. On the other hand, CAT activity in PKU patients showed no significant difference from the controls.

As can be seen in Fig. 3, a significant negative correlation was verified between free L-carnitine levels and lipid peroxidation ($r = -0.560$; $P < 0.01$). On the other hand, a highly significant positive correlation between plasma selenium levels and erythrocyte glutathione peroxidase

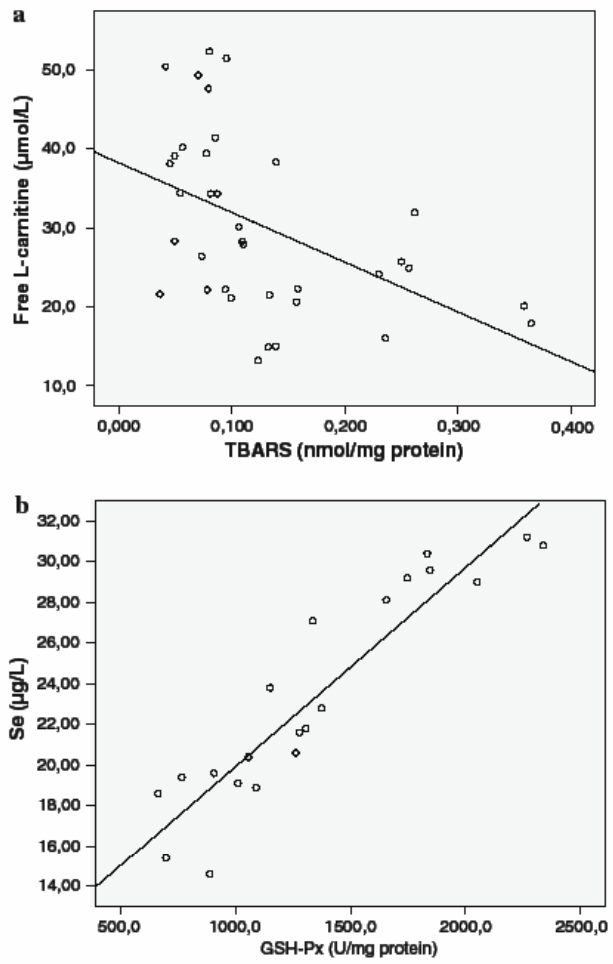

Fig. 3 Correlation between oxidative stress parameters and antioxidants in PKU patients. **a** Correlation between TBARS and free L-carnitine levels. **b** Correlation between GSH-Px activity and selenium levels

activity had been verified in treated PKU patients ($r = 0.945$; $P < 0.01$).

Discussion

PKU is one of the most common inborn errors of metabolism (IEM) and is also considered the first successfully treated IEM (Bickel et al. 1953). Dietary therapy is the main treatment for PKU patients. To maintain plasma levels of phenylalanine within nearly normal levels, the recommended therapy is a low-protein diet poor in animal products including controlled amounts of cereal, fruit, and vegetables, in addition to a protein supplementation with phenylalanine-free synthetic formulas (Start 1998). Untreated PKU patients usually present with severe mental retardation, seizures, microcephaly, spasticity, and developmental problems, whereas a well-controlled diet prevents these clinical manifestations (Huttenlocher 2000).

Although necessary to avoid mental retardation in PKU patients, the low ingestion of proteins with a high biologic value decreases the bioavailability of essential nutrients, including antioxidant compounds (Acosta 1996). Thus, it was reported that PKU patients under restricted diet present low levels of selenium, L-carnitine, and coenzyme Q10 substances that are necessary for a normal antioxidant capacity (Sitta et al. 2009b; Wilke et al. 1992; Artuch et al. 1999, 2001; Schulpis et al. 1990). In this context, recent studies carried out in animal models and also in PKU patients have emphasized the role of oxidative stress in the pathophysiology of PKU, which represent a disequilibrium between tissue antioxidant and reactive species formation in favor of the latter (Sierra et al. 1998; Sirtori et al. 2005; Sitta et al. 2006, 2009a, b; Ercal et al. 2002; Hagen et al. 2002; Martinez-Cruz et al. 2002). In this study, we evaluated the effect of supplementation with a special formula containing L-carnitine and selenium on various oxidative stress parameters in patients with classic PKU under treatment.

Initially, we verified that lipid peroxidation was significantly increased in PKU patients treated with low-protein diet and Phe-free synthetic formula not containing LC and Se. Furthermore, the supplementation of antioxidants LC and Se was capable of correcting this pathological process, reducing malondialdehyde levels, measured by the thiobarbituric acid-reactive species assay. Lipid peroxidation has received special attention since this process may damage cell structures by altering the integrity, fluidity, permeability, and functional loss of biomembranes, modifying low density lipoprotein and generating potentially toxic products (Greenberg et al. 2008) being, therefore, associated to a crescent number of pathological conditions, including neurological diseases (Adibhatla and Hatcher 2010). In this context, lipid oxidative damage has been reported in various neurodegenerative disorders including various inborn errors of metabolism (Deon et al. 2007; Ribas et al. 2010; Barschak et al. 2009; Mc Guire et al. 2009). This is probably because brain is particularly vulnerable to lipid oxidation since it contains high concentrations of polyunsaturated fatty acids and has relatively low antioxidant capacity compared to other organs (Smith et al. 2007; Markesbery and Lovell 2007).

Enhanced lipid peroxidation in PKU patients could be associated to high levels of the toxic metabolites accumulating in the disease, particularly Phe that could lead to an increased production of free radicals. The increase in plasma lipid peroxides could also result from a reduction in enzymatic and non-enzymatic antioxidant defenses, common in patients under restricted diets.

Our findings showing a marked diminution of plasma L-carnitine levels and significantly increased TBARS (lipid

oxidation) in PKU patients that were reestablished with L-carnitine supplementation, as well as a significant inverse correlation between L-carnitine and malondialdehyde (MDA) blood levels, indicate that lipid peroxidation in PKU patients was mainly due to shortage of L-carnitine. In particular, it is presumed that L-carnitine has a protective role against ROS by scavenging hydroxyl radicals, formed in the Fenton reaction system (Pietta 2000; Derin et al. 2004). L-Carnitine can also reduce MDA levels by facilitating fatty acid transport thereby lowering the availability of lipids for peroxidation (Rajasekar et al. 2005).

In addition to lipid peroxidation, high levels of protein oxidative damage (high levels of carbonyl formation and reduced levels of sulfhydryl groups) were also observed in treated PKU patients not receiving LC and Se. Oxidative damage to proteins, lipids, or DNA may all be deleterious, however, proteins are the most important targets for ROS and secondary by-products of oxidative stress when these are formed in vivo, as they are the major component of most biological systems and can scavenge 50–75% of reactive radicals such as hydroxyl (Davies et al. 1999). For this reason, in the last decade, there has been a considerable growth in the number of articles reporting increased levels of protein damage in various human diseases often correlating well with the progression of the disease (Dalle-Donne et al. 2003). Oxidative damage to proteins is induced either directly by reactive species or indirectly by reaction of secondary products of oxidative stress. Some ROS-induced protein modifications can result in unfolding or alteration of protein structure, and some are essentially harmless events. However, not all proteins are equally sensitive to oxidative damage, and oxidation susceptibility depends on the structure of the protein (Dalle-Donne et al. 2005).

Furthermore, L-carnitine and selenium treatment reversed oxidation of thiol groups but did not alter the increase of carbonyls in PKU patients. This is probably because thiol groups are easily oxidized and reduced pending on the redox status of the cell. These groups can be oxidized by reactive species, especially at protein cysteine residues that may mediate regulatory processes of protein, potentially leading to alterations of protein function. In this context, mild oxidation of cysteines can generate sulfenic acid, inter- or intra-molecular disulfides, protein-mixed disulfides with low molecular weight thiols, and S-nitrosothiols, all reversible modifications (Woo et al. 2003). On the other hand, carbonylation of proteins, a widespread indicator of severe oxidative damage and disease-derived protein dysfunction is irreversible since carbonyls tend to form high-molecular-weight aggregates that are resistant to degradation and accumulate as damaged or unfolded proteins (Dalle-Donne et al. 2006).

In our study, we found a decrease of the enzymatic antioxidant capacity in erythrocytes of PKU patients not receiving Se and LC, as verified by a decrease of SOD and GSH-Px activities. As regard to GSH-Px activity, it is possible that Se deficiency found in PKU patients could be responsible for the decrease in this activity, which is dependent on this micronutrient. In fact, Se supplementation restored the activity of this enzyme and the concentrations of plasma selenium were strongly correlated with the GSH-Px activity in erythrocytes, which reinforces this presumption and is in agreement with previous studies (Sierra et al. 1998; van Backel et al. 2000). On the other hand, it may be speculated that because of the reduction of these antioxidant activities, these PKU patients might have a lower capacity to scavenge hydrogen peroxide and superoxide reactive species. On the other hand, LC and Se supplementation did not alter SOD activity, which does not depend on Se.

We used erythrocytes in the present study because selenium-dependent glutathione peroxidase is expressed in these cells and they are highly susceptible to oxidative stress because their membranes are rich in polyunsaturated fatty acids and they have a large content of oxygen and iron (Carmagnol et al. 1983). Furthermore, selenium has a higher affinity for erythrocyte glutathione peroxidase than for plasma glutathione peroxidase (Lombeck et al. 1996).

In conclusion, this report corroborates previous studies showing that PKU patients are susceptible to oxidative stress caused by an increase in free radical production and a depletion in antioxidant capacity. More importantly, to the best of our knowledge this is the first report describing that the supplementation of a mixture containing selenium and L-carnitine to PKU patients for a long period was capable of correcting lipid and protein oxidative damage and restoring the GSH-Px activity. For this reason, selenium and L-carnitine supplementation might be an adjuvant therapy for PKU patients consuming artificial low-protein diets.

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DISCUSSÃO

A PKU é causada pela deficiência da PAH, uma enzima hepática que promove a hidroxilação da Phe à Tyr. Quando a doença não é tratada, os pacientes apresentam altas concentrações de Phe em seus tecidos e líquidos biológicos, que é sabido relacionar-se com o retardo mental progressivo (Scriver et al., 2001). Quando a doença é detectada e tratada precocemente com uma dieta restrita em Phe, o retardo mental pode ser prevenido, apesar de alterações neurofisiológicas e comportamentais persistirem (Channon et al., 2004, Huijbregts et al., 2002).

Mesmo após várias décadas de investigação, seja em pacientes portadores de PKU, em modelos animais de hiperfenilalaninemia farmacologicamente induzida e, mais recentemente, em um modelo de camundongo *knockout* para a doença (PAH^{enu2}), os mecanismos fisiopatológicos envolvidos no dano neuronal presente na PKU permanecem ainda não completamente elucidados (Sierra et al., 1998; Hagen et al., 2002; Ercal et al., 2002). Cabe salientar que parece não se tratar de apenas um mecanismo neurotóxico, mas um somatório deles, a causar alterações no SNC dos pacientes afetados. Na figura 4, estão apresentados possíveis mecanismos patológicos que poderiam contribuir para o dano neurológico que ocorre na PKU.

O estresse oxidativo vem sendo proposto como um dos mecanismos causadores do dano cerebral na PKU. Nesse contexto, a ação deletéria das espécies reativas já está extensivamente documentada como participante da fisiopatologia de doenças neurodegenerativas. Na PKU acumulam-se

metabólitos tóxicos que potencialmente representam uma fonte produtora de radicais livres. Por outro lado, as dietas com restrição protéica às quais os pacientes são submetidos poderiam diminuir a disponibilidade de antioxidantes, reduzindo a sua concentração no organismo.

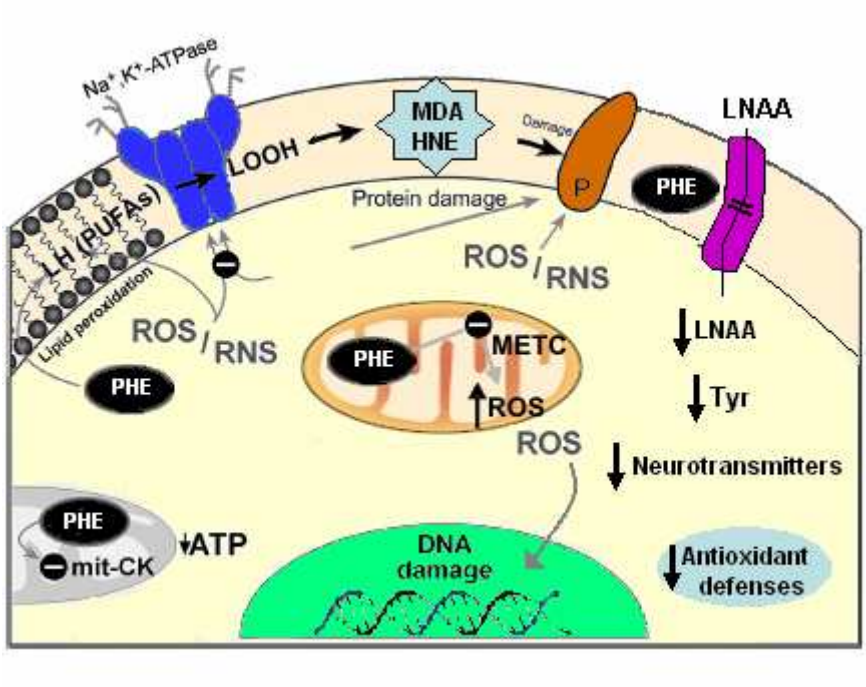

Figura 4. Possíveis mecanismos causadores do dano neurológico na PKU (Adaptado de Ribas et al., 2011). A Phe impede o transporte de vários aminoácidos neutros (LNAA) para dentro do cérebro, o que, em conjunto com a redução dos níveis de tirosina, reduz a síntese de neurotransmissores, como a dopamina e a noradrenalina. A Phe também pode inibir enzimas como a Na+,K+-ATPase, a isoforma mitocondrial da creatina quinase (mit-CK) e a cadeia mitocondrial de transferência de elétrons (METC), importantes para o metabolismo energético. A Phe (e também seus metabólitos) pode ainda levar à produção de espécies reativas de oxigênio (ROS) e de nitrogênio (RNS) e reduzir as defesas antioxidantes, causando dano oxidativo a biomoléculas, como proteínas, lipídios e DNA.

Estudos nessa área iniciaram-se com a investigação de deficiências nutricionais que levariam à diminuição da disponibilidade de antioxidantes. A dieta restrita em fenilalanina em pacientes fenilcetonúricos leva à redução na concentração de selênio eritrocitário, de Q10, do *status* antioxidante total e da atividade das enzimas CAT, SOD e GSH-Px (Wilke, 1992; Lombeck, I., Jochum, F., Terwolbeck, 1996; Van Bakel et al., 2000; Artuch et al., 2004). Sierra e

colaboradores (1998) verificaram que distúrbios neurológicos foram observados com maior frequência em pacientes fenilcetonúricos que apresentavam diminuição na atividade da GSH-Px.

Estudos em modelos animais de hiperfenilalaninemia induzida quimicamente demonstraram que filhotes de ratas fenilcetonúricas apresentavam aumento da lipoperoxidação, que foi revertida pelo tratamento com melatonina e vitaminas C e E (Martinez-Cruz et al., 2002). Também foi demonstrado que a fenilalanina tanto *in vitro* como *in vivo* aumenta a quimioluminescência (parâmetro de peroxidação lipídica) e reduz o potencial antioxidante total no tecido cerebral de ratos (Hagen et al., 2002). Em um modelo de camundongo *knockout* para a PKU (PAH^{enu2}), verificou-se que a lipoperoxidação estava aumentada, enquanto o conteúdo de tióis (parâmetro de oxidação a proteínas) estava diminuído (Ercal et al., 2002). Em um trabalho recente, foi demonstrado que o tratamento com ácido lipóico *in vitro* e *in vivo* parece ter papel neuroprotetor ao restaurar a atividade de antioxidantes e prevenir o dano oxidativo a lipídios em cérebro de ratos (Moraes et al., 2010).

Em um estudo prévio, nosso grupo de pesquisa verificou que pacientes fenilcetonúricos no momento do diagnóstico (tardio), apresentavam uma indução da lipoperoxidação (medida pela medida das espécies reativas ao ácido tiobarbitúrico – TBARS), bem como uma diminuição da reatividade antioxidante total e da atividade da enzima GSH-Px (Sirtori et al., 2005). Em seguimento a este trabalho, verificou-se que os pacientes fenilcetonúricos, ainda que já estivessem sendo tratados, permaneciam com as mesmas alterações na lipoperoxidação e na redução de antioxidantes, e que estas

alterações não eram relacionadas diretamente com as concentrações de Phe séricas (Sitta et al., 2006).

O presente estudo visa ampliar os conhecimentos com relação à participação do estresse oxidativo e da diminuição do conteúdo dos antioxidantes na PKU e inicia-se com a investigação de dano ao DNA em pacientes fenilcetonúricos *in vivo* e também aquele produzido pela Phe *in vitro*, que foi determinado através do método do ensaio cometa.

O ensaio cometa é uma técnica amplamente utilizada para avaliar dano ao DNA por se tratar de uma metodologia simples e rápida que verifica quebras simples e duplas no DNA (Singh et al., 1988). As células sanguíneas individualizadas, englobadas em gel e espalhadas sobre uma lâmina, são submetidas a uma corrente elétrica que proporciona a migração dos segmentos de DNA livres, resultantes de quebras, para fora do núcleo. Após a eletroforese, as células que apresentam um núcleo redondo são identificadas como normais, sem possuir dano detectável ao DNA. Por outro lado, as células lesadas são identificadas por uma espécie de cauda formada pelos fragmentos do DNA, gerando, ao serem visualizadas em microscópio, a imagem de um cometa, que dá o nome ao ensaio. A identificação do dano ao DNA pode ser realizada de diversas maneiras, como, por exemplo, pela medida do comprimento da cauda do cometa com uma ocular de medidas, ou ainda, pela classificação virtual, em diferentes níveis de dano, das células analisadas, podendo obter-se um valor arbitrário que expresse o dano geral sofrido por uma população de células, método que foi empregado neste estudo.

Foi verificado neste trabalho que a adição de concentrações crescentes de Phe ao meio de células sanguíneas, produziu dano crescente, até a

concentração de 1000 µmol/L e que concentrações de Phe de 250 µmol/L já foram capazes de produzir dano significativo ao DNA. É importante salientar que uma das mais preconizadas metas a serem atingidas no tratamento da PKU é a manutenção dos níveis sanguíneos de Phe abaixo de 360 µmol/L e, portanto, conforme verificado no ensaio *in vitro*, mesmo alcançando nestas concentrações, os pacientes já estariam sofrendo maiores lesões ao DNA relativamente a indivíduos saudáveis (Phe 100 µmol/L).

Os resultados de dano ao DNA *in vivo* encontrados em pacientes fenilcetonúricos corroboram com os resultados encontrados com a Phe *in vitro*, uma vez que pacientes com níveis sanguíneos de Phe bastante elevados (acima de 600 µmol/L) apresentaram maior dano do que aqueles pacientes com um “adequado” controle da Phe, que, por sua vez, já apresentavam dano significativo em relação aos controles. Desta forma, pode-se inferir que as concentrações sanguíneas de Phe e, talvez, de seus metabólitos, estejam diretamente relacionadas ao dano ao DNA.

Embora o ensaio cometa não seja uma técnica que determine especificamente dano oxidativo ao DNA, existe um número substancial de evidências na literatura indicando que processos oxidativos geralmente lesam o DNA. Além disso, já está bem documentada a ocorrência de dano oxidativo a outras biomoléculas, como proteínas e lipídios na PKU. Inclusive, verificamos uma correlação positiva entre a peroxidação lipídica (medida através do método do TBARS) e o dano ao DNA medido pelo ensaio cometa nos pacientes fenilcetonúricos (dados não mostrados) e, portanto, poderíamos inferir que as quebras ao DNA produzidas na doença poderiam ser geradas pelo ataque de espécies reativas.

Neste particular, o dano oxidativo ao DNA é considerado o mais significativo dano oriundo do metabolismo celular representando, portanto, um fator determinante de disfunção e morte celular. Estima-se que aproximadamente $2x10^4$ lesões oxidativas ao DNA ocorram no genoma humano por dia (Ames e Shigenoga, 1992). Desta maneira, acredita-se que o reparo destas lesões possua um papel central na prevenção do aumento de mutações nos organismos vivos (Maluf, 2004). Muitas evidências sugerem que os danos cumulativos ao DNA causados por espécies reativas contribuem para diversas situações clínicas como o câncer (Palyvoda et al., 2003), a esquizofrenia (Psimadas et al., 2004) e a doença de Alzheimer (Migliore et al., 2005).

Assim, com este trabalho, propomos que também na PKU o dano oxidativo ao DNA possa contribuir para a fisiopatologia da doença e que estratégias para minimizar este dano devam ser empregadas, como, por exemplo, o uso de antioxidantes. Nesse sentido, uma das mais promissoras aplicações do ensaio cometa está mesmo no ramo da nutrição, uma vez que a eficácia de ensaios clínicos com antioxidantes tem sido testada com o uso deste método, pela avaliação da diminuição dos índices de dano ao DNA que os antioxidantes propiciam (Cemeli, Baumgartner e Anderson, 2009; Collins, 2007).

A PKU talvez seja o erro inato do metabolismo mais estudado, provavelmente por ser um transtorno de relativamente alta prevalência. Além da elevada prevalência, a PKU apresenta possibilidade de diagnóstico rápido e um tratamento efetivo. Por este motivo, a doença é indicada a fazer parte dos programas de triagem neonatal, que visam diagnosticar precocemente diversas doenças congênitas através de exames realizados em massa em recém-

nascidos. O objetivo de um programa de triagem neonatal, mais conhecido no Brasil como teste do pezinho, é intervir no curso natural das doenças por meio do tratamento precoce, evitando, assim, sequelas graves ao desenvolvimento físico e mental da criança (Meirelles, 2002).

De fato, os programas de triagem neonatal iniciaram-se com a PKU, através dos estudos de Guthrie (1963). No Brasil, o teste do pezinho em sua versão mais simples foi introduzido ainda na década de 70, mas somente em 1992, por meio de lei federal, o teste tornou-se obrigatório em todo o país. Entretanto, ainda hoje a cobertura do teste não atinge 100% dos recém-nascidos e muitos fenilcetonúricos são “perdidos” no período neonatal. O diagnóstico tardio impede o tratamento precoce, o que muitas vezes leva ao retardo mental, causado pela exposição às altas concentrações de Phe e seus metabólitos por um longo período de tempo.

Devido a esta realidade, nosso grupo de pesquisa teve acesso a material biológico de pacientes fenilcetonúricos (em tratamento) que foram diagnosticados tanto tardiamente como no período neonatal, o que nos motivou a investigar se havia alguma diferença entre estes dois grupos, no que se refere ao estresse oxidativo e às defesas antioxidantes.

Investigou-se inicialmente a capacidade antioxidante enzimática (GSH-Px) e não enzimática (TAR e GSH). Nenhuma diferença foi observada entre os dois grupos de pacientes no que se refere a essas defesas antioxidantes. Pacientes fenilcetonúricos com diagnóstico tardio e com diagnóstico precoce apresentaram, de maneira similar, redução nos antioxidantes, quando comparados com indivíduos saudáveis. Estes resultados estão em concordância com estudos prévios que demonstraram que pacientes

fenilcetonúricos sob dieta restrita apresentam redução na concentração sanguínea de diversos antioxidantes, que é atribuída provavelmente à redução do aporte de substâncias com capacidade antioxidante pela dieta (Artuch et al., 2004; Van Bakel et al., 2000; Wilke et al., 1992).

Entretanto, o resultado mais interessante neste trabalho foi encontrado quando se avaliou dano oxidativo a lipídios (TBARS) e a proteínas (conteúdo de grupamentos carbonilas e sulfidrilas) nos dois grupos de pacientes com fenilcetonúria. Verificamos que apenas os pacientes fenilcetonúricos que foram diagnosticados tardiamente apresentaram oxidação destas biomoléculas significativamente maior em comparação aos controles. Cabe salientar que os dois grupos de pacientes utilizados no estudo apresentavam idade e níveis de fenilalanina sanguínea comparáveis entre si e que estudos prévios do nosso grupo de pesquisa foram desenvolvidos utilizando-se apenas amostras de pacientes fenilcetonúricos cujo diagnóstico da doença foi realizado tardiamente (Sirtori et al., 2005; Sitta et al., 2006).

Podemos inferir com os achados deste estudo, que a exposição dos pacientes fenilcetonúricos com diagnóstico tardio, por um período longo de tempo, a altas concentrações de fenilalanina e seus metabólitos levou a um dano oxidativo incapaz de ser completamente prevenido pelos antioxidantes teciduais, que, por sua vez, estão sabidamente reduzidos nos pacientes. A maneira pela qual os metabólitos acumulados na PKU induzem à oxidação de biomoléculas ainda não pode ser totalmente esclarecida. Entretanto, é possível que esteja relacionada à própria superprodução, direta ou indireta, de espécies reativas ou, então, à depleção de substâncias antioxidantes (Wajner et al., 2004).

Como previamente mencionado, o tecido cerebral é bastante suscetível ao ataque de espécies reativas que provocam injúria ao SNC. Um aumento na produção de ERO e uma redução nos níveis de GSH são frequentemente observados e associados a processos apoptóticos (Slater et al., 1995; van den Dobbelsteen et al., 1996). Podemos, então, especular, que o acúmulo de metabólitos tóxicos na PKU possa mesmo levar à morte celular por apoptose. Isto poderia explicar, pelo menos parcialmente, as alterações neurológicas apresentadas pelos pacientes expostos a altas concentrações de Phe por um período prolongado. Ainda, o fato de termos observado dano oxidativo apenas nos pacientes que não foram tratados precocemente, que são os mais suscetíveis a desenvolver retardo mental, nos dá mais subsídios para sugerir que o estresse oxidativo esteja mesmo relacionado com a fisiopatologia da PKU.

Dando continuidade a este trabalho, passamos a investigar o *status* antioxidante nos pacientes fenilcetonúricos e como ele poderia estar relacionado com o dano neurológico presente na PKU.

Para manter a Phe dentro de níveis considerados seguros, os pacientes fenilcetonúricos são recomendados a seguir uma dieta especial, com baixa quantidade de proteína, que exclui, quase que completamente, produtos de origem animal e inclui quantidades controladas de cereais, frutas e vegetais. A baixa ingestão de proteínas com alto valor biológico diminui, entretanto, a biodisponibilidade de diversos nutrientes, dentre eles, substâncias com ação antioxidante, como o selênio, a L-carnitina (LC) e a Q10 (Artuch et al., 1999; van Bakel et al., 2000; Vilaseca et al., 1993).

Embora a LC seja sintetizada endogenamente em humanos, o maior conteúdo presente no organismo (75%) é adquirido exogenamente, através da alimentação (Bremer, 1983). Uma vez que os alimentos mais ricos em LC são o leite e derivados, e também a carne vermelha, pacientes fenilcetonúricos em tratamento com restrição dietética frequentemente apresentam deficiências neste composto (Schulpis et al., 1990; Vilaseca et al., 1993).

Neste estudo, ao analisar as concentrações de carnitina total no sangue dos pacientes fenilcetonúricos, verificamos uma diminuição significativa deste composto apenas no grupo de pacientes que seguia estritamente a dieta prescrita, e que possuía, portanto, níveis de Phe sérica considerados satisfatórios. Tendo sido encontrada uma correlação positiva entre os níveis sanguíneos de LC e os níveis de Phe, a redução deste composto pôde ser atribuída à restrição protéica a que os pacientes eram submetidos.

Também verificamos uma correlação significativa negativa entre os níveis sanguíneos de LC e a peroxidação lipídica, bem como uma correlação significativa positiva entre os níveis de LC e a reatividade antioxidante total nos pacientes com bom controle dietético. É possível inferir, portanto, que, ao menos nestes pacientes, a deficiência da LC possa estar contribuindo para a ocorrência de dano oxidativo a biomoléculas.

Nos últimos anos, diversos estudos têm investigado o papel da LC (ácido 4-n-trimetilamônio-3-hidiroxibutírico) no organismo, sendo que diversas e importantes funções já foram atribuídas a ela. A LC é uma substância altamente polar e hidrofílica, cujo principal papel no metabolismo é o transporte de ácidos graxos de cadeia longa para dentro da matriz mitocondrial, atravessando a

membrana interna da organela, para que esses ácidos graxos possam ser oxidados para a produção de energia (Bahl e Bressler 1987).

Além disso, a LC possui papel modulatório da função neural por mediar a transferência de grupamentos acetila para a síntese de acetilcolina, bem como influenciar vias de transdução de sinal e expressão gênica (Nalecz e Nalecz, 1996; Binienda e Ali, 2001). Ela também atua como cofator na oxidação peroxissomal de ácidos graxos de cadeia muito longa, estimula a respiração celular e a função do peroxissomo e é frequentemente utilizada como um suplemento alimentar por, supostamente, aumentar o desempenho físico e promover a perda de peso. Também tem sido atribuído à LC papel na melhora das funções imunológicas e ela tem sido sugerida como um agente terapêutico para diversas desordens neurodegenerativas (Solarska et al., 2010).

Os efeitos benéficos da LC parecem ser mediados, pelo menos em parte, por suas propriedades antioxidantes. A LC tem sido descrita como um "sequestrador" de radicais superóxido (Gulçin, 2006). Por sua vez, os radicais superóxido desempenham um importante papel na formação de outros ROS, como o peróxido de hidrogênio e os radicais hidroxila, os quais induzem dano oxidativo a lipídios, proteínas e DNA (Pietta, 2000). Também tem sido descrito que a LC seria capaz de sequestrar o peróxido de hidrogênio e os radicais hidroxila (considerados os mais deletérios ao organismo) e também poderia inibir a produção dos radicais hidroxila (Derin et al., 2004; Gulçin, 2006; Reznick, Kagan e Ramsey,1992). Além disso, a LC também apresenta um efeito antiperoxidativo que está relacionado à sua ação como um quelante de metais que leva a uma diminuição na concentração de íons metálicos e, consequentemente, na geração de radicais livres (Muthuswamy et al., 2006).

A atividade antioxidante da LC também se relaciona ao seu papel de proteção aos antioxidantes teciduais, tanto enzimáticos quanto não-enzimáticos. Esse papel inclui a regulação dos níveis de glutationa e a elevação na atividade de enzimas antioxidantes (Tanghasamy et al., 2009; Calabrese et al., 2006). Assim, os efeitos *in vivo* da LC podem ser mediados pela ação direta da carnitina ou, então, pela biossíntese de enzimas antioxidantes e outras proteínas (Solarska et al., 2010).

Estando bem definidas as importantes funções que a LC exerce no organismo, incluindo aquelas de proteção ao dano oxidativo, e estando descrita a deficiência deste composto em pacientes fenilcetonúricos sob dieta restrita, nos últimos anos, diversas fórmulas sintéticas utilizadas no tratamento dos pacientes com PKU têm incluído em sua composição a LC.

Outra substância que já foi descrito estar deficiente em pacientes fenilcetonúricos sob tratamento é o selênio (Wilke, 1992; Lombeck, I., Jochum, F., Terwolbeck, 1996; Van Bakel et al., 2000). Por esse motivo, nos últimos anos, sua suplementação vem sendo recomendada aos pacientes e ele também vem sendo incorporado às fórmulas utilizadas na terapia dietética da fenilcetonúria.

O selênio é um micronutriente essencial, que é adquirido pelo ser humano através da dieta, principalmente a partir de cereais, peixes e carnes (Steinbrenner e Sies, 2009). É hoje bem estabelecido que o selênio desempenha importantes funções biológicas nos organismos vivos, principalmente através da sua incorporação em uma família de proteínas chamadas selenoproteínas, nas quais o selênio é especificamente incorporado

ao aminoácido cisteína, resultando na selenocisteína (Patrick, 2004; Moghadaszadeh e Beggs, 2006).

Até hoje, todas as selenoproteínas cujas funções já são conhecidas desempenham atividade catalítica, sendo que o resíduo de Se está localizado no sítio catalítico da enzima, onde participa de reações redox. As enzimas contendo resíduos de Se podem estar envolvidas em diversas funções, como no desenvolvimento muscular, no sistema imunológico, na fertilidade, na regulação do metabolismo da tireoide e no sistema de defesa antioxidante. Consequentemente, a quantidade de patologias associadas a defeitos primários ou secundários na atividade de selenoproteínas é bastante grande (Moghadaszadeh e Beggs, 2006). Além disso, quer em níveis nutricionais, ou supranutricionais, o selênio é reconhecido tanto como agente de prevenção a determinados cânceres, como por apresentar efeitos antitumorigênicos em cânceres já iniciados (Patrick, 2004).

Foi verificado no presente trabalho que os pacientes fenilcetonúricos submetidos à dieta com restrição protéica e que recebiam suplementação com uma fórmula rica em micronutrientes, mas não contendo LC e selênio, eram deficientes nestas substâncias. No entanto, a suplementação por um período de 6 meses com uma fórmula contendo LC (98 mg/dia) e selênio (31,5 µg/dia) foi capaz de restabelecer os níveis desses compostos no sangue dos pacientes, a valores comparáveis aos encontrados em indivíduos controles.

Verificamos também que antes de iniciarem a suplementação com LC e selênio, os pacientes com PKU apresentavam aumento significativo na oxidação a lipídios e proteínas, bem como uma diminuição significativa na atividade das enzimas antioxidantes SOD e GSH-Px. A suplementação, no

entanto, foi capaz de restabelecer a atividade da enzima GSH-Px e também corrigiu completamente a oxidação aos lipídios.

Além disso, foi verificada uma correlação fortemente positiva entre os níveis de selênio sérico e a atividade da enzima GSH-Px. Este achado já era esperado, visto que a GSH-Px é uma selenoproteína que catalisa a oxidação da glutationa reduzida e permite a redução do peróxido de hidrogênio à água, advindo daí sua ação antioxidante, por impedir processos peroxidativos, e protegendo, portanto, o organismo do dano celular.

Adicionalmente à bem documentada função do selênio como antioxidante, estudos recentes têm demonstrado um papel do selênio na manutenção da função cerebral (Zhang et al., 2010). De fato, o selênio é amplamente distribuído através do corpo, mas é particularmente mantido em altas concentrações no cérebro, mesmo quando da sua deficiência prolongada no organismo (Schweizer, Schomberg e Savaskan, 2004). Várias evidências sugerem que há uma ligação entre os níveis de selênio e alterações cognitivas, depressão, ansiedade e hostilidade em humanos (Rayman, 2002). A diminuição na expressão de diversas selenoproteínas vem sendo associada a diversas doenças neurológicas como as doenças de Parkinson, Alzheimer e epilepsia, e recentes avanços usando modelos animais geneticamente modificados demonstraram que as selenoproteínas oferecem proteção contra a neurodegeneração, principalmente através da regulação redox (Zhang et al., 2010). Dessa forma, podemos inferir que também na PKU a deficiência de selênio possa contribuir significativamente para os sintomas neurológicos apresentados pelos pacientes e, portanto, deve ser evitada.

Tendo sido encontrada uma correlação negativa significativa entre os níveis de carnitina livre e a peroxidação lipídica (medida pelo método do TBARS) nos pacientes fenilcetonúricos, pudemos inferir que a suplementação com LC desempenhe papel fundamental na diminuição da oxidação aos lipídios verificada neste estudo. De fato, diversos trabalhos vêm demonstrando uma ação protetora da LC sobre o dano aos lipídios. Isso pode dever-se, pelo menos parcialmente, à principal atividade biológica da carnitina que é a de transportar ácidos graxos para dentro da mitocôndria para que possam ser oxidados, diminuindo, dessa forma, a disponibilidade de lipídios para sofrerem oxidação. Deve-se ainda enfatizar que a formação de EROs como o ânion superóxido, o peróxido de hidrogênio e o radical hidroxila é também catalisada pelo ferro livre, através da reação de Haber-Weiss. Nesse particular, foi também demonstrado que a L-carnitina e seus ésteres também são capazes de inibir a peroxidação lipídica induzida por ferro, através da formação de complexos com o metal (Gulçin, 2006). Este poderia ser, então, outro mecanismo importante na diminuição do dano a lipídios proporcionado pela LC e verificado em nossos pacientes.

É importante salientar que a peroxidação lipídica causa efeitos deletérios aos organismos, principalmente por alterar a organização e, portanto, a função de membranas, tendo sido, dessa forma, associada à patogênese de diversas doenças (Niki, 2009). Assim, a administração de substâncias que inibam a lipoperoxidação, como a L-carnitina, principalmente quando estiverem deficientes, como no caso de pacientes fenilcetonúricos, deve ser fortemente recomendada.

CONCLUSÕES

O estresse oxidativo é um processo patológico que vem sendo descrito em um número crescente de doenças em que há comprometimento neurológico, incluindo diversos erros inatos do metabolismo.

Neste trabalho, realizado a partir de sangue de pacientes fenilcetonúricos, confirmamos e agregamos novas evidências de que o estresse oxidativo de fato ocorre na PKU. Verificou-se uma diminuição das defesas antioxidantes, que possivelmente foi causada pela restrição dietética de compostos com ação antioxidante, como a L-carnitina e o selênio. Além disso, foi demonstrado um aumento nos biomarcadores que refletem dano a proteínas, lipídios e DNA, principalmente nos pacientes sem controle adequado dos níveis sanguíneos de Phe. O dano às biomoléculas ocorre, provavelmente, devido a um aumento na formação de espécies reativas, a partir do acúmulo de Phe. A deficiência nas defesas antioxidantes também poderia resultar em uma predisposição maior ao dano oxidativo, como foi observado.

Assim, considerando que os mesmos achados verificados em sangue, também ocorram no cérebro dos pacientes fenilcetonúricos, podemos presumir que o estresse oxidativo participa do dano cerebral encontrado na PKU, possivelmente contribuindo para os sintomas e anormalidades neurológicas característicos da doença.

Desta forma, sugerimos que a terapia com antioxidantes deva ser cada vez mais considerada como uma ferramenta auxiliar no tratamento dos pacientes com PKU, devendo ser associada à terapia já preconizada baseada na restrição de alimentos ricos em proteínas na dieta.

PERSPECTIVAS

Pretendemos dar continuidade a esse trabalho, expandindo nossos resultados. Desta forma, são nossas perspectivas imediatas:

a) Quantificar os metabólitos tóxicos fenil-lactato, fenilpiruvato e fenilacetato excretados na urina de pacientes com PKU, a fim de correlacioná-los a parâmetros de estresse oxidativo e com as defesas antioxidantes determinados na urina dos pacientes;

b) Investigar o efeito dos metabólitos acumulados na PKU (fenil-lactato, fenilpiruvato e fenilacetato) sobre o dano ao DNA através da técnica do ensaio cometa;

c) Avaliar parâmetros de estresse oxidativo em pacientes com outras formas clínicas de PKU (forma moderada, forma leve e a causada pela deficiência do cofator tetra-hidrobiopterina) separadamente;

d) Correlacionar parâmetros de estresse oxidativo e quantidade de antioxidantes com testes cognitivos, por exemplo, o de quociente de inteligência (QI).

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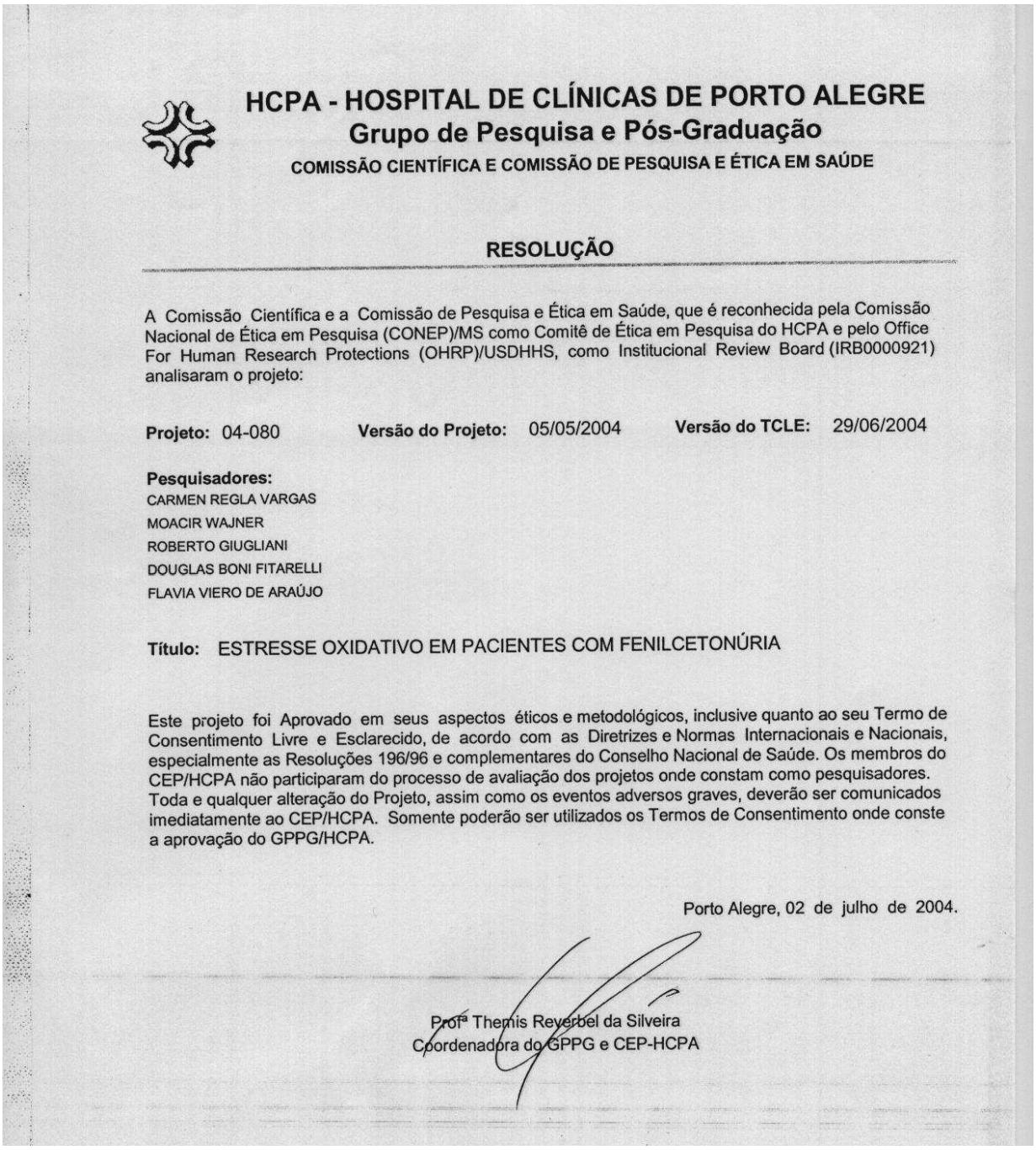
ANEXO 1 – Lista de figuras

ANEXO 2 – Parecer da comissão científica e comissão de pesquisa e ética em saúde do HCPA



HCPA - HOSPITAL DE CLÍNICAS DE PORTO ALEGRE
Grupo de Pesquisa e Pós-Graduação
COMISSÃO CIENTÍFICA E COMISSÃO DE PESQUISA E ÉTICA EM SAÚDE

RESOLUÇÃO

A Comissão Científica e a Comissão de Pesquisa e Ética em Saúde, que é reconhecida pela Comissão Nacional de Ética em Pesquisa (CONEP)/MS como Comitê de Ética em Pesquisa do HCPA e pelo Office For Human Research Protections (OHRP)/USDHHS, como Institucional Review Board (IRB0000921) analisaram o projeto:

Projeto: 04-080 **Versão do Projeto:** 05/05/2004 **Versão do TCLE:** 29/06/2004

Pesquisadores:
CARMEN REGLA VARGAS
MOACIR WAJNER
ROBERTO GIUGLIANI
DOUGLAS BONI FITARELLI
FLAVIA VIERO DE ARAÚJO

Título: ESTRESSE OXIDATIVO EM PACIENTES COM FENILCETONÚRIA

Este projeto foi Aprovado em seus aspectos éticos e metodológicos, inclusive quanto ao seu Termo de Consentimento Livre e Esclarecido, de acordo com as Diretrizes e Normas Internacionais e Nacionais, especialmente as Resoluções 196/96 e complementares do Conselho Nacional de Saúde. Os membros do CEP/HCPA não participaram do processo de avaliação dos projetos onde constam como pesquisadores. Toda e qualquer alteração do Projeto, assim como os eventos adversos graves, deverão ser comunicados imediatamente ao CEP/HCPA. Somente poderão ser utilizados os Termos de Consentimento onde conste a aprovação do GPPG/HCPA.

Porto Alegre, 02 de julho de 2004.

Profª Themis Reverbel da Silveira
Coordenadora do GPPG e CEP-HCPA

ANEXO 3 – Termo de consentimento livre e esclarecido para pacientes

Termo de Consentimento Livre e Esclarecido

Estamos através deste convidando você a participar do trabalho de pesquisa cujo objetivo é verificar os efeitos da ação dos radicais livres (por exemplo, radiação solar) e de substâncias antioxidantes (por exemplo, vitaminas) em pacientes fenilcetonúricos.

Para participar você fará exames de sangue e/ou de urina, que serão coletados juntamente com as amostras que você coleta para os seus exames de acompanhamento, solicitados rotineiramente pelo seu médico. Não será realizada nenhuma alteração no tratamento prescrito pelo seu médico. Os dados que virão com a sua doação são de importância científica relevante para o melhor entendimento da fenilcetonúria. Os riscos e desconfortos causados pela coleta de sangue para o estudo são semelhantes aos envolvidos na coleta de sangue para exames de laboratoriais de rotina. O material coletado será única e exclusivamente utilizado para fins do projeto de pesquisa, sendo reservado ao doador acesso às mesmas.

As informações individuais levantadas pela pesquisa são confidenciais. Os resultados obtidos serão agrupados e expressos através de resultados numéricos, sem qualquer referência a elementos que possam identificar as pessoas que participaram do estudo.

Todas as despesas relacionadas ao custo dos exames laboratoriais serão cobertas por verbas do próprio Projeto de Pesquisa, completamente gratuitas para o paciente.

Caso você queira se retirar em definitivo da pesquisa, terá total liberdade para fazê-lo. O seu material (sangue e /ou urina) coletado será posteriormente destruído e os seus dados excluídos do nosso banco de dados.

Os pesquisadores responsáveis pelo estudo estão à disposição para o esclarecimento de qualquer dúvida durante todo o andamento da pesquisa.

Telefone de contato: 51 3359 8011 (Pesquisador Responsável: Profa. Dra. Carmen Regla Vargas).

Pela presente, declaro que fui devidamente informado sobre o projeto de pesquisa, de forma clara e detalhada, da liberdade de não participar do estudo e tive minhas dúvidas esclarecidas.

Data:_____

Nome:_____

Nome do responsável legal:_____

Assinatura:_____

Pesquisador responsável: Profa. Dra. Carmen Regla Vargas (Serviço de Genética Médica/Hospital de Clínicas de Porto Alegre – Fone: 51 3359 8011)

Assinatura do pesquisador:_____