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Investigação de estresse oxidativo em pacientes tratados e não tratados com Doença do Xarope do Bordo

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RESUMO

A Doença do Xarope do Bordo (DXB) é um erro inato do metabolismo causado pela deficiência na atividade do complexo da desidrogenase dos α-cetoácidos de cadeia ramificada. Como conseqüência deste bloqueio ocorre o acúmulo dos aminoácidos de cadeia ramificada leucina, isoleucina e valina, bem como de seus respectivos α-cetoácidos e α-hidroxiácidos de cadeia ramificada. A manifestação clínica da DXB varia da forma clássica severa a formas variantes moderadas. Os principais sinais clínicos laboratoriais apresentados pelos pacientes com DXB incluem cetoacidose, hipoglicemia, recusa alimentar, opistótono, apnéia, ataxia, convulsões, coma, atraso no desenvolvimento psicomotor e retardo mental. No entanto, os mecanismos responsáveis pelos sintomas neurológicos apresentados pelos pacientes com DXB ainda são pouco conhecidos. O tratamento para DXB consiste na restrição da ingestão de proteínas, bem como dos aminoácidos leucina isoleucina e valina, suplementada com uma fórmula semi-sintética contendo aminoácidos essenciais, minerais e vitaminas. Estudos in vitro têm demonstrado que os aminoácidos de cadeia ramificada e seus respectivos α-cetoácidos acumulados na DXB estimulam a lipoperoxidação e reduzem as defesas antioxidantes em córtex cerebral de ratos. O objetivo deste estudo foi investigar se o estresse oxidativo ocorre em pacientes com DXB em tratamento ou ainda não tratados. Inicialmente, foram analisados parâmetros de estresse oxidativo em amostras de plasma de pacientes com DXB obtidas antes do início do tratamento (no diagnóstico). Foi observado um aumento significativo das espécies reativas ao ácido tiobarbitúrico (TBARS) e uma diminuição significativa da reatividade antioxidante total (TAR). Em seguida foram

avaliados parâmetros de estresse oxidativo em plasma de dois grupos de pacientes com DXB em tratamento dietético com níveis plasmáticos baixos e altos de leucina. Verificou-se um marcado aumento do TBARS e uma diminuição significativa do TAR em ambos os grupos de pacientes tratados, porém a redução do TAR foi mais pronunciada nos pacientes com baixos níveis sanguíneos de leucina. Além disso, não foi observada correlação entre os níveis de leucina, isoleucina ou valina com os parâmetros de estresse oxidativo. Em continuidade ao trabalho, foram estudados os níveis de selênio no plasma de pacientes com DXB antes e durante o tratamento dietético, bem como a atividade de enzimas antioxidantes em eritrócitos de pacientes em tratamento. Foi verificado que os pacientes com DXB apresentam uma deficiência moderada de selênio no momento do diagnóstico (antes do tratamento) da doença e que essa deficiência se agrava com o tratamento dietético. Ainda, verificou-se que a atividade da enzima glutationa peroxidase está diminuída em eritrócitos de pacientes em tratamento. Posteriormente, foram medidos vários parâmetros bioquímicos em plasma de pacientes com DXB tratados, com o objetivo de verificar se havia correlação destes com parâmetros de estresse oxidativo. Foi demonstrado que os pacientes com DXB em tratamento apresentam redução nos níveis séricos de glicose, colesterol total, creatinina sérica e albumina e aumento na atividade enzimática da aspartato aminotransferase e da lactato desidrogenase. Entretanto, não foram observadas alterações nos níveis de colesterol HDL, colesterol LDL, triglicerídeos, alanina aminotransferase, creatina quinase, uréia e ácido úrico. Foi ainda verificado um aumento significativo de TBARS e uma diminuição significativa de TAR, bem como uma correlação positiva entre a medida de

TBARS e os níveis de triglicerídeos. Concluindo, nossos resultados indicam que existe um desequilíbrio entre a produção de radicais livres (aumento) e as defesas antioxidantes (diminuição) gerando estresse oxidativo em pacientes com DXB não tratados e em tratamento e que esse processo não está correlacionado com os níveis plasmáticos dos aminoácidos leucina, isoleucina e valina acumulados nesta doença. Além disso, verificamos que a deficiência de selênio e a concomitante diminuição da atividade da glutationa peroxidase podem estar associados ao desenvolvimento do estresse oxidativo nesta doença. É, portanto, possível inferir que o dano oxidativo contribua, pelo menos em parte, para a fisiopatologia desta doença, explicando o dano neurológico que ocorre nos pacientes afetados. Portanto, é possível que a administração de antioxidantes possa ser útil na DXB.

ABSTRACT

Maple Syrup Urine Disease (MSUD) is an inborn error of metabolism caused by a deficiency of the branched-chain α-keto acid dehydrogenase complex activity. This blockage leads to accumulation of the branched-chain amino acids leucine, isoleucine and valine, as well as their corresponding α-keto acids and α-hydroxy acids. Clinical manifestations are variable, pending on the variants classical severe form to milder variants. The major clinical features presented by MSUD patients include ketoacidosis, hypoglycemia, poor feeding, opisthotonos, apnea, ataxia, convulsions, coma, psychomotor delay and mental retardation. However, the mechanisms of the neurological symptoms presented by MSUD patients are still poorly understood. MSUD treatment consists of a low protein diet and a semi-synthetic formula poor in leucine, isoleucine, and valina and supplemented by essential amino acids minerals and vitamins. Studies have been showed that the branched-chain amino acids and their corresponding brached-chain α-keto acids accumulating in MSUD stimulate in vitro lipid peroxidation and reduce antioxidant defences in cerebral cortex of rats. The objective of the present study was to investigate oxidative stress parameters in MSUD patients treated and non-treated. First, it was analyzed parameters of oxidative stress in plasma from non-treated MSUD patients. It was observed a significant increase of thiobarbituric acid-reactive species (TBARS) and a marked reduction in total antioxidant reactivity (TAR). Parameters of oxidative stress in plasma from two groups of MSUD patients under dietetic treatment with low and high blood leucine levels were also evaluated. It was verified an increase of TBARS and a significant decrease of TAR in both groups of MSUD patients. However, TAR reduction was higher in

the group presenting low leucine levels. On the other hand, it was not found a correlation between leucine, valine and isoleucine levels and oxidative stress parameters. Selenium levels in plasma from MSUD patients at diagnosis (non-treated) and under treatment, as well as the activities of antioxidant enzymes in erythrocytes from treated patients were also determined. MSUD patients presented a significant selenium deficiency at diagnosis which becomes more pronounced during treatment and a decrease of erythrocyte glutathione peroxidase activity during treatment. Subsequently, it was evaluated various biochemical parameters in plasma from MSUD patients during treatment in order to verify if there was correlation between these parameters and oxidative stress. It was demonstrated that MSUD patients under treatment present a reduction of glucose, total cholesterol, albumin and creatinine levels and an increased activity of aspartate aminotransferase and lactate dehydrogenase. However, HDL cholesterol, LDL cholesterol, triglycerides, alanine aminotransferase, creatine kinase, urea and uric acid measurements were not altered. Besides, it was verified a significant increase of TBARS and a significant decrease of TAR, as well as a positive correlation between TBARS and triglycerides levels. In summary, our results suggest an imbalance between the production of free radicals and the antioxidant defenses in MSUD patients leading to oxidative stress at diagnosis and during treatment and that this is not correlated with the amino acids leucine, isoleucine and valine accumulating in this disease. Besides, the selenium deficiency and the decrease of erythrocyte glutathione peroxidase activity could be associated to the development of oxidative stress. Finally, it is possible that oxidative damage may contribute, at

least in part, to the pathophysiology of this disorder. If that is the case, antioxidants may serve as an adjuvant therapy in MSUD

LISTA DE ABREVIATURAS

AACR	aminoácidos de cadeia ramificada
Alolleu	aloisoleucina
ALT	alanina aminotransferase
AST	aspartato aminotransferase
CACR	α-cetoácidos de cadeia ramificada
CAT	catalase
CDCR	desidrogenase dos α-cetoácido de cadeia ramificada
CIC	ácido α-cetoisocapróico
CIV	ácido α-cetoisovalérico
CK	creatina quinase
CK-MM	isoenzima muscular da creatina quinase
CMV	ácido α-ceto-β-metilvalérico
DXB	doença do xarope do bordo
EIM	erros inatos do metabolismo
ERN	espécies reativas de nitrogênio
ERO	espécies reativas de oxigênio
GR	glutationa redutase
GSH	glutationa reduzida
GSH-Px	glutationa peroxidase
GSSG	glutationa oxidada
HACR	α-hidroxiácidos de cadeia ramificada
HIC	ácido α-hidroxiisocapróico
HIV	ácido α-hidroxiisovalérico

HMV	ácido α-hidroxi-β-metilvalérico
Ileu	isoleucina
LDH	lactato desidrogenase
Leu	leucina
MDA	malondialdeído
NADPH	nicotinamida adenina dinucleotídeo fosfato
SNC	sistema nervoso central
SOD	superóxido dismutase
TAR	reatividade antioxidante total
TAS	status antioxidante total
TBARS	espécies reativas ao ácido tiobarbitúrico
Val	valina

I. INTRODUÇAO

I.1 Erros inatos do metabolismo

O termo erros inatos do metabolismo (EIM) foi proposto pela primeira vez por Archibald Garrod em 1908, a partir de seus estudos em pacientes com alcaptonúria. Garrod observou que os indivíduos afetados por esta doença excretavam na urina quantidades aumentadas de ácido homogentísico e que esta peculiaridade era encontrada em diversos membros de uma mesma família. Além disso, os pais dos afetados tinham, geralmente, parentesco consangüíneo próximo (Waber, 1990). Baseado nessas observações, Garrod, juntamente com o geneticista inglês Bateson, sugeriu um modelo de herança autossômica recessiva para esse distúrbio, e, ainda, que a alcaptonúria e outras anormalidades metabólicas herdadas eram raras e incomuns (Waber, 1990; Scriver et al., 2001).

Desde os estudos de Garrod, muitos pesquisadores têm detectado novas doenças metabólicas hereditárias e os erros inatos do metabolismo já foram descritos em todas as áreas do metabolismo humano (aminoácidos, lipídios, ácidos orgânicos, carboidratos etc.) (Scriver et al., 2001).

Os EIM são individualmente raros, porém são freqüentes quando analisados em conjunto, atingindo um a cada mil nascidos vivos (Gimenez-Sanchez et al., 2001). Estas doenças correspondem a cerca de 10% de todas as doenças genéticas e, atualmente, já foram descritos aproximadamente 500 distúrbios envolvendo defeitos na síntese, degradação, transporte e armazenamento de moléculas no organismo (Gimenez-Sanchez et al., 2001).

Esses defeitos hereditários do metabolismo devem-se a anormalidades na síntese de uma proteína, geralmente um enzima, alterando suas funções. A ausência ou a deficiência severa de atividade enzimática leva a um bloqueio metabólico com acúmulo de substratos e seus derivados. Dependendo da importância da rota afetada, este bloqueio repercute de forma clínica variável, geralmente provocando sintomatologia grave, que na maioria das vezes afeta o sistema nervoso central (SNC) (Scriver et al, 2001).

Os EIM podem ser classificados de diversas maneiras, como pela idade de apresentação ou pela área do metabolismo afetada. A classificação descrita por Saudubray e Charpentier (2001) estabelece três grandes grupos de EIM: distúrbios de síntese ou degradação de macromoléculas complexas, incluindo as doenças lisossômicas de depósito (ex: doença de Gaucher, doença de Fabry etc.) e as desordens peroxissomais (ex: adrenoleucodistrofia ligada ao X, doença de Refsum etc.); doenças com deficiência de energia, incluindo doenças de depósito de glicogênio, defeitos de gliconeogênese, acidemias lácticas congênitas, defeitos de oxidação de ácidos graxos e doenças mitocondriais de cadeia respiratória; e erros inatos do metabolismo intermediário, que incluem as aminoacidopatias (ex: doença do xarope do bordo, fenilcetonúria etc.), as acidemias orgânicas (ex: acidemia propiônica, acidemia metilmalônica etc.), os defeitos no ciclo da uréia (ex: citrulinemia, argininemia etc.) e as intolerâncias a açúcares (ex: galactosemia etc.). Neste último grupo de doenças pode ocorrer intoxicação aguda ou crônica, causada pelo acúmulo de componentes tóxicos e de metabólitos produzidos devido ao bloqueio de rotas metabólicas. Estudos revelam que aproximadamente um

terço dos EIM correspondem a aminoacidopatias, outro terço a acidemias orgânicas e o terço final a todos os outros EIM (Hoffmann, 1994).

I.2 Doença do xarope do bordo

Menkes e colaboradores descreveram em 1954 quatro pacientes com uma doença neurológica degenerativa, caracterizada por edema cerebral, convulsões, espasticidade e sofrimento respiratório, com início na primeira semana de vida e morte dentro de três meses. A característica mais proeminente foi o forte odor de açúcar queimado na urina que deu origem ao nome Doença do Xarope do Bordo (DXB). No final dos anos 1950**,** Dancis e colaboradores**,** identificaram os compostos acumulados na urina e plasma dos pacientes como os aminoácidos de cadeia ramificada leucina (Leu), isoleucina (Ileu) e valina (Val) e seus correspondentes α-cetoácidos. Dessa forma, a doença também é chamada cetoacidúria de cadeia ramificada (Dancis, 1959; Dancis et al., 1959). Posteriormente, Dancis e colaboradores, demonstraram que o bloqueio metabólico na DXB ocorre na descarboxilação dos α-cetoácidos de cadeia ramificada (Dancis et al., 1960).

A DXB é, portanto, um erro inato do metabolismo causado pela deficiência na atividade do complexo da desidrogenase dos α-cetoácidos de cadeia ramificada (CDCR). Como conseqüência deste bloqueio ocorre o acúmulo dos aminoácidos de cadeia ramificada (AACR) leucina, isoleucina e valina, bem como de seus respectivos α-cetoácidos de cadeia ramificada (CACR) α-cetoisocapróico (CIC), α-ceto-β-metilvalérico (CMV) e α-

cetoisovalérico (CIV) e α-hidroxiácidos de cadeia ramificada (HACR) α-hidroxiisocapróico (HIC), α-hidroxi-β-metilvalérico (HMV) e α-hidroxiisovalérico (HIV) (Figura 1). A DXB é uma desordem autossômica recessiva e apresenta freqüência mundial estimada em 1:185000 nascidos vivos (Chuang e Shih, 2001).

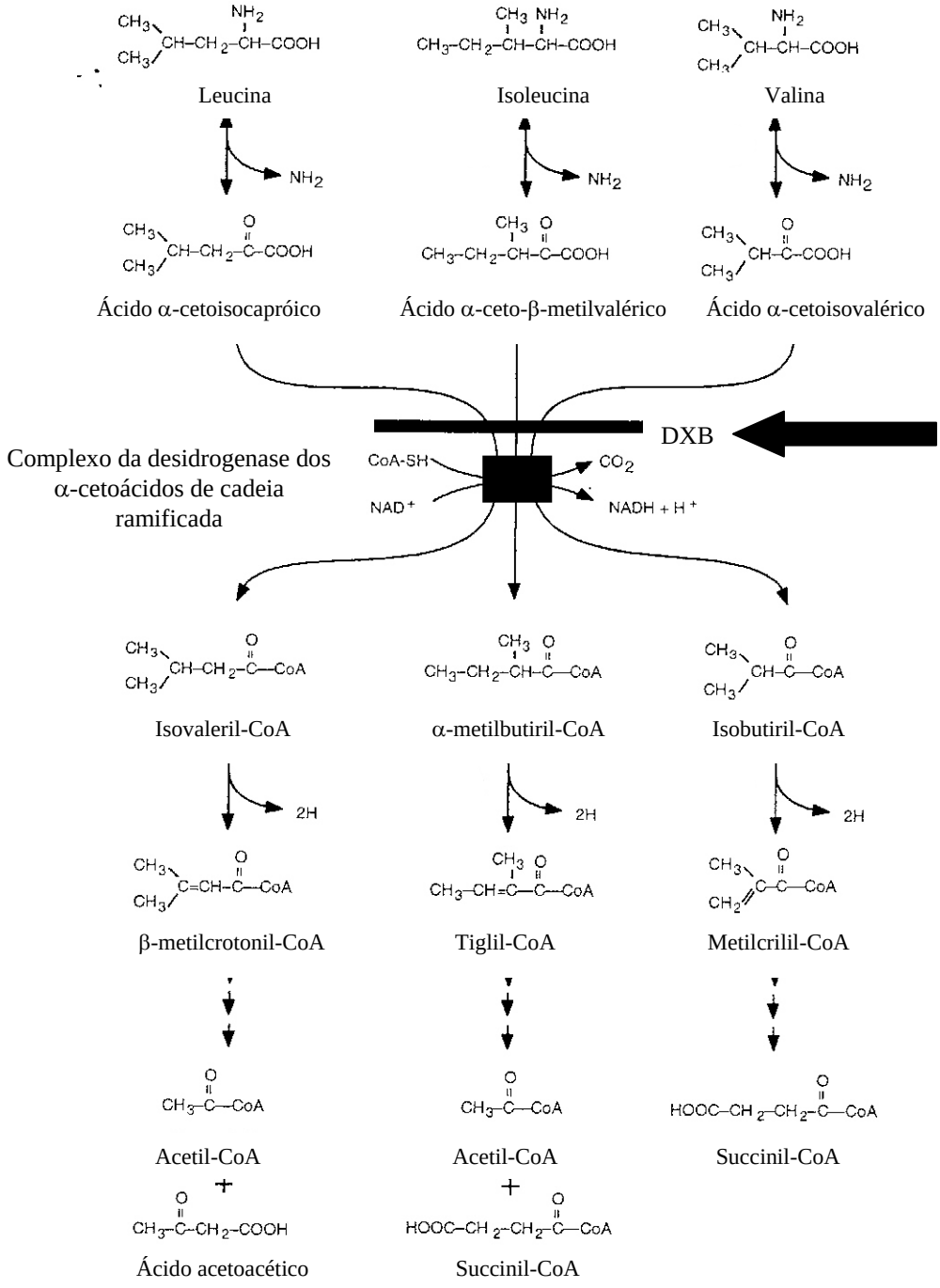

Figura 1. Rota metabólica dos aminoácidos de cadeia ramificada leucina, isoleucina e valina, indicando o bloqueio metabólico que ocorre na Doença do Xarope do Bordo. (Adaptado de Scriver et al., 2001).

O complexo multienzimático da desidrogenase dos α-cetoácido de cadeia ramificada está localizado no lado interno da membrana mitocondrial interna. Esse complexo enzimático compreende três sítios catalíticos: uma descarboxilase dependente de tiamina pirofosfato, ou E1; uma transacilase, ou E2; e uma desidrogenase, ou E3. Esse complexo possui duas enzimas reguladoras: uma quinase (quinase da desidrogenase dos α-cetoácidos de cadeia ramificada) e uma fosfatase (fosfatase da desidrogenase dos α-cetoácidos de cadeia ramificada) (Figura 2) (Peinemann e Danner, 1994; Chuang, 1998; Chuang e Shih, 2001).

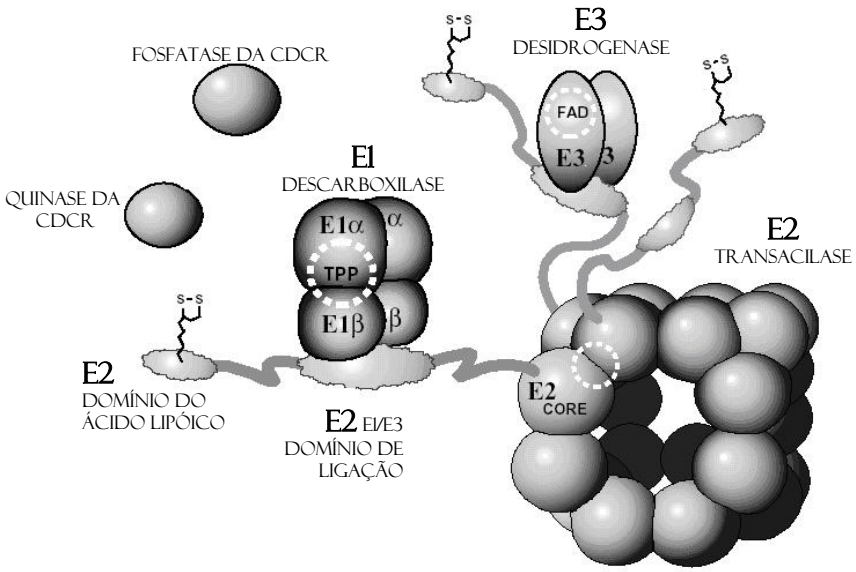

Figura 2. Subunidades e organização do complexo da desidrogenase dos α-cetoácido de cadeia ramificada (CDCR). O complexo da CDCR mitocondrial é organizado em torno de um centro cúbico que compreende a subunidade transacilase (E2). A descarboxilase dependente de tiamina pirofosfato (E1) e a desidrogenase ligada ao FAD (E3) estão ligadas de forma não covalente ao domínio de ligação E1/E3 da subunidade E2. A quinase específica se liga ao domínio do ácido lipóico da E2. O sítio de ligação específico para a fosfatase é desconhecido. Os três domínios da E2 são ligados por regiões flexíveis. Os sítios ativos ou de ligação ao cofator em cada componente da enzima estão circulados. (Adaptado de Chuang, 1998).

O catabolismo dos AACR inicia por sua transaminação reversível catalisada pela aminotransferase de cadeia ramificada formando os α-cetoácidos de cadeia ramificada que são internalizados na mitocôndria através de uma proteína transportadora específica (sistema L de transporte não dependente de sódio). No interior da mitocôndria os CACR sofrem descarboxilação oxidativa irreversível pelo complexo da desidrogenase dos α-cetoácidos de cadeia ramificada. A reação produz respectivos acil CoA de cadeia ramificada que são metabolizados por vias específicas. Os produtos finais do catabolismo da leucina são acetil-CoA e acetoacetato, da valina succinil-CoA e da isoleucina acetil-CoA e succinil-CoA (Schadewaldt e Wendel, 1997; Chuang e Shih, 2001).

A manifestação clínica da DXB é variável, dependendo da variante da doença (forma clássica severa a formas variantes moderadas). O tratamento envolve restrição na ingestão de proteínas e utilização de fórmulas específicas com redução dos AACR, suplementada por aminoácidos essenciais e associada ou não a administração do cofator tiamina. A classificação dos pacientes é baseada em parâmetros como idade de início dos sintomas, tolerância à leucina, atividade residual do complexo da desidrogenase dos α-cetoácidos de cadeia ramificada e resposta a administração de tiamina. Os pacientes com DXB podem ser classificados em cinco fenótipos: clássica, intermediária, intermitente, responsiva à tiamina e deficiência de lipoamida desidrogenase (Chuang e Shih, 2001; Simon et al., 2006a).

A forma clássica da doença se caracteriza por início neonatal e é a forma mais severa e freqüente da doença, representando aproximadamente 80% dos casos de DXB. A atividade residual do complexo da desidrogenase

dos α-cetoácidos de cadeia ramificada é extremamente baixa (<2% do normal), e os níveis dos AACR é extremamente elevado no sangue, líquor e urina. Os recém nascidos são aparentemente normais, desenvolvendo os sintomas entre o 4º e o 7º dias de vida. Inicialmente a doença se manifesta por letargia e recusa alimentar, evoluindo para perda de peso, cetoacidose, sinais neurológicos progressivos, convulsões, encefalopatia aguda e coma. A maioria dos pacientes clássicos não tratados morre nos primeiros meses de vida devido a crises metabólicas agudas e deterioração neurológica (Peinemann e Danner, 1994; Chuang e Shih, 2001).

Os pacientes com a forma intermediária da DXB apresentam uma elevação persistente dos AACR, porém menos pronunciada que na forma clássica. A atividade residual do complexo da desidrogenase dos α-cetoácidos de cadeia ramificada varia de 3 a 30% do normal e os pacientes não apresentam manifestações agudas no período neonatal. O diagnóstico, normalmente, é feito em torno dos dois anos de vida e os pacientes apresentam atraso no desenvolvimento, convulsões e episódios de cetoacidose (Chuang e Shih, 2001).

Os pacientes com a forma intermitente apresentam desenvolvimento neuropsicomotor normal. No entanto, apresentam risco elevado de descompensação metabólica durante situações de estresse. A atividade do complexo da α-cetoácido desidrogenase de cadeia ramificada varia de 5 a 20% do normal. Enquanto assintomáticos os níveis dos AACR encontram-se normais. O início dos sintomas ocorre entre os cinco meses e os dois anos de vida, em geral, associados a algum tipo de infecção (Chuang e Shih, 2001).

Em geral os pacientes com a forma responsiva à tiamina não apresentam doença neonatal aguda, e o curso inicial da doença se caracteriza por atraso no desenvolvimento psicomotor. Nesses pacientes a administração de tiamina associada à dieta restrita em proteínas pode reduzir os altos níveis dos AACR para valores normais. As doses de tiamina utilizadas variam de 10 a 1000 mg/dia (Chuang e Shih, 2001).

A deficiência de lipoamida desidrogenase (E3 - deficiente) é uma desordem rara, caracterizada por atraso no desenvolvimento psicomotor e acidose lática. Os AACR no plasma estão levemente ou moderadamente aumentados. Esses pacientes apresentam uma deficiência combinada das desidrogenases dos α-cetoácidos de cadeia ramificada, do piruvato e do α-cetoglutarato. O curso da doença é caracterizado por deterioração neurológica progressiva, incluindo hipotonia, atraso no desenvolvimento e desmielinização (Chuang e Shih, 2001).

Os principais sinais clínicos apresentados pelos pacientes com a forma clássica da DXB incluem cetoacidose, hipoglicemia, recusa alimentar, opistótono, apnéia, ataxia, convulsões, coma, atraso no desenvolvimento psicomotor e retardo mental. A encefalopatia também é um achado bastante importante nos pacientes com DXB. Além disso, pode ser observada a presença de edema generalizado e hipomielinização/desmielinização no sistema nervoso central desses pacientes, principalmente durante as crises de descompensação metabólica (Chuang e Shih, 2001; Schönberger et al., 2004; Sener, 2007).

A DXB pode ser diagnosticada pela detecção de altas concentrações de leucina, isoleucina e valina no plasma, e de níveis elevados dos CACR na

urina. O perfil de aminoácidos pode ser determinado através de cromatografia líquida ou cromatografia líquida acoplada a espectrometria de massas (CL/EM/EM), enquanto o perfil de ácidos orgânicos é determinado através de cromatografia gasosa acoplada a espectrometria de massa (CG/EM) (Chuang e Shih, 2001). O principal metabólito acumulado na doença é a leucina que pode atingir níveis plasmáticos de até 5 mM, sendo a quantificação deste aminoácido o principal critério para diagnóstico e avaliação do tratamento, enquanto que isoleucina e valina atingem 1 mM no momento do diagnóstico (Bremer et al., 1981). A presença de aloisoleucina (AloIleu) no plasma é considerada patognomônico da DXB. Esse aminoácido não protéico é um produto da racemização da isoleucina e apresenta uma depuração lenta. Altos níveis de AloIleu no plasma persistem por vários dias após o episódio de descompensação metabólica, sendo permanentemente detectável no plasma dos pacientes com a forma clássica da DXB (Chuang e Shih, 2001).

Atualmente, muitos países têm utilizado testes de triagem neonatal baseados na espectrometria de massa em Tandem para a identificação precoce da DXB, permitindo o diagnóstico quando os pacientes são ainda assintomáticos ou apresentam poucos sintomas, usualmente no 5º ou 6º dia de vida (Peinemann e Danner, 1994; Simon et al., 2006b).

O diagnóstico da DXB é confirmado através da medida da atividade do complexo da desidrogenase dos α-cetoácidos de cadeia ramificada em leucócitos periféricos, cultura de fibroblastos ou cultura de linfoblastos de pacientes (Peinemann e Danner, 1994; Chuang e Shih, 2001; Chaung et al., 2006).

O diagnóstico pré-natal da DXB pode ser realizado através da análise enzimática direta do tecido de vilosidades coriônicas ou através de cultura de células do fluído amniótico ou das vilosidades coriônicas, coletados entre a 14º e 18º semanas de gestação (Chuang e Shih, 2001).

O tratamento para DXB consiste na restrição da ingestão de proteínas, o que minimiza o acúmulo dos AACR e, consequentemente, seus efeitos tóxicos, principalmente ao sistema nervoso central. O objetivo do tratamento é normalizar as concentrações dos AACR, sem prejudicar o crescimento e desenvolvimento dos pacientes. O tratamento deve ser iniciado o mais precocemente possível, ainda no período neonatal, e deve ser mantido por toda a vida do paciente. A terapia com tiamina (50-300mg/dia) é empregada por 3 semanas no início do tratamento para detecção de pacientes com a forma responsiva a tiamina. A dieta prescrita aos pacientes com DXB deve ser hipoproteica e hipercalórica, praticamente isenta de proteínas de origem animal combinada com uma fórmula semi-sintética contendo aminoácidos essenciais, exceto os AACR, e suplementada com minerais (molibdênio, manganês...) e vitaminas (vitaminas C, D, E...) (Chuang e Shih, 2001). Sem o diagnóstico precoce e o tratamento efetivo as crianças desenvolvem problemas cerebrais severos e permanentes, podendo até mesmo morrer nos primeiros meses de vida (Morton et al., 2002).

Durante a fase aguda da doença medidas mais agressivas precisam ser empregadas para reduzir rapidamente os níveis dos AACR, que podem levar a deterioração das funções cerebrais. A descompensação metabólica aguda pode ser desencadeada por períodos de estresse como, infecções, febre ou outras doenças. Dois aspectos são importantes no manejo das crises

metabólicas: a rápida remoção dos metabólitos tóxicos e a supressão do catabolismo e/ou a promoção do anabolismo (Chuang e Shih, 2001; Yoshino et al., 1999).

Entre as medidas utilizadas para a rápida remoção dos metabólitos acumulados estão a diálise peritoneal, a transfusão exsangüínea e a hemodiálise, as quais apresentam bons resultados, aumentando significativamente a depuração desses compostos. Entre os tratamentos extra corporais, a hemodiálise apresenta melhor desempenho que a hemofiltração, devendo ser considerada a primeira opção de tratamento. Quando esta medida não é disponível, a transfusão exsangüínea pode ser considerada a melhor escolha. A administração parenteral de uma solução contendo eletrólitos, glicose, lipídios, vitaminas e uma mistura de aminoácidos isenta de AACR, sozinha ou combinada com a administração de insulina, tem sido utilizada como terapia durante a descompensação metabólica visando suprimir o catabolismo e/ou promover o anabolismo (Chuang e Shih, 2001; Yoshino et al., 1999).

A maioria das crianças afetadas que são prospectivamente monitoradas e controladas apresentam bons resultados de desenvolvimento mental; contudo, a intoxicação metabólica aguda e a deterioração neurológica podem se desenvolver rapidamente em qualquer idade. Cada episódio de descompensação metabólica está associado com um risco para edema e comprometimento cerebral (Chuang e Shih, 2001; Strauss e Morton, 2003).

A leucina e/ou seu respectivo α-cetoácido (α-cetoisocapróico) tem sido considerados os principais metabólitos neurotóxicos na DXB, uma vez que o aumento na concentração plasmática desses compostos tem sido associado ao

aparecimento dos sintomas neurológicos (Chuang e Shih, 2001; Snyderman et al., 1964). No entanto, os mecanismos responsáveis pelos sintomas neurológicos apresentados pelos pacientes com DXB ainda são pouco conhecidos.

I.3 Radicais livres

Radicais livres são estruturas químicas que possuem um elétron desemparelhado, ou seja, ocupando um orbital atômico ou molecular sozinho. Isso os torna muito instáveis, extraordinariamente reativos e com uma enorme capacidade para combinar-se inespecificamente com diversas moléculas integrantes da estrutura celular (Halliwell e Gutteridge, 2007).

Existem diversas fontes geradoras de radicais livres nos sistemas biológicos. Os radicais livres podem ser formados endogenamente (subprodutos do metabolismo aeróbico) ou por influências externas (dieta inadequada, consumo exagerado de álcool, fumo, uso de quimioterápicos e outras drogas, exposição às radiações ionizante e eletromagnética, poluição atmosférica, etc.) (Ames et al., 1993; Halliwell, 1994; Dröge, 2002).

O termo espécies reativas de oxigênio (ERO) inclui não apenas os radicais formados pela redução do oxigênio (superóxido ($O_2^{\bullet -}$) e hidroxila ($OH^{\bullet}$)), mas também alguns não radicais derivados do oxigênio, como o peróxido de hidrogênio (H_2O_2) e o oxigênio *singlet* ($^1O_2$). Além das espécies reativas de oxigênio, existem ainda as espécies reativas de nitrogênio (ERN),

representadas principalmente pelo óxido nítrico ($NO^{\bullet}$) e peroxinitrito ($ONOO^{-}$) (Halliwell e Gutteridge, 2007) (tabela 1).

Tabela 1. Algumas espécies reativas de oxigênio (ERO) e de nitrogênio (ERN).

	Radicais	Não Radicais
ERO	Superóxido, $O_2^{\bullet-}$	Peróxido de hidrogênio, H_2O_2
	Hidroxila, $OH^{\bullet}$	Oxigênio singlet, $^1O_2$
	Peroxila, $RO_2^{\bullet}$	Ácido hipocloroso, HOCl
	Hidroperoxila, $HO_2^{\bullet}$	Peroxinitrito, $ONOO^{-}$
		Ozônio, O_3
ERN	Óxido nítrico, $NO^{\bullet}$	Ácido nitroso, HNO_2
	Dióxido de nitrogênio, $NO^{\bullet}_2$	Peroxinitrito, $ONOO^{-}$
		Peroxinitrato, O_2NOO^{-}

(Adaptado de Halliwell e Gutteridge, 2007)

O radical superóxido é formado normalmente no organismo, principalmente através da cadeia transportadora de elétrons mitocondrial ou por ação de células fagocitárias durante o processo de defesa. Apesar do nome, este radical é fracamente reativo. O radical de oxigênio mais reativo é o radical hidroxila ($OH^{\bullet}$), que uma vez formado reage rápida e inespecificamente, podendo atacar e lesar qualquer biomolécula. O radical hidroxila é formado pela reação entre o radical superóxido e o H_2O_2 ou pela reação entre o H_2O_2 e metais de transição como o ferro (Reação de Fenton). O H_2O_2 é formado em

praticamente todos os tecidos do organismo e apesar de fracamente reativo sua importância está relacionada à sua capacidade de formar o radical hidroxila (Halliwell, 1996; Halliwell, 2001).

O oxido nítrico é um radical pouco reativo que apresenta grande importância biológica atuando na vasorregulação e neurotransmissão, porém em excesso pode ser citotóxico. A reação entre os radicais óxido nítrico e superóxido leva a formação de peroxinitrito, o qual apresenta maior reatividade podendo oxidar lipídios, DNA e aminoácidos (Halliwell, 1996; Halliwell, 2001).

As ERO e as ERN, em baixos níveis, são indispensáveis em muitos processos bioquímicos, incluindo a comunicação intracelular, a apoptose, a defesa do organismo contra agentes infecciosos, entre outros. Entretanto, uma produção excessiva dessas espécies ou uma deficiência na sua remoção podem gerar um estado pró-oxidante que favorece a ocorrência de lesões oxidativas em macromoléculas e estruturas celulares como proteínas, lipídios e DNA. Os radicais livres podem promover a lipoperoxidação, causar a oxidação de proteínas levando a sua inativação e podem também reagir com DNA e RNA causando mutações ou distúrbios de transcrição (Halliwell, 1996; Delanty e Dichter, 1998; Halliwell e Gutteridge, 2007).

I.4 Defesas antioxidantes

Os antioxidantes são substâncias endógenas ou exógenas que reduzem a formação de radicais livres ou reagem promovendo sua inativação. Para evitar o dano celular que pode ser causado pela presença de radicais livres, o

organismo possui defesas antioxidantes enzimáticas e não-enzimáticas (Halliwell e Gutteridge, 2007).

O organismo sintetiza uma série de compostos não-enzimáticos que apresentam grande capacidade de defesa antioxidante, atuando para manter o equilíbrio celular. Dentre esses compostos podemos citar, a bilirrubina, a melatonina, o ácido lipóico, a coenzima Q, o acido úrico, a glutationa e os estrógenos. Esses antioxidantes atuam de diferentes maneiras combatendo diretamente os radicais livres ou indiretamente, ligando íons como ferro e cobre, tornando-os menos reativos ou mesmo estimulando a produção de outras defesas antioxidantes (Halliwell e Gutteridge, 2007). Além dos antioxidantes sintetizados endogenamente, alguns importantes antioxidantes são obtidos através da dieta, incluindo as vitamina A, C e E, e polifenóis (Salvador e Henriques, 2004; Halliwell e Gutteridge, 2007).

Os antioxidantes enzimáticos também são importantes na detoxificação celular dos radicais livres. Dentre os antioxidantes enzimáticos podemos destacar a enzima superóxido dismutase (SOD) que catalisa a dismutação do radical superóxido a H_2O_2, a enzima catalase (CAT) que é responsável pela decomposição direta do H_2O_2 formando água (H_2O) e oxigênio (O_2) e a enzima glutationa peroxidase (GSH-Px) que catalisa a decomposição de peróxidos através da oxidação da glutationa reduzida (GSH) formando glutationa oxidada (GSSG). Fisiologicamente a GSH-Px atua acoplada a enzima glutationa redutase (GR) que, por sua vez, catalisa a redução da GSSG, usando NADPH como coenzima (Halliwell, 2001; Bonnefoy et al., 2002; Salvador e Henriques 2004). A figura 3 mostra os sítios de formação de radicais livres de oxigênio e as correspondentes defesas antioxidantes.

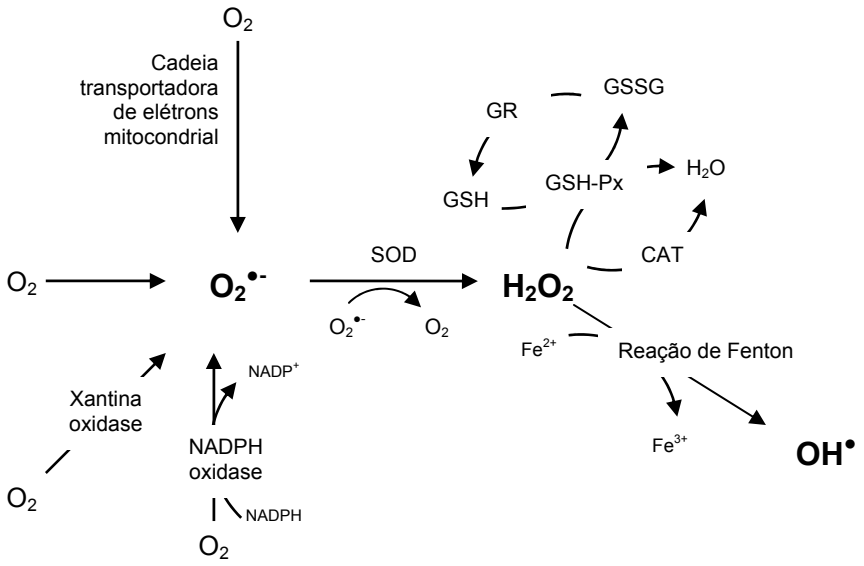

Figura 3. Geração de espécies reativas de oxigênio e as correspondentes defesas antioxidantes. $O_2^{\bullet-}$, radical superóxido; H_2O_2, peróxido de hidrogênio; $OH^{\bullet}$, radical hidroxila; GSH, glutationa reduzida; GSSG, glutationa oxidada; GSH-Px, glutationa peroxidase; GR, glutationa redutase; SOD, superóxido dismutase; CAT, catalase (Adaptado de Wajnet et al., 2004).

I.5 Estresse oxidativo

Normalmente, nos organismos saudáveis existe um equilíbrio entre a produção de espécies reativas e as defesas antioxidantes. As defesas antioxidantes controlam os níveis de espécies reativas, permitindo que estas desempenhem seu papel dentro do metabolismo normal. No entanto, situações patológicas podem causar o rompimento deste equilíbrio, seja através da diminuição das defesas antioxidantes, seja pelo aumento na produção de espécies reativas ou mesmo da combinação de ambos, resultando no que chamamos de estresse oxidativo. O termo estresse oxidativo se refere ao desequilíbrio entre a capacidade antioxidante e as espécies reativas formadas (pró-oxidante), em favor destas, podendo resultar em dano oxidativo a componentes celulares (Halliwell e Gutteridge, 2007; Halliwell, 2001). O estado pró-oxidante gerado por esse desequilíbrio favorece a ocorrência de lesões oxidativas em biomoléculas (lipídios, DNA e proteínas). As conseqüências do estresse oxidativo incluem a lipoperoxidação na membrana celular, a oxidação de proteínas, lesões ao DNA e RNA e a morte celular, dependendo do tipo celular e da severidade do estresse oxidativo (Halliwell e Gutteridge, 2007, Halliwell, 2001).

Todos os tecidos humanos são suscetíveis ao dano oxidativo. No entanto, o cérebro parece ser especialmente sensível a este tipo de lesão. Uma razão importante para isso seria o alto consumo de oxigênio apresentado por este tecido. Além disso, as membranas neuronais apresentam grande quantidade de lipídios poliinsaturados, altamente suscetíveis a lipoperoxidação. Ainda, a autooxidação de muitos neurotransmissores, como dopamina e

noradrenalina, gera espécies reativas, sendo que esta autooxidação pode ser acelerada pela presença de ferro, amplamente distribuído no cérebro. Por fim, o tecido cerebral apresenta um baixo nível de defesas antioxidantes (Halliwell e Gutteridge, 2007).

Existem evidências crescentes sugerindo que as espécies reativas desempenham um papel importante na patogênese de muitas doenças, como diabetes, neoplasias, aterosclerose, doenças inflamatórias e doenças neurodegenerativas, em particular a doença de Alzheimer, a doença de Parkinson e a esclerose lateral amiotrófica (Reznick e Packer, 1993; Przedborski *et* al., 1996; Bem–Menachem et al., 2000).

Tem também sido recentemente demonstrado que o estresse oxidativo atua em vários erros inatos do metabolismo (Colomé et al., 2000, Wajner et al., 2004). A produção excessiva de radicais livres e a redução das defesas antioxidantes ocorrem em algumas acidemias orgânicas, como nas acidemias glutárica (Kolker et al., 2001; Latini et al., 2005; Latini et al., 2007), propiônica e metilmalônica (Fontella et al., 2000), bem como em aminoacidopatias como na homocistinúria (Streck et al., 2003; Stefanello et al., 2005), tirosinemia tipo I (Bird et al., 1995) e fenilcetonúria (Sierra et al., 1998; Artuch et al., 2001; Hagen et al., 2002; Artuch et al., 2004; Sirtori et al., 2005; Sitta et al., 2006) e também na doença peroxissomal adrenoleucodistrofia ligada ao X (Vargas et al., 2004; Deon et al., 2006), sugerindo que o estresse oxidativo possa estar envolvido no dano neurológico observado nessas doenças.

Estudos em animais demonstraram que os aminoácidos de cadeia ramificada (leucina, isoleucina e valina) e seus respectivos α-cetoácidos acumulados na DXB estimulam a lipoperoxidação em homogeneizado de

cérebro de ratos. (Fontella et al., 2002). Ainda, foi demonstrado que estes compostos, principalmente a leucina e o ácido α-cetoisocapróico, reduzem a capacidade do cérebro em modular o dano associado ao aumento na produção de radicais livres e que a lipoperoxidação estimulada pela leucina pode ser atenuada por antioxidantes como vitaminas C e E, glutationa reduzida e superóxido dismutase (Bridi et al., 2003; Bridi et al., 2005a; Bridi et al., 2005b).

Embora o mecanismo responsável pelo estresse oxidativo nos erros inatos do metabolismo não seja totalmente compreendido, é possível que o acúmulo de metabólitos tóxicos induza a formação excessiva de radicais livres. Além disso, é provável que a restrição dietética a qual muitos dos pacientes com erros inatos do metabolismo são submetidos, cause redução nas defesas antioxidantes devido à deficiência de nutrientes essenciais, como vitaminas e minerais (Artuch et al., 2004).

II. OBJETIVOS

II.1 Objetivo geral

Considerando que os mecanismos envolvidos no dano neurológico apresentado pelos pacientes com DXB ainda não estão totalmente esclarecidos e que estudos em animais têm demonstrado que o estresse oxidativo é induzido pelos metabólitos acumulados na DXB, o objetivo deste estudo foi investigar vários parâmetros de estresse oxidativo em amostras de plasma e eritrócitos de pacientes com DXB, antes e durante o tratamento, no intuito de melhor entender a fisiopatologia e o efeito do tratamento nestes indivíduos.

II.2 Objetivos específicos

Capitulo I - Avaliar parâmetros de estresse oxidativo, como a medida das espécies reativas ao ácido tiobarbitúrico (TBARS), a medida da reatividade antioxidante total (TAR) e a medida do *status* antioxidante total (TAS) em plasma de pacientes não tratados (no diagnóstico) com doença do xarope do bordo.

Capitulo II - Avaliar parâmetros de estresse oxidativo, como a medida das espécies reativas ao ácido tiobarbitúrico (TBARS), a medida da reatividade antioxidante total (TAR) e a medida do *status* antioxidante total (TAS) em

plasma de pacientes com doença do xarope do bordo sob tratamento dietético com níveis baixos ou níveis altos de leucina.

Capitulo III – Avaliar os níveis de selênio no plasma de pacientes com doença do xarope do bordo não tratados (no diagnóstico) e durante o tratamento dietético, bem como a atividade das enzimas glutationa peroxidase (GSH-Px), catalase (CAT) e superóxido dismutase (SOD) em eritrócitos de pacientes em tratamento.

Capitulo IV – Avaliar parâmetros de estresse oxidativo, como a medida das espécies reativas ao ácido tiobarbitúrico (TBARS) e a medida da reatividade antioxidante total (TAR), e outros parâmetros bioquímicos (glicose, colesterol total e frações, triglicerídeos, albumina, transaminases, creatina quinase, lactato desidrogenase, uréia, creatinina e ácido úrico) em plasma de pacientes com doença do xarope do bordo em tratamento dietético, a fim de verificar se há correlação entre os parâmetros de estresse oxidativo e os parâmetros bioquímicos.

III. RESULTADOS

Os resultados estão apresentados na forma de artigos científicos.

III.1 Capítulo I – Artigo 01

Evidence that oxidative stress is increased in plasma from patients with maple syrup urine disease

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ORIGINAL PAPER

Evidence that oxidative stress is increased in plasma from patients with maple syrup urine disease

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Abstract Maple syrup urine disease (MSUD) or branched-chain α-keto aciduria (BCKA) is an inherited disorder caused by a deficiency of the branched-chain α-keto acid dehydrogenase complex (BCKAD) activity. The blockage of this pathway leads to tissue accumulation of the branched-chain amino acids (BCAA) leucine, isoleucine and valine and their respective keto-acids. The clinical features presented by MSUD patients include ketoacidosis, convulsions, coma, psychomotor delay and mental retardation. The mechanism of brain damage in this disease is still poorly understood. However, an increase in lipid peroxidation *in vitro* in cerebral cortex of young rats as well as a decrease in the antioxidant defenses has been previously observed. In the present work we evaluated different oxidative stress parameters, named reactive species of thiobarbituric acid (TBARS), total antioxidant reactivity (TAR) and total antioxidant status (TAS) in plasma of MSUD patients in order to evaluate whether oxidative stress is involved in this disorder. We verified a marked increase of plasma TBARS measurements, which is indicative of increased lipid peroxidation, as well as a decrease on plasma TAR reflecting a deficient capacity to efficiently modulate the damage associated with an increased production of reactive species. In contrast, TAS was not changed indicating that the total content of antioxidants in plasma of patients affected by MSUD was not altered.

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These results suggest that free radical generation is elicited in MSUD and is possibly involved in the pathophysiology of the tissue damage found in this disorder.

Keywords Maple syrup urine disease · Oxidative stress · Lipid peroxidation · Antioxidant defenses

Introduction

Maple syrup urine disease (MSUD) or branched-chain α-keto aciduria (BCKA) is an autosomal recessive metabolic disorder caused by a severe deficiency of the branched-chain α-keto acid dehydrogenase complex (BCKAD) activity. The blockage in this enzyme complex leads to tissue accumulation of the branched-chain amino acids (BCAA) leucine, isoleucine and valine as well as their corresponding branched chain α-keto acids (BCKA) α-ketoisocaproate, α-keto-β-methylvalerate and α-ketoisovalerate, respectively (Chuang and Shih, 2001; Treacy *et al*., 1992).

The major clinical features presented by MSUD patients include ketoacidosis, hypoglycemia, poor feeding, apnea, ataxia, convulsions, coma, psychomotor delay and mental retardation. Magnetic resonance imaging studies have demonstrated generalized edema and hypomyelination/demyelination in central nervous system (CNS) of MSUD patients (Chuang and Shih, 2001; Schönberger *et al*., 2004).

MSUD presents heterogeneous molecular and clinical phenotypes range from a severe classic form with neonatal onset to milder variant forms with later onset, and presenting different residual enzyme activity (Chuang and Shih, 2001; Schadewaldt and Wendel, 1997).

The aim of MSUD treatment is to keep the BCAA plasma concentrations in the normal range, protecting the brain from injury. The treatment consists of a low protein diet and a semi-synthetic formula restricted in BCAA and supplemented with essential amino acids. Metabolic intoxication may cause a fatal outcome in untreated patients (Chuang and Shih, 2001; Danner and Elsas, 1989).

The mechanisms of the neurological symptoms presented by MSUD patients are still poorly understood. However, considering that increased concentrations of leucine and/or α-ketoisocaproate were associated with the appearance of neurological symptoms, these compounds seem to be the main important neurotoxic metabolites in MSUD (Chuang and Shih, 2001; Snyderman *et al*., 1964). Furthermore, it has been demonstrated that the metabolities accumulating in MSUD cause impairment of energy metabolism by inhibiting the electron transport chain (Sgaravatti *et al*., 2003) and creatine kinase activity in rat brain (Pilla *et al*., 2003). Other investigators demonstrated that the BCAA and/or BCKA that accumulate in MSUD provoke neuronal apoptosis (Jouvet *et al*., 2000), as well as convulsions (Coitinho *et al*., 2001), impairment of neurotransmitter synthesis and function (Zielke *et al*., 1996; Tavares *et al*., 2000), myelin alteration (Treacy *et al*., 1992; Tribble and Shapira, 1983; Taketomi *et al*., 1983) and reduced uptake of essential amino acids by the brain (Araújo *et al*., 2001).

Free radicals seem to be involved in a large number of human diseases. Increasing evidence has shown that damage caused by free radicals is an important contributing factor in chronic-inflammatory, vasculary, neoplastic and neurodegenerative diseases (Halliwell, 1994; Reznick and Packer, 1993; Przedborski *et al*., 1996; Bem-Menachem *et al*., 2000).

Oxidative stress has been observed in some inborn errors of intermediary metabolism owing to the accumulation of toxic metabolites which leads to excessive free radical production (Colome *et al*., 2000). Restricted diets utilized to treat patients affected by metabolic disorders may result in a low antioxidant status (Colome *et al*., 2000).

Recently, it was demonstrated that the BCAA and their respective BCKA that accumulate in MSUD stimulate *in vitro* lipid peroxidation in brain homogenates of rats (Fontella *et al*., 2002). It was later demonstrated that these compounds, particularly leucine and α-ketoisocaproate, not only stimulate *in vitro* lipid peroxidation but also reduce the cerebral capacity to modulate the damage associated with the increased free radical production (Bridi *et al*., 2003, 2005a). Furthermore, it was shown that the increased lipid peroxidation induced by leucine could be attenuated by the free radicals scavengers ascorbic acid, α-tocopherol, gluthatione and superoxide dismutase (Bridi *et al*., 2005b).

The aim of the present work was to evaluate some parameters of oxidative stress, namely thiobarbituric acid-reactive substances (TBARS), total antioxidant reactivity (TAR) and total antioxidant status (TAS), in plasma of MSUD patients at the time of diagnosis in order to verify whether free radicals could be involved in the pathophysiology of this disease.

Material and methods

Patients and controls

Plasma from five MSUD patients (classic form) aged between 15 days and 4 months at diagnosis were used to evaluate the parameters of oxidative stress. The most common clinical features presented by these patients were convulsions, hypoglycemia, poor feeding, ketoacidosis and psychomotor delay. Samples were obtained at the time of the diagnosis, which was made by increased plasma levels of leucine ($2{,}346.1 \pm 810.7$ μmol/L), isoleucine (304.8 ± 185.2 μmol/L) and valine (456.5 ± 275.1 μmol/L) by HPLC method (Joseph and Marsden, 1986). Control group was composed of healthy age matched individuals (leucine 158.33 ± 37.63 μmol/L; isoleucine 76.54 ± 18.02 μmol/L; valine 260.73 ± 39.79 μmol/L).

Reagents

All chemicals were of PA purity and were purchased from Sigma (St. Louis, MO, USA) except for thiobarbituric acid, which was purchased from Merck (Darmstadt, Germany) and a kit for TAS measurement that was purchased from Randox Laboratories (Antrim, United Kingdom). TAR was assayed using a beta liquid scintillation spectrometer (Wallac model 1409) and TBARS was measured with a spectrofluorimeter (Hitachi F2000).

Plasma preparation

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

Thiobarbituric Acid-Reactive Species (TBARS)

Thiobarbituric acid-reactive substances (TBARS) were determined according to the method described by Buege and Aust (1978). Briefly, 250 μL of 10% trichloroacetic acid were added to 125 μL of plasma, then 375 μl 0.67% thiobarbituric acid (in 7.1% sodium sulphate) were added and incubated at 100°C for 30 min. After the incubation, the mixture was extracted with 750 μL butanol. The resulting pink stained TBARS were determined in a spectrofluorimeter at 515 nm. Calibration curve was performed using 1,1,3,3-tetramethoxypropane subjected

to the same treatment as that of the samples. TBARS were calculated as nmol TBARS/mg protein.

Total Antioxidant Reactivity (TAR)

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*. (1992). The background chemiluminescence was measured by adding 4 mL of 2 mM ABAP (in 0.1 M glycine buffer, pH 8.6) into a glass scintillation vial. Ten microliters of luminol (4 mM) were added to each vial and the chemiluminescence was measured. This was considered to be the basal value. Ten microliters of 25–200 μM Trolox (curve calibration) or plasma was then added and the chemiluminescence was measured during 60 s. The Trolox and plasma addition reduces the chemiluminescence. The rapid reduction in luminol intensity is considered as a measure of the TAR capacity. TAR measurement was calculated as nmol Trolox/mg protein.

Total Antioxidant Status (TAS)

TAS, which represents the quantity of the tissue antioxidants, was determined by using a kit from RANDOX Laboratories. The plasma sample was incubated with ABTS (2,2′-azino-di-[3-ethylbenzthiazoline sulphonate]) plus a peroxidase (metmyoglobin) and H_2O_2 to produce the cation ABTS+. A relatively stable blue–green color occurred and was measured at 37°C at 600 nm. Antioxidants in the added sample cause suppression of this color production to a degree which was proportional to their concentration (Miller *et al*., 1993; Yu and Ong, 1999). The results were expressed in mmol/L plasma.

Protein determination

Protein concentrations were determined by the biuret method from Labtest® (Gornall *et al*., 1949), using albumin as standard.

Statistical analysis

The Student *t* test was used to compare results from controls and MSUD patients. A p value less 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 1 shows that TBARS was significantly increased in plasma of MSUD patients when compared to control group [$t(8) = -4.552$, $p < 0.01$], indicating that lipid peroxidation is stimulated in MSUD patients.

TAR measurement, which is a measure of the tissue capacity to react with free radicals, was markedly reduced [$t(8) = 3.021$, $p < 0.05$] in plasma of MSUD patients (Fig. 2). These results suggest a deficient capacity of plasma to modulate the damage associated with the enhanced production of reactive species in these MSUD patients. Finally, it was also observed that TAS measurement, which represents the quantity of the tissue antioxidants,

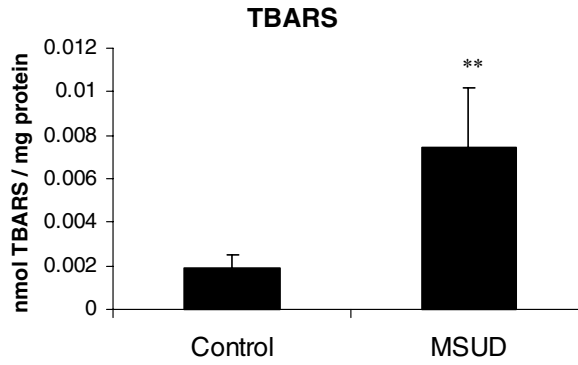

Fig. 1 Plasma thiobarbituric acid reactive species (TBARS) from MSUD patients and controls. Data represent the mean ± SD ($n = 5$). $^{**}p < 0.01$ (Student' t test for unpaired samples) compared to control

was not altered in plasma of MSUD patients [$t(6) = -0.713$, $p > 0.05$] (Fig. 3), suggesting that the total nonenzymatic antioxidant defenses were not altered.

Discussion

Neurological symptoms are frequent in MSUD patients and untreated patients normally have a fatal outcome (Chuang and Shih, 2001; Danner and Elsas, 1989). Leucine and/or α-ketoisocaproate are considered the main neurotoxic metabolites in these disease (Chuang and Shih, 2001; Snyderman *et al*., 1964). However, the mechanisms underlying the sequelae presented by these patients are not well understood.

It was previously demonstrated that the BCAA and BCKA accumulating in MSUD stimulate *in vitro* lipid peroxidation and reduce the antioxidant defenses in cerebral homogenates of young rats (Fontella *et al*., 2002; Bridi *et al*., 2003, 2005a). However, to our knowledge there is no report in the literature assessing whether oxidative stress occurs in tissues from MSUD patients. Therefore, in the present study we investigated some parameters of oxidative stress in plasma from these patients which were not under any therapy. So our results cannot be attributed to any medication.

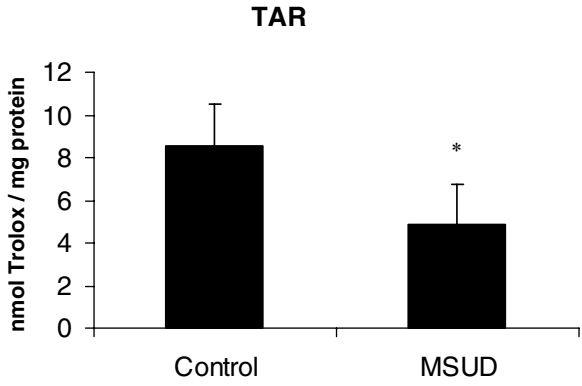

Fig. 2 Plasma total antioxidant reactivity (TAR) from MSUD patients and controls. Data represent the mean ± SD ($n = 5$). $^{*}p < 0.05$ (Student' t test for unpaired samples) compared to control

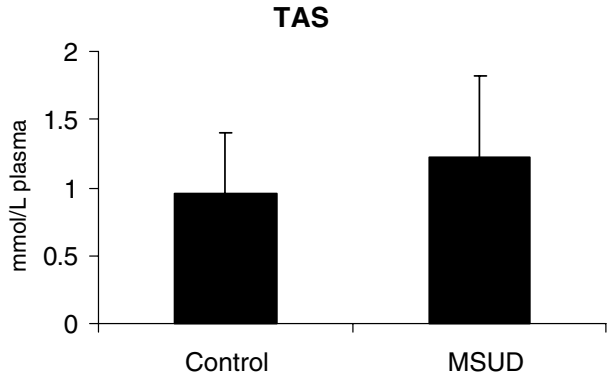

Fig. 3 Plasma total antioxidant status (TAS) from MSUD patients and controls. Data represent the mean ± SD ($n = 4$). No significant differences between means were found (Student' t test for unpaired samples)

We demonstrated a significant increase of TBARS in plasma from MSUD patients. Considering that TBARS reflects the content of malondialdehyde, an end product of lipid breakdown due to lipid peroxidation (Halliwell and Gutteridge, 2001; Esterbauer and Cheeseman, 1990), our data indicate that lipid peroxidation is induced in MSUD patients, probably secondary to free radical generation. Despite the fact that we did not find any decrease of total antioxidant defenses in plasma from these patients as indicated by TAS values TAR, which corresponds to a useful index of the capacity of a given tissue to modulate the damage associated with an increased production of free radicals and mainly reflects the quality of antioxidants (Lissi *et al*., 1995), was significantly decreased in the patients studied. These results indicate a deficient capacity of plasma from MSUD patients to rapidly handle an increase of reactive species. Considering that an imbalance between the total antioxidant defenses and the reactive species formed in the tissues are indicative of oxidative stress (Halliwell and Gutteridge, 2001), it is proposed that free radical generation is involved in the pathophysiology of the tissue damage found in MSUD.

Oxidative stress has been considered an important contributor to brain damage in neurodegenerative diseases, seizures and demyelination (Halliwell, 2001; Méndez-Álvarez *et al*., 2001; Karelson *et al*., 2001), probably because brain has relatively low levels of antioxidant defenses (Halliwell and Gutteridge, 2001), as well as high lipid content, specially unsaturated fatty acids, and iron that stimulates the Fenton reaction being therefore highly susceptible to reactive species attack. Taken together our present *in vivo* results and those demonstrating in studies *in vitro* with rats that the metabolities accumulated in MSUD cause a stimulation of lipid peroxidation and a reduction of brain antioxidant defenses, (Fontella *et al*., 2002; Bridi *et al*., 2003, 2005a), suggest that oxidative stress is probably involved in the pathophysiology of MSUD.

To our knowledge this is the first report demonstrating increased oxidative stress in patients affected by MSUD. Our results should, however, be taken with caution and confirmed with a higher number of patients and with other techniques to measure oxidative stress. In this context, CSF specimens may be useful in order to evaluate if the brain is also a target for reactive species. In case the present results are confirmed, we may conclude that oxidative stress contributes at least in part to the severe neurological dysfunction found in MSUD.

Acknowledgements This work was supported by grants from Brazilian National Research Council (CNPq), FAPERGS, and FIPE/HCPA—Brazil.

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III.2 Capítulo II – Artigo 02

Oxidative stress in plasma from maple syrup urine disease patients during treatment

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ORIGINAL PAPER

Oxidative stress in plasma from maple syrup urine disease patients during treatment

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Abstract Maple Syrup Urine Disease (MSUD) is an autossomal recessive metabolic disorder caused by a deficiency of branched-chain α-keto acid dehydrogenase complex activity leading to accumulation of the branched-chain amino acids leucine, isoleucine and valine and their corresponding branched-chain α-keto acids. Affected patients usually present hypoglycemia, ketoacidosis, convulsions, poor feeding, coma, psychomotor delay and mental retardation. Considering that the pathophysiology of MSUD is still poorly understood, in this study we evaluated some parameters of oxidative stress, namely thiobarbituric acid-reactive substances (TBARS), total antioxidant reactivity (TAR) and total antioxidant status (TAS) in plasma from treated MSUD patients presenting high and low plasma leucine levels. We verified a significant increase of TBARS (lipid peroxidation) and a decrease of TAR (capacity to rapidly react with free radicals) in plasma from treated MSUD patients with low and with high plasma levels of leucine compared to the control group. It was also verified that TAS (quantity of tissue antioxidants) was not altered in plasma from treated MSUD patients with low and high blood leucine levels. Finally, we found no correlation between leucine, valine and isoleucine levels with the various parameters of oxidative stress. These results are indicative that increased lipid oxidative damage and decreased antioxidant defenses occur in plasma of MSUD patients and that the accumulating branched-chain amino acids are probably not directly associated to oxidative stress in this disorder.

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Keywords MSUD · Oxidative stress · Free radicals · Leucine

Introduction

Maple Syrup Urine Disease (MSUD) is an inherited disorder affecting the metabolism of branched-chain amino acids (BCAA) leucine (Leu), isoleucine (Ile) and valine (Val). The activity of the branched-chain α-keto acid dehydrogenase complex (BCKAD) is deficient in MSUD leading to tissue accumulation of BCAA (Leu, Ile e Val), as well as their corresponding transaminated branched-chain α-keto acids (BCKA) α-ketoisocaproate, α-keto-β-methylvalerate and α-ketoisovalerate (Chuang and Shih 2001; Treacy *et al.* 1992).

The clinical and biochemical phenotypes of MSUD patients are heterogeneous. Patients can be divided into five phenotypes ranging from the classical form with a neonatal onset to milder variants with later onset (Chuang and Shih 2001; Schadewaldt and Wendel 1997). Individuals with MSUD usually present poor feeding, convulsions, ketoacidosis, apnea, hypoglycemia, coma, ataxia, psychomotor delay and mental retardation, as well as generalized edema and hypomyelination/demyelination on magnetic resonance imaging studies of the central nervous system (CNS) (Chuang and Shih 2001; Schönberger *et al.* 2004). Therapy for this disease is based on a natural protein restricted diet with low BCAA supplemented with a semi-synthetic formula of essential amino acids, vitamins and minerals. This treatment minimizes the accumulation of the toxic metabolites and contributes to the survival of the affected individuals, but do not prevent a variable degree of neurological dysfunction evidenced by developmental delay and mental retardation whose pathogenesis is poorly known (Chuang and Shih 2001). Leucine and/or α-ketoisocaproate are thought to be the main neurotoxic metabolites in this disorder once their increased concentrations have been associated to the appearance of neurological symptoms (Chuang and Shih 2001; Snyderman *et al.* 1964).

Free radicals and oxidative stress have been associated with a large number of diseases including various neurodegenerative disorders, epileptic seizures, demyelination and dementia (Halliwell 1994; Reznick and Packer 1993; Przedborski *et al.* 1996; Ben-Menachem *et al.* 2000). This may occur because the CNS is highly susceptible to oxidative damage due to the relatively low activity of antioxidant defenses, high iron content, high lipid content, specially unsaturated fatty acids, and high oxygen consumption (Halliwell and Gutteridge 2001). Oxidative stress has been also observed in some inborn errors of intermediary metabolism, and this has been attributed to the accumulation of toxic metabolites which are able to induce excessive free radical generation (Colome *et al.* 2000; Wajner *et al.* 2004). We cannot also exclude the possibility that the restricted diets used to treat patients with metabolic disorders may decrease the tissue antioxidant defenses since they potentially deplete essential nutrients involved in the antioxidant defenses (Halliwell and Gutteridge 2001).

With regards to MSUD, experimental animal studies have demonstrated that lipid peroxidation is stimulated in vitro by the BCAA and their respective BCKA in brain homogenates of rats (Fontella *et al.* 2002). It was also verified that these metabolites reduce in vitro the cerebral capacity to modulate the damage associated to increased free radical production (Bridi *et al.* 2003; Bridi *et al.* 2005a) and that the increased

lipid peroxidation induced in vitro in cerebral cortex of rats by leucine is attenuated by the free radicals scavengers ascorbic acid, α-tocopherol, gluthatione and superoxide dismutase (Bridi *et al.* 2005b).

On the other hand, we recently verified that the antioxidant status and lipid peroxidation were significantly altered in plasma of MSUD patients at diagnosis, suggesting the involvement of oxidative stress in the pathogenesis of this disease (Barschak *et al.* 2006). In the present study, we evaluated various parameters of oxidative stress in blood from treated MSUD patients showing high and low blood leucine levels, in order to test whether this amino acid could be involved in the oxidative stress. The parameters analyzed were thiobarbituric acid-reactive substances (TBARS), total antioxidant reactivity (TAR) and total antioxidant status (TAS).

Materials and methods

Reagents

All chemicals were of PA purity and were purchased from Sigma (St. Louis, MO, USA) except thiobarbituric acid that was purchased from Merck (Darmstadt, Germany) and a kit for TAS measurement that was purchased from Randox Laboratories (Antrim, United Kingdom). TAR was assayed using a beta liquid scintillation spectrometer (Wallac model 1409), TBARS was measured in a spectrofluorimeter (Hitachi F2000) and TAS in a double-bean spectrophotometer with temperature control (Hitachi U-2001).

Subjects

Plasma specimens from ten treated MSUD patients with the classic form and five age matched controls were used to evaluate the parameters of oxidative stress. The patients were aged between 15 days and 4 months at diagnosis and followed a treatment that consisted of a natural protein restricted diet with low BCAA and supplemented with a semi-synthetic formula of essential amino acids, vitamins and minerals. The patients ingested the following amounts of Leu (before 12 months of age: 40–80 mg kg^{-1} day^{-1}; after 1 year of age: 275–535 mg/day), Ile (before 12 months of age: 20–50 mg kg^{-1} day^{-1}; after 1 year of age: 165–325 mg/day) and Val (before 12 months of age: 20–60 mg kg^{-1} day^{-1}; after 1 year of age: 190–375 mg/day). Plasma samples obtained from MSUD patients under treatment were divided into two groups depending on blood Leu levels. In group I plasma Leu levels were lower than 100 μmol/l (36.3±17.1 μmol/l, treatment duration was 18.6±12.9 months), whereas in group II plasma Leu levels were higher than 600 μmol/l (1,314±914 μmol/l, treatment duration was 17.2±20.1 months). The control group corresponded to healthy age matched individuals (leucine 158±37.6 μmol/l; isoleucine 76.5±18.0 μmol/l; valine 260±39.8 μmol/l) (Table 1).

The present study was approved by the Ethical Committee of Hospital de Clínicas de Porto Alegre, RS, Brazil (protocol number 04-256). The parents of the patients included in the present study gave informed consent.

Table 1 Plasma concentrations of leucine (Leu), isoleucine (Ile) and valine (Val) in controls and MSUD patients during treatment

	Leu (μmol/l)	Ile (μmol/l)	Val (μmol/l)
Controls	158±16.8	76.5±8.0	260±17.7
MSUD patients group I	36.3±7.66	112±42.2	341±169
MSUD patients group II	1,314±408*	198±62.6	384±154

Values represent mean±SE (n=5)
*p<0.01, different from controls (ANOVA followed by the Duncan multiple range test)

Plasma preparation and amino acids determination

Plasma was prepared from whole blood samples obtained from fasting individuals (controls and MSUD 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. Blood amino acids levels were measured by HPLC (Joseph and Marsden 1986), with slight modifications (Wajner *et al*. 2000).

Thiobarbituric acid-reactive substances (TBARS)

Thiobarbituric acid-reactive substances (TBARS) were determined according to the method described by Buege and Aust 1978. Briefly, 250 μl of 10% trichloroacetic acid were added to 125 μl of plasma. Then 375 μl 0.67% thiobarbituric acid (in 7.1% sodium sulphate) were added and incubated at 100°C for 30 min. After the incubation, the mixture was extracted with 750 μl butanol. The resulting pink stained TBARS were determined in a spectrofluorimeter at 515 nm. Calibration curve was performed using 1,1,3,3-tetramethoxypropane subjected to the same treatment as that of the samples. TBARS were calculated as nmol TBARS/mg protein.

Total antioxidant reactivity (TAR)

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.* (1992). The background chemiluminescence was measured by adding 4 ml of 2 mM ABAP (in 0.1 M glycine buffer, pH 8.6) into a glass scintillation vial. Ten microliters of luminol (4 mM) were added to each vial and the chemiluminescence was measured. This was considered to be the basal value. Ten microliters of 25–200 μM Trolox (curve calibration) or plasma was then added and the chemiluminescence was measured during 60 s. The Trolox and plasma addition reduce the chemiluminescence. The rapid reduction in luminol intensity is considered as a measure of the TAR capacity. TAR measurement was calculated as nmol Trolox/mg protein.

Total antioxidant status (TAS)

TAS, which represents the quantity of the tissue antioxidants, was determined by using a kit from RANDOX Laboratories. The plasma sample was incubated with ABTS (2,2′-

azino-di-[3-ethylbenzthiazoline sulphonate]) plus a peroxidase (metmyoglobin) and H_2O_2 to produce the cation ABTS+. A relatively stable blue–green color occurred and was measured at 37°C at 600 nm. Antioxidants in the added sample cause suppression of this color production to a degree which is proportional to their concentration (Miller *et al.* 1993; Yu and Ong 1999). The results were expressed in millimole per liter.

Protein determination

Protein concentrations were determined by the Biuret method from Labtest® (Gornall *et al.* 1949), using bovine serum albumin as standard.

Statistical analysis

Results were analyzed by one-way ANOVA, followed by the Duncan multiple range test when appropriated (Altman 1991). Only significant F values are shown in the text. Correlations of plasma Leu, Val and Ile levels with TBARS, TAR or TAS were carried out using the Pearson correlation coefficient. A p value less 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 1 shows that TBARS measurement was significantly increased in plasma from MSUD patients both with low (group I) and high (group II) Leu levels, as compared to the control group [$F(2,12)=4.289$, $p<0.05$]. These data indicate that lipid peroxidation is stimulated in plasma from MSUD patients.

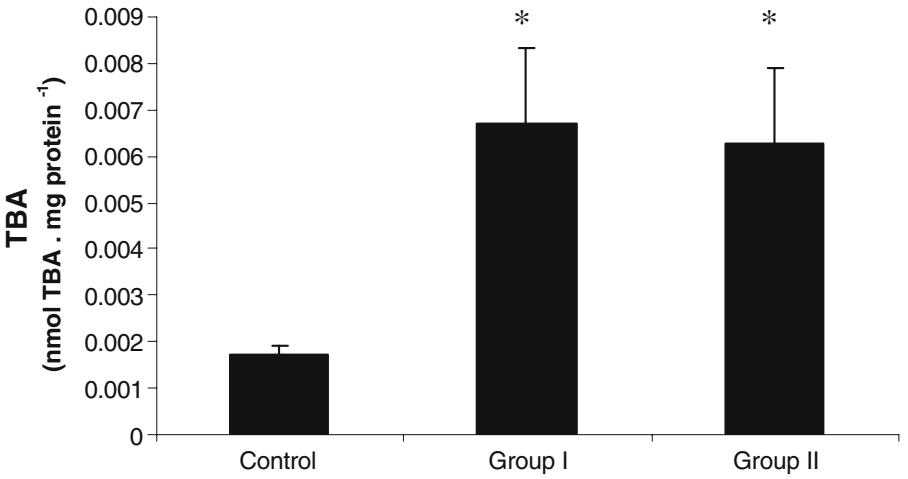

Fig. 1 Comparison between thiobarbituric acid reactive substances (TBARS) in plasma from MSUD patients during treatment and controls. Data represent the mean±SE ($n=5$). *Single asterisk* $p<0.05$, compared to controls (ANOVA, followed by the Duncan multiple range test). Group I: low plasma leucine levels; group II: high plasma leucine levels

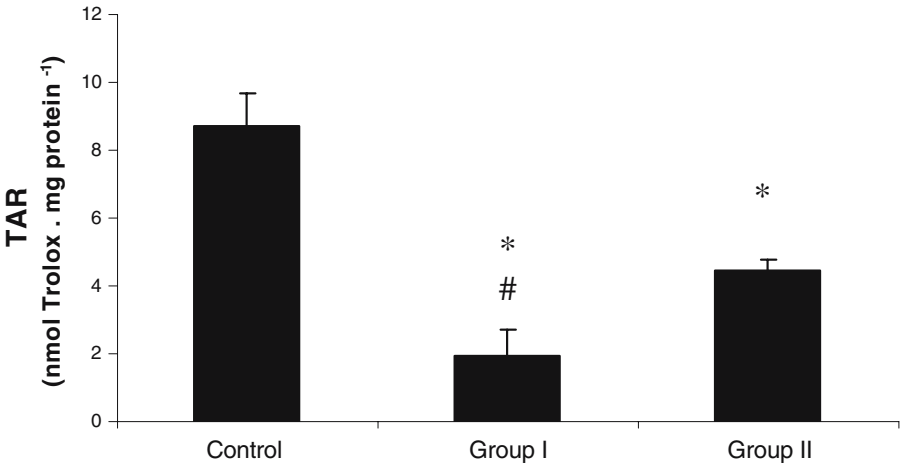

Fig. 2 Comparison between total antioxidant reactivity (TAR) in plasma from MSUD patients during treatment and controls. Data represent the mean±SE (n=5). *Single asterisk* $p<0.001$, compared to controls (ANOVA, followed by the Duncan multiple range test). *Pound sign* $p<0.001$, compared to group II (ANOVA, followed by the Duncan multiple range test). Group I: low plasma leucine levels; group II: high plasma leucine levels

Furthermore, TAR measurement was significantly reduced in plasma of MSUD patients independently of the concentration of Leu (high and low Leu levels), as compared to the control group [$F(2,12)=30.196$, $p<0.001$] (Fig. 2). It can be also observed in the figure that group I is different from group II. Considering that TAR measurement reflects the tissue capacity to react with free radicals, these results

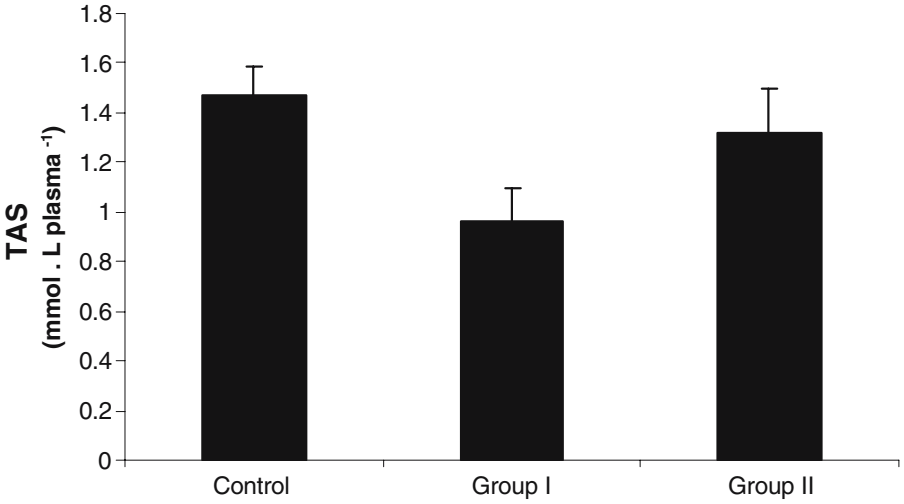

Fig. 3 Comparison between total antioxidant status (TAS) in plasma from MSUD patients during treatment and controls. Data represent the mean±SE (n=5). No significant differences between means were found (ANOVA). Group I: low plasma leucine levels; group II: high plasma leucine levels

suggest a deficient capacity of plasma from treated MSUD patients to modulate the damage associated with the enhanced production of reactive species.

We also observed that TAS measurement, which represents the quantity of tissue antioxidants, was not altered in plasma from treated MSUD patients (group I and group II), as compared to the control group [$F(2,12)=3.352$, $p>0.05$] (Fig. 3).

Finally we found no correlation between Leu, Ile and Val blood levels and TBARS, TAR and TAS measurements (results not shown).

Discussion

MSUD treatment is directed to minimize the accumulation of the toxic metabolites BCAA and BCKA and decisively contributes to the survival of affected individuals. However, some patients present a variable degree of developmental delay and mental retardation that are attributed to a poor adherence to the restricted diet (Chuang and Shih 2001).

Although the mechanisms underlying the pathophysiology of the brain damage in MSUD are still poorly understood, high plasma levels of Leu and its ketoacid derivative α-ketoisocaproate have been correlated with the appearance of neurological symptoms (Chuang and Shih 2001). Furthermore, it has been demonstrated that the metabolites accumulating in MSUD cause impairment of energy metabolism by inhibiting the electron transport chain (Sgaravatti *et al.* 2003) and creatine kinase activity (Pilla *et al.* 2003), induce neuronal apoptosis (Jouvet *et al.* 2000), convulsions (Coitinho *et al.* 2001), impairment of neurotransmitter synthesis and function (Zielke *et al.* 1996; Tavares *et al.* 2000), induce alterations of myelin synthesis or degradation (Treacy *et al.* 1992; Tribble and Shapira 1983; Taketomi *et al.* 1983) and also reduce the uptake of essential amino acids by the brain (Araújo *et al.* 2001).

Human and animal studies have indicated that metabolites accumulating in various inborn errors of metabolism induce excessive free radical production and reduce the tissue antioxidant defenses (Colome *et al.* 2000; Wajner *et al.* 2004). In this context, oxidative stress has been demonstrated in patients with phenylketonuria (Sirtori *et al.* 2005) and adrenoleukodystrophy (Vargas *et al.* 2004) and leucine and α-ketoisocaproate were shown to stimulate in vitro lipid peroxidation and reduce the cerebral antioxidant reactivity in cerebral homogenates from young rats (Fontella *et al.* 2002; Bridi *et al.* 2003; Bridi *et al.* 2005a).

We have recently verified that plasma from MSUD patients at diagnosis present increased lipid peroxidation and decreased antioxidant reactivity, indicating the involvement of oxidative stress in the pathogenesis of this disease (Barschak *et al.* 2006). In order to extend this investigation and better understand the involvement of oxidative stress in the pathophysiology of MSUD, in the present study we measured TBARS, TAR and TAS in plasma from MSUD patients with high and low levels of leucine and compared to plasma from normal age matched individuals. We also investigated whether alterations of those parameters were correlated with plasma leucine concentrations, as well as, isoleucine and valine.

We demonstrated that TBARS measurement was significantly increased in plasma from treated MSUD patients with both high and low leucine levels. Since TBARS reflects the formation of malondialdehyde, an end product of membrane fatty acid

peroxidation (Halliwell and Gutteridge 2001; Esterbauer and Cheeseman 1990), these data suggest that patients under treatment present increased lipid oxidative damage (lipid peroxidation) independently of plasma Leu levels. Furthermore, we did not find a correlation between plasma concentrations of leucine, valine and isoleucine with the lipid peroxidation parameter TBARS. Taken together these observations, it may be presumed that leucine and the other BCAA are not directly associated to free radical production in MSUD.

We also observed that TAR measurement, which represents the capacity of a tissue to modulate the damage associated with an increased production of free radicals, reflecting the quality of non enzymatic antioxidants (Lissi *et al.* 1995), was significantly decreased in plasma from treated MSUD patients and that this decrease was not dependent on the plasma concentrations of leucine. In addition, TAS (total antioxidant status) measurement, which corresponds to the total quantity of tissue non enzymatic antioxidants, was not altered plasma of MSUD patients presenting low and high leucine levels. It may be therefore presumed that the dietetic treatment (low protein diet) contributed to reduce the tissue antioxidants in these patients since strict protein ingest may secondarily deplete essential substances involved in the antioxidant system, like minerals, vitamins and selenium. We believe that lack of nutrients may be involved in the reduction of antioxidant defenses observed in our present study since patients with low blood leucine levels (more adherent to treatment) would theoretically have greater nutrient deficiency than those with high plasma leucine concentrations and consequently much lower TAR, what was observed in ours patients. These observations, allied to the fact that no correlation was found between leucine, valine and isoleucine with TBARS, TAR and TAS values, indicate that other factors than the BCAA should be investigated to explain our present results.

In summary, taken together the present and previous findings (Barschak *et al.* 2006), it can be concluded that MSUD patients present increased lipid peroxidation and decreased antioxidant defenses at diagnosis and after treatment, which is strongly indicative that oxidative stress may be an underlying mechanism of tissue damage in this disorder. We also observed that the alterations observed were not associated to the plasma levels of leucine, suggesting that other factors, including the BCKA, which are primarily accumulated in these patients, are potentially responsible for the oxidative damage in this disorder. It could be therefore presumed that oxidative stress may contribute at least in part to the chronic progressive neurological damage observed in MSUD patients. Thus, it is desirable that more studies involving other oxidative stress parameters and a larger number of treated patients are carried out in order to better understand the contribution of oxidative stress in MSUD pathophysiology.

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III.3 Capítulo III – Artigo 03

Erythrocyte glutathione peroxidase activity and plasma selenium concentration are reduced in maple syrup urine disease patients during treatment

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Short communication

Erythrocyte glutathione peroxidase activity and plasma selenium concentration are reduced in maple syrup urine disease patients during treatment

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Abstract

Maple syrup urine disease (MSUD) is an inherited disorder caused by a deficiency of the branched-chain α-keto acid dehydrogenase complex activity. In the present study we evaluated selenium levels in plasma from MSUD patients at diagnosis and under treatment and the activities of glutathione peroxidase, catalase and superoxide dismutase in erythrocytes from treated patients. We verified that MSUD patients present a significant selenium deficiency at diagnosis, which becomes more pronounced during treatment, as well as a decrease of erythrocyte glutathione peroxidase activity during treatment. In contrast, erythrocyte catalase and superoxide dismutase activities were not altered in these patients. Our present results suggest that the reduction of an important antioxidant enzyme activity may be partially involved in the pathomechanisms of this disorder and that plasma selenium levels must be corrected through dietary supplementation in MSUD patients.

Keywords: MSUD; Selenium; Glutathione peroxidase; Oxidative stress

Maple syrup urine disease (MSUD) is an inherited disorder caused by a deficiency of the branched-chain α-keto acid dehydrogenase complex (BCKAD) activity. The blockage of this pathway leads to tissue accumulation of the branched-chain amino acids (BCAA) leucine (Leu), isoleucine (Ile) and valine (Val) and their, respective α-keto-acids (Chuang and Shih, 2001; Treacy et al., 1992). MSUD treatment consists of a low protein diet and a semi-synthetic formula restricted in BCAA and supplemented with essential amino acids. This treatment reduces the BCAA plasma concentrations and minimizes the toxic effect of the accumulating metabolites (Chuang and Shih, 2001).

Animal studies have shown that excessive free radical production and reduced tissue antioxidant defenses are induced by the metabolites accumulating in various inborn errors of metabolism (Colome et al., 2000; Fontella et al., 2000; Latini et al., 2002; de Oliveira Marques et al., 2003; Fontella et al., 2002; Fighera et al., 2003; Bridi et al., 2003; Wajner et al., 2004; Bridi et al., 2005). Furthermore, human studies have demonstrated that oxidative stress occurs in patients with phenylketonuria (Sierra et al., 1998; Sirtori et al., 2005) and adrenoleukodystrophy (Vargas et al., 2004). Furthermore, we have recently demonstrated that plasma from MSUD patients at diagnosis present increased lipid peroxidation and decreased antioxidant defenses (Barschak et al., 2006), indicating the involvement of oxidative stress in the pathogenesis of this disease.

In the present study we evaluated selenium (Se) levels in plasma from MSUD patients at diagnosis and under treatment and the activities of glutathione peroxidase (GSH-Px), catalase (CAT) and superoxide dismutase (SOD) in erythrocytes from treated patients.

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Table 1
Age at diagnosis, age at testing, length of treatment and clinical findings of MSUD treated patients

Patients	Age at diagnosis	Age at testing	Length of treatment	Clinical features	
				At presentation	Under treatment
1	1 month	1 year and 7 months	1 year and 6 months	Poor feeding, hypotonia, seizures	Severe psychomotor retardation
2	1 month	2 years and 4 months	2 years and 3 months	Ketoacidosis, hypertonia	Moderate psychomotor retardation
3	16 days	3 years and 9 months	3 years and 9 months	Poor feeding, hypotonia	Severe developmental delay
4	1 month	3 years and 11 months	3 years and 10 months	Poor feeding, hypotonia, coma, hypoglycemia	Moderate psychomotor retardation
5	12 days	7 years and 6 months	7 years and 6 months	Poor feeding, hypotonia, hypoglycemia	Severe mental retardation

Table 2
Plasma concentrations of leucine (Leu), isoleucine (Ile), valine (Val) and alloisoleucine (Allo) in controls and MSUD patients at diagnosis and during treatment

	Leu (μmol/L)	Ile (μmol/L)	Val (μmol/L)	Allo (μmol/L)
Control	158 ± 37.6 (5)	76.5 ± 18.0 (5)	260 ± 39.7 (5)	Nd (5)
MSUD patients at diagnosis	2746 ± 939* (4)	415 ± 192* (4)	710 ± 358* (4)	69.3 ± 42.7 (4)
Treated MSUD patients	485 ± 280 (5)	187 ± 138 (5)	227 ± 159 (5)	44.8 ± 31.1 (5)

Values represent mean ± S.D. (number of cases). *$p < 0.05$, different from controls and treated patients (ANOVA followed by the Duncan multiple range test). Nd: not detected

1. Materials and methods

We obtained blood samples from nine distinct MSUD patients with the classic form of the disease, four at diagnosis and five under treatment. The group of non-treated MSUD patients whose samples were obtained at diagnosis aged between 12 days and 10 months. The most common clinical features of these patients were convulsions, coma, hypoglycemia, poor feeding, ketoacidosis and psychomotor delay. Table 1 displays age of diagnosis, age at testing, length of treatment and clinical profile of MSUD patients under treatment. The treatment consisted of a natural protein restricted diet with low BCAA (Leu: until 12-months-old: 40–80 mg/kg/day, after 1 year of age: 275–535 mg/day; Ile: until 12-months-old: 20–50 mg/kg/day, after 1 year of age: 165–325 mg/day; Val: until 12-months-old: 20–60 mg/kg/day, after 1 year of age: 190–375 mg/day) and supplemented with a semi-synthetic formula of essential amino acids (MSUD 1 or MSUD 2 Milupa®) containing small amounts of vitamins C (0.8–1.6 mg/g), D (0.05–0.21 μg/g) and E (0.18–0.27 mg/g) as well as molibdenium (1.0–2.0 μg/g) and manganese (24 μg/g). It is important to emphasize that this semi-synthetic diet do not contain selenium. Table 2 shows plasma Val, Ile, Leu and alloisoleucine (Allo) concentrations in MSUD patients at diagnosis and under treatment and in normal healthy age matched individuals (controls). Blood amino acids levels were measured by HPLC according Joseph and Marsden (1986), with slight modifications (Wajner et al., 2000). Atomic absorption spectrophotometry was used for plasma selenium determination. Plasma selenium concentrations were determined in controls and MSUD patients at diagnosis and under treatment. The activities of the antioxidant enzymes glutathione peroxidase (GSH-Px), catalase (CAT) and superoxide dismutase (SOD) were determined in erythrocytes from treated MSUD patients. GSH-Px activity was measured by the method of Wendel (1981), CAT activity was assayed by the method of Aebi (1983) and SOD activity was determined using the RANSOD kit (Randox, United Kingdom). For the statistical analysis, Student's *t*-test and one-way ANOVA, followed by the Duncan multiple range test were utilized. Correlations were carried out using the Pearson correlation coefficient. A *p*-value less than 0.05 was considered significant.

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. Results

Table 3 shows plasma selenium values in MSUD patients and controls. It was observed that plasma Se levels in MSUD patients at diagnosis and under treatment were significantly different from controls [$F(2, 11) = 20.751, p < 0.001$]. It can be also seen in the table that plasma Se levels were reduced (around 50%), but not significantly, in treated MSUD patients, as compared to MSUD patients at diagnosis.

Table 3
Plasma selenium levels from MSUD patients and controls

	Selenium (μg/dL)
Control	7.88 ± 1.31 (5)
MSUD patients at diagnosis	4.05 ± 2.26* (4)
Treated MSUD patients	2.14 ± 0.20* (5)

Values represent mean ± S.D. (number of cases). *$p < 0.001$, different from controls (ANOVA followed by the Duncan multiple range test).

Table 4 shows that the activity of GSH-Px was significantly decreased in treated MSUD patients [$t(7) = 3.502$, $p < 0.05$], whereas CAT [$t(8) = -0.353, p > 0.05$] and SOD [$t(7) = 4.782$, $p > 0.05$] activities were not altered, as compared to the controls.

Finally, we found no significant correlation between plasma selenium and Leu [$r = 0.316, p > 0.05$], Ile [$r = 0.108, p > 0.05$] or Val [$r = 0.071, p > 0.05$] levels and between plasma Se levels and erythrocyte GSH-Px activity [$r = 0.223$, $p > 0.05$].

Table 4
Antioxidant enzyme activities in erythrocytes from MSUD patients and controls

	GSH-Px	CAT	SOD
Control	0.64 ± 0.04 (5)	5.10 ± 1.52 (5)	4.35 ± 0.95 (4)
Treated MSUD patients	0.47 ± 0.09* (4)	5.34 ± 0.38 (5)	6.35 ± 3.35 (5)

CAT: catalase (pmol/mg prot); GSH-Px: glutathione peroxidase (mU/mg prot); SOD: superoxide dismutase (U/mg protein). Data represent the mean ± S.D. (number of cases). One U is defined as 1 μmol of NADPH consumed per min for GSH-Px. *$p < 0.05$, different from controls (Student's *t*-test).

3. Discussion

Our objective in the present study was to evaluate the selenium levels in plasma from MSUD patients at diagnosis and under treatment, as well as to verify the activities of glutathione peroxidase (GSH-Px), catalase (CAT) and superoxide dismutase (SOD) in erythrocytes from treated patients, trying to correlate GSH-Px activity with selenium levels. GSH-Px functions as a part of the antioxidant system that protects membranes and essential proteins from the potentially damaging effects of reactive oxygen and lipid peroxides (Lombeck et al., 1996; Schulz et al., 2000). Selenium is an important trace element and diet (predominantly grains, cereals and meat) represents the only source of selenium in humans (Brenneisen et al., 2005). Dietary selenium is important to provide the selenium-containing cofactor for the glutathione peroxidase enzyme (Halliwell and Gutteridge, 2001).

CAT is a ferric heme protein that directly catalyses the decomposition of hydrogen peroxide, whereas GSH-Px presents a similar function as to CAT breaking predominantly lipid peroxides (Halliwell and Gutteridge, 2001). SOD removes the anion superoxide by accelerating the rate of its dismutation to hydrogen peroxide (Halliwell and Gutteridge, 2001).

We verified that MSUD patients present a moderate selenium deficiency at diagnosis and a marked deficiency of this element under treatment. This may possibly be secondary to a protein restricted diet that implies less amount of dietary selenium. In this context, patients presenting inborn errors of amino acid metabolism are potentially at risk for selenium deficiency once their usual treatment consists of a protein restricted diet (Chuang and Shih, 2001).

However, there was no correlation between the plasma branched-chain amino acids (BCAA) leucine, isoleucine and valine with plasma selenium levels, suggesting that the major accumulating metabolites in MSUD are not directly associated to selenium deficiency. These findings do not necessarily rule out a causative association, so that the inclusion of a larger sample size would be important to better evaluate this association.

We also observed in the present study a significant decrease of erythrocyte glutathione peroxidase (GSH-Px) activity in treated MSUD patients, but we did not find a correlation between erythrocyte GSH-Px activity and plasma Se concentrations. Thus, it is presumed that Se deficiency was not responsible for the deficient activity of this enzyme, implying that other factors may be involved in this effect. We also verified that erythrocyte CAT and SOD activities were not altered in treated MSUD patients, indicating that GSH-Px deficient activity was a specific finding in these MSUD patients.

In summary, our data shows that the plasma BCAA and Allo concentrations were decreased in treated MSUD patients, but not to near normal levels indicating that the patients were not strictly adherent to the therapy. Furthermore, plasma Se levels were decreased in non-treated and particularly in patients under treatment. These findings, allied to a poor adherence to the protein restricted diet with low BCAA, may possibly explain why all treated patients presented a variable degree of psychomotor delay/mental retardation. However, it must be emphasized that the overall clinical manifestations of MSUD patients at diagnosis were more severe, presenting mainly hypotonia, poor feeding, hypoglycemia ketoacidosis, convulsions and coma.

Recently, we demonstrated an increased lipid peroxidation and decreased antioxidant reactivity in plasma of MSUD patients at diagnosis (Barschak et al., 2006). Our present results therefore indicate that an important antioxidant activity (GSH-Px) is decreased in MSUD patients under treatment, further indicating that oxidative stress may be considered as one of the pathomechanisms involved in this disorder. Furthermore, since Se levels were markedly deficient in MSUD treated patients, it is proposed that dietary Se supplementation should be incorporated as an adjuvant therapy in MSUD.

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III.4 Capítulo IV – Artigo 04

Maple Syrup Urine Disease in treated patients: Biochemical and oxidative stress profiles

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Maple syrup urine disease in treated patients: Biochemical and oxidative stress profiles

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Abstract

Objective: The objective of this study was to evaluate and correlate the biochemical and oxidative stress profiles in MSUD patients during the dietary treatment.

Design and methods: Plasma samples from treated MSUD patients were used to evaluate the biochemical profile and oxidative stress parameters.

Results: It was observed that glucose, total cholesterol, albumin and creatinine are reduced and that aspartate aminotransferase and lactate dehydrogenase activities are increased in plasma from MSUD patients under treatment. Besides, it was verified an increase of thiobarbituric acid-reactive species (TBARS) and a decrease of total antioxidant reactivity (TAR).

Conclusions: Our results suggest that oxidative stress occurs in treated MSUD patients and that dietary treatment and clinical conditions associated to the disease can lead to biochemical alterations in these patients.

Keywords: Maple syrup urine disease; Biochemical profile; Lipid peroxidation; Antioxidant reactivity

Introduction

Maple syrup urine disease (MSUD) or branched-chain ketoaciduria is an inborn error of metabolism caused by a deficiency of the branched-chain α-keto acid dehydrogenase complex (BCKAD) activity. This blockage leads to accumulation of the branched-chain amino acids (BCAA) leucine, isoleucine, and valine, and their corresponding α-keto acids (α-ketoisocaproic, α-keto-β-methylvaleric and α-ketoisovaleric, respectively). MSUD is an autosomal recessive metabolic disorder with a world frequency estimated in approximately 1 in 185,000 newborns [1].

Based on the clinical presentation and biochemical responses to thiamine administration, MSUD can be divided into five phenotypes: classic, intermediate, intermittent, thiamine-responsive and dihydrolipoyl dehydrogenase (E3)-deficient [1,2]. The classic form has a neonatal onset of encephalopathy and is the most severe and common form. The main clinical signals presented by MSUD patients include ketoacidosis, hypoglycemia, opisthotonos, poor feeding, apnea, ataxia, convulsions, coma, psychomotor delay and mental retardation. Severe brain edema is usually seen in MSUD patients who died during acute metabolic crisis [1,3,4].

The levels of the BCAAs leucine, isoleucine and valine are greatly increased in tissues and biological fluids of the patients, and with the presence of alloisoleucine, they are diagnostic of MSUD. High levels of leucine and α-ketoisocaproic acid seem to be the main important neurotoxic metabolites in MSUD [1,5]. The treatment involves restriction in the protein ingestion and a specific formula of essential amino acids except those accumulated in the DXB, as well as the aggressive intervention during acute metabolic decompensation. The majority of untreated classic patients die

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within the early months of life from recurrent metabolic crisis and neurologic deterioration. The age of diagnosis and the subsequent metabolic control are the most important determinants of prognostic.

Free radicals are molecules or atoms presenting unpaired electrons in their outer orbitals, which make them very unstable and reactive. Free radicals present an enormous capacity to react with diverse integrant molecules of the cellular structure and derived from each one of them [6]. The free radicals can be produced by exogenous (radiation, tobacco and stress, for example) and endogenous sources [7]. In vivo they are produced in all cells as by-products of normal metabolism, and endogenous mechanisms exist to reduce their formation or increase their inactivation [8]. The enzymatic antioxidant defenses involve mainly the superoxide dismutase, catalase and glutathione peroxidase enzymes, whereas the nonenzymatic antioxidant systems involve small molecules as vitamins, glutathione, and uric acid [6].

The imbalance between the formation and the removal of the free radicals in the organism, decurrent of the reduction of the endogenous antioxidants and/or of the increase of the generation of oxidant species generates a prooxidant state defined as oxidative stress [6]. Increasing evidences show that the oxidative stress is involved in a large number of diseases, for example, arterioscleroses, diabetes, neurodegenerative disorders, such as Alzheimer's disease and Parkinson's disease, cancer and aging, among others [8,9]. In this context, studies in animals have showed that the excessive production of free radicals and the decrease of antioxidant defenses are induced by metabolites accumulated in several inborn errors of metabolism [10–15]. Besides, studies in humans showed that oxidative stress occurs in patients with phenylketonuria [16,17] and X-linked adrenoleukodystrophy [18,19]. Recently, it was demonstrated an increase of the lipid peroxidation and a decrease of the antioxidant defenses in MSUD untreated patients [20], indicating that oxidative stress is involved in the pathophysiology of this disease.

Thus, the aim of the present study was to evaluate and correlate the biochemical profile and oxidative stress parameters in plasma from MSUD patients during the dietary treatment.

Material and methods

Patients and controls

Plasma samples obtained from seven treated MSUD patients (classic form) were used to evaluate the biochemical profile and oxidative stress parameters. The treatment consisted of a natural protein restricted diet with low BCAA and supplemented with a semi-synthetic formula of essential amino acids containing small amounts of vitamins and minerals. The average duration of treatment at the test time was 3 years and 5 months. Table 1 displays age of diagnosis, age at testing, length of treatment and clinical profile of MSUD patients under treatment. Plasma levels of leucine, isoleucine, valine and alloisoleucine at the test were 365.9±287.9 μmol/L, 99.0±65.3 μmol/L, 197.7±184.2 μmol/L and 25.6±14.0 μmol/L, respectively. Blood amino acids were determined by HPLC method [21]. Control group was composed of eighth healthy individuals with similar age to the patients (1 to 8 years) (leucine 158.3±37.6 μmol/L, isoleucine 76.5±18.0 μmol/L, valine 260.7±39.7 μmol/L and alloisoleucine were not detected).

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.

Plasma preparation

Plasma was prepared from whole blood samples obtained from fasting individuals (controls and MSUD patients) by venous puncture using vials with EDTA. Whole blood was centrifuged at 1000×*g* and plasma was removed by aspiration and frozen at −80 °C until determinations.

Determination of the biochemical parameters

Glucose

Glycemia measurement was carried out using the method of the glucose oxidase that catalyzes the oxidation of the glucose and hydrogen peroxide formation, which reacts with 4-aminoantipyrine and phenol under the catalytic action of peroxidase, to form a colored complex whose intensity of color is proportional to the glucose concentration in the sample (Glucose PAP kit, Diagnostic Labtest).

Total cholesterol

Total cholesterol was determined in accordance with the method of the cholesterol esterase. The formed free cholesterol is oxidated producing hydrogen peroxide that reacts with 4-aminoantipyrine to form a red complex, whose intensity of color is proportional to the cholesterol concentration in the sample (Cholesterol kit, Diagnostic Labtest).

HDL cholesterol

Cholesterol HDL was measured through cholesterol esterase method for dosage of described total cholesterol above, after the precipitation of lipoproteins of low and very low density with phosphotungstic acid and magnesium chloride (Cholesterol HDL kit, Diagnostic Labtest).

LDL cholesterol

Cholesterol LDL was determined through calculation, using the Friedewald formula (cholesterol LDL = total cholesterol − (cholesterol HDL + triglycerides / 5)) for values of triglycerides until 400 mg/dL.

Triglycerides

Triglycerides had been measured in accordance with the method of lipoprotein lipase, where glycerol set free is converted to glycerol-3-phosphate by the action of glycerolkinase, and it is oxidated to form hydrogen peroxide. This reacts with the 4-aminoantipyrine producing a violet complex whose intensity of color is proportional to the concentration of

Table 1
Age at diagnosis, age at testing, length of treatment and clinical findings of MSUD patients

Patients	Age at diagnosis	Age at testing	Length of treatment	Clinical Features	
				At presentation	Under treatment
1	1 month	1 year and 7 months	1 year and 6 months	Poor feeding, hypotonia, seizures	Severe psychomotor retardation
2	1 month	2 years and 4 months	2 years and 3 months	Ketoacidosis, hypertonia	Moderate psychomotor retardation
3	16 days	3 years and 9 months	3 years and 9 months	Poor feeding, hypotonia	Severe developmental delay
4	1 month	3 years and 11 months	3 years and 10 months	Poor feeding, hypotonia, coma, hypoglycemia	Moderate psychomotor retardation
5	12 days	7 years and 6 months	7 years and 6 months	Poor feeding, hypotonia, hypoglycemia	Severe mental retardation
6	4 months	1 year and 7 months	1 year and 3 months	Hypotonia, seizures	Severe psychomotor retardation
7	2 months	1 year and 7 months	1 year and 5 months	Poor feeding, hypotonia, seizures	Moderate developmental delay

triglycerides in the sample (Triglycerides GPO-ANA kit, Diagnostic Labtest).

Transaminases

Transaminases (alanine aminotransferase — ALT and aspartate aminotransferase — AST) had been determined in accordance with the kinetic method, which is based on the transference of the amine group of amino acids L-alanine (ALT) or L-aspartate (AST) for the alpha-ketoglutarato producing its corresponding α-keto acid, which reacts with a secondary enzyme (lactate dehydrogenase or malate dehydrogenase) producing one hydrazone in alkaline way, which is proportional to the activity of the ALT or AST, respectively in the sample (AST and ALT kit, Diagnostic Labtest).

Creatine kinase

Creatine kinase (CK) was determined in accordance with the kinetic method. Creatine phosphate was dephosphorylated leading to the formation of adenosine triphosphate (ATP), which reacts with glucose producing glucose-6-phosphate that was oxidated to 6-phosphogluconate reducing NAD to NADH. This reduction is proportional to CK activity in the sample (CK-NAC kit, Diagnostic Labtest).

Lactate dehydrogenase

Lactate dehydrogenase (LDH) was measured through colorimetric method. LDH catalyzes the reversible reaction of lactate-pyruvate in the presence of NAD, which is reduced to NADH. NADH reduces *p*-iodonitrotetrazolium leading to the formation of a colored product proportional to LDH activity in the sample (Lactate dehydrogenase kit, Diagnostic Labtest).

Albumin

Albumin was measured in accordance with the method of the bromo cresol green. The albumin interacts with the buffered bromocresol green, and due to proteinic error of pointers, green color formation occurs, which is proportional to the albumin concentration in the sample (Albumin Kit, Diagnostic Labtest).

Creatinine

Creatinine was measured by the method of the picrate, which reacts with the creatinine in the sample, after addition of acid reagent for elimination of pseudo-creatinines, producing a red color complex, which intensity is proportional to the concentration of creatinine in the sample (Creatinine K Kit, Diagnostic Labtest).

Urea

Urea determination was carried out by the method of urease, in which the urea in the sample is hydrolyzed to form ammonium, which reacts with salicylate and sodium hypochlorite, in alkaline pH and using nitroprusside as catalytic agent, to form blue of indofenol. The intensity of the formed color is proportional to the amount of urea in the sample (Urea CE kit, Diagnostic Labtest).

Uric acid

Uric acid was determined by the method of uricase, in which it was oxidated to alantoine and hydrogen peroxide, which reacts with 4-aminoantipyrine and dichloro hydroxy benzene sulfonate (DHBS) in the presence of peroxidase producing a red complex, whose intensity of color is proportional to the concentration of uric acid in the sample (Uric acid kit, Diagnostic Labtest).

Determination of oxidative stress parameters

Thiobarbituric acid-reactive species (TBARS)

Thiobarbituric acid-reactive species (TBARS) were determined according to the method described by Buege and Aust [22]. Briefly, 250 μL of 10% trichloroacetic acid was added to 125 μL of plasma, then 375 μL 0.67% thiobarbituric acid (in 7.1% sodium sulfate) was added and incubated at 100 °C for 30 min. After the incubation, the mixture was extracted with 750 μL butanol. The resulting pink stained TBARS were determined in a spectrofluorimeter at 515 nm. Calibration curve was performed using 1,1,3,3-tetramethoxypropane subjected to the same treatment as that of the samples. TBARS were calculated as nmol TBARS/mg protein.

Total antioxidant reactivity (TAR)

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. [23]. The background chemiluminescence was measured by adding 4 mL of 2 mM ABAP (in 0.1 M glycine buffer, pH 8.6) into a glass scintillation vial. Ten microliters of luminol (4 mM) was added to each vial and the chemiluminescence was measured. This was considered

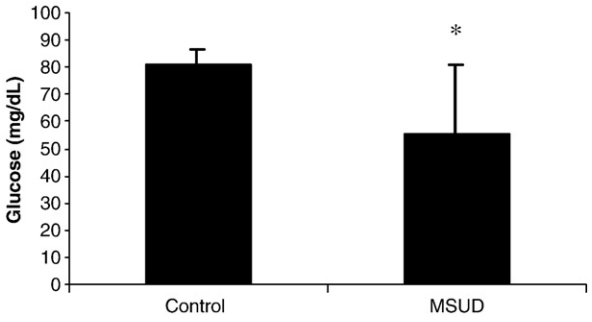

Fig. 1. Plasma glucose from MSUD patients and controls. Data represent the mean±SD (n=6–7). *p<0.05 (Student's t test for unpaired samples) compared to control.

to be the basal value. Ten microliters of 25–200 μM Trolox (curve calibration) or plasma was then added and the chemiluminescence was measured during 60 s. The Trolox and plasma addition reduces the chemiluminescence. The rapid reduction in luminol intensity is considered as a measure of the TAR capacity. TAR measurement was calculated as nmol Trolox/mg protein.

Total proteins

Protein concentrations were determined by the Biuret method (Total protein kit, Diagnostic Labtest), using albumin as standard.

Statistical analysis

The Student's t test for unpaired samples was used to compare means between controls and MSUD patients. Correlations were carried out using the Pearson correlation coefficient. Only significant values are shown in the text. A p value less 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

Fig. 1 shows glycemia measurement in controls and MSUD patients under dietary treatment. It can be observed that MSUD patients presented a significant reduction in the plasma levels of glucose [$t(11)=2.508$, $p<0.05$] as compared to the controls. The glucose levels presented by these patients are below reference values (70–105 mg/dL).

Fig. 2 shows the lipidic profile in controls and treated MSUD patients. It can be seen that the concentrations of total cholesterol (A) [$t(13)=2.894$, $p<0.05$] were significantly reduced in MSUD patients as compared to the control group. However, both groups presented values in the reference range (112–205 mg/dL). The plasma levels of HDL cholesterol (B) [$t(12)=0.591$, $p>0.05$], LDL cholesterol (C) [$t(11)=0.140$, $p>0.05$] and triglycerides (D) [$t(13)=0.988$, $p>0.05$] in the MSUD patients

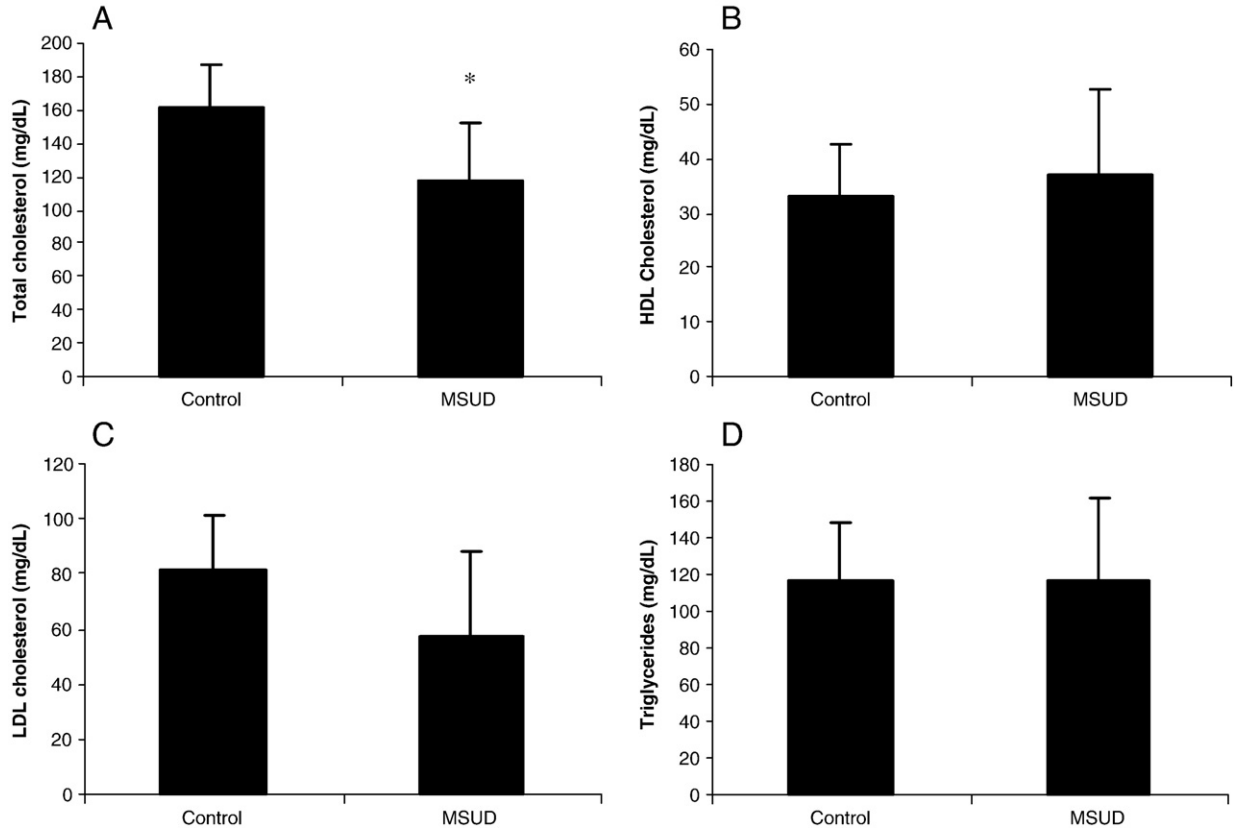

Fig. 2. Lipidic profile in plasma from MSUD patients and controls [total cholesterol (A), HDL cholesterol (B), LDL cholesterol (C) and triglycerides (D)]. Data represent the mean±SD (n=7–8). *p<0.05 (Student's t test for unpaired samples) compared to control.

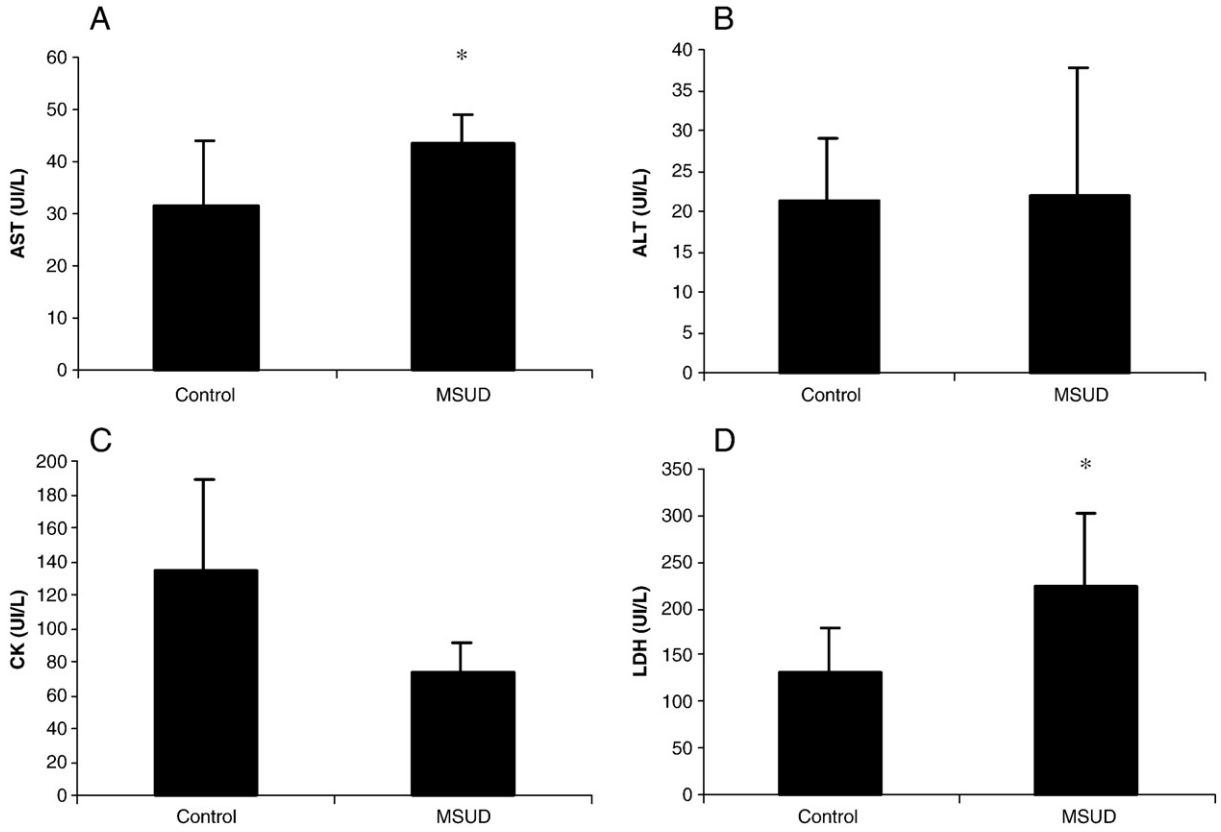

Fig. 3. Plasma transaminases [aspartate aminotransferase (AST) (A) and alanine aminotransferase (ALT) (B)], creatine kinase (CK) (C) and lactate dehydrogenase (D) from MSUD patients and controls. Data represent the mean±SD (n=5–7). *p<0.05 (Student's t test for unpaired samples) compared to control.

were not significantly different when compared to the control group.

Fig. 3 shows enzymatic activities of AST, ALT, CK and LDH in plasma of MSUD patients. These patients presented an increase of AST activity (A) [$t(12)=-2.281$, $p<0.05$] when compared to the control group, lightly increased in relation to the reference range (10–40 UI/L). The levels of ALT (B) had not been significantly different from the controls [$t(12)=-0.107$, $p>0.05$]. We can also observe that LDH activity (D) was increased in plasma of MSUD patients [$t(10)=-2.364$, $p<0.05$] when compared to the control group, both groups presented values in the reference range (110–295 UI/L). However, CK activity (C) was not altered in these patients [$t(9)=2.470$, $p>0.05$].

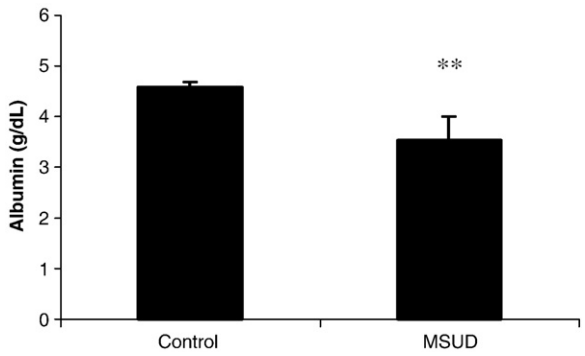

Fig. 4. Plasma albumin from MSUD patients and controls. Data represent the mean±SD (n=5–7). **p<0.01 (Student's t test for unpaired samples) compared to control.

It can be observed in Fig. 4 that MSUD patients presented a reduction in the plasma levels of albumin [$t(10)=5.685$, $p<0.01$] when compared to the control group, these levels being below reference range (3.8–5.4 g/dL).

The concentrations of creatinine, urea and uric acid in the plasma of controls and MSUD patients were determined. As it can be observed, in Fig. 5, MSUD patients presented a significant reduction in plasma levels of creatinine (A) [$t(12)=4.076$, $p<0.01$] relatively to the control group. The plasma levels of creatinine in these patients were below the reference values (0.3–0.7 mg/dL). We did not observe alterations in the urea (B) [$t(12)=-1.813$, $p>0.05$] and uric acid levels (C) [$t(10)=-0.391$, $p>0.05$] when compared to the control group.

Fig. 6 shows the measure of thiobarbituric acid-reactive species (TBARS) and total antioxidant reactivity (TAR) in plasma from controls and MSUD patients. It was observed a significant increase of TBARS (A) [$t(11)=-3.112$, $p<0.05$], while TAR was markedly reduced (B) [$t(9)=2.273$, $p<0.05$], when compared to the controls.

A significant positive correlation was observed between the plasma levels of triglycerides and TBARS measurement [$r=0.838$, $p<0.05$]. Correlation between the other biochemical parameters and the oxidative stress parameters was not observed.

Discussion

The deficiency in the activity of the branched-chain α-keto acid dehydrogenase complex is clinically characterized by ketoacidosis, convulsions, coma, psychomotor delay and mental retardation. The mechanisms of the neurological symptoms presented by MSUD patients are still poorly understood. However, considering that high concentrations of leucine and/or α-ketoisocaproate were associated with the appearance of the neurological symptoms, these compounds seem to be the main neurotoxic metabolites in the illness [1,5]. Animal studies have shown that excessive free radical production and reduced tissue antioxidant defenses are induced by the metabolites accumulating in MSUD [13,14]. Recently we have demonstrated that oxidative stress is induced in MSUD patients at diagnosis [20].

In the present study we evaluated and correlated the biochemical and oxidative profiles in the plasma of MSUD patients under dietary treatment. To our knowledge this is the first report demonstrating the biochemical and oxidative profiles of treated MSUD patients.

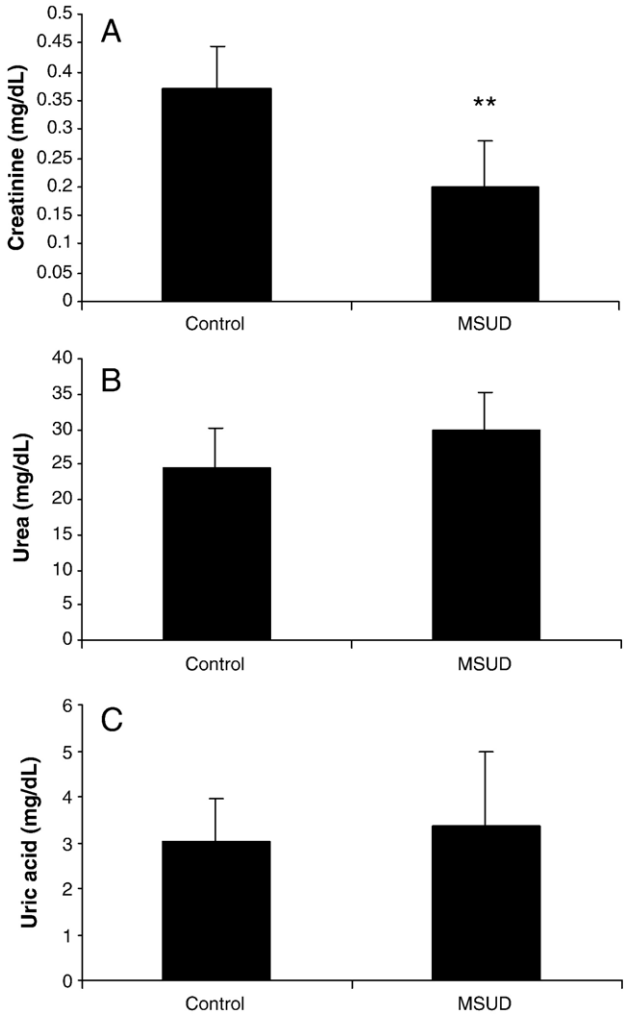

Fig. 5. Plasma creatinine (A), urea (B) and uric acid (C) from MSUD patients and controls. Data represent the mean ± SD (n = 5–7). **p < 0.01 (Student's t test for unpaired samples) compared to control.

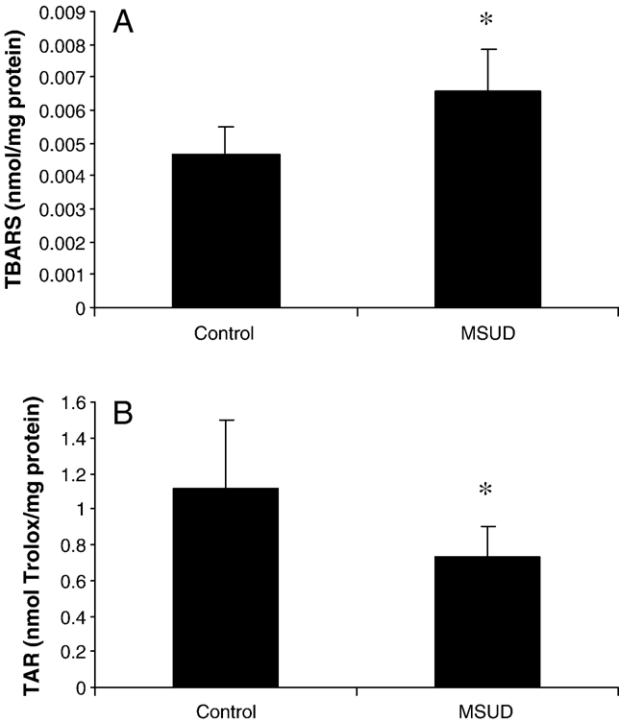

Fig. 6. Plasma thiobarbituric acid-reactive species (TBARS) (A) and total antioxidant reactivity (TAR) (B) from MSUD patients and controls. Data represent the mean ± SD (n = 5–7). *p < 0.05 (Student's t test for unpaired samples) compared to control.

Our results showed that glucose levels are reduced in MSUD patients. The hypoglycemia is a common finding in these patients, and this alteration is possibly associated with an increased secretion of insulin stimulated by high concentrations of leucine presented by these patients [1].

The evaluation of the lipidic profile showed a significant reduction in the total cholesterol levels in MSUD patients in comparison to the control group. Once the patients are submitted to a low protein diet of animal origin and consequently poor in saturated fat, it is possible that this reduction could be secondary to dietary treatment. However, HDL cholesterol, LDL cholesterol and triglycerides were not different from controls.

It was also observed an increase in the AST activity in MSUD patients, whereas ALT activity was not modified. Hepatic illnesses in general provoke concomitant increase of ALT and AST levels, being ALT the most specific enzyme of the liver. Increases in the serum ALT activity are rarely observed in conditions other than hepatic illness [24]. This suggests that the isolated increase of AST observed in the studied patients could be related to other causes. In addition, we verified that LDH activity was increased in MSUD which could be associated to muscular damage [24]. MSUD patients present clinical symptoms as lethargy, hypotonia, hypertonia and convulsions [1]. Studies have demonstrated association of muscular damages (dystrophies and polymyosite) with increased levels of the enzymatic activities of AST, CK and LDH [24–26]. Our results indicate that the increased activities of AST and LDH could be

associated to the characteristic muscular alterations presented by MSUD patients. However, total CK activity was not altered in MSUD suggesting that additional studies evaluating the activity of CK-MM (muscular isoenzyme) could contribute in better understanding these findings.

It was also evaluated the plasma concentrations of creatinine, urea and uric acid. The MSUD patients presented a significant reduction in plasma creatinine levels, but urea and uric acid concentrations were not altered. The low concentration of creatinine could be associated to muscular alterations presented by patients in the course of the illness. It is important to emphasize that creatinine is formed through free creatine in the muscle, so the produced amount of endogenous creatinine is proportional to muscular mass [24].

MSUD treatment consists of a restricted ingestion of proteins, supplemented with a semi-synthetic formula of essential amino acids except leucine, isoleucine and valine. The aim of the treatment is to normalize the plasma levels of the BCAA, minimizing the neurological damage associated mainly to increase in leucine levels [1]. It was observed that MSUD patients presented a reduction in plasma albumin levels, which probably reflects deficiencies associated to low protein diet that these patients are submitted to.

It was observed a significant increase of TBARS in plasma from MSUD patients. Considering that TBARS reflects the amount of malondialdehyde, an end product of lipid peroxidation [6,27], our data indicate that lipid peroxidation is stimulated in MSUD patients, probably secondary to free radical generation. Already, TAR measurement, which reflects the quality of antioxidant substances [28], was markedly reduced in MSUD patients, suggesting a deficient capacity of plasma to modulate the damage associated with the increased production of reactive species. The increase of lipid peroxidation and the reduction of the antioxidant defenses suggest that oxidative stress occurs in treated MSUD and can explain at least in part the pathophysiology of this illness. It was previously verified an increase in TBARS and a decrease in TAR in MSUD patients at diagnosis [20].

A positive correlation was observed between plasma triglycerides levels and TBARS measurement. Triglycerides are composed by the union of three fat acids and glycerol. Fat acids are easily oxidated by free radicals, leading to lipid peroxidation. Considering that malondialdehyde (MDA) is an end product of lipid breakdown due to lipid peroxidation, and TBARS reflects the content of MDA (27), it is possible that lipid peroxidation could be related to triglycerides amount.

In conclusion, our results show that glucose, total cholesterol, albumin and creatinine are reduced and that AST and LDH activities are increased in plasma from MSUD patients under dietary treatment. It is probable that these findings are secondary to low protein diet and/or to the characteristic clinical manifestations of the illness. These results suggest that serum biochemical profile should be evaluated in all MSUD patients during treatment. However, our results must be taken with caution since our experiments were conducted with samples collected at a single time point during treatment. Therefore, these samples might not represent patients' metabolic control as a whole. The increased TBARS and the reduction of the TAR are in accordance with our previous studies, suggesting that oxidative stress occurs also in MSUD patients under treatment. In this context, it is possible that dietary antioxidant supplementation could be important as an adjuvant therapy in MSUD.

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IV. DISCUSSÃO

Os mecanismos responsáveis pelos sintomas neurológicos apresentados pelos pacientes com DXB ainda são pouco conhecidos. Nesse sentido, muitos estudos vêm sendo desenvolvidos no intuito de melhor entender a fisiopatologia da DXB. Existem muitas evidências associando a deficiência no metabolismo da leucina e os sintomas neurológicos nesses pacientes. Estudos mostraram que a administração crônica de leucina a ratos jovens induz a déficit no aprendizado/memória na idade adulta, indicando que os altos níveis de leucina podem contribuir para a deterioração neurológica observada na doença (Mello et al., 1999). Foi também demonstrado que o ácido α-cetoisovalérico apresenta propriedades convulsivantes, sugerindo que este metabólito esteja envolvido na gênese das convulsões características da DXB (Coitinho et al., 2001). Ainda, altas concentrações dos AACR e dos CACR causam déficit no metabolismo energético através da inibição da cadeia transportadora de elétrons (Sgaravatti et al., 2003) e da enzima creatina quinase (Pilla et al., 2003), sugerindo que o déficit energético possa estar envolvido nos sintomas neurológicos apresentados na doença. Outros autores demonstraram que os AACR e/ou CACR acumulados na DXB provocam apoptose neuronal (Jouvet et al., 2000), redução na síntese e função de neurotransmissores (Zielke et al., 1997; Tavares et al., 2000), alterações na mielina (Schönberger, 2004; Treacy et al., 1992; Tribble e Shapira, 1983; Taketomi et al., 1983) e redução na captação de aminoácidos essenciais pelo cérebro (Araújo et al., 2001).

Nos últimos anos tem crescido o número de evidências associando estresse oxidativo e erros inatos do metabolismo com manifestação neurológica, sugerindo que o dano oxidativo possa contribuir para a disfunção neurológica nessas doenças (Colomé et al., 2000; Wajner et al., 2004). Estudos in vivo e in vitro mostraram que os ácidos metilmalônico e propiônico, principais metabólitos acumulados nas acidemias metilmalônica e propiônica, respectivamente, estimulam a lipoperoxidação e reduzem as defesas antioxidantes em cérebro de ratos (Figuera et al., 2003; Fontella et al., 2000). Além disso, estudos têm demonstrado que o ácido 3-hidroxiglutárico estimula o estresse oxidativo em cérebro de ratos (Latini et al., 2002; Latini et al., 2005). Estudos em humanos mostraram que as defesas antioxidantes estão diminuídas no plasma de pacientes com acidemias propiônica e metilmalônica (Moyano et al., 1997). Ainda, estudos recentes têm demonstrado que o estresse oxidativo está induzido em pacientes com fenilcetonúria (Artuch et al., 2004). Foi verificado que a lipoperoxidação está induzida e as defesas antioxidantes não–enzimáticas estão diminuídas no plasma, bem como a atividade da enzima glutationa peroxidase está diminuída em eritrócitos de pacientes fenilcetonúricos ao diagnóstico e durante o tratamento dietético (Sirtori et al., 2005; Sitta et al., 2006). Ainda, estes autores verificaram que os níveis plasmáticos de fenilalanina não se correlacionam com o estresse oxidativo.

Vários estudos em animais têm demonstrado o envolvimento do estresse oxidativo na DXB. Fontella e colaboradores (2002) mostraram que os aminoácidos, α-cetoácidos e α-hidroxiácidos acumulados na DXB estimulam a lipoperoxidação em cérebro de ratos jovens. Corroborando com esses dados,

Bridi e colaboradores (2003; 2005a; 2005b) verificaram que os AACR e os CACR estimulam a lipoperoxidação e reduzem as defesas antioxidantes, e, ainda, que o ácido α-cetoisocapróico inibe a atividade da enzima glutationa peroxidase no córtex cerebral de ratos. No entanto, não encontramos na literatura relatos de estudos sobre estresse oxidativo em pacientes com DXB.

Dessa forma, o presente trabalho teve por objetivo investigar vários parâmetros de estresse oxidativo em pacientes com DXB antes e durante o tratamento dietético a fim de melhor entender a fisiopatologia da DXB, bem como o efeito do tratamento atualmente preconizado para estes pacientes. Esse projeto de pesquisa foi aprovado pelo Comitê de Ética em Pesquisa do Hospital de Clínicas de Porto Alegre, cujo parecer encontra-se em anexo (anexo 3).

Inicialmente, foram analisadas amostras de plasma de pacientes com DXB obtidas antes da instituição do tratamento (no diagnóstico). Foi demonstrado um aumento significativo do TBARS nos pacientes com DXB quando comparado ao grupo controle. Uma vez que o TBARS reflete a quantidade de malondialdeído formado, um produto final da peroxidação de lipídios de membrana (Halliwell e Gutteridge, 2007), este resultado sugere que a lipoperoxidação está induzida em plasma de pacientes com DXB, provavelmente devido ao aumento da formação de radicais livres. Apesar de não ter sido observado redução no conteúdo total de antioxidantes no plasma dos pacientes, como indicado pelos valores de TAS, foi observado uma diminuição significativa nos níveis de TAR. O TAR corresponde a um índice da capacidade de um determinado tecido em modular o dano associado à produção aumentada de radicais livres e reflete não só a quantidade dos

antioxidantes, mas principalmente a qualidade desses antioxidantes (demonstrada por sua reatividade) (Lissi et al., 1995).

Estudos em animais mostraram que os aminoácidos leucina, isoleucina e valina acumulados na DXB reduzem as defesas antioxidantes e estimulam a lipoperoxidação em córtex cerebral de ratos (Bridi et al., 2003). Ainda, foi demonstrado que os α-cetoácidos e os α-hidroxiácidos acumulados na DXB estimulam a lipoperoxidação em cérebro de ratos (Fontella et al., 2002; Bridi et al., 2005a).

Assim, os resultados observados nos pacientes com DXB antes do tratamento (aumento da lipoperoxidação e a diminuição da capacidade de lidar com os radicais livres) sugerem que existe um aumento na produção de radicais livres e uma diminuição da reatividade antioxidante, gerando o processo conhecido com o estresse oxidativo.

O tratamento preconizado para a DXB consiste em uma dieta pobre em proteínas, com baixo conteúdo de AACR e suplementada com uma fórmula semi-sintética contendo aminoácidos essenciais (exceto os AACR), vitaminas e minerais. Esse tratamento diminui o acúmulo de metabólitos tóxicos, principalmente do aminoácido leucina e seu respectivo α-cetoácido (ácido α-cetoisocapróico), minimizando seus efeitos deletérios ao SNC. O objetivo do tratamento é manter os níveis plasmáticos de leucina o mais próximo possível dos valores de referência, que se encontram entre 77 e 153 μmol/L (Lepage et al., 1997) ou, preferencialmente, entre 100 e 300 μmol/L que são limites aceitáveis no sentido de evitar danos (Morton et al., 2002). No entanto, a disfunção neurológica pode ser observada em muitos pacientes, uma vez que o desenvolvimento mental está relacionado à idade de início da terapia (que deve

ser o mais precoce possível), à duração dos períodos de descompensação metabólica e, ainda, ao estrito cumprimento da dieta a longo prazo (Chuang e Shih, 2001; Morton et al., 2002).

Considerando os resultados obtidos inicialmente, indicando o envolvimento do estresse oxidativo na DXB, buscamos estender essas investigações para melhor entender a participação do estresse oxidativo na DXB. Determinamos os mesmos parâmetros de estresse oxidativo (TBARS, TAR e TAS) em pacientes com DXB em tratamento dietético. Os pacientes tratados foram divididos em dois grupos, um grupo com níveis de leucina muito elevados e outro com níveis de leucina baixos para verificar o efeito da terapia sobre o estresse oxidativo e também se existe correlação entre o estresse oxidativo nesta doença e os níveis de leucina, isoleucina e valina, os aminoácidos que se encontram acumulados nestes pacientes.

A medida do TBARS mostrou-se igualmente aumentada nos dois grupos de pacientes com DXB tratados, indicando que a terapia dietética não altera a lipoperoxidação induzida na doença. Além disso, não foi observada correlação entre os níveis plasmáticos de leucina, isoleucina ou valina e a medida de TBARS. Foi observada ainda uma diminuição significativa na medida do TAR em ambos os grupos de pacientes tratados comparativamente aos controles. No entanto, os pacientes com baixos níveis de leucina apresentaram níveis de TAR mais baixos que do grupo de pacientes com níveis elevados de leucina. Além disso, não foi verificada associação entre essa medida antioxidante e os níveis séricos de leucina, isoleucina ou valina. Por outro lado, não observamos alteração da medida de TAS nos dois grupos de pacientes tratados, o que

sugere que o conteúdo de antioxidantes não esteja alterado nos pacientes sob tratamento, mas apenas a reatividade antioxidante.

O organismo faz uso de uma série de compostos para neutralizar os radicais livres formados durante o metabolismo aeróbico normal. Esses compostos em conjunto formam o sistema antioxidante que inclui enzimas, vitaminas e oligoelementos. Para que o sistema antioxidante seja eficiente e evite o dano oxidativo, é necessário que esses elementos atuem de forma sinérgica neutralizando os radicais livres formados. A deficiência em qualquer parte do sistema antioxidante pode levar ao desequilíbrio gerando estresse oxidativo (Davies, 1995).

Considerando que muitas substâncias antioxidantes são provenientes da dieta, é possível que a restrição dietética a qual os pacientes com DXB são submetidos possa causar a deficiência de vitaminas e micronutrientes envolvidos nas defesas antioxidantes, como observado na fenilcetonúria (Acosta, 1996; Artuch et al., 2004; Sitta et al., 2006). Dessa forma, é possível que o grupo de pacientes com baixos níveis de leucina, o qual parece ser mais aderente ao tratamento, apresente uma maior deficiência nutricional que aquele grupo com altos níveis de leucina e, como conseqüência, tenha atividade antioxidante (TAR) menor. Nos pacientes estudados verificou-se diminuição mais acentuada da reatividade antioxidante no grupo com baixos níveis de leucina.

O selênio é um importante micronutriente, essencial para a atividade da enzima glutationa peroxidase e a dieta (principalmente grãos, cereais e carnes) representa a única fonte de selênio para o ser humano (Brenneisen et al., 2005). Sendo assim, em continuidade a nossa investigação medimos as

concentrações de selênio no plasma de pacientes com DXB antes e durante o tratamento dietético e também avaliamos as atividades das enzimas antioxidantes glutationa peroxidase (GSH-Px), catalase (CAT) e superóxido dismutase (SOD) em eritrócitos de pacientes com DXB em tratamento.

Verificamos que os pacientes com DXB apresentam uma deficiência moderada de selênio no momento do diagnóstico da doença, ou seja, antes do tratamento, e que essa deficiência se agrava com o tratamento dietético. Estudos com pacientes fenilcetonúricos em tratamento dietético têm demonstrado que a deficiência de selênio ocorre na fenilcetonúria (Lombeck et al., 1996). Como a dieta representa a única fonte de selênio nos seres humanos, é provável que a deficiência desse micronutriente possa ser secundária à restrição protéica e que essa deficiência possa contribuir para a ocorrência de estresse oxidativo nesse pacientes. Verificamos também que não foi observada correlação entre os níveis plasmáticos dos AACR leucina, isoleucina e valina e a concentração plasmática de selênio, sugerindo que os principais metabólitos acumulados na DXB não estão diretamente associados à deficiência de selênio.

Paralelamente, mostramos que a enzima GSH-Px apresentou atividade significativamente diminuída nos eritrócitos de pacientes com DXB em tratamento, porém não foi observada correlação entre a atividade GSH-Px em eritrócitos e os níveis de selênio no plasma. As atividades das enzimas CAT e SOD em eritrócitos não estavam alteradas em pacientes com DXB em tratamento. De forma semelhante foi observado em pacientes fenilcetonúricos ao diagnóstico e durante o tratamento uma diminuição significativa na atividade da GSH-Px em eritrócitos (Sirtori et al., 2005; Sitta et al., 2006).

A enzima GSH-Px funciona como parte de um sistema antioxidante que protege membranas e proteínas essenciais dos efeitos potencialmente danosos das espécies reativas e de lipoperóxidos (Schulz et al., 2000). Nos eritrócitos, a ação desse sistema é particularmente importante, já que essas células são altamente suscetíveis ao estresse oxidativo por suas membranas serem ricas em ácidos graxos poliinsaturados e pelo seu conteúdo ser rico em ferro e oxigênio. O selênio está presente no sítio ativo da GSH-Px e desempenha um papel importante na atividade dessa enzima. Estudos mostraram que somente existe uma boa correlação entre os níveis de selênio e a atividade da GSH-Px em sangue total quando as concentrações de selênio são muito baixas (Halliwell e Gutteridge, 2007). Dessa forma, apesar de não ter sido observada correlação entre os níveis de selênio e a atividade da GSH-Px, é provável que além do selênio outros fatores estejam associados à deficiência na atividade dessa enzima.

Não foi observado nos pacientes com DXB em tratamento, por nós estudados, correlação entre os parâmetros de estresse oxidativo (TBARS e TAR) e as concentrações plasmáticas dos AACR leucina, isoleucina e valina, o que sugere que esses aminoácidos não estejam diretamente envolvidos no dano oxidativo observado nos pacientes DXB.

Os pacientes com DXB apresentam sintomas graves incluindo cetoacidose, hipoglicemia, opistótono, dificuldade de alimentação, apnéia, ataxia, hipotonia/hipertonia, convulsões, encefalopatia e coma (Chuang e Shih, 2001; Schönberger et al., 2004). A instituição do tratamento permite uma melhora significativa, atenuando as manifestações clínicas. No entanto, o bom

controle metabólico a longo prazo é essencial para o desenvolvimento neuropsicomotor dos pacientes (Hoffmann et al., 2006; Simon et al., 2006b).

Não existem relatos na literatura mostrando o perfil bioquímico em pacientes com DXB e sua correlação com parâmetros de estresse oxidativo. Assim, estudamos o perfil bioquímico em pacientes com DXB sob tratamento dietético. Avaliamos os níveis plasmáticos de glicose, colesterol total, colesterol HDL, colesterol LDL, triglicerídeos, alanina aminotransferase, aspartato aminotransferase, creatina quinase, lactato desidrogenase, albumina, creatinina, uréia e ácido úrico, objetivando verificar a existência de uma possível correlação entre essas medidas e os parâmetros de estresse oxidativo (TBARS e TAR).

Nossos resultados mostraram que os níveis glicêmicos estão diminuídos nos pacientes com DXB. A hipoglicemia é um achado comum nestes pacientes, e essa alteração está possivelmente associada a um aumento na secreção de insulina estimulada pela alta concentração de leucina apresentada pelos pacientes (Chuang e Shih, 2001). Ainda, é possível que a diminuição da glicemia destes pacientes possa ser explicada por uma baixa aderência ao tratamento, uma vez que eles apresentam níveis plasmáticos de leucina aumentados.

Na avaliação do perfil lipídico foi possível observar uma redução significativa na concentração de colesterol total, porém não verificamos alteração nos níveis plasmáticos de triglicerídeos e de colesterol HDL e LDL. Os pacientes estudados foram submetidos a uma dieta pobre em proteínas de origem animal e, consequentemente, pobre em gorduras. Dessa forma é

possível que a redução de colesterol observada seja secundária ao tratamento dietético.

Foi verificado, ainda, um aumento na atividade enzimática da aspartato aminotransferase (AST) nos pacientes com DXB em tratamento, sem alteração na atividade da alanina aminotransferase (ALT). As doenças hepáticas de uma forma geral promovem o aumento concomitante dos níveis de AST e ALT. A ALT é considerada a enzima mais específica do tecido hepático e raramente são observadas elevações na sua atividade que não tenham como causa doenças hepáticas. Isso nos permite sugerir que o aumento isolado da AST apresentado pelos pacientes estudados possa estar relacionado a outras causas (Burtis e Ashwood, 1998). Também, verificamos que a atividade da lactato desidrogenase (LDH) está aumentada nos pacientes DXB sob tratamento. Os pacientes com DXB apresentam sinais clínicos como letargia, hipotonia/hipertonia e convulsões (Chuang e Shih, 2001). Estudos têm demonstrado a associação de dano muscular (doenças distróficas e polimiosite) com aumento na atividade da AST, da creatina quinase (CK) e da lactato desidrogenase (LDH) (Lott e Landesman, 1984; Burtis e Ashwood, 1998; Nathwani et al., 2005). Assim, pode-se supor que o aumento na atividade da AST e da LDH nos pacientes por nós estudados poderia estar relacionado às alterações musculares características da DXB. Entretanto, não foi verificada alteração na atividade da creatina quinase total (CK) no plasma desses pacientes, sugerindo que estudos adicionais avaliando a atividade da isoenzima muscular da CK (CK-MM) poderiam contribuir para o esclarecimento desses achados.

Foram também avaliadas as concentrações plasmáticas de creatinina, uréia e ácido úrico. Os pacientes DXB apresentaram uma redução nos níveis de creatinina sérica, porém os níveis de uréia e ácido úrico não estavam alterados, sugerindo que a função renal está normal nesses pacientes. A diminuição da concentração sérica de creatinina, por sua vez, pode também estar associada às alterações musculares manifestadas pelos pacientes no curso da doença, assim como as atividades alteradas das enzimas AST e LDH. A creatinina endógena é formada através da creatina livre no músculo, ou seja, a creatinina produzida é proporcional à massa muscular (Burtis e Ashwood, 1998).

Foi demonstrado ainda que os níveis plasmáticos de albumina estão diminuídos nos pacientes com DXB. Essa redução poderia estar relacionada a dieta hipoproteica a que eles estão submetidos. Na ausência de doença hepática, a diminuição de concentração de albumina plasmática indica uma disponibilidade insuficiente de aminoácidos para a síntese protéica endógena (Marks et al., 1996).

Uma correlação positiva entre a medida de TBARS e os níveis plasmáticos de triglicerídeos, foi observada nos pacientes DXB sob tratamento dietético. Entretanto, não foi observada correlação entre os demais parâmetros bioquímicos e os parâmetros de estresse oxidativo. Os triglicerídeos são compostos formados pela união de três ácidos graxos com glicerol. Os ácidos graxos, principalmente os poliinsaturados, são facilmente oxidados por radicais livres, levando a peroxidação lipídica. Entre os produtos finais da lipoperoxidação está o malondialdeído (MDA), que é determinado através da medida do TBARS. Sendo assim, o TBARS reflete a quantidade de ácidos

graxos oxidados por radicais livres, podendo ser influenciado pela concentração de triglicerídeos.

Enfim, nossos resultados mostrando aumento da lipoperoxidação e redução da reatividade antioxidante e da atividade da GSH-Px, indicam que existe um desequilíbrio entre a produção de radicais livres e as defesas antioxidantes na DXB, gerando estresse oxidativo antes e durante o tratamento. Esses resultados permitem supor que o dano oxidativo pode estar contribuindo, pelo menos em parte, para a fisiopatologia desta doença.

Os resultados obtidos em pacientes com DXB antes e durante o tratamento dietético indicam que os aminoácidos acumulados na DXB (leucina, isoleucina e valina) não estão diretamente envolvidos no estresse oxidativo, uma vez que não foi observada correlação entre esses compostos e os parâmetros de estresse oxidativo estudados. No entanto, essas observações devem ser analisadas com cuidado uma vez que os experimentos foram realizados com amostras coletadas em um único momento durante o tratamento e as concentrações dos metabólitos acumulados podem não ser representativas do real controle metabólico dos pacientes.

Por outro lado, é possível supor que a deficiência de micronutrientes importantes ao sistema antioxidante possa contribuir para o estresse oxidativo observado nos pacientes. Como foi verificado, a deficiência de selênio se torna mais pronunciada nos pacientes durante o tratamento dietético, que envolve uma dieta restrita em proteínas naturais. Além desse, é possível que outros micronutrientes e vitaminas estejam deficientes nos pacientes tratados, permitindo sugerir que a suplementação com antioxidantes e oligoelementos deva ser considerada como uma terapia adjuvante para os pacientes com DXB.

Ainda, é possível que a investigação de parâmetros de estresse oxidativo em líquor de pacientes com DXB poderia ser mais apropriado para melhor entender a relação entre os metabólitos envolvidos na DXB, o estresse oxidativo e a disfunção neurológica apresentada pelos pacientes. É possível que o início tardio do tratamento, a baixa aderência ao tratamento e as deficiências causadas pela dieta, em conjunto com o estresse oxidativo possam ajudar a explicar o variável grau de retardo mental e/ou atraso psicomotor apresentados pelos pacientes estudados. Por fim, cabe salientar a importância da monitorização do perfil bioquímico durante a terapêutica de pacientes com DXB, no intuito de bem adequar o tratamento destes pacientes incluindo, se necessário, suplementação dietética.

V. CONCLUSÕES

As conclusões abaixo descritas estão agrupadas conforme os objetivos específicos propostos neste trabalho, seguido a seqüência dos capítulos apresentados.

Capítulo I

- A medida de lipoperoxidação TBARS está significativamente aumentada no plasma de pacientes com DXB antes do início do tratamento.
- A medida da reatividade antioxidante total (TAR) está significativamente diminuída no plasma de pacientes com DXB antes do início do tratamento.
- A medida do status antioxidante total (TAS) nos pacientes DXB antes do início do tratamento não diferiu do grupo controle.

Capítulo II

- A medida de TBARS está significativamente aumentada no plasma de pacientes com DXB durante o tratamento dietético preconizado para a doença.
- Não foi observada correlação entre os níveis plasmáticos de leucina, isoleucina ou valina e a medida de TBARS nos pacientes durante o tratamento.
- A medida do TAR está significativamente diminuída no plasma de pacientes com DXB durante o tratamento dietético preconizado para

a doença, sendo mais pronunciada nos pacientes com níveis de leucina mais baixos.

- Não foi observada correlação entre os níveis plasmáticos de leucina, isoleucina ou valina e a medida de TAR nos pacientes durante o tratamento.
- A medida do status antioxidante total (TAS) nos pacientes DXB durante o tratamento dietético preconizado para a doença não diferiu do grupo controle.

Capítulo III

- Os níveis de selênio estão significativamente diminuídos no plasma dos pacientes com DXB antes e durante o tratamento dietético, sendo está redução mais pronunciada durante o tratamento.
- Não foi observada correlação entre os níveis plasmáticos de leucina, isoleucina e valina e a concentração plasmática de selênio nos pacientes DXB.
- A atividade da enzima antioxidante GSH-Px em eritrócitos está diminuída em pacientes com DXB durante o tratamento dietético, e não mostrou correlação com os níveis de selênio.
- As atividades das enzimas SOD e CAT em eritrócitos de pacientes com DXB em tratamento não diferiram do grupo controle.

Capítulo IV

- A medida da glicemia está significativamente diminuída nos pacientes com DXB durante o tratamento.
- A concentração de colesterol total está significativamente diminuída nos pacientes com DXB durante o tratamento, enquanto os níveis de colesterol HDL, colesterol LDL e triglicerídeos não diferem do grupo controle.
- As atividades da AST e da LDH estão significativamente aumentadas no plasma de pacientes com DXB durante o tratamento, enquanto que as atividades da ALT e da CK não diferem do grupo controle.
- A concentração plasmática de creatinina está diminuída nos pacientes com DXB durante o tratamento, enquanto as concentrações plasmáticas de uréia e ácido úrico não diferem do grupo controle.
- A concentração de albumina plasmática está significativamente diminuída nos pacientes DXB durante o tratamento.
- A medida de TBARS está significativamente aumentada e a medida do TAR está significativamente diminuída no plasma de pacientes com DXB durante o tratamento.
- Foi observada uma correlação positiva entre a medida de TBARS e os níveis plasmáticos de triglicerídeos, enquanto que não foi observada correlação entre os demais parâmetros bioquímicos e as medidas de TBARS ou TAR.

Conclusão Geral

Enfim, podemos concluir que ocorre estresse oxidativo na doença do xarope do bordo antes e durante o tratamento dietético, porém este processo não está diretamente associado com os níveis plasmáticos dos principais metabólitos acumulados na doença (Leu, Ileu e Val). Ainda, os pacientes com doença do xarope do bordo apresentam deficiência de selênio antes e durante o tratamento e diminuição da atividade da glutationa peroxidase durante o tratamento, sugerindo que a suplementação com antioxidantes e oligoelementos poderia ser benéfica como um adjuvante na terapêutica da doença.

VI. PERSPECTIVAS

Pretendemos dar continuidade a este trabalho, através dos seguintes estudos:

1. Avaliar, nos pacientes com DXB, outros parâmetros de estresse oxidativo como medida de sulfidrilas e carbonilas em plasma, bem como a medida de glutationa em eritrócitos.
2. Avaliar os níveis séricos de outros micronutrientes e oligoelementos, como ubiquinona-10, vitamina E, vitamina C e ferro, em pacientes com DXB.
3. Avaliar em pacientes com DXB os níveis séricos de carnitina e homocisteína.
4. Avaliar os níveis dos α-cetoácidos e α-hidroxiácidos de cadeia ramificada em plasma de pacientes com DXB.
5. Avaliar o estresse oxidativo em outros líquidos biológicos de pacientes com DXB como urina e líquor.
6. Avaliar, se possível, a terapêutica com antioxidantes (selênio, vitaminas, carnitina) em pacientes com DXB.

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ANEXO 1 – Lista de Figuras

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ANEXO 2 – Lista de Tabelas

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ANEXO 3 – 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: 04-256 **Versão do Projeto:** 14/10/2004 **Versão do TCLE:** 16/11/2004

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ALETHEA GATTO BARSCHAK

Título: AVALIAÇÃO DE ESTRESSE OXIDATIVO EM PACIENTES COM DOENÇA DO XAROPE DO BORDO ANTES E DURANTE O TRATAMENTO DIETÉTICO

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, 16 de novembro de 2004.

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

ANEXO 4 – Termo de Consentimento

TERMO DE CONSENTIMENTO LIVRE E ESCLARECIDO –

PACIENTES COM DOENÇA DA URINA DO XAROPE DO BORDO

O presente projeto de pesquisa tem por objetivo verificar os efeitos de substâncias danosas (radicais livres) ao organismo em pacientes portadores da Doença da Urina do Xarope do Bordo. Esta doença é caracterizada pelo aumento das concentrações dos aminoácidos leucina, isoleucina e valina no sangue.

Os dados necessários para a realização do projeto serão obtidos através da análise de aminoácidos e dos parâmetros de avaliação de estresse oxidativo em plasma e eritrócitos obtidos pela coleta de sangue periférico. Estes dados serão coletados nos dias de consultas rotineiras dos pacientes, não sendo necessário comparecimento dos pacientes em consultas extras.

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 utilizado única e exclusivamente para fins do projeto de pesquisa, sendo garantido o sigilo dos dados de identificação dos participantes e que os mesmos terão acesso aos resultados obtidos.

Pelo presente Consentimento, declaro que fui informado, de forma clara e detalhada, sobre o presente Projeto de Pesquisa. Os pesquisadores responsáveis pelo projeto são a Prof.Dra. Carmen Regla Vargas e a farmacêutica-bioquimica Alethéa Gatto Barschak.

Fui igualmente informado da garantia de receber resposta a qualquer pergunta ou esclarecimento a qualquer dúvida acerca da pesquisa; da liberdade de não participar do estudo, da segurança da preservação da privacidade .

Data:

Indivíduo:

Responsável Legal:

Pesquisador:

Telefone para contato: 2101 8309

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