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Avaliação do efeito preventivo da suplementação de creatina, piruvato e taurina sobre parâmetros de estresse oxidativo e de metabolismo energético induzidos pela administração prolongada de leucina a ratos Wistar

Elenara Rieger

Orientador: Prof. Dr. Clovis Milton Duval Wannmacher

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Dedico este trabalho aos meus filhos,

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“A mente que se abre a uma nova ideia

jamais voltará ao seu tamanho original”

Albert Einstein

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SUMÁRIO

LISTA DE ABREVIATURAS

AACR – aminoácidos de cadeia ramificada

ADP - difosfato de adenosina

AK - adenilato cinase

AMP – monofosfato de adenosina

ATP - trifosfato de adenosina

CACR - α-cetoácidos de cadeia ramificada

CAT - catalase

CK - creatina cinase

CK Cit – creatina cinase citosólica

CK Mit – creatina cinase mitocondrial

CK-BB – creatina cinase citosólica isoforma cerebral

CK-MB – creatina cinase citosólica heterodímero cardíaca

CK-Mis – creatina cinase mitocondrial forma sarcomérica

CK-Miu – creatina cinase mitocondrial forma ubíqua

CK-MM – creatina cinase citosólica isoforma muscular

Cr - creatina

CuZnSOD - cobre-zinco superóxido dismutase

DXB - doença da urina do xarope do bordo

E3 – lipoamina desidrogenase

EIM - erros inatos do metabolismo

ER – espécies reativas

ERON - espécies reativas de oxigênio e nitrogênio

FAD - flavina adenina dinucleotideo

FBP – frutose 1,6 bifosfato

GAPDH- gliceraldeído-3-fosfato desidrogenase

GPx - glutationa peroxidase

GSH – glutationa reduzida

GSSH – glutationa reduzida

GTP – trifosfato de guanosina

H_2O_2 - peróxido de hidrogênio

HK – hexocinase

HOCl – ácido hipocloroso

MnSOD - manganês superóxido dismutase

NAD^+ - nicotiamina adenina dinucleotídeo

NMDA – N-metil-D-aspartato

$O_2 \cdot^-$ - radical ânion superóxido

OH• - radical hidroxil

$OONO^-$ - peroxinitrito

PCr – fosfocreatina

PEP – fosfoenolpiruvato

Pi – fosfato inorgânico

PK – piruvato cinase

SLC6A8 – transportador de creatina

SNC - sistema nervoso central

TCA – ciclo do ácido tricarboxílico

TnCl – taurina cloroamina

RESUMO

A doença do xarope do bordo é causada pela deficiência na atividade do complexo desidrogenase dos α-cetoácidos de cadeia ramificada, levando ao acúmulo dos aminoácidos de cadeia ramificada leucina, isoleucina e valina e de seus α-cetoácidos correspondentes nos tecidos e fluidos corporais. Os pacientes podem apresentar atraso no desenvolvimento psicomotor e retardo mental. A patofisiologia da doença não é bem compreendida, contudo, o acúmulo do metabólito tóxico leucina induz alterações metabólicas associadas aos sintomas clínicos. Além disso, a capacidade antioxidante está reduzida nestes pacientes. Creatina, piruvato e taurina são potentes antioxidantes em sistemas biológicos e protegem a célula contra os danos causados pelo estresse oxidativo. Portanto, neste estudo, investigamos os efeitos da administração de leucina e da coadministração de creatina mais piruvato e de taurina em alguns parâmetros da rede de fosforiltransferência e de estresse oxidativo em córtex e hipocampo de ratos. A administração de leucina induziu estresse oxidativo com o aumento de radicais livres, e diminuiu a atividade das enzimas piruvato cinase, adenilato cinase, hexocinase, gliceraldeído-3-fosfato desidrogenase, creatina cinase citosólica e creatina cinase mitocondrial. A coadministração de creatina mais piruvato e de taurina foi capaz de prevenir as alterações causadas pela administração de leucina sobre o estresse oxidativo e as enzimas tiólicas da rede de fosforiltransferência. As correlações significativas entre alguns parâmetros de estresse oxidativo e as enzimas da rede de fosforiltranferência sugerem que a diminuição na atividade das enzimas ocorre devido ao aumento das espécies reativas. Portanto, é possível sugerir que os efeitos nocivos que a leucina causa no cérebro possa contribuir para a disfunção neurológica observada em pacientes com a doença do xarope do bordo, e é possível que a suplementação com creatina, piruvato e taurina possa ser benéfica a estes pacientes.

ABSTRACT

Maple Syrup Urine Disease is caused by the 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 in tissues and body fluids. The affected patients may present psychomotor development delay and mental retardation. The pathophysiology of this disease is not entirely understood. However, the accumulation of toxic metabolite leucine induces profound metabolic alterations and impairment of energy homeostasis associated with clinical symptoms. Moreover, the antioxidant capacity is decreased in these patients. Creatine, pyruvate, and taurine plays a critical role in brain function and are potent antioxidants in biological systems, contributing to cellular protection against oxidative stress. In this study, we investigated the effects of leucine administration and coadministration of creatine plus pyruvate and taurine on some parameters of phosphoryl transfer network and oxidative stress in cerebral cortex and hippocampus of rats. Leucine-induced oxidative stress and diminished the activities of pyruvate kinase, adenylate kinase, hexokinase, glyceraldehyde-3-phosphate dehydrogenase, cytosolic creatine kinase, and mitochondrial creatine kinase. Coadministration of creatine plus pyruvate and taurine prevented the alterations provoked by leucine administration on the oxidative stress and the thiol-containing enzymes of phosphoryl transfer network. Significant correlations between some parameters of oxidative stress and the enzymes of the phosphoryl transfer network suggest that the diminution of the enzymes activities is possible due to increase in free radicals. It is possible that these effects of leucine may contribute to the neurological dysfunction observed in patients affected by Maple Syrup Urine Disease. In this case, it is possible that creatine plus pyruvate and taurine supplementation could benefit to the patients.

PARTE I

INTRODUÇÃO E OBJETIVOS

I. INTRODUÇÃO

I.1 Erros Inatos do Metabolismo

Os erros inatos do metabolismo (EIM) são distúrbios genéticos ocasionados por uma deficiência na atividade de uma enzima. A diminuição na atividade enzimática leva a um bloqueio total ou parcial de uma rota metabólica que tem como consequência o acúmulo do substrato e a ausência do produto final. Os indivíduos afetados apresentam sintomatologia variada e a gravidade da doença depende da rota metabólica afetada, bem como do metabólito acumulado ou deficiente. Geralmente são doenças graves que na maioria das vezes afetam o Sistema Nervoso Central e que podem levar o paciente à óbito quando não tratado precocemente (Scriver et al., 2001).

I.1.2 Doença da Urina do Xarope do Bordo

A Doença da Urina do Xarope do Bordo (DXB) foi descrita inicialmente em 1954, por Menkes e colaboradores, que identificaram quatro casos de uma doença degenerativa cerebral em uma mesma família, caracterizada por edema cerebral, convulsões, espasticidade e sofrimento respiratório. A manifestação do quadro clínico ocorreu na primeira semana de vida e o prognóstico fatal em três meses. O achado mais proeminente foi encontrado na urina que tinha um forte odor de xarope do bordo, cheiro parecido ao de açúcar queimado, e esta síndrome foi chamada de Doença da Urina do Xarope do Bordo. Em 1959, Dancis e colaboradores identificaram que os compostos acumulados na doença eram os aminoácidos de cadeia ramificada (AACR) e seus α-cetoácidos de cadeia ramificada (CACR). E, em 1960, o mesmo grupo de pesquisa identificou através de estudos enzimáticos em leucócitos e fibroblastos que a doença é causada pela deficiência na atividade do complexo desidrogenase dos α-cetoácidos de

cadeia ramificada. O primeiro tratamento foi proposto em 1964 por Snyderman e colaboradores, que sugeriram uma dieta restrita em aminoácidos de cadeia ramificada.

Portanto, a DXB é um Erro Inato do Metabolismo causado por uma deficiência na atividade do complexo da desidrogenase dos α-cetoácidos de cadeia ramificada como ilustrado na Figura 1. A incapacidade deste complexo enzimático em descarboxilar os α-cetoácidos de cadeia ramificada, α-cetoisocapróico, α-ceto-β-metilvalérico e α-cetoisovalérico leva ao acúmulo tecidual desses metabólitos e de seus aminoácidos precursores de cadeia ramificada, leucina, isoleucina e valina (Chuang e Shih, 2001).

A DXB é uma doença metabólica de caráter autossômico recessivo cuja frequência mundial é de aproximadamente 1 para cada 185.000 nascidos vivos (Chuang e Shih, 2001). Em comunidades menonitas dos Estados Unidos, a incidência estimada é de 1:200 nascidos vivos (Morton et al., 2002). No Brasil, a frequência da DXB pode ser maior do que a literatura internacional comenta, pois em um levantamento realizado em recém-nascidos brasileiros, foi estimada uma frequência aproximada de 1:84.000, (Jardim et al., 1995).

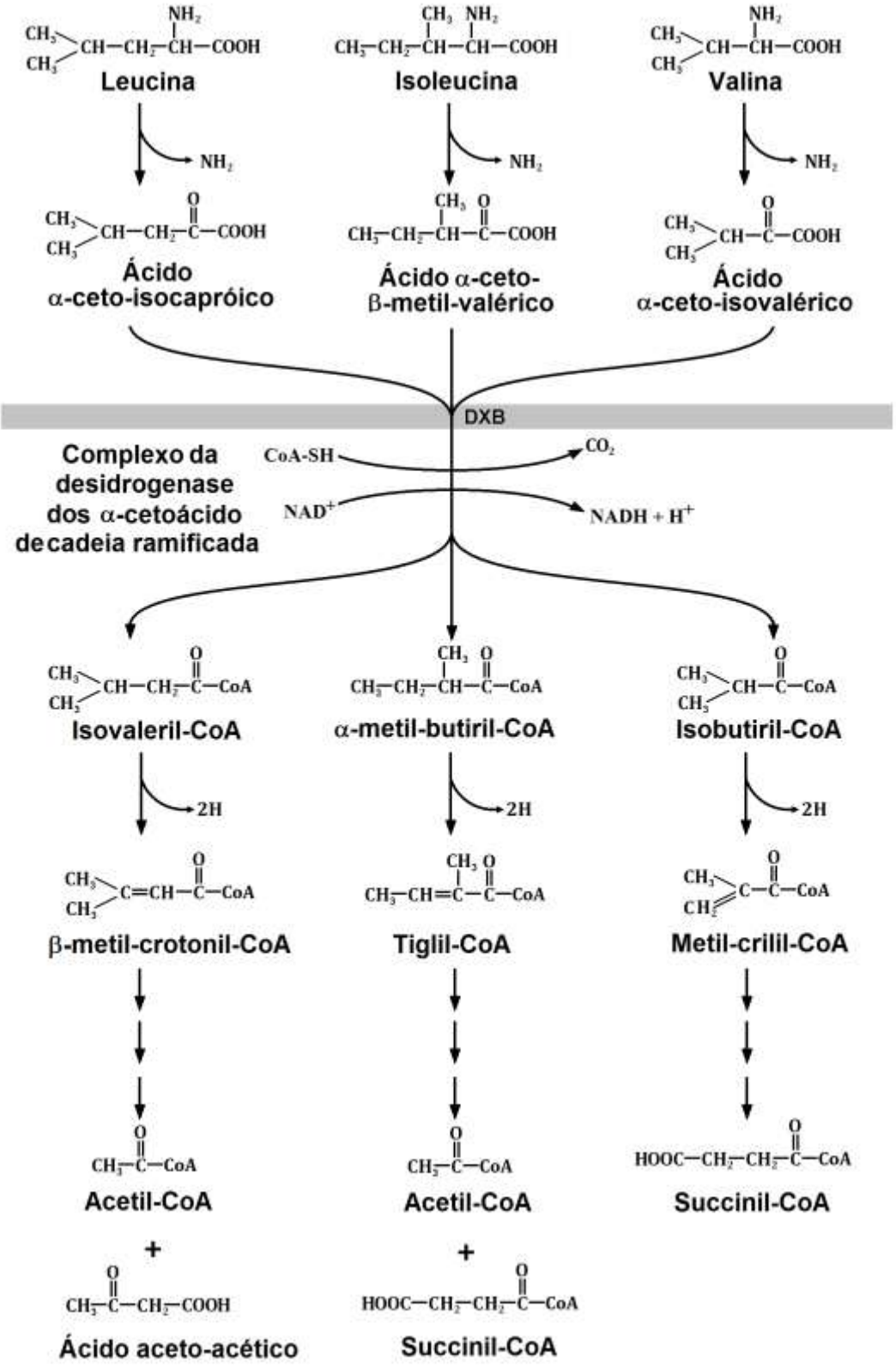

Figura 1. Rota metabólica dos aminoácidos de cadeia ramificada leucina, isoleucina e valina, indicando o bloqueio metabólico que ocorre na doença da urina do xarope do bordo (Adaptado de Scriver et al., 2001).

I.1.2.1 Diagnóstico

O diagnóstico da doença é feito através da identificação de altas concentrações dos AACR no sangue e seus derivados na urina através de cromatografia de aminoácidos e de ácidos orgânicos, respectivamente (Chuang e Shih, 2001). O método de espectrometria de massa em Tandem permite a identificação precoce da DXB quando os pacientes ainda são assintomáticos ou apresentam poucos sintomas (Peinemman e Danner, 1994; Simon et al., 2006). A leucina, principal metabólito acumulado, pode atingir concentrações séricas de 5 mM, e isoleucina e valina chegam a concentrações de 1mM (Bremer et al., 1981). A confirmação do diagnóstico se dá pela medida da atividade do complexo enzimático α-cetoácido desidrogenase de cadeia ramificada em cultura de leucócitos periféricos de pacientes (Peinemman e Danner, 1994; Chuang e shih, 2001).

I.1.2.2 Classificação fenotípica

A DXB apresenta cinco fenótipos clínicos que variam conforme a apresentação clinica, a atividade residual da enzima e na resposta à administração de tiamina. Estes são: clássico, intermediário, intermitente, tiamina-responsivo e deficiente em lipoamina desidrogenase (E3) (Chuang e Shih, 2001).

I.1.2.2.1 Clássico

A forma clássica da DXB corresponde a 80% dos casos e representa o fenótipo mais grave e comum da doença. Os níveis de leucina e seu α-cetoácido chegam a concentração de 5 mM no sangue. A atividade residual do complexo enzimático é menor que 2%. Os primeiros sinais clínicos da doença se manifestam entre o 4^{o} e o 7^{o} dia de vida, e incluem cetoacidose, hipoglicemia, dificuldade de alimentação, apnéia, ataxia, convulsões, coma, letargia, deterioração neurológica, odor de açúcar queimado

na urina. A maioria dos pacientes, quando não tratados, morre nos primeiros meses de vida em decorrência de crises metabólicas decorrentes e pela deterioração neurológica, precipitada por eventos relacionados ao catabolismo, como infecções, cirurgias ou vacinas (Chuang e Shih, 2001).

I.1.2.2.2 Intermediário

A forma intermediária apresenta elevações persistentes dos AACR, porém, menores do que as verificadas na forma clássica. A atividade residual da enzima varia de 3 a 30%. Este fenótipo não apresenta sintomas severos no período neonatal e em muitos casos não ocorre descompensação metabólica aguda. O diagnóstico é tardio, ocorre entre 5 meses a 7 anos de idade em decorrência do atraso no desenvolvimento acompanhado de cetoacidose e convulsões (Chuang e Shih, 2001).

I.1.2.2.3 Intermitente

Pacientes apresentam um desenvolvimento normal, com níveis de AACR dentro da faixa de normalidade ou com discretas elevações; no entanto, apresentam risco de sofrerem descompensação metabólica durante situações de estresse. A atividade enzimática residual é de 5 a 20%. Os sintomas surgem entre os 5 meses e os 2 anos de idade em associação com infecções, ocorrem mudanças agudas no comportamento e dificuldade para andar, podendo evoluir para o coma (Chuang e Shih, 2001).

I.1.2.2.4 Tiamina-responsivo

Neste fenótipo, a atividade residual do complexo é de 2 a 40% e os níveis de AACR (2 – 5 mM) são reduzidos para níveis normais com doses de 10 a 1000 mg/dia de tiamina e com dieta restrita em proteínas. As manifestações clínicas são a descompensação metabólica, cetoacidose e atraso no desenvolvimento psicomotor (Chuang e Shih, 2001).

I.1.2.2.5 Deficiente em lipoamina desidrogenase (E3)

A deficiência de E3 é a forma mais rara e apresenta sinais clínicos semelhantes aos da forma intermediária, porém, acompanhada de acidose láctica. A atividade residual da enzima é de 0 a 25% e há o aumento de lactato, piruvato, α-cetoglutarato, α-hidroxiisovalerato e α-hidroxiglutarato. Ocorre a deficiência das três enzimas em que o E3 é componente, a α-cetoglutarato desidrogenase, piruvato desidrogenase e o complexo desidrogenase dos α-cetoácidos de cadeia ramificada. O diagnóstico ocorre entre 8 semanas e 2 anos de idade com acidose láctica persistente, acompanhada de deterioração neurológica progressiva característica e hipotonia (Chuang e Shih, 2001).

I.1.2.3 Tratamento

O tratamento dos pacientes com DXB consiste em uma dieta hipercalórica com restrição na ingestão de proteínas e suplementada com fórmulas específicas contendo aminoácidos essenciais, exceto aqueles acumulados na doença, além de carboidratos, vitaminas e sais minerais (Strauss et al., 2010). Essencialmente o tratamento visa restaurar a homeostase do metabolismo intermediário e evitar a descompensação metabólica através da manutenção da síntese proteica normal e prevenção do catabolismo proteico, da prevenção de desequilíbrios ou deficiências de aminoácidos e intermediários metabólicos, da atenuação da disfunção celular, da restauração da homeostase energética e da promoção do anabolismo. Espera-se assim normalizar as concentrações de AACR e contribuir para o bom desenvolvimento do SNC dos indivíduos afetados, pois quanto maior o tempo que SNC fica exposto a esse aminoácido, maiores serão as sequelas deste paciente. No entanto, o tratamento não impede que haja um grau variável de disfunção neurológica, que é evidenciado pelo atraso no desenvolvimento e pelo retardo mental. Portanto, um diagnóstico e tratamento

precoces, antes dos 15 dias de vida, são cruciais para o desenvolvimento e a sobrevida destes pacientes, já que os danos neurológicos causados pelo acúmulo dos metabólitos envolvidos na doença são irreversíveis e podem levar à morte nos primeiros meses de vida (Chuang e Shih, 2001; Morton et al., 2002). As necessidades de AACR variam em função da idade, da taxa de crescimento e do déficit enzimático. Para que o paciente tenha um crescimento adequado, a dieta deve ser calculada a partir dos níveis séricos de AACR para que se tenha um aporte calórico e proteico adequado. Além disso, é provável que a restrição dietética a qual se submetem os pacientes cause diminuição nas defesas antioxidantes devido à deficiência de nutrientes essenciais, como vitaminas e sais minerais, e provoque atraso no crescimento e desenvolvimento global, anemia, imunodeficiência, desmielinização e lesões de pele (Morton et al., 2002; Artuch et al., 2004). Para detecção dos pacientes pertencentes ao fenótipo responsivo à tiamina, é administrada tiamina (50-300 mg/dia) nas primeiras três semanas do início do tratamento. A expectativa de vida dos pacientes DXB vem melhorando ao longo dos anos, dados da literatura relatam que um terço dos pacientes diagnosticados tem desenvolvimento psicomotor normal. Destes, a maioria iniciou o tratamento antes dos 10 dias de vida (Serra et al., 2010).

I.1.2.4 Neuropatologia

Quanto aos achados neuropatológicos da doença observa-se edema generalizado e atrofia dos hemisférios cerebrais, bem como hipomielinização e desmielinização do Sistema Nervoso Central, principalmente durante as crises de descompensação metabólica (Chuang e Shih, 2001; Schönberger et al., 2004). Também ocorrem alterações neurocognitivas, com déficit no raciocínio não-verbal e visual-espacial. Além

disso, os pacientes apresentam alta taxa de distúrbios neuropsiquiátricos, como transtorno de déficit de atenção, depressão e ansiedade (Walsh e Scott, 2010).

Os AACR competem com o sistema L de transporte de aminoácidos na barreira hematoencefálica. Assim, o aumento plasmático de AACR leva ao aumento na entrada destes aminoácidos para o cérebro, diminuindo o transporte de outros aminoácidos como tirosina, triptofano e fenilalanina, e como consequência afeta a biossíntese de neurotransmissores como a serotonina e as catecolaminas. A combinação desses fatores contribui para o dano cerebral na DXB (Strauss et al., 2010; Fernstrom 2005, Huang et al., 1996; Yuwiler e Geller, 1965). Essas deficiências neuroquímicas podem persistir no estado crônico, embora em menor grau, e cumulativamente, contribuir para a morbidade neuropsiquiátrica (Muelly et al., 2013).

I.1.2.5 Fisiopatologia do dano neurológico

Os mecanismos pelos quais os AACR e seus CACR são tóxicos ao SNC ainda não são totalmente compreendidos. Alguns fatores, tais como a complexidade do desenvolvimento cerebral, as concentrações alcançadas pelas toxinas e o estágio do desenvolvimento do cérebro no momento em que elas atuam prejudicam o esclarecimento desses efeitos. A fisiopatologia da DXB é multifatorial, há o acúmulo de metabólitos com efeitos tóxicos à célula. Dentre os aminoácidos acumulados na doença, a leucina é considerado o mais tóxico; ocorrem efeitos secundários como a deficiência de metabólitos intermediários e micronutrientes; e ocorrem alterações mitocondriais associadas com distúrbio na produção de ATP e estresse oxidativo (Knerr et al., 2012). O acúmulo de leucina causa profundas alterações metabólicas e prejudica a homeostase energética por comprometer a atividade de diversas enzimas, como as enzimas da

cadeia respiratória e do ciclo do ácido tricarboxílico (TCA), o que leva a efeitos deletérios ao metabolismo aeróbico do cérebro (Ribeiro et al., 2008).

Estudos realizados pelo nosso grupo de pesquisa demonstraram que a administração de leucina compromete o metabolismo energético através da inibição da atividade das enzimas creatina cinase (CK) e piruvato cinase (PK), (Pilla et al., 2003; Feksa et al., 2005), como consequência pode haver a morte de neurônios (Tomimoto et al., 1993), e a depleção celular de antioxidantes importantes como o piruvato (Das, 2006). Esta inibição também está associada ao surgimento de algumas doenças neurodegenerativas (Akzenov et al., 2000). Quanto ao estresse oxidativo, a administração de leucina provocou uma disfunção na atividade mitocondrial (Amaral et al., 2010), aumentou a peroxidação de lipídios e diminuiu as defesas antioxidantes no cérebro de ratos (Bridi et al., 2003 e 2005), além de causar danos à molécula de DNA via produção de radicais livres (Mescka et al., 2014). Estresse oxidativo tambem foi observado no plasma (Barschak et al., 2008 A e B; Barschak et al., 2006 e 2009, Mescka et al., 2013) e na urina de pacientes com DXB (Guerreiro et al, 2015). Em outro estudo no qual a leucina foi administrada em ratas prenhas e tambem durante o aleitamento materno, foi observada a presença de estresse oxidativo e alteração dos parâmetros da rede de fosforiltransferência no córtex cerebral e hipocampo da prole (de Franceschi et al., 2013).

I.1.3 Metabolismo energético

I.1.3.1 Rede de fosforiltransferência

Os mecanismos responsáveis envolvidos no equilíbrio entre o consumo e a produção de ATP são finamente regulados por processos intracelulares. Para um ótimo funcionamento do sistema bioenergético celular, é necessário que compostos ricos em

energia, como o ATP, sejam produzidos e entregues aos locais de consumo em uma velocidade correspondente à sua taxa de consumo (Dzeja et al, 2000, 2003).

Uma rede intracelular enzimática, catalisada principalmente pela creatina cinase (CK), adenilato cinase (AK) e enzimas glicolíticas (especialmente a piruvato cinase – PK), atua em processos de produção e consumo de ATP (Wallimann et al, 1994; Saks et al, 1996; Dzeja et al, 1998). A sinalização metabólica é dinâmica e as enzimas da rede de transferência de grupos fosforil contribuem para a eficiente comunicação energética intracelular na manutenção dos altos níveis de ATP celular (Dzeja et al, 2000; Neumann et al , 2003). A CK é a principal responsável pela produção do ATP (Dzeja et al , 1999). A AK produz 10% do total da renovação de ATP no tecido muscular cardíaco, e 4% no músculo esquelético em repouso. No entanto, a AK pode transferir e utilizar duas moléculas de ADP, podendo assim, em momentos de alta demanda energética fornecer ATP, além de AMP que estimula a via glicolítica por alosteria na enzima fosfofrutocinase (Pucar et al., 2000). Além disso, as enzimas glicolíticas como a hexocinase (HK), gliceraldeído-3-fosfato desidrogenase e PK podem contribuir para a transferência intracelular de grupos fosforil (Dzeja et al, 2003). Em média, as concentrações de ATP, ADP e AMP são de 5, 1 e 0,1 mM, respectivamente, e são mantidas em equilíbrio constante dentro da célula (Noma, 2005). Mudanças nessas concentrações podem alterar a atividade de importantes enzimas em diversas rotas oxidativas e de biossíntese (Dzeja et al, 2000). O ATP sozinho não é responsável pelo fluxo de energia na célula. O mecanismo que supre a demanda energética é a rede de fosforiltransferência, da qual fazem parte as enzimas AK, HK, GAPDH, CK e PK (Dzeja et al, 1999).

I.1.3.1.1 Creatina cinase

A CK catalisa a transferência reversível de um grupo N-fosforil da fosfocreatina (PCr) para o ADP, formando ATP e creatina (Cr), conforme a reação que é dependente de magnésio: PCr + MgADP $\leftrightarrow$ MgATP + Cr. Essa enzima participa na homeostasia de células com necessidades energéticas variáveis e intermitentes como, por exemplo, músculo esquelético e cardíaco, tecidos neurais como cérebro, retina e fotorreceptores e nos eritrócitos (Wallimann, 1992). O pH ótimo para a catálise enzimática varia de 6,0 a 7,0 na direção de formação de ATP e 7,5 a 9,0 na direção de formação de PCr.

Há quatro principais isoenzimas da CK e os nomes são dados em função dos tecidos em que foram historicamente isoladas. Existem duas isoformas citosólicas, a muscular (CK-MM) e a cerebral (CK-BB), ambas existindo como homodímeros sob condições fisiológicas, podendo se apresentar como um heterodímero CK-MB no coração. As isoformas mitocondriais também são duas, a forma ubíqua (CK-Miu) e a sarcomérica (CK-Mis), as quais são expressas predominantemente no cérebro e no músculo estriado, respectivamente (Wallimann et al., 1992). As isoformas da creatina cinase mitocondrial (CK-Mi) geralmente existem como octâmeros, mas podem ser transformadas rapidamente em dímeros (McLeish et al., 2005). As isoformas citosólicas são sempre co-expressadas em tecido específico junto com a isoformas mitocondriais, e essa interação é de fundamental importância para a homeostasia energética celular (Silva et al., 2003).

As isoenzimas da CK são extremamente solúveis, elas são compartimentadas subcelularmente e unidas funcional e/ou estruturalmente com sítios de produção de ATP (glicólise e mitocôndria) ou consumo de energia (ATPases celulares, como ATPase actinomiosina e ATPase retículo sarcoplasmático). Elas formam um sistema de distribuição de energia altamente regulado e intrincado, o circuito da fosfocreatina

(PCr). Diferentes funções têm sido sugeridas para a comunicação entre as isoformas de creatina cinase citosólica e mitocondrial por PCr e Cr (Wallimann, 1994), levando à proposta do modelo de circuito da PCr (Rojo et al., 1991; Wallimann et al., 1992; Wyss et al., 1992). O circuito CK/PCr parece cumprir todas as necessidades de um sistema altamente organizado de transporte e tamponamento de energia, assim como um sistema regulatório para controle das razões subcelulares de ATP/ADP, levando juntos a uma utilização de energia mais eficiente em termos termodinâmicos. Dependendo das necessidades metabólicas da célula ou tecido, uma dessas diferentes funções do circuito CK/PCr pode estar dominante. O circuito PCr serve para melhorar a eficiência termodinâmica da hidrólise do ATP, para manter baixa a concentração de ADP e manter alta a razão ATP/ADP nos sítios subcelulares onde a CK está ligada à processos que consomem ATP, como por exemplo, bombas de íons (Wallimann et al., 1994). O circuito também serve como tampão de energia flutuante, mantendo as concentrações de ATP e ADP estáveis e tamponando os H^+ gerados. Nesse papel, a PCr tem a função de transportadora de energia, conectando sítios de produção de ATP, como fosforilação oxidativa mitocondrial, com sítios de utilização de ATP, onde a CK-Mi tem um papel eminente (Wallimann et al., 1992).

As isoenzimas da CK contêm um grupamento sulfidrílico no sítio ativo: cisteína 278 na CK mitocondrial e cisteína 283 na CK citosólica (Furter, 1993). Modificações que ocorrem nesses grupos tiólicos causadas por espécies reativas de oxigênio e nitrogênio (ERON) diminuem a atividade da enzima, sendo assim, esse grupamento tem um importante envolvimento na sua atividade catalítica (Kaneko, 1993; Sukuki et al., 1992). Essa diminuição de atividade produz deficiência energética, acúmulo de ADP e excesso de cálcio intracelular (Wallimann et al., 1994). As ERON elevam os níveis de cálcio intracelular (Ca^{2+}) e induzem apoptose (Mattson, 1992). A CK parece estar

envolvida em certas condições patológicas relacionadas com déficit de energia cerebral. Em condições anóxicas, a adição de creatina ao meio de incubação contendo fatias de cérebro de ratos protege a transmissão sináptica e mantém o potencial de ação via Na^+, K^+-ATPase (Whittingham, 1981); creatina aumenta os níveis de PCr, reduzindo a queda de ATP, a liberação de Ca^{2+} e a morte celular no SNC (Carter et al., 1995). Também foi observado que o processo de mielinização e as demais atividades do sistema nervoso dependem da maturação funcional da CK durante o desenvolvimento do encéfalo do rato com expressão sequencial das isoenzimas (Holtzman et al., 1993).

I.1.3.1.2 Piruvato cinase

A última etapa da via glicolítica é a transferência do grupo fosforil do fosfoenolpiruvato (PEP) ao ADP, catalisada pela PK, que requer K^+ e Mg^{2+} ou Mn^{2+} (Oberfelder et al., 1984). As enzimas glicolíticas estão presentes em todas as células ou tecidos dos mamíferos, a via glicolítica é um sistema fundamental para o metabolismo energético (Heyduk et al., 1992). Entretanto, cada tipo de tecido utiliza essas enzimas de uma maneira específica para seu metabolismo (Friesen et al., 1998). A manutenção do fluxo de energia e de nutrientes para dentro da célula é essencial para a homeostasia e o funcionamento celular.

São conhecidas 4 isoenzimas da PK que são: M1, L, R e M2 (Consler et al.,1989). Essas isoenzimas diferem em suas propriedades químicas, físicas, cinéticas, eletroforéticas e imunológicas, refletindo a sua importância no suprimento das necessidades metabólicas de cada tecido. A isoenzima tipo M_1 é o principal tipo encontrado no músculo esquelético, coração e cérebro adulto (Consler et al, 1988). O tipo L é predominante em tecidos gliconeogênicos, como o fígado e o rim (Oskamet al., 1985). O tipo R está presente nos eritrócitos e tecidos hematopoiéticos. A M_2–PK é

encontrada no tecido fetal, em células proliferativas e tumorais (Friesen et al.,1998). As expressões dessas isoenzimas são reguladas durante o desenvolvimento. A M_2–PK é a única detectável precocemente em tecido fetal, sendo gradualmente substituída pelos tipos L, R e M_1, durante o desenvolvimento. Em contraste, em células transformadas e na regeneração do fígado, as isoenzimas tecido-específicas estão presentes em níveis diminuídos ou completamente ausentes, sendo substituídas pela isoenzima M_2–PK (Heyduk et al., 1992; Consler et al., 1992). A PK é tipicamente uma enzima alostérica e participa com uma maior função no controle do fluxo metabólico da frutose-1,6-bifosfato (FBP) a piruvato, o qual está envolvido em uma variedade de rotas metabólicas, indicando que a PK pode ser considerada uma enzima chave não somente para a rota glicolítica, mas também para o metabolismo celular (Valentini et al., 2000).

I.1.3.1.3 Adelinato cinase

A adenilato cinase (AK) é uma fosfotransferase que catalisa a reação de transferência de fosfatos de alta energia entre os nucleotídeos de adenina, ATP, ADP e AMP, conforme a reação: 2ADP ↔ATP + AMP. A AK é responsável pela interconversão de ATP, ADP e AMP (Pucar et al., 2000). Essa enzima potencializa o papel energético do ATP por ter habilidade de regenerar o ATP a partir de dois ADP e também pela regulação dos processos envolvendo os nucleotídeos de adenina, desempenhando assim um importante papel na homeostasia energética celular (Dzeja et al., 1999).

A AK gera sinais metabólicos para a célula, como o AMP, ativando rotas dependentes de AMP (Dzeja et al., 2002). A estrutura da AK sofre grandes mudanças conformacionais durante seu ciclo catalítico. A enzima possui três domínios bem descritos: o CORE, o domínio de ligação do AMP e o domínio de ligação do ATP. Os

dois domínios de ligação do AMP e ATP são descritos como passo limitante da catálise da AK e o CORE é responsável pelas propriedades alostéricas da enzima (Noma et al., 2005; Dzeja et al., 2002).

São descritas até o momento sete isoenzimas da AK (AK1 a AK7), distribuídas em todos os compartimentos intracelulares, espaços intersticiais e líquidos corporais, com a finalidade de regular o metabolismo energético, proporcionando uma eficiente economia energética celular (Dzeja e Terzic, 2009). A AK1 existe no citoplasma e a AK2 está localizada principalmente no espaço intermembrana mitocondrial; ambas utilizam os nucleotídeos de adenina (Dzeja et al., 1999). A AK3 utiliza GTP para formação de ADP ou GDP devido à localização subcelular, pois é encontrada na matriz mitocondrial, onde há a formação de GTP no ciclo do ácido cítrico. A AK4 está localizada na matriz mitocondrial e AK5 no citosol. A AK6 é encontrada no núcleo celular (Noma et al., 2005). A AK7 está relacionada com a motilidade celular, é altamente expressa no epitélio brônquico e parece estar associada com o movimento ciliar (Dzeja e Terzic, 2009).

I.1.3.1.4 Hexocinase

A hexocinase (HK) está localizada no citosol e catalisa a primeira reação da via glicolítica (Marks et al., 2007). A enzima utiliza o ATP como doador de fosforil ao carbono 6 da glicose, formando a glicose-6-fosfato (Nelson e Cox, 2008). A hexocinase requer magnésio como cofator para exercer sua atividade catalítica, no qual forma um complexo com o ATP (complexo $MgATP^{2-}$). Ela está presente em quase todas as células do organismo, ainda que sob outras formas, como a glicocinase, localizada no hepatócito. Essas isoenzimas são codificadas por genes diferentes, tendo propriedades cinéticas e regulatórias variadas (Nelson e Cox, 2008). A hexocinase cerebral está

associada à membrana mitocondrial externa e o ADP produzido pela enzima controla o potencial de membrana e a geração de espécies reativas. Além disso, a atividade da hexocinase controla a síntese de ATP e a apoptose no cérebro (Da Silva et al., 2004).

I.1.3.1.5 Gliceraldeído-3-fosfato-desidrogenase

A gliceraldeído-3-fosfato desidrogenase (GAPDH) é a primeira enzima que catalisa a fase de pagamento da glicólise (Nelson e Cox, 2008). A GAPDH oxida o gliceraldeído-3-fosfato em 1,3-bifosfoglicerato, sendo adicionado fosfato inorgânico na molécula em formação, juntamente com a redução do NAD^+. O grupo aldeído do gliceraldeído-3-fosfato reage com o grupo sulfidrila de um resíduo essencial da cisteína no sítio ativo da GAPDH. A reação com metal pesado no resíduo de cisteína, tal como o mercúrio, leva à inibição irreversível da enzima (Marks et al., 2007). O aumento na expressão da GAPDH é encontrado em alguns tumores e em células proliferativas e diferenciadas (Perrota et al., 2014).

I.1.4 Radicais livres

Radicais livres são qualquer átomo ou molécula que contém um ou mais elétrons não pareados nos orbitais externos. Este não pareamento pode torná-lo altamente instável e reativo, capaz de reagir com qualquer composto situado próximo à sua órbita, passando a ter uma função oxidante ou redutora de elétrons (Valko et al., 2007).

Existem diversas fontes geradoras de radicais livres nos sistemas biológicos. Os radicais livres podem ser formados endogenamente como subprodutos do metabolismo aeróbico ou por influências externas como uma dieta inadequada, consumo exagerado de álcool, fumo, exposição às radiações ionizante e eletromagnética, poluição atmosférica e etc (Halliwell, 1996).

As espécies reativas (ER) são átomos ou moléculas produzidas constantemente durante os processos metabólicos e atuam como mediadores de transferência de elétrons em várias reações bioquímicas. Sendo assim, desempenham funções relevantes no metabolismo, como a comunicação intracelular, apoptose, defesa contra agentes infecciosos, dentre outras (Halliwell e Gutteridge, 2007).

As principais fontes das ER são as organelas citoplasmáticas que metabolizam o oxigênio, o nitrogênio e o cloro, gerando grande quantidade de metabólitos (Shami e Moreira, 2004). Em condições fisiológicas, o oxigênio molecular (O_2), através do citocromo oxidase mitocondrial, sofre redução e resulta na formação de água (Bergendi et al, 1999) no entanto, aproximadamente 5% deste O_2 não é reduzido a H_2O, e pode ser convertido em reativos intermediários (Boveris, 1998). Entre as principais formas de espécies reativas de oxigênio estão: ânion superóxido (O^{2-}), que é decorrente de processos metabólicos ou ativação do oxigênio por irradiação, sendo teoricamente a primeira espécie reativa formada e apresenta baixa capacidade de oxidação quando comparado com outras espécies reativas (Valko et al., 2007); radical hidroxil (OH•) que é bastante reativo, uma vez formado age rápido e inespecificamente, podendo atacar e lesar qualquer biomolécula; peróxido de hidrogênio (H_2O_2) que é fracamente reativo, porém, é capaz de atravessar a membrana nuclear e reagir com outros radicais induzindo danos a moléculas por meio de reações enzimáticas. A sua importância está na capacidade de formar o radical hidroxil através de duas reações: a primeira é a reação de Fenton, onde H_2O_2 reage com Fe^{2+} formando Fe^{3+} e OH• e a segunda é a reação de Haber-Weiss, em que o H_2O_2 mais o O^{2-} também produzem OH• (Anderson, 1996). Além das espécies reativas de oxigênio, existem ainda as espécies reativas de nitrogênio, representadas principalmente pelo óxido nitríco (NO•) e peroxinitrito ($ONOO^-$). O óxido nítrico é pouco reativo e tem grande importância biológica atuando

na vasorregulação e neurotransmissão, porém em excesso pode ser citotóxico. E o peroxinitrito é bastante reativo e pode oxidar lipídios, DNA e aminoácidos (Halliwell, 2001).

As altas concentrações de ERON podem causar danos à estrutura celular, ácidos nucleicos, lipídeos e proteínas. O OH• reage com bases púricas e pirimídicas. Os ácidos graxos poli-insaturados também são muito sensíveis a esse radical, que ataca estes resíduos nos fosfolipídeos (Halliwell, 2000). A cadeia lateral dos aminoácidos de resíduos de proteínas também são os alvos de oxidação, principalmente os resíduos de cisteína e metionina, que são mais suscetíveis à ação das ERON. As oxidações nos resíduos de cisteína formam pontes de dissulfeto reversíveis entre os grupos tiólicos das proteínas, diminuindo as concentrações moleculares de tióis, em particular a glutationa reduzida (GSH), e ainda, ao quebrar essas pontes de dissulfeto, causam a inativação de proteínas (Valko et al., 2007). A concentração de grupos carbonil são bons indicativos da presença de espécies reativas.

I.1.4.1 Estresse oxidativo

O termo estresse oxidativo se refere a um distúrbio do equilíbrio pró-oxidante/antioxidante em favor do pró-oxidante, podendo resultar em dano oxidativo a componentes celulares. Em princípio, o estresse oxidativo pode resultar de uma diminuição dos antioxidantes e/ou da produção aumentada de ERON, gerando assim um acúmulo das espécies reativas que causam danos à estrutura das biomoléculas de DNA, lipídios, carboidratos e proteínas. O excesso de espécies reativas no organismo é neutralizado por antioxidantes produzidos pelo corpo ou absorvidos da dieta. A diminuição dos antioxidantes pode ser causada pela diminuição da atividade das enzimas antioxidantes: cobre-zinco superóxido dismutase (CuZnSOD), manganês

superóxido dismutase (MnSOD), catalase (CAT) ou glutationa peroxidase (GPx) ou pela deficiência nutricional de antioxidantes e/ou outros constituintes dietéticos essenciais: alfa-tocoferol, ácido ascórbico, aminoácidos contendo enxofre necessário para a síntese de glutationa, ou riboflavina necessária para a produção de flavina adenina dinucleotideo (FAD), um cofator da glutationa redutase (Halliwell e Gutteridge, 1999).

Todos os tecidos são suscetíveis ao dano oxidativo. No entanto, o cérebro parece ser especialmente sensível a este tipo de lesão, uma razão para isso seria o alto consumo de oxigênio apresentado por este tecido, e a grande quantidade de lipídios poliinsaturados presentes nas membranas neuronais, altamente suscetíveis a lipoperoxidação. Além disso, a auto-oxidação de neurotransmissores como dopamina e noradrenalina tambem gera espécies reativas e por fim, o tecido cerebral apresenta baixos níveis de defesas antioxidantes (Halliwell e Gutteridge, 2007).

Evidências sugerem que o estresse oxidativo tem um papel importante na patogênese de muitas doenças neurodegenerativas, como mal de Alzheimer, doença de Parkinson e esclerose lateral amiotrófica (Reznick e Packer, 1993). Além disso, o estresse oxidativo atua em diversos Erros Inatos do Metabolismo, como nas acidemias orgânicas (Latini et al., 2005; Fontella et al., 2000;) e aminoacidopatias (Streck et al., 2003; Artuch et al., 2001; Bird et al., 1995), dentre outras, sugerindo o seu envolvimento com o dano neurológico observado nessas doenças.

I.1.5 Defesas antioxidantes

O organismo possui mecanismos de defesa contra a ação tóxica das ERON, diminuindo ou eliminando as consequências negativas de seus efeitos no organismo (Marks et al, 2007). Esse sistema de proteção é constituído de defesas enzimáticas e não

enzimáticas. As defesas antioxidantes enzimáticas são as enzimas: catalase , responsável pela decomposição direta do H_2O_2 formando H_2O e O_2; a superóxido dismutase que catalisa a dismutação do radical superóxido a H_2O_2; e a glutationa peroxidase que catalisa a decomposição de peróxidos através da oxidação da glutationa reduzida (GSH) formando glutationa oxidada (GSSH). E as defesas não enzimáticas são as vitaminas (como o ácido ascórbico, vitamina E e vitamina A) e a GSH (Halliwell, 2001). Do ponto de vista biológico, um antioxidante é um composto que, quando presente em baixas concentrações em relação ao substrato oxidável, reage com as espécies reativas, regenerando o substrato ou prevenindo significativamente a oxidação do mesmo, e assim impedindo a propagação da reação em cadeia (Bergendi et al., 1999; Halliwell, 2000).

I.1.5.1 Creatina

A creatina é encontrada no músculo esquelético e sintetizada endogenamente pelo fígado, rins e pâncreas a partir dos aminoácidos glicina, arginina e metionina e é armazenada em outros tecidos, como coração, músculo liso, testículos e cérebro (Wyss & Schulze, 2002). Nas células humanas, 60% a 70%, aproximadamente, correspondem à forma livre e 30% a 40%, à forma fosforilada (fosfocreatina). A creatina tem um papel crítico no metabolismo energético, pois sua principal função ocorre quando se encontra na forma fosforilada, agindo como uma doadora de grupo fosforil para moléculas de ADP, regenerando o ATP, principalmente no músculo esquelético, onde é degradada em condições de alta demanda energética dentro da célula (Wallimann et al., 1998). O transportador de creatina (SLC6A8) é expresso na barreira hematoencefálica e regula a concentração de creatina no cérebro, representando uma via para o fornecimento de creatina da circulação sanguínea para o cérebro (Tarnopolsky, 2007). Estudos recentes

com a suplementação de creatina demonstram as propriedades antioxidantes desse composto (Sestili et al., 2006; Hersch et al., 2006). A creatina desempenha a tarefa de eliminar espécies reativas e melhorar o estado energético da célula (Wyss e Schulze, 2002), portanto, desempenha um papel antioxidante primário significativo, havendo uma relação direta da concentração de creatina com a neutralização do radical ânion superóxido (O^{2-}) e com o peroxinitrito ($OONO^-$) (Lawler et al., 2002). Além disso, a creatina não possui atividade antioxidante contra hidroperóxidos como o H_2O_2, sugerindo que a creatina apresenta uma capacidade antioxidante seletiva (Wyss & Schulze, 2002). O composto exerce uma atividade citoprotetora antioxidante direta em diferentes linhagens de células contra diversos agentes oxidativos (Sestili et al., 2006). A creatina também regula o crescimento de axônios e a densidade sináptica, reduz o acúmulo de glutamato extracelular, estabiliza os níveis de cálcio dentro da célula e inibe a transição de permeabilidade mitocondrial, prevenindo assim a apoptose em doenças neurodegenerativas, neuromusculares e neurometabólicas (Bender et al., 2006; Hersch et al., 2006; Tarnopolsky, 2007; Bolaños et al., 2009).

I.1.5.2 Piruvato

O piruvato desempenha um papel chave no metabolismo intermediário como um produto da glicólise e fonte de acetil-CoA, conectando a via glicolítica com o ciclo do ácido tricarboxílico. O piruvato é considerado um poderoso agente antioxidante e neuroprotetor, pois é capaz de oferecer resistência ao estresse oxidativo e ao insulto mitocondrial. Isso ocorre devido a sua capacidade de sequestrar H_2O_2 impedindo os efeitos danosos deste radical na célula. O H_2O_2 é pouco reativo, porém penetra facilmente na membrana celular induzindo a produção de outras espécies reativas, e causa apoptose devido ao aumento na atividade da caspase 3 e a perda do potencial de

membrana na mitocôndria (Mazzio e Soliman, 2003; Andrae et al, 1985; Japtag et al, 2003).

O piruvato é um substrato energético que produz ATP mesmo na ausência de glicose, atua na homeostase energética restaurando os níveis de ATP na célula. Desse modo, protege neurônios contra a toxicidade e morte celular induzida pelo glutamato e receptores N-metil-D-aspartato (NMDA), e previne o acúmulo de glutamato em células submetidas a privação de glicose (Maus, 1999).

I.1.5.3 Taurina

A taurina é um β-aminoácido condicionalmente essencial, obtido diretamente da dieta e indiretamente, pela sua biossíntese a partir dos aminoácidos metionina e cisteína no fígado, sendo de grande necessidade na fase de desenvolvimento do sistema nervoso, rins e retina. Encontrado de forma livre no líquido intracelular atinge concentração de 10 a 100μM no plasma, 30 a 40 mM no cérebro e concentrações de 50 e 70 mM nos neutrófilos e na retina, respectivamente (Green et al., 1991; Sturman, 1993). As concentrações mais altas de taurina ocorrem no cérebro em desenvolvimento, sendo que o nível de taurina no indivíduo adulto é de aproximadamente um terço dos neonatos (Huxtable, 1992).

Muitos trabalhos demonstram que a taurina possui propriedades antioxidantes e exerce funções citoprotetoras e de vital importância para o organismo, (Bouckenooghe et al., 2006; Cozzi, 1995; Sturman, 1993). Taurina possui habilidade de neutralizar o ácido hipocloroso (HOCl), um potente oxidante, e formar um composto estável, a taurina-cloramina (TnCl), sendo assim capaz de reduzir o dano ao DNA causado por compostos aromáticos de amina *in vitro* (Kozumbo et al., 1992). A taurina também

pode modular o grau de peroxidação lipídica regulando os íons Ca^{2+} envolvidos na patogênese do dano celular mediada por radicais livres (Braughter, 2005, Wang et al., 2005). A taurina possui ação antioxidante e de 'scavenger' contra a maioria das espécies oxidativas, como ânions superóxidos (Kilic et al., 1999), óxido nítrico, peroxinitrito, radical peroxil (Oliveira et al., 2010) e no entanto, não possui reatividade contra o H_2O_2 (Wang et al., 1995; Arouma et al., 1988).

A taurina tem sido usada no tratamento de doenças cardiovasculares, hepáticas e neurodegenerativas como a Doença de Alzheimer, Parkinson e Huntington (Sturman, 1993). Também é adicionada a fórmulas alimentícias de bebês prematuros e neonatos mantidos durante muito tempo em nutrição parenteral total (Chesney, 1988).

2. OBJETIVOS

I.2.1 Objetivo geral

Considerando que pacientes com a doença da urina do xarope do bordo manifestam sintomas neurológicos na infância, o objetivo geral deste trabalho foi investigar, através de um modelo crônico de hiperleucinemia, o efeito da leucina sobre parâmetros bioquímicos do metabolismo energético e de estresse oxidativo em córtex cerebral e hipocampo de ratos Wistar. Além disso, investigamos o papel protetor da creatina, piruvato e taurina sobre possíveis alterações bioquímicas observadas no modelo de hiperleucinemia.

I.2.2 Objetivos específicos

1 - Determinar o efeito da administração subcutânea de 4,8 μmol/g de leucina sobre parâmetros do metabolismo energético e de estresse oxidativo em córtex cerebral e hipocampo de ratos Wistar de 21 dias de idade.

2 - Determinar o efeito neuroprotetor da administração intraperitoneal de 0,4 μmol/g de creatina e 0,2 μmol/g de piruvato sobre parâmetros do metabolismo energético e de estresse oxidativo em córtex cerebral e hipocampo de ratos Wistar de 21 dias de idade.

3 - Determinar o efeito neuroprotetor da administração intraperitoneal de 1,6 μmol/g de taurina sobre parâmetros do metabolismo energético e de estresse oxidativo em córtex cerebral e hipocampo de ratos Wistar de 21 dias de idade.

PARTE II

ARTIGOS CIENTÍFICOS

II. CAPÍTULO 1 – Artigo 1

Neuroprotective effect of creatine and pyruvate on enzymes activities of phosphoryl transfer network and oxidative stress alterations caused by leucine administration in Wistar rats

Elenara Rieger, Itiane Diehl de Franceschi, Thales Preissler, Clovis Milton Duval Wannmacher

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Neuroprotective effect of creatine and pyruvate on enzymes activities of phosphoryl transfer network and oxidative stress alterations caused by leucine administration in Wistar rats

Elenara Rieger, Itiane Diehl de Franceschi, Thales Preissler, Clovis Milton Duval Wannmacher

Departamento de Bioquímica, Instituto de Ciências Básicas da Saúde, Universidade Federal do Rio Grande do Sul, Porto Alegre, Rua Ramiro Barcelos 2600, CEP 90.035-003, RS, Brazil

Corresponding Author: Clovis M. D. Wannmacher
Departamento de Bioquímica, Instituto de Ciências Básicas da Saúde, UFRGS, Rua Ramiro Barcelos 2600, CEP 90.035-003, Porto Alegre, RS, Brazil
Telephone 55 51 33085576
Fax 55 51 33085535
E-mail. clovisdw@ufrgs.br

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Abstract

Maple Syrup Urine Disease is an autosomal metabolic disease caused by a deficiency of branched-chain α-keto acid dehydrogenase complex activity. In this disease occur the accumulation of the branched-chain amino acids leucine, isoleucine and valine and their corresponding branched-chain α-keto acids in the tissues and body fluids. The affected patients may present psychomotor development delay and mental retardation. The pathophysiology of Maple Syrup Urine Disease is not entirely understood, but leucine seems to be the primary neurotoxic metabolite. Creatine and pyruvate are energetics and antioxidants substances. In this study, we investigated the effects of leucine administration and co-administration of creatine plus pyruvate on several parameters of oxidative stress and phosphoryl transfer network in cerebral cortex and hippocampus of Wistar rats treated from the 8^{th} to the 21^{st} postpartum day. Leucine induced oxidative stress and diminished the activities of pyruvate kinase, adenylate kinase, cytosolic and mitochondrial creatine kinase. Co-administration of creatine plus pyruvate prevented the alterations provoked by leucine administration on the oxidative stress and the enzymes of phosphoryltransfer network. These results indicate that chronic administration of leucine may stimulate oxidative stress and alters the enzymes of phosphoryltransfer network in the cerebral cortex and hippocampus of the rats. It is possible that these effects may contribute, along with other mechanisms, to the neurological dysfunction found in patients affected by Maple Syrup Urine Disease. In this case, it is possible that creatine plus pyruvate supplementation could benefit to the patients.

Introduction

Maple Syrup Urine Disease (MSUD) is caused by a severe deficiency in the activity of the branched-chain α-keto acid dehydrogenase complex (BCKAD). As a consequence, the branched-chain amino acids (BCAA) leucine (Leu), isoleucine (Ile) and valine (Val), and their corresponding transaminated branched-chain α-keto acids (BCKAs) α-ketoisocaproic, α-keto-β-methylvaleric acid and α-ketoisovaleric acid accumulate in tissue and body fluids (Chuang and Shih 2001).

MSUD presents heterogeneous molecular and clinical phenotypes ranging from a classic severe form with neonatal onset to milder variants forms with later onset and presenting different residual enzyme activity (Chuang and Shih 2001). Individuals with classical MSUD usually present poor feeding, convulsions, ketoacidosis, apnea, hypoglycemia, coma, ataxia, psychomotor delay and mental retardation. They may show generalized edema and hypomyelination/demyelination on magnetic resonance imaging studies of the central nervous system (CNS) (Chuang and Shih 2001; Schönberger 2004). Therapy for this disease is based on a protein-restricted diet with low BCAA supplemented with a semi-synthetic formula of essential amino acids, vitamins, and minerals (Strauss et al. 2010). This treatment minimizes the accumulation of the toxic metabolites and contributes to the survival of the affected individuals. However, this treatment does not prevent a variable degree of neurological dysfunction evidenced by development delay and mental retardation whose pathogenesis is poorly known (Chuang and Shih 2001). It is known that leucine and/or α-ketoisocaproate are the main important neurotoxic metabolites in this disorder once their increased concentrations have been associated with the appearance of neurological symptoms (Chuang and Shih 2001; Snyderman et al. 1964).

Several studies show that oxidative stress and alteration of brain energy metabolism are associated with the pathophysiology of several neurodegenerative disorders (Halliwell 2001; Mochel 2012; Beal 1995. 2000; Hayashi 2012; Reed 2011). Oxidative stress and alteration of brain energy also occur in animal models of inborn errors of metabolism, as hyperprolinemia (Kessler 2003), hyperphenylalaninemia (Costabeber 2003), tyrosinemia (de Andrade 2012), tryptofanemia (Cornelio 2004; Feksa 2008), and maternal hyperleucinemia (de Franceschi et al. 2013).

Previous studies demonstrated that leucine impairment of energy metabolism by inhibiting creatine kinase (CK) and pyruvate kinase (PK) activities (Pilla et al. 2003; Feksa et al. 2005). The CK activity is severely reduced in some neurodegenerative diseases (Akzenov et al. 2000), and this inhibition may lead to neuronal loss in the brain (Tomimoto et al. 1993). Inhibition of PK activity could reduce pyruvate content, an important antioxidant agent (Das 2006). Leucine also provokes an impairment mitochondrial function in rat brain (Amaral et al. 2010) and increases lipid peroxidation and decreases antioxidant defenses in rat brain (Bridi et al. 2003, 2005). Leucine administration to female Wistar rats during pregnancy and lactation induces oxidative stress and alters parameters of phosphoryl transfer network in the brain cortex and hippocampus of the offspring (de Franceschi et al. 2013). Leucine causes DNA damage via free radical production (Mescka et al. 2014), and oxidative damage was observed in plasma (Barschak 2006, 2008a, 2008b, 2009; Mescka et al. 2013) and urine of MSUD patients (Guerreiro et al. 2015).

As a consequence of the high cost of treatment, novel alternative therapies for MSUD have been sought. Creatine (Cr) and pyruvate (Pyr) are important substrates energetics and antioxidants. Cr has antioxidant properties protecting cells against superoxide anion and peroxynitrite (Lawler et al. 2002; Matthews et al. 1998; Sartini 2012; Sestili 2006). Also, Cre supplementation can protect neurons against neurotoxins in vitro (Brewer and Wallimann 2000) and has a neuroprotective effect on neurodegenerative diseases, such as in Huntington´s and Parkinson´s disease (Beal 2011; Ferrante 2000; Tarnopolsky 2007; Bender et al. 2006). Pyruvate is an antioxidant, anti-inflammatory and neuroprotective agent (Desagher et al. 1997; Das 2006; Kim et al. 2005). Pyr readily scavenges hydrogen peroxide in cultured cells (O'Donnell-Tormey 1987; Jagtap 2003); prevents energy decline (Vlassenko et al. 2006) and neuronal apoptosis (Araki et al. 2004; Zeng et al. 2007; Mukherjee et al. 1997). Pyr enriched-diet can prevent neuronal malfunctions and energy metabolism deficiency early Alzheimer's disease-related (Zilberter et al. 2013). Furthermore, Pyr therapy showed clinical benefits in patients with Leigh syndrome, a mitochondrial disorder (Koga et al. 2012). It is conceivable that the combination of creatine and pyruvate can prevent damage in some diseases that alter brain energy metabolism and/or oxidative stress, such as some inborn errors of metabolism. The association of creatine and pyruvate prevented some damage in animal models of phenylketonuria (Berti et al.

2012; dos Reis et al. 2013), tryptofanemia (Andrade et al. 2012) and maternal hyperleucinemia (de Franceschi et al. 2013).

Considering that the mechanisms responsible for the neurological dysfunction in patients with maple syrup urine disease are not fully known and that oxidative stress and deficiency of energy metabolism have been implicated in various inborn errors of metabolism (de Andrade et al. 2015; Gemelli et al. 2013; Mescka et al. 2011; Artuch et al. 2004) and neurodegenerative disorders (Halliwell 2006), in the present study, we investigated the effects of leucine administration and the possible preventive effect of the co-administration of the association of creatine and pyruvate on some parameters of oxidative stress and of some enzymes activities of phosphoryl transfer network in the cerebral cortex and hippocampus of the Wistar rats with 21-days-old.

Materials and Methods

Animals and Reagents

Wistar rats were obtained from the Central Animal House of Biochemistry Department, Institute of Basic Health Sciences, Universidade Federal of Rio Grande do Sul, Porto Alegre, Rio Grande do Sul, Brazil. They were maintained on a 12:12-h light/dark cycle in a room acclimatized at constant temperature (22±1oC), with free access to water and commercial chow. This chow contains 20.5% protein (predominantly soybean supplemented with methionine), 54% carbohydrate, 4.5%, fiber 4%, lipid 7%, and 10% moisture. Animal care followed the ‘‘Principles of Laboratory Animal Care’’ (NIH publication no. 80–23, revised 1996; http://www.nap.edu/readingroom/books/labrats/) and was approved by the UFRGS Ethical Committee. All efforts were made to minimize animal suffering and to use only the number of animals necessary to produce reliable scientific data. All chemicals were purchased from Sigma

Treatment of rats

Rats (n=24) were randomly divided into four groups: saline, leucine, creatine+pyruvate, and leucine+creatine+pyruvate. Before the administration, the solutions were buffered to pH 7.4.The rats received twice a day, at a 12-h interval, during 14 days (from the 8th to the 21st day of life), subcutaneous administration of L-leucine or saline and intraperitoneal administration of creatine+pyruvate or saline.

Doses used were: 4.8 µmol of L-leucine per g of body weight (Pilla et al. 2003); 0.4 mg of creatine per g of body weight (Stokler et al. 1994); 0.2 mg of pyruvate per g of body weight (Ryu et al. 2006). The rats were euthanized at 22 days of age.

Tissue Preparation

The animals were decapitated. The brain was immediately removed, and cerebral cortex and hippocampus were dissected on a glass dish over ice. For the assays of the oxidative stress parameters, the cerebral cortex and hippocampus were homogenized separately with (1:10 w/v) of 20 mM sodium phosphate and 140 mM KCl buffer (pH 7.4) using a Potter-Elvehjem glass homogenizer and centrifuged at 800 x g for 10 min at 4^{o}C. The supernatant was collected and frozen at -70^{o}C for no more than 1 week until a determination of the parameters of oxidative stress and protein.

For assays of the enzymes of phosphoryl transfer network, the cerebral cortex and hippocampus were washed in SET buffer (0.32 M sucrose, 1 mM EGTA, 10 mM Tris–HCl, pH 7.4) and homogenized (1:10 w/v) in SET buffer with a Potter-Elvehjem glass homogenizer. The homogenate was centrifuged at 800xg for 10 min at 4^{o}C. Part of the supernatant was used for determination of AK activity and the rest was centrifuged at 10,000xg for 15 min at 4^{o}C. The supernatant of this second centrifugation, containing cytosol and others cellular components was used for determination of PK and CKcyt activities. The pellet was washed twice with the same SET buffer, resuspended in 100 mM Trizma–15 mM MgSO4–buffer, pH 7.5, and used for determination of CKmit activity. The supernatants were stored for no more than 1 week at -70^{o}C when the assay was not carried out immediately.

Measurements of Oxidative Stress Parameters

Thiobarbituric Acid-Reactive Substances

TBA-RS were measured as described previously by Ohkawa et al. (1979). TBA-RS determines malondialdehyde (MDA) and others dialdehydes, the product of lipoperoxidation caused by hydroxyls free radicals. These free radicals are formed from H_2O_2 by the iron-catalyzed Fenton reaction or by the Haber–Weiss reaction (Kehrer JP, 2000). TBA-RS were expressed as nmol of TBA per mg of protein.

2`7`-Dihydrodichlorofluorescein Oxidation Assay

The production of reactive oxygen and nitrogen species was assessed according to LeBel et al. (1992) by using reduced 2`7`-dihydro dichlorofluorescein diacetate (DCF-DA). Reduced 2`7`-Dichlorofluorescein diacetate (H2DCF-DA) is enzymatically hydrolyzed by intracellular esterases, and the H2DCF formed is oxidized by reactive oxygen species (ROS) or reactive nitrogen species (RNS) presenting in samples to DCF. The DCF fluorescence intensity parallels to some reactive species formed. The content of reactive species was expressed as nmol of DCF formed per mg of protein.

Total Sulfhydryl Content

The sulfhydryl assay is based on the reduction of 5,5-0-dithio-bis (2-nitrobenzoic acid) (DTNB) by sulfhydryls generating TNB, whose absorption is measured spectrophotometrically at 412 nm (Aksenov and Markesbery 2001).The sulfhydryl content is indicative of oxidative damage to proteins. Results were expressed as nmol of TNB per mg of protein.

Superoxide Dismutase (SOD)

SOD is responsible for the dismutation of superoxide free radicals in hydrogen peroxide, a less reactive substance. SOD activity was determined according to Marklund (1985). This assay is based on the capacity of pyrogallol to autoxidize, a process highly dependent on the superoxide radical in the presence of SOD, whose activity can be indirectly assayed spectrophotometrically at 420 nm. The specific activity was expressed as SOD units per mg of protein.

Glutathione Peroxidase

GPx activity was measured according to Wendel (1981) using tert-butyl-hydroperoxide as substrate. One GPx unit is defined as 1 µmol of NADPH consumed per minute, and specific activity was expressed as µmol of NADPH consumed per min per mg of protein.

Measurements of Parameters of Phosphoryltransfer Network

Pyruvate kinase activity

PK activity was assayed as described by Leong et al (1981). The incubation medium consisted of 0.1 M Tris/ HCl buffer, pH 7.5, 10 mM MgCl2 , 0.16 mM NADH, 75 mM KCl, 5.0 mM ADP, 7 U of L-lactate dehydrogenase, 0.1% (v/v) Triton X-100, and 10 ml of the mitochondria-free supernatant in a final volume of 0.5 ml. After 30 min of pre-incubation at 37°C, the reaction was started by the addition of 1.0 mM phosphoenolpyruvate. The oxidation of NADH was followed for 3 min at 340 nm in a spectrophotometer (Spectronic Genesys 8, Spectronic Instruments, Rochester, New York, USA). All assays were performed in duplicate at 25°C. The results were expressed as µmol of pyruvate per min per mg of protein.

Adenylate kinase activity

AK activity was measured with hexokinase (HK) and glucose 6-phosphate dehydrogenase (G6PD), according to Dzeja et al (1999). The reaction assay contained 100 mM KCl, 20 mM HEPES, 4 mM MgCl2, 20 mM glucose, 1 mM EDTA, 2 mM NADP+, 4.5 U/ml of HK, 2 U/mL of G6PD, and 1 µg of protein homogenate. After 0–90 min of pre-incubation at 37°C, the reaction was initiated by the addition of 2 mM ADP. The reduction of NADP+ was followed for 3 min at 340 nm in a spectrophotometer (Spectronic Genesys 8, Spectronic Instruments, Rochester, New York, USA). ADP, NADP+, G6PD, and HK were dissolved in water. The reagents concentration and the assay time (3 min) were chosen to assure the linearity of the reaction. GSH did not interfere with HK or G6PD activities or with spectrophotometric readings. The results were expressed in µmol of ATP formed per min per mg of protein.

Creatine kinase activity

CK activity was assayed in the reaction mixture contained the following final concentrations: 65 mM Tris–HCl buffer, pH 7.5, 7.0 mM phosphocreatine, 9.0 mM MgSO4, and 1 µg of protein in a final volume of 0.1 ml. After 5 min of pre-incubation at 37°C, the reaction was started by the addition of 0.3 µmol of ADP. The reaction was stopped after 10 min by the addition of 1 µmol of p-hydroxymercuribenzoic acid. The incubation time and the reagent concentrations were chosen to assure linearity of the enzymatic reaction. Appropriate controls were carried out to measure chemical hydrolysis of phosphocreatine. The creatine formed was determined according to the method of Hughes (1962). The color was developed by the addition of 0.1 ml 2% α-

naphthol and 0.1 ml 0.05% diacetyl in a final volume of 1 ml and read after 20 min at 540 nm. None of the substances added to the assay medium interfered with the color development or spectrophotometer readings. Results were expressed as μmol of creatine formed per min per mg of protein.

Protein Determination

The protein content of hippocampus or cerebral cortex homogenates was determined by the method of Lowry et al. (1951), using bovine serum albumin as the standard.

Statistical Analysis

Data were expressed as mean±SD and were analyzed by two-way ANOVA (leucine and creatine+pyruvate as factors). When appropriate, the comparison between means was analyzed by the Tukey test when the F value was significant. Correlation between some parameters of oxidative stress and the activities of the enzymes of the phosphoryl transfer network was performed by the Spearman's correlation coefficient. Data were analyzed by the Statistical Package for the Social Sciences software (SPSS 20.0 for Windows). P values lower than 0.05 were considered significant.

Results

Data of the experiments are showed in Table 1 and the Spearman's correlation coefficient in Table 2. The effect of the treatments on the generation of reactive species was assessed by DCFH oxidation. Leucine administration increased reactive species formation in the cerebral cortex (11%) and hippocampus (11%). A significant interaction between leucine and creatine plus pyruvate in the cerebral cortex [$F(1,20) = 28.1$; $\rho<0.05$] and the hippocampus [$F(1,20) = 6.18$; $\rho<0.05$] indicated that co-administration of leucine and creatine plus pyruvate prevented reactive species formation.

The effect of the treatments on lipid damage was evaluated by TBARS levels. Leucine administration caused a significant increase in the cerebral cortex (13%) and hippocampus (13%). Two-way ANOVA showed a significant interaction between leucine and creatine plus pyruvate in the cerebral cortex [$F(1,20) = 5.5$; $\rho<0.05$] and in

the hippocampus [$F(1,20) = 5.8$; $\rho<0.05$], indicating that co-administration of creatine plus pyruvate was effective to prevent the lipid peroxidation.

Reduced power was assessed by total sulfhydryls content. Leucine administration increased the sulfhydryls content significantly in the cerebral cortex (13%) and hippocampus (12%). Significant interaction between leucine and creatine plus pyruvate showed that and co-administration of creatine plus pyruvate prevented this increase in cerebral cortex [$F(1,20) = 5.6$; $\rho<0.05$] and hippocampus [$F(1,20) = 13.5$; $\rho<0.05$].

In respect to the antioxidant enzymes, GPx and SOD activities were evaluated. GPx activity was reduced by leucine administration in the cerebral cortex (20%) and hippocampus (20%) of the rats. However, SOD activity was significantly decreased only in the cerebral cortex (17%) by leucine administration. The significant interaction between leucine and creatine plus pyruvate indicated that the diminution of the activities of GPx in the cortex [$F(1,20) = 4.9$; $\rho<0.05$] and hippocampus [$F(1,20) = 32.1$; $\rho<0.05$] and SOD in the cerebral cortex [$F(1,20) = 4.2$; $\rho<0.05$] were prevented by co-administration of creatine plus pyruvate .

Thiol-containing enzymes of the phosphoryl transfer network were also evaluated. Chronic administration of leucine decreased the activities of AK (18%), CKcyt (20%) and CKmit (28%) in the brain cortex and hippocampus AK (36%), CKcyt (20%) and CKmit (27%). However, PK activity was diminished only in the hippocampus (31%). Significant interactions between leucine and creatine plus pyruvate were found for AK activity in the cortex [$F(1,20) = 11.1$; $\rho<0.05$] and hippocampus [$F(1,20) = 8.9$; $\rho<0.05$]; for CKcyt in the cortex [$F(1,20) = 6.7$; $\rho<0.05$] and hippocampus [$F(1,20) = 4.7$; $\rho<0.05$]; for CKmit in the cerebral cortex [$F(1,20) = 8.7$; $\rho<0.05$] and hippocampus [$F(1,20) = 5.3$; $\rho<0.05$]; for PK in the hippocampus [$F(1,20) = 5.3$; $\rho<0.05$]. These interactions indicated that co-administration of creatine plus pyruvate prevented the reduction of the enzymes activities. Spearman's correlation coefficients between DCFH oxidation and total sulfhydryl content were 0.598 ($p<0.05$) for the cerebral cortex and 0.491 ($p<0.05$) for the hippocampus, suggesting that the diminution of sulfhydryl content could be due to the oxidation of the thiol groups by reactive species. On the other hand, Spearman's correlation coefficients between DCFH oxidation or total sulfhydryl content and the enzymes activities of the phosphoryl

transfer network were significant, except for PK activity in the cerebral cortex (Table 2).

Discussion

Animal models are only partially similar to human diseases in all its complexity. However, chemical animal models have been used because they have the advantage of studying every substance known to accumulate in human disease against adequate control. Therefore, animal models are important in the investigation of pathophysiologic mechanisms of the diseases, helping to suggest preventive measures and new drugs for treatment (Skvorak 2009).

Leucine and its keto acid (KIC) may achieve up to 5 mM in the plasma of MSUD patients and are considered the main neurotoxic metabolites (Chuang and Shih, 2001). However, KIC is rapidly taken up by neurons and actively transaminated to Leu, increasing the concentration of this amino acid in the brain tissue (Yudkoff, 1997). In the present investigation, the rats present normal BCKD activity, indicating that the others amino acids and keto acids have normal levels. Therefore, the observed effects of Leu administration are possible due to this amino acid.

Leucine administration induces oxidative damage and inhibits critical enzymes of metabolism cellular in the cerebral cortex of rats (Bridi et al. 2005) and offspring of maternal hyperleucinemia (de Francheschi et al. 2013). Therefore, we investigated the possible effects of chronic administration of leucine on the enzymes of phosphoryl transfer network that are crucial for energy homeostasis and oxidative stress parameters in cerebral cortex and hippocampus from 21-day-old Wistar rats. Moreover, we investigated the possible preventive effect of the co-administration of creatine plus pyruvate, two important energetic and antioxidants substances, on these parameters.

The oxidative stress is an imbalance between the generation of reactive species and antioxidant mechanisms in favor for the former, causing excessive oxidative damage (Halliwell and Gutteridge, 2015). The reactive species may contribute significantly to the neurodegenerative diseases (Halliwell 2013). The brain is particularly vulnerable to oxidative stress, due to its high rate of oxidative metabolism, the high content of polyunsaturated compounds, and the moderate levels of antioxidant defenses compared with other tissue (Halliwell and Gutteridge, 1996; Halliwell, 1999). We observed an increased in DCFH oxidation, suggesting that leucine provokes an increase in the reactive species generation in cerebral cortex and hippocampus. Co-

administration of creatine plus pyruvate prevents this increase. The influence of leucine administration on lipid oxidation was investigated by TBA-RS determination. TBA-RS reflect the content of malondialdehyde, the most abundant individual aldehyde resulting from lipid peroxidation (Esterbauer and Cheeseman 1990). We have demonstrated that leucine can increase TBA-RS levels in cerebral cortex and hippocampus of rats, suggesting that oxidative damage to lipids has occurred. Moreover, co-administration of creatine plus pyruvate prevented the lipid peroxidation.

Leucine administration was able to increase total thiol levels in the hippocampus. This increase can be explained by the increase of reactive species that react with disulfide bonds (S-S) in proteins, as protein sulfhydryl content is a useful index of protein oxidation (Stadtman 2003) or to increase in GSH synthesis since oxidative stress is the major stimulus for GSH synthesis (Lu 2009). Moreover, co-administration of creatine plus pyruvate was able to prevent the increase of total thiol levels.

Regarding the activities of antioxidant enzymes, SOD plays a vital role in the protection of aerobic cells against the effects of superoxide anion, catalyzing the transfer of superoxide free radicals to hydrogen peroxide (H_2O_2) (Marklund 1985), which in turn is removed by GPx by coupling the oxidation reaction of GSH (Leong 1981). In the cerebral cortex, the administration of leucine diminished SOD activity, which may suggest higher superoxide radical, since this radical inhibits SOD activity, and decreases GPx activity, suggesting high liberation of H_2O_2. In this context, it is possible that hydroxyl radical, the most toxic in vivo reactive specie, might be formed via the Haber-Weiss/Fenton reaction (Change 1979; Halliwell 2013). On the other hand, GPx activity was reduced by leucine administration in the two tissues, probably because anion superoxide can inhibit this enzyme activity (Macedo 2013). Moreover, co-administration of creatine plus pyruvate prevented these alterations.

We evaluated the activity of four kinases of phosphoryltransfer network: cytosolic creatine kinase (CKcyt), mitochondrial creatine kinase (CKmit), adenylate kinase (AK) and pyruvate kinase (PK). This enzymatic network is important to support high-energy phosphoryl transfer between ATP-generating and ATP-consuming processes cellular. (Wallimann et al. 1992; Dzeja and Terzic 1998). This dynamic metabolic signaling maintains the balance between cellular ATP consumption and production, maintaining the energetic homeostasis for preserving cell survival (Dzeja

and Terzic 2003; Dzeja et al. 2000). The creatine/phosphocreatine/creatine kinase (Cr/PCr/CK) system plays essential roles to maintain the high energy levels necessary for central nervous system (Wyss 2000). The deficit in the central nervous system energy metabolism play critical role in the progression of neurological diseases (Beal 2000; Chaturvedi 2008). AK is the main responsible for the enzymatic phosphoryl transfer network, catalyzing the reversible transfer of phosphate between ATP and AMP from mitochondria to the cytosol (Dzeja and Terzic 2003; Price et al. 1975). PK is a key enzyme of glucose metabolism, catalyzing the irreversible conversion of PEP to pyruvate and forming ATP from ADP (Valentini et al. 2000).

We observed that AK, CKcyt, and CKmit activities were inhibited by leucine administration in the cerebral cortex and hippocampus, and PK in the hippocampus. Moreover, co-administration of creatine plus pyruvate was able to prevent the inhibition of the kinases in cerebral cortex and hippocampus.

A diminished activity of a single enzyme of the phosphoryl transfer network may be compensated by the others. However, a decrease in the activity of two or more enzymes of this network could lead to a significant alteration in the communication between ATP-generating and ATP-consuming sites (Dzeja et al. 2000). Kinases are enzymes containing thiol groups in their structure, may alter the activities by oxidative stress (Gilbert 1984). For example, CK activity can be inactivated by anion superoxide (Wendt et al. 2003) and PK activity can be inactivated by hydrogen peroxide (Halliwell and Gutteridge 2007). Several amino acids can induce oxidative stress (Halliwell and Gutteridge, 2015). The amino acids cystine (Feksa et al. 2004; Figueiredo et al. 2009; Fleck 2005), phenylalanine (Feksa et al. 2003; Costabeber et al. 2003), tyrosine (de Andrade et al. 2012) and β-alanine (Gemelli et al. 2013) also inhibit these kinases, possibly by oxidation of the sulfhydryl groups of the enzymes. In this study, a significant correlation was found between the formation of reactive species (DCFH oxidation) and the inhibition of the kinases (except for PK in cerebral cortex). Besides, we found a significant correlation between total sufhydryl content and the inhibition of the kinases. These data suggest that it is possible that the inhibition of the kinases activities might occur through the action of the reactive species on the thiol groups of the kinases. Therefore, it is possible that the induction of oxidative stress and the consequent alteration of the energy homeostasis may significantly contribute to the brain damage observed in many inborn errors of the metabolism of the amino acids.

Therefore, the inhibition of enzymes activities in the phosphoryltransfer network is possibly caused by oxidative stress induced by administration of leucine. Alteration of energy homeostasis and oxidative stress may induce apoptosis and brain cells death (Burlacu et al. 2001; Park and Thoene 2005). Moreover, co-administration of creatine plus pyruvate prevented the effects elicited by leucine administration on oxidative stress and phosphoryl transfer network parameters in cerebral cortex and hippocampus, suggesting that creatine and pyruvate acted as antioxidants and energy-supplying substances. The antioxidant properties of creatine may result from different mechanisms of action: direct scavenging of radical species, iron chelation (Halliwell 2001), and amelioration of cellular energy charge (Persky and Brazeau 2001). Creatine supplementation has been effective in a variety of animal/cellular models of neurodegenerative diseases, such as Alzheimer's, Parkinson's, and Huntington's disease (Klein and Ferrante 2007). Pyruvate is scavenging of H_2O_2, protects from oxidative stress and mitochondrial insult because of it is an effective neuronal energy substrate from glycolysis and acts as a powerful antioxidant (Mazzio and Soliman 2003). Besides, Pyruvate protects neurons by normalizing cellular energy charge (Andrae et al. 1985).

In conclusion, more studies are needed to elucidate if these mechanisms are involved in the neurological dysfunction and toxicity of leucine presented by patients with MSUD and the possible benefits of creatine plus pyruvate supplementation as a potential adjuvant therapy for this disease.

All procedures performed in studies involving animals were by the ethical standards of the institution or practice at which the studies were conducted.

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Table 1

Effect of leucine and creatine plus pyruvate administration on parameters of oxidative stress and enzymes activities of phosphoryl transfer network in cerebral cortex and hippocampus of rats.

Groups	saline	creatine+pyruvate	leucine	leucine+creatine +pyruvate
CEREBRAL CORTEX				
DCFH	29.4 ± 0.6	31.7 ± 2.1	33.2 ± 0.9*	29.8 ± 1.2
TBARS	1.4 ± 0.2	1.5 ± 0.1	1.8 ± 0.2*	1.6 ± 0.9
Total Sulfhydryls	44.8 ± 5.4	44.7 ± 4.6	56.8 ± 5.9*	46.8 ± 5.5
GPx	7.8 ± 0.3	7.6 ± 0.7	6.3 ± 0.9*	7.3 ± 0.5
SOD	13.4 ± 1.4	13.1 ± 1.1	11.1 ± 0.5*	13.1 ± 1.1
CK mit	1.8 ± 0.2	1.7 ± 0.2	1.3 ± 0.2*	1.8 ± 0.4
CK cyt	2.8 ± 0.4	2.8 ± 0.3	2.2 ± 0.2*	3.1 ± 0.5
AK	1.7 ± 0.4	1.8 ± 0.3	1.4 ± 0.1*	1.9 ± 0.1
PK	1.3 ± 0.3	1.2 ± 0.4	1.1 ± 0.3	1.2 ± 0.3
HIPPOCAMPUS				
DCFH	41.2 ± 2.9	39.1 ± 2.1	45.9 ± 1.1*	40.2 ± 0.5
BARS	1.6 ± 0.1	1.7 ± 0.2	2.1 ± 0.2*	1.7 ± 0.2
Total Sulfhydryls	49.5 ± 3.7	52.1 ± 2.2	59.5 ± 4.4*	49.2 ± 5.6
GPx	10.5 ± 0.9	9.8 ± 0.4	8.4 ± 0.8*	11.2 ± 0.8
SOD	11.6 ± 1.4	12.1 ± 1.1	11.1 ± 1.1	11.6 ± 0.9
CK mit	1.5 ± 0.2	1.5 ± 0.2	1.1 ± 0.2*	1.4 ± 0.3
CK cyt	3.4 ± 0.2	3.5 ± 0.4	2.7 ± 0.4*	3.3 ± 0.3
AK	1.1 ± 0.3	0.9 ± 0.1	0.7 ± 0.1*	1 ± 0.1
PK	1.7 ± 0.3	1.6 ± 0.3	1.1 ± 0.2*	1.7 ± 0.4

Data are expressed as mean ± SD for 6 animals per group. *$\rho<0.05$ compared to the other groups (two way ANOVA followed by the Tukey test). Abbreviations: DCFH (2`7`-dihydrodichlorofluorescein oxidation assay) expressed as nmol of DCF per mg of protein; TBARS (thiobarbituric acid reactive substances) expressed as nmol per mg of protein; Sulfhydryl content expressed as nmol of TNB per mg of protein; GPx (glutathione peroxidase) expressed as μmol of NADPH consumed per min per mg of protein; SOD (superoxide dismutase) expressed as SOD U per mg of protein; CKcyt (cytosolic creatine kinase) and CKmit (mitochondrial creatine kinase) expressed as μmol of creatine formed per min per mg of protein; PK (pyruvate kinase) expressed as μmol of pyruvate formed per min per mg of protein; AK (adenylate kinase), expressed as μmol of ATP formed per min per mg of protein.

Table 2

Spearman's correlation coefficient between oxidative stress parameters and enzymes activities of phosphoryl transfer network in cerebral cortex and hippocampus of rats.

Groups	DCFH	Total Sulfhydryl
Cerebral cortex		
PK	.303	.433*
AK	.461*	.407*
CKcyt	.552*	.559*
CKmit	.492*	.429*
Hippocampus		
PK	.475*	.420*
AK	.615*	.599*
CKcyt	.601*	.473*
CKmit	.427*	.604*

*$\rho<0.05$ for Spearman's correlation coefficient. Abbreviations: DCFH (2`7`-dihydrodichlorofluorescein; PK (pyruvate kinase); AK (adenylate kinase).CKcyt (cytosolic creatine kinase); CKmit (mitochondrial creatine kinase);

II. CAPÍTULO 2 – Artigo 2

Taurine prevents the effects of leucine administration to Wistar rats on oxidative stress parameters and enzymes activities of phosphoryl transfer network in Wistar rats

Elenara Rieger, Itiane Diehl de Franceschi, Vanessa Trindade Bortoluzzi, Clovis Milton Duval Wannmacher

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Taurine prevents the effects of leucine administration on oxidative stress parameters and enzymes activities of phosphoryl transfer network in Wistar rats

Elenara Rieger, Itiane Diehl de Franceschi, Vanessa Trindade Bortoluzzi, Clovis Milton Duval Wannmacher

Departamento de Bioquímica, Instituto de Ciências Básicas da Saúde, Universidade Federal do Rio Grande do Sul, Porto Alegre, Rua Ramiro Barcelos 2600, CEP 90.035-003, RS, Brazil

Corresponding author: Clovis Milton Duval Wannmacher, Universidade Federal do Rio Grande do Sul, Instituto de Ciências Básicas da Saúde, Departamento de Bioquímica. Rua Ramiro Barcelos 2600-Anexo - Porto Alegre – RS, Brasil, 90035-003. Tel: 555133085575; E-mail: clovisdw@ufrgs.br

Running title: taurine prevents changes in redox state and energy metabolism provoked by leucine

Abstract

The affected patients of Maple Syrup Urine Disease present psychomotor development delay and mental retardation. The pathophysiology of this disease is not entirely understood. However, leucine seems to be the main neurotoxic metabolite. Taurine is an amino acid that plays a critical role in brain function and is a potent antioxidant in biological systems, contributing to cellular protection against oxidative stress. In this study, we investigated the effects of leucine administration and coadministration of taurine on some parameters of phosphoryl transfer network and oxidative stress in cerebral cortex and hippocampus of Wistar rats. The animals were treated from the 8th to the 21st postpartum day. Leucine induced oxidative stress and diminished the activities of pyruvate kinase, adenylate kinase, hexokinase, glyceraldehyde-3-phosphate dehydrogenase, cytosolic creatine kinase, and mitochondrial creatine kinase. Coadministration of taurine prevented the alterations provoked by leucine administration on the oxidative stress and the thiol-containing enzymes of phosphoryl transfer network. Significant correlations between some parameters of oxidative stress and the enzymes of the phosphoryl transfer network suggest that the diminution of the enzymes activities is possible due to oxidative stress. It is possible that these effects of leucine may contribute to the neurological dysfunction observed in patients affected by Maple Syrup Urine Disease. In this case, it is possible that taurine supplementation could benefit to the patients.

Keyword: taurine, oxidative stress, leucine, phosphoryl transfer network, MSUD.

1. Introduction

Maple Syrup Urine Disease (MSUD) or branched-chain α-keto aciduria is an autosomal recessive metabolic disease due to 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 (Leu), valine (Val), and isoleucine (Ile), as well as their corresponding branched-chain α-keto acids (BCKA) α-ketoisocaproic acid, α-keto-β-methyl valeric acid and α-ketoisovaleric acid, respectively [1]. The major clinical features presented by MSUD patients include ketoacidosis, apnea, hypoglycemia, coma, ataxia, mental retardation, and psychomotor delay. Edema and hypomyelination/demyelination in central nervous system (CNS) of MSUD patients are common findings [1; 2]. MSUD presents heterogeneous molecular and clinical phenotypes ranging from a severe classic form with neonatal onset to milder variants forms with later onset, and showing different residual enzyme activity [1]. The treatment of the patients consists in to keep the BCAA plasma concentrations in the normal range, protecting the brain from damage. The treatment consisted of a low protein diet, restriction in BCAA and supplementation with essential amino acids. Metabolic intoxication may lead to a fatal outcome in untreated patients [1; 3].

The pathomechanisms of the neurological symptoms presented by MSUD patients are still poorly understood. However, considering that increased concentrations of leucine and α-ketoisocaproate were associated with the appearance of neurological symptoms, these compounds seem to be the primary important neurotoxic metabolites in MSUD [1; 4]. It has been demonstrated that the substances accumulating in MSUD cause impairment of energy metabolism. This impairment is partially caused by inhibiting the electron transport chain [5] creatine kinase (CK) and pyruvate kinase (PK) activities in rat brain [6, 7]. CK activity is severely reduced in some neurodegenerative diseases [8], and this inhibition may lead to the neuronal loss in the brain [9]. Inhibition of PK activity could reduce pyruvate content, an important antioxidant agent [10]. Other investigators demonstrated that the BCAA and BCKA that accumulate in MSUD provoke neuronal apoptosis [11], as well as impairment of neurotransmitter synthesis and function [12, 13]. Besides, reduced uptake of essential amino acids by the brain is observed [14].

Free radicals and oxidative stress seem to be involved in a large number of human diseases. Increased evidence has shown that damage caused by free radicals is an important contributing factor in chronic-inflammatory, vascular, neoplastic and neurodegenerative diseases [15, 16, 17]. As well as in some inborn errors of intermediary metabolism owing to the accumulation of toxic metabolites, this leads to excessive free radical production. Restricted diets utilized to treat patients affected by metabolic disorders may result in a low antioxidant status [18].

Studies demonstrated that the leucine, not only stimulates *in vitro* lipid peroxidation, but also reduces antioxidant defenses and the cerebral capacity to modulate the damage associated with the increased free radical production [19, 20]. Furthermore, it was shown that the increased lipid peroxidation induced by leucine could be attenuated by the free radicals scavengers ascorbic acid, α-tocopherol, glutathione and superoxide dismutase [21]. Leucine administration to female Wistar rats during pregnancy and lactation induces oxidative stress and alters parameters of phosphoryl transfer network in the brain cortex and hippocampus of the offspring [22]. Leucine also causes DNA damage via free radical production [23] and oxidative damage was observed in plasma [24, 25, 26, 27, 28] and urine of MSUD patients [29].

Taurine is an amino acid involved in several physiological processes like bile acid conjugation, osmoregulation, cell membrane stabilization detoxification of xenobiotics, modulation of cellular calcium flux and of neuronal excitability [30], cellular homeostasis, ischemia/reperfusion states, humoral defense [31], normal development of the brain and retina [32]. In the central nervous system (CNS), taurine plays a critical role in brain function, being implicated in cell volume regulation and also in neuromodulation or inhibitory neurotransmission [33]. Moreover, taurine is considered as an antioxidant in biological systems, contributing to cellular protection against oxidative stress. This protection is attributed to its ability to scavenger reactive oxygen species (ROS), protecting from lipid peroxidation and sparing sulfhydryl groups from oxidation [34, 35, 36]. Besides, taurine protects mitochondrial enzymes from the action of free radicals [37].

Leucine is neurotoxic by its ability to induce oxidative stress and alters parameters of phosphoryl transfer network in the brain homogenates of rats. Besides, the restricted diets used to treat patients with metabolic disorders may decrease the tissue antioxidant defenses due to depletion of essential nutrients. Therefore, in the

present work, we investigated the effects of leucine administration and the possible preventive effect of the co-administration of taurine on some parameters of oxidative stress and enzymatic phosphoryl transfer network (PK, AK, HK, CK and GAPDH activities) in the cerebral cortex and hippocampus homogenates of young rats.

2. Materials and methods

2.1 Animals

Wistar rats were obtained from the Central Animal House of Biochemistry Department, Institute of Basic Health Sciences, Universidade Federal of Rio Grande do Sul, Porto Alegre, Rio Grande do Sul, Brazil. The animals were maintained on a 12:12-h light/dark cycle in a room acclimatized at constant temperature (22±1^{o}C). They had free access to water and commercial chow containing 20.5% protein (predominantly soybean supplemented with methionine), 54% carbohydrate, 4.5%, fiber 4%, lipid 7%, and 10% moisture. Animal care followed the ‘‘Principles of Laboratory Animal Care’’ (NIH publication no. 80–23, revised 1996; http://www.nap.edu/readingroom/books/labrats/) and was approved by the UFRGS Ethical Committee. All efforts were made to minimize animal suffering and to use only the number of animals necessary to produce reliable scientific data. All chemicals were purchased from Sigma

2. Treatment of the rats

Wistar rats (n=28) were randomly divided into four groups: saline, leucine, taurine, and leucine+taurine. Solutions were buffered to pH 7.4 immediately before administration. Rats received twice a day at 12-h interval, during 14 days (from the 8th to the 21st day of life), subcutaneous administration of L-leucine or saline or intraperitoneal administration of taurine or saline. Doses used were: 4.8 μmol of L-leucine per g of body weight [6]; 1.6 μmol of taurine per g of body weight [38]. Rats were euthanized at 22 days of age, and the cerebral cortex and hipocamppus homogenate were collected for evaluation of oxidative stress and energy metabolism parameters.

2.3 Tissue Preparation

Treated animals were sacrificed by decapitation without anesthesia, and the brain was immediately removed, and cerebral cortex and hippocampus were dissected on a glass dish over ice. The cerebellum, olfactory bulbs, and pons/medulla were discarded. For

the measurements of the oxidative stress parameters, the cerebral cortex and hippocampus were homogenized separately with (1:10 w/v) of 20 mM sodium phosphate and 140 mM KCl buffer (pH 7.4) using a Potter-Elvehjem glass homogenizer and centrifuged at 800 x g for 10 min at 4^{o}C. The supernatant was collected and frozen at -70^{o}C for no more than 1 week until a determination of the parameters of oxidative stress and protein.

For assays of the enzymes of phosphoryl transfer network, the cerebral cortex and hippocampus were washed in SET buffer (0.32 M sucrose, 1 mM EGTA, 10 mM Tris–HCl, pH 7.4) and homogenized (1:10 w/v) in the same SET buffer with a Potter-Elvehjem glass homogenizer. The homogenate was centrifuged at 800 x g for 10 min at 4^{o}C. Part of the supernatant was used for determination of AK, GAPDH and HK activities; the pellet was discarded, and the rest of the supernatant was centrifuged at 10,000 x g for 15 min at 4^{o}C. The supernatant of this second centrifugation, containing cytosol and others cellular components as endoplasmatic reticulum, was collected for determination of PK and cytosolic CK activity. The pellet, containing mitochondria, myelin, synaptosomes and membrane fragments was washed twice with the same SET buffer, resuspended in 100 mM Trizma–15 mM MgSO4–buffer, pH 7.5, for determination of mitochondrial CK activity. The supernatants were stored for no more than 1 week at -70^{o}C when the assay was not carried out immediately.

2.4 Measurements of Oxidative Stress Parameters

2.4.1 Thiobarbituric Acid-Reactive Substances

TBA-RS were measured as described previously by Ohkawa et al. [39] with slight modifications. TBA-RS measures mainly malondialdehyde (MDA), a product of lipoperoxidation caused mostly by hydroxyls free radicals. Hydroxyl free radicals are mainly formed from H_2O_2 by the iron-catalyzed Fenton reaction or by the Haber–Weiss reaction [40]. Briefly, to glass tubes samples and reagents were added in the following order: 200 µL of tissue supernatant, 50 µL of SDS 8.1 %, 375 µL of 20 % acetic acid in aqueous solution (v/v), pH 3.5; and 375 µL of 0.8 % thiobarbituric acid. The mixture was mixed, and the reaction was carried out in a boiling water bath for 1-h. The mixture was allowed to cool on water for 5 min and was centrifuged at 850 x g for 10 min. The

resulting pink-stained TBA-RS obtained were determined spectrophotometrically at 532 nm. TBA-RS were represented as nmol/mg protein.

2.4.2 2`7`-Dihydrodichlorofluorescein oxidation assay

The production of reactive oxygen and nitrogen species was assessed according to LeBel et al. [41] by using reduced 2`7`-dihydro dichloro fluorescein diacetate (DCF-DA). Briefly, 30 µL samples were incubated for 30 min at 37^{o}C in the dark with 30 µL of 20 mM sodium phosphate buffer pH 7.4 with 140 mM KCl and 240 µL of 100 µM reduced 2`7`-Dichlorofluorescein diacetate (H_2DCF-DA) solution in a 96 wells plate. H_2DCF-DA is enzymatically hydrolyzed by intracellular esterases, and the H_2DCF formed is oxidized by reactive oxygen species (ROS) or reactive nitrogen species (RNS) presenting in samples to DCF. The DCF fluorescence intensity parallels to the number of reactive species formed. Fluorescence was measured using excitation and emission wavelengths of 480 and 535 nm, respectively. A calibration curve was performed with standard DCF (0.25–10 µM), and the levels of reactive species were expressed as nmol of DCF formed per mg of protein.

2.4.3 Total sulfhydryl content

The sulfhydryl assay is based on the reduction of 5,5-0-dithio-bis (2-nitrobenzoic acid) (DTNB) by thiols generating a yellow derivative (TNB) whose absorption is measured spectrophotometrically at 412 nm [42].The sulfhydryl content is usually inversely correlated to oxidative damage to proteins. Results were calculated as nmol of TNB per mg of protein.

2.4.4 Superoxide dismutase (SOD)

SOD is responsible for the dismutation of superoxide free radicals in hydrogen peroxide, a less reactive substance. SOD activity was determined as described by Marklund [43]. This assay is based on the capacity of pyrogallol to autoxidize, a process highly dependent on superoxide radical. The inhibition of autoxidation of this compound occurs in the presence of SOD, whose activity can be indirectly assayed spectrophotometrically at 420 nm. A calibration curve was performed with purified SOD as standard. A 50% inhibition of pyrogallol autoxidation is defined as one unit of SOD, and the specific activity was expressed as SOD units per mg of protein.

2.4.5 Glutathione peroxidase (GPx)

GPx activity was measured according to Wendel [44] using tert-butyl-hydroperoxide as substrate. The enzyme activity was determined by monitoring the nicotinamide adenosine dinucleotide phosphate (NADPH) disappearance at 340 nm in a medium containing 100 mM potassium phosphate buffer/1 mM ethylene diamine tetra acetic acid (EDTA), pH 7.7, 2 mM glutathione, 0.15 U/mL glutathione reductase, 0.4 mM azide, 0.5 mM tert-butyl-hydroperoxide, 0.1 mM NADPH, and 10 µL supernatants. One GPx unit is defined as 1 µmol of NADPH consumed per minute, and specific activity was expressed as micromole of NADPH consumed per minute per mg of protein.

2.4.6 Reduced glutathione (GSH) content

The most important non-enzymatic antioxidant present in the cells is glutathione (GSH). GSH reduces peroxides, mainly through GPx activity, scavengers superoxide and hydroxyl radicals, and regenerates oxidized C vitamin. GSH levels were measured according to Browne and Armstrong [45] which is based on the reaction of GSH with the fluorophore ophtalaldehyde (OPT) after the sample be deproteinized with meta-phosphoric acid. Then, 150 uL of the samples were incubated with an equal volume of o-phthaldialdehyde (1 mg/ml of methanol) for 15 min at 37^{o}C. After that, fluorescence was measured using excitation of 350 nm and emission of 420 nm wavelengths. A calibration curve was performed with standard GSH (1 mM), and the tissue supernatant concentrations were expressed as nmol of GSH per mg of protein.

2.5 Measurements of Parameters of Phosphoryl Transfer Network

2.5.1 Creatine kinase activity

CK activity was assayed in the reaction mixture contained the following final concentrations: 65 mM Tris–HCl buffer, pH 7.5, 7 mM phosphocreatine (PCr), 9 mM MgSO4, and 1 µg of protein in a final volume of 0.1 mL. After 5 min of pre-incubation at 37^{o}C, the reaction was started by the addition of 0.3 µmol of ADP. The reaction was stopped after 10 min by the addition of 1 µmol of ρ-hydroxymercuribenzoic acid. The reagent concentrations and the incubation time were defined to assure linearity of the enzymatic reaction. Controls were carried out to discount chemical hydrolysis of PCr. The creatine (Cr) formed was estimated according to the colorimetric method of Hughes [46]. The color was developed by the addition of 0.1 mL 2 % α-naphthol and 0.1 mL

0.05 % diacetyl in a final volume of 1 mL and read after 20 min at 540 nm. Results were expressed as µmol of creatine formed per min per mg of protein.

2.5.2 Pyruvate kinase activity

PK activity was assayed as described by Leong [47]. The incubation medium consisted of 0.1 M Tris/HCl buffer, pH 7.5, 10 mM MgCl, 0.16 mM NADH, 75 mM KCl, 5.0 mM ADP, 7 units of L-lactate dehydrogenase, 0.1% (v/v) Triton X-100, and 10 µL of the mitochondrial- free supernatant in a final volume of 0.5 mL. After 30 min of pre-incubation at 37^{o}C, the reaction was started by the addition of 1 mM phosphoenolpyruvate (PEP). All assays were performed in duplicate at 25^{o}C. Results were expressed as µmol of pyruvate formed per min per mg of protein.

2.5.3 Adenylate kinase activity

AK activity was measured with a coupled enzyme assay with hexokinase (HK) and glucose 6-phosphate dehydrogenase (G6PD), according to Dzeja [48]. The reaction mixture contained 100 mM KCl, 20 mM HEPES, 20 mM glucose, 4 mM MgCl2, 2 mM NADP+, 1 mM EDTA, 4.5 U/mL of HK, 2 U/mL of G6PD, and 1 µg of protein homogenate. The reaction was initiated by the addition of 2 mM ADP, and the reduction of $NADP^{+}$ was followed at 340 nm for 3 min in a spectrophotometer. ADP, NADP+, G6PD, and HK were dissolved in water. Reagents concentration and assay time (3 min) were chosen to assure the linearity of the reaction. The results were expressed in µmol of ATP formed per min per mg of protein.

2.5.4 Hexokinase activity

The activity of mitochondrial bound hexokinase was determined based on a previously described method with minor modifications [49]. The mitochondrial protein used in this assay varied from 0.03 to 0.08 mg/mL. The activity of hexokinase was determined by NADH formation, following the absorbance at 340 nm. The assay contained 10 mM Tris–HCl, pH 7.4, 10 mM MgCl2, 5 mM D-glucose, 1 mM ATP, 1 mM NAD^{+} , 1 unit/mL Glc-6-P dehydrogenase (Leuconostoc mesenteroides), and 0.1 mM Ap5A as an inhibitor of AK, in a final volume of 1 mL. The reaction temperature was 37°C. Results were expressed as µmol of NADH per min/mg protein.

2.5.5 Glyceraldehyde 3-fosfate dehydrogenase activity

GAPDH activity was measured as described by Mazzola and Sirover with minor modifications [50]. Reaction mixtures contained 2.2 mM glyceraldehyde-3-phosphate, 0.25 mM NAD^+ , and buffer medium, pH 8.9 (100 mM glycine, 100 mM potassium phosphate monobasic, and 5 mM EDTA). The sample was mixed and preincubated for 15 min at room temperature before inclusion of glyceraldehyde-3-phosphate in the reaction mixture. The activity of GAPDH was determined by NADH formation following the absorbance at 340 nm. Assays were performed in triplicate. Results were expressed as μmol of NADH per min/mg protein.

2.6 Protein determination

The protein content of hippocampus or cerebral cortex homogenates was determined by the method of Lowry et al. [51], using serum bovine albumin as the standard.

2.7 Statistical analysis

Data were expressed as mean±SD and were analyzed by two-way ANOVA (leucine and taurine as factors). When appropriate, the comparison between means was analyzed by the Tukey test when the F value was significant. Correlation between some parameters of oxidative stress and the activities of the enzymes of the phosphoryl transfer network was performed by the Spearman's correlation coefficient. All data were analyzed by the Statistical Package for the Social Sciences software (SPSS 20.0 for Windows). Values were considered significant when $p<0.05$.

3. Results

The effect of treatments on the generation of reactive species was assessed by DCFH oxidation. Leucine administration increases reactive species formation in the cerebral cortex and hippocampus. Two-way ANOVA showed a significant interaction between leucine and taurine in the cerebral cortex [$F(1,25) = 23.01$; $\rho<0.05$] (Table 1) and hippocampus [$F(1,25) = 9.96$; $\rho<0.05$] (Table 2). The interactions suggest that coadministration of taurine was effective in preventing the increased reactive species formation by Leu administration.

The effect of treatments on lipid damage was evaluated by TBARS levels. Leucine administration caused a significant increase in TBARS levels in cerebral cortex

and hippocampus. A significant interaction between leucine and taurine in cerebral cortex [$F(1,25) = 11.67$; $\rho<0.05$] (Table 1) and hippocampus [$F(1,25) = 6.18$; $\rho<0.05$] (Table 2) indicates that co-administration of taurine prevented the lipid peroxidation elicited by Leu administration.

Reduced power was assessed by total sulfhydryl content. Leucine administration increased significantly the sulfhydryl content in the cerebral cortex and hippocampus. Significant interaction between leucine and taurine shows that coadministration of taurine prevented this increased in cerebral cortex [$F(1,25) = 6.43$; $\rho<0.05$] (Table 1) and hippocampus [$F(1,25) = 4.67$; $\rho<0.05$] (Table 2).

Leucine administration increased the GSH content in cerebral cortex and hippocampus. Two-way ANOVA showed a significant interaction between leucine and taurine in the cerebral cortex [$F(1,25) = 5.23$; $\rho<0.05$] (Table 1) and in the hippocampus [$F(1,25) = 8.60$; $\rho<0.05$] (Table 2). These interactions suggest that coadministration of taurine prevented the increased of GSH content.

In respect to the antioxidant enzymes, GPx and SOD activities were evaluated. GPx activity was significantly decreased by administration of leucine in cerebral cortex and hippocampus. However, SOD activity was decreased only in the cerebral cortex and increased in hippocampus by leucine administration. The significant interaction between leucine and taurine indicates that the diminution of the activities of GPx in the cerebral cortex [$F(1,25) = 5.83$; $\rho<0.05$] (Table 1) and hippocampus [$F(1,25) = 6.71$; $\rho<0.05$] (Table 2) and SOD in the cerebral cortex [$F(1,25) = 8.88$; $\rho<0.05$] (Table 1) were prevented by co-administration of taurine.

The thiol-containing enzymes of the phosphoryl transfer network were also evaluated. Chronic administration of leucine decreased the activities of CK cyt, CK mit, PK, AK, and GAPDH in the cerebral cortex (15-30%) and hippocampus (17-49%). In respect to the HK activity, Leu administration increased in the cerebral cortex (70%) and decreased in the hippocampus (46%). Significant interactions between leucine and taurine were found for all the enzymes activities in the two structures, indicating that co-administration of taurine prevented the effects induced by Leu administration (Tables 1 and 2). In the cerebral cortex the interactions between Leu and taurine were: [$F(1,25) = 6.21$; $\rho<0.05$] for CK cyt; [$F(1,25) = 6.41$; $\rho<0.05$] for CK mit; [$F(1,25) = 5.21$; $\rho<0.05$] for PK; [$F(1,25) = 7.21$; $\rho<0.05$] for AK; [$F(1,25) = 8.21$; $\rho<0.05$] for HK; [$F(1,25) = 5.81$; $\rho<0.05$] for GAPDH. In the hippocampus the interactions between

Leu and taurine were: [$F(1,25) = 7.52$; $\rho<0.05$] for CK cyt; [$F(1,25) = 6.32$; $\rho<0.05$] for CK mit; [$F(1,25) = 6.21$; $\rho<0.05$] for PK; [$F(1,25) = 6.35$; $\rho<0.05$] for AK; [$F(1,25) = 8.31$; $\rho<0.05$] for HK; [$F(1,25) = 5.95$; $\rho<0.05$] for GAPDH.

Spearman's correlation coefficients between DCFH oxidation and total sulfhydryl content were 0.464 ($p<0.05$) for the cerebral cortex and 0.475 ($p<0.05$) for the hippocampus; Spearman's correlation coefficients between DCFH oxidation and total GSH content were 0.601 ($p<0.05$) for the cerebral cortex and 0.927 ($p<0.05$) for the hippocampus. The significant correlations between the increase in free radical and in GSH or total sulfhydryl content suggest that the increase of total sulfhydryl and GSH content could be a cellular response induced by the oxidative stress. On the other hand, Spearman's correlations coefficients between total sulfhydryl content and the enzymes activities of the phosphoryl transfer network were significant, suggesting that alteration of the thiol-groups of the enzymes might be related to the alteration of their activities (Table 3).

4. Discussion

Several studies correlate oxidative stress and maple syrup urine disease in an animal model [19, 20, 21, 52, 53]. In an earlier study, we observed that chronic administration of leucine was able to induce oxidative stress damaging lipids and proteins, and causes inhibition of enzymes in rat brain and the association of creatine plus pyruvate prevented these effects [52]. For this reason, in this study, we decided to evaluate a possible preventive role of taurine in a pathological increase of free radicals caused by chronic administration of leucine. Studies also have shown that oxidative stress is induced in MSUD patients at diagnosis and during treatment [24; 25]. Altogether, these results strongly suggest that oxidative stress may be involved in the pathophysiology of MSUD.

According to Halliwell and Gutteridge [54], antioxidants are substances that at low concentrations, delays, prevents, or removes oxidative damage to a target molecule, competing with oxidizable substrates inhibiting their oxidation. In the CNS, taurine is an inducer of inhibitory neurotransmission, antioxidant, neuromodulator, regulator of calcium homeostasis, and neuroprotector, making it an putative therapeutic agent for many neurological disorders and neurodegenerative diseases, including Alzheimer's, Parkinson's, and Huntington's Disease.

We observed an increase in the generation of reactive species by DCFH oxidation in cerebral cortex and hippocampus, suggesting that leucine provokes the increase of reactive species generation, and the coadministration of taurine prevented these increase induced by leucine administration. Taurine acts as a good scavenger of reactive oxygen and nitrogen species [34, 55, 56]. The interaction of taurine with NO^- is important to prevent the superoxide anions generation in mitochondrial electron transport chain, avoid nitration, and inactivation of enzymes such as glyceraldehyde-3-phosphate-dehydrogenase (GAPDH), and to avoid damage of iron-sulfur proteins [57]. TBA-RS reflects the content of dialdehydes, mainly malondialdehyde (MDA), the most abundant individual aldehyde resulting from lipid breakdown due to lipid peroxidation process. MDA can impair several physiological mechanisms of the human body through its ability to react with molecules such as DNA and protein [58]. The concentration of thiol groups are a good indicator of redox balance in the cell [59], unaltered thiol groups are crucial for the catalytic and structural functions of many proteins [60]. We have shown that leucine administration was able to cause lipoperoxidation, as verified by increased TBA-RS levels, and oxidative protein damage, as analyzed by significant alterations of sulfhydryl content in cortex and hippocampus. The increase of total sulfhydryl GSH content is possibly a cellular reaction to the oxidation of the thiol groups due to their interaction with the reactive species. The treatment with taurine prevented lipoperoxidation and was efficient in preserving thiol groups, as well as in protecting the total thiol pool.

SOD and GPX activities were also evaluated. SOD is responsible for removing anion superoxide by accelerating the rate of its dismutation to hydrogen peroxide and water, and GPx removes hydrogen peroxide and other peroxides by coupling its reduction to H_2O with oxidation of reduced glutathione (GSH), using selenium as a cofactor. GPx is responsible for hydrogen peroxide detoxification and the inhibition its activity may lead to an impairment of detoxification of this substrate [57; 61]. Leucine administration decreased GPx activity in cerebral cortex and hippocampus, SOD activity decreased in cerebral cortex and increased in hippocampus. The decrease of this antioxidant system in the cerebral cortex could cause the accumulation of H_2O_2 or products of its decomposition, like O_2^- [61]. The increase of observed in the hippocampus is possibly due to a cellular reaction to intial decrease of enzyme activity. Moreover, the simultaneous treatment with taurine prevented the alterations observed in

SOD and GPx activities. Studies have shown that, in an oxidative environment, taurine is able to induce activity of endogenous antioxidant, catalase and glutathione peroxidase [62, 63]. So, taurine acts as a scavenger of ROS [64]. An important non-enzymatic antioxidant present in the cells, GSH, was found to be increased by leucine in cerebral cortex and hippocampus. This is possible since oxidative stress is the major stimulus for GSH synthesis [65] and, considering that GSH comprises one-third of total sulfhydryl groups [66], we have seen that the SH content also was increased by leucine administration. Administration of taurine prevented the increased GSH and sulfhydryl content.

Considering that derangement in cellular energy flow and distribution has been implicated in neurodegenerative diseases [67], we evaluated the activity of some thiol-containing enzymes important for the phosphoryl transfer network and maintenance of energy balance: HK, GAPDH, CKcyt, CKmit, AK, and PK. We observed that Leu administration decreased any enzyme activity in the cerebral cortex, and hippocampus. Moreover, coadministration of taurine was able to prevent the inhibition of these enzymes.

The significant Spearman's correlation coefficients between DCFH oxidation and total sulfhydryl and GSH content suggest that the increase of total sulfhydryl and GSH content could be a cellular response induced by oxidative stress. On the other hand, the significant Spearman's correlation coefficients between total sulfhydryl content and the thiol-containing enzymes activities of the phosphoryl transfer network suggest that the inhibition of the enzymes activities might be caused by alteration of the thiol-groups of these enzymes.

The phosphoryl transfer network contributes to efficient intracellular energetic communication, maintaining the balance between cellular ATP consumption and production, preserving cell phenotype and survival under stress [57, 42]. The adenylate kinase system makes a direct transmission of high-energy phosphoryls from mitochondria to the nucleus and cytosol, maintaining the optimal nucleotide ratios required for active transport [68]. When transport is inhibited by disruption of AK another alternative phosphoryl transfer pathways can be activated, such as the creatine kinase system [68, 69, 70]. Creatine kinase is the major phosphoryl transfer system in cells with high-energy demand [68]. Mitochondrial isoforms are necessary to maintain normal high-energy phosphate metabolite levels in the brain during stress [71]. High

energy phosphoryls generated by glycolytic enzymes, HK, GAPDH, and PK, can be used to support some specific cellular functions, such as maintenance of membrane ionic gradients and nuclear processes [72, 73]. In this regard, disequilibrium created at one specific intracellular local of the glycolytic network could be transferred to other cellular compartments [74, 75].

Therefore, it is possible to hypothesize that oxidative stress and deficient phosphoryl transfer network may impair brain energy homeostasis altering brain functioning. Deficit in the central nervous system energy metabolism plays a critical role in the progression of neurological diseases [76, 77]. Taurine was able to prevent all effects of oxidative stress caused by leucine administration reported now by improving activity of antioxidant enzymes and by reducing free radicals production and lipid and oxidative protein damage.

Through the chemically-induced chronic model of MSUD conducted in this work, it can be presumed that oxidative stress and the consequent inhibition of enzymes activities in the phosphoryl transfer network may contribute, along with other mechanisms, to the pathophysiology of the brain dysfunction characteristic of MSUD. Data presented here support an antioxidant/scavenger function for taurine, indicating a possible neuroprotective role for taurine as a potential adjuvant therapy to the MSUD affected patients. Thereby, these data may contribute to the understanding of the mechanisms of action of the cytotoxic effects of leucine accumulated in MSUD and the antioxidant effect of taurine upon this process.

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Table 1. Effect of leucine and taurine administration on parameters of oxidative stress and thiol-containing enzymes activities of phosphoryl transfer network in cerebral cortex of Wistar rats

Groups	saline	taurine	leucine	leucine + taurine
DCFH	27.8 ± 1.1	26.4 ± 0.9	31 ± 1.2*	27.4 ± 0.6
TBARS	1.8 ± 0.4	1.8 ± 0.1	2.4 ± 0.4*	1.7 ± 0.2
Total Sulfhydryl	45.7 ± 3.2	44.6 ± 2.8	53.6 ± 6*	46.7 ± 4.7
GSH	0.81 ± 0.09	0.74 ± 0.08	0.98 ± 0.05*	0.82 ± 0.03
GPx	11.4 ± 0.8	10.6 ± 0.4	9.9 ± 0.9*	10.9 ± 0.4
SOD	12.9 ± 0.4	11.8 ± 0.8	10.6 ± 1.2*	11.9 ± 0.6
CK mit	2.6 ± 0.2	2.4 ± 0.3	2 ± 0.6*	2.3 ± 0.3
CK cyt	3.1 ± 0.3	2.7 ± 0.6	2.4 ± 0.3*	3.1 ± 0.7
AK	1.7 ± 0.4	1.6 ± 0.3	1.2 ± 0.2*	1.5 ± 0.1
PK	1.4 ± 0.05	1.3 ± 0.1	1.1 ± 0.1*	1.2 ± 0.2
HK	0.05 ± 0.5	0.08 ± 0.003	0.14 ± 0.2*	0.06 ± 0.02
GAPDH	0.7 ± 0.05	0.6 ± 0.05	0.6 ± 0.07*	0.7 ± 0.05

Data are express as mean ± SEM for 7 animals per group. *$\rho<0.05$ compared to other groups (two-way ANOVA followed by Tukey test). Abbreviations: DCFH (2`7`-dihydrodichloro fluorescein oxidation), expressed as nmol of DCF per mg of protein; TBARS (thiobarbituric acid reactive substances), expressed as nmol of TBA per mg of protein; Sulfhydryl content expressed as nmol TNB/mg protein. GSH (reduced glutathione), expressed as nmol of GSH per mg of protein. Data from GPx (glutathione peroxidase) are expressed as μmol of NADPH per min per mg of protein. SOD (superoxide dismutase), expressed as SOD U per mg of protein. CK cyt (cytosolic creatine kinase) and CK mit (mitochondrial creatine kinase) are expressed as μmol of creatine per min per mg of protein. PK (pyruvate kinase) expressed as μmol of pyruvate per min per mg of protein. AK (adenylate kinase) expressed as μmol of ATP per min per mg of protein. HK (hexokinase) expressed as μmol of NADH per min per mg of protein. GAPDH (glyceraldehyde-3-phosphate dehydrogenase), expressed as μmol of NADH per min per mg of protein.

Table 2. Effect of leucine and taurine administration on parameters of oxidative stress and thiol-containing enzymes activities of phosphoryl transfer network in hippocampus of Wistar rats

Groups	saline	taurine	leucine	leucine + taurine
DCFH	32.4 ± 1.6	32.1 ± 2.3	37.2 ± 1.4*	33.4 ± 1.4
TBARS	2.1 ± 0.1	1.9 ± 0.3	2.8 ± 0.2*	2.1 ± 0.4
Total Sulfhydryl	54.8 ± 7.1	56.4 ± 5.6	65.2 ± 4.1*	51.8 ± 5
GSH	0.84 ± 0.1	0.91 ± 0.1	1.1 ± 0.2*	0.89 ± 0.1
GPx	12.8 ± 0.8	11.9 ± 1	11.4 ± 0.8*	13.3 ± 0.5
SOD	13.1 ± 1.6	12.4 ± 1.6	14.2 ± 0.6*	13.4 ± 1.6
CK mit	2.9 ± 0.4	2.7 ± 0.4	1.6 ± 0.3*	2.5 ± 0.9
CK cyt	4.1 ± 0.4	3.4 ± 0.2	2.1 ± 0.4*	3.7 ± 0.4
AK	2.6 ± 0.4	2.4 ± 0.5	1.9 ± 0.4*	2.5 ± 0.4
PK	2.3 ± 0.3	2.3 ± 0.2	1.6 ± 0.4*	2.1 ± 0.4
HK	0.35 ±0.04	0.32 ± 0.06	0.19 ± 0.04*	0.28 ± 0.03
GAPDH	1.8 ± 0.1	1.9 ± 0.4	1.5 ± 0.1*	1.7 ± 0.3

Data are express as mean ± SEM for 7 animals per group. *$\rho<0.05$ compared to the other groups (two way ANOVA followed by the Tukey test). Abbreviations: DCFH (2`7`-dihydrodichloro fluorescein oxidation), expressed as nmol of DCF per mg of protein. TBARS (thiobarbituric acid reactive substances), expressed as nmol of TBA per mg of protein. Sulfhydryl content expressed as nmol TNB/mg protein. GSH (reduced glutathione), expressed as nmol of GSH per mg of protein. Data from GPx (glutathione peroxidase) are expressed as µmol of NADPH per min per mg of protein. SOD (superoxide dismutase), expressed as SOD U per mg of protein. CK cyt (cytosolic creatine kinase) and CK mit (mitochondrial creatine kinase) are expressed as µmol of creatine per min per mg of protein. PK (pyruvate kinase), expressed as µmol of pyruvate per min per mg of protein. AK (adenylate kinase), expressed as µmol of ATP per min per mg of protein. HK (hexokinase), expressed as µmol of NADH per min per mg of protein. GAPDH (glyceraldehyde-3-phosphate dehydrogenase), expressed as µmol of NADH per min per mg of protein.

Table 3. Spearman's correlation coefficient between total sulfhydryl content and thiol-containing enzymes activities of phosphoryl transfer network in cerebral cortex and hippocampus of rats.

Enzymes	Cerebral Cortex	Hippocampus
PK	0.346*	0.535*
AK	0.461*	0.367*
CK cyt	0.500*	0.431*
CK mit	0.368*	0.451*
HK	0.480*	0.800*
GAPDH	0.443*	0.414*

*$\rho < 0.05$ for Spearman's correlation coefficient. Abbreviations: PK (pyruvate kinase); AK (adenylate kinase); CK cyt (cytosolic creatine kinase); CK mit (mitochondrial creatine kinase); HK (hexokinase); GAPDH (glyceraldehyde-3-phosphate dehydrogenase).

PARTE III

DISCUSSÃO, CONCLUSÃO E PERSPECTIVAS

III.1 DISCUSSÃO

Os mecanismos responsáveis pelos sintomas neurológicos apresentados pelos pacientes com DXB são pouco conhecidos. Nesse sentido, muitos estudos estão sendo desenvolvidos para entender melhor a fisiopatologia da DXB. Existem evidências associando o acúmulo do aminoácido leucina ao surgimento dos sintomas neurológicos nesses pacientes (Chuang e Shih, 2001). Mello e colaboradores (1999) mostraram que a administração crônica de leucina a ratos jovens induz déficit no aprendizado e memória, contribuindo para a deterioração neurológica. Estudos em animais e em pacientes já demonstraram o envolvimento do estresse oxidativo na DXB, sugerindo um aumento na produção de radicais livres e/ou uma diminuição das defesas antioxidantes na doença (Barschak et al., 2006, 2008a, 2008b, 2009; Mescka et al., 2013; Colomé et al., 2000, Fontella et al., 2002; Bridi et al., 2003). Além do dano oxidativo, ocorre a redução da homeostase energética pela inibição de importantes enzimas do metabolismo energético (Bridi et al., 2005; Pilla et al., 2003; de Franceschi et al., 2013), o que contribui para a disfunção neurológica da doença. O estudo do possível efeito protetor de uma terapia antioxidante na DXB pode levar a resultados promissores na terapêutica de portadores desta patologia. Portanto, o presente trabalho teve por objetivo investigar os possíveis efeitos da administração crônica de leucina sobre a atividade de enzimas da rede de fosforiltransferência e sobre parâmetros de estresse oxidativo em córtex e hipocampo de ratos Wistar de 21 dias de vida. Além disso, investigamos o possível efeito preventivo da administração de creatina e piruvato e da administração de taurina sobre esses parâmetros.

O estresse oxidativo é causado por um desequilíbrio entre a geração de espécies reativas e os antioxidantes, em favor da sua formação, causando o dano oxidativo

(Halliwell, 2013). O cérebro é particularmente vulnerável ao dano oxidativo devido à sua alta taxa de utilização de oxigênio, grande conteúdo de ácidos graxos poli-insaturados e níveis moderados de defesas antioxidantes quando comparado a outros tecidos (Butterfield e Stadtman, 1997; Halliwell, 1996). O estresse oxidativo é considerado um evento importante relacionado com a patogênese de algumas doenças neurodegenerativas (Reznick a Packer, 1993; Halliwell e Gutteridge, 1985). Portanto, em ambos os trabalhos encontramos resultados semelhantes em relação aos parâmetros de estresse oxidativo causados pela administração crônica de leucina. Observamos que a administração de leucina causou um aumento na geração de espécies reativas através da oxidação do DCFH em córtex cerebral e hipocampo. A oxidação de lipídios foi determinada através dos níveis de substâncias reativas ao ácido tiobarbitúrico (TBARS), esta técnica reflete o conteúdo de malondialdeído (MDA), o produto mais abundante resultante da lipoperoxidação. O MDA pode prejudicar vários mecanismos fisiológicos devido a sua capacidade de reagir com as proteínas e com o DNA (Del Rio et al., 2005). Foi observado que a administração crônica de leucina aumentou os níveis de TBARS no córtex e hipocampo dos ratos, sugerindo que houve peroxidação lipídica. A concentração de grupos tiólicos reflete o estado redox da célula (Ying et al., 2007), os grupos tiólicos são fundamentais para manter a estrutura proteica e para que as proteínas desenvolvam suas funções catalíticas (Kim et al., 2000). A administração de leucina provocou aumento no conteúdo de sulfidrilas, esse aumento foi observado somente no hipocampo no primeiro trabalho e em ambas as estruturas, córtex e hipocampo, no segundo trabalho. O aumento nos níveis de tióis ocorre devido ao aumento das espécies reativas que reagem com a ligação dissulfeto (S-S) nas proteínas levando ao dano oxidativo (Stadtman e Levine, 2003). Além disso, considerando que um terço dos grupos sulfidrilas totais são encontrados na molécula de GSH, o aumento de tióis pode

ocorrer devido ao aumento na síntese de GSH, e o estresse oxidativo estimula a produção de GSH na célula (Lu, 2009). Com relação a esses parâmetros, a administração de creatina e piruvato no primeiro trabalho e a administração de taurina no segundo trabalho preveniram o aumento das espécies reativas e dos grupos sulfidrilas, e preveniram a lipoperoxidação. O conteúdo de GSH foi avaliado somente no segundo trabalho e observamos que a administração de leucina aumentou os níveis de glutationa no córtex e hipocampo e a administração de taurina preveniu este aumento.

A atividade das enzimas antioxidantes SOD e GPx também foram avaliadas. A SOD é responsável pela remoção de ânions superóxidos através da dismutação do H_2O_2, e assim protege as células contra os efeitos nocivos deste radical livre (Marklund, 1985). E a GPx faz a remoção do H_2O_2 e de outros peróxidos através da reação de oxidação da GSH (Leong et al., 1981). Em ambos os trabalhos, a administração de leucina diminuiu a atividade da GPx no córtex e no hipocampo e a atividade da SOD foi reduzida somente no córtex. Com relação à atividade da SOD no hipocampo, no primeiro trabalho não houve alteração na atividade e no segundo trabalho houve um aumento na atividade após a administração crônica de leucina. É possível que este aumento se deva a uma reação de compensação da célula devido à diminuição na atividade das enzimas. Portanto, a diminuição na atividade deste sistema antioxidante pode levar ao acúmulo de H_2O_2 e de produtos da sua decomposição como o ânion superóxido (Diplock, 1994). O ânion superóxido é capaz de inibir a atividade da GPx (Macedo et al., 2013), assim como o H_2O_2 pode inibir a atividade da SOD (Change et al., 1979). Além disso, os efeitos citotóxicos das espécies reativas de oxigênio podem ser causados pelo radical hidroxila (OH•) que é gerado a partir da reação de Fenton, o aumento deste radical pode inibir direta ou indiretamente a atividade das enzimas

antioxidantes (Halliwell, 2000). Com relação ao tratamento com os antioxidantes, observamos que a administração de creatina e piruvato no primeiro trabalho e a administração taurina no segundo trabalho foram capazes de prevenir estas alterações.

Considerando que em doenças neurodegenerativas ocorrem perturbações na distribuição e no fluxo energético das células (Ames, 2000), nós avaliamos a atividade de enzimas da rede de fosforiltransferência. Esta rede é formada por enzimas tiólicas que são responsáveis pela transferência de grupos fosforilas de alta energia entre os locais de produção e consumo de ATP, mantendo o equilíbrio energético na célula e contribuindo para a sua sobrevivência sobre condições de estresse (Wallimann et al., 1992; Dzeja e Terzic, 2003). As enzimas avaliadas foram: creatina cinase citosólica (CKcit), creatina cinase mitocondrial (CKmit), adenilato cinase (AK), piruvato cinase (PK), hexocinase (HK) e gliceraldeído-3-fosfato-desidrogenase (GAPDH).

O sistema creatina/fosfocreatina/creatina cinase (Cr/PCr/CK) é o maior sistema de transferência de grupos fosfato de alta energia nas células com alta demanda energética (Dzeja e Terzic, 2003), e desempenha uma importante função de manutenção dos altos níveis de energia necessários para o funcionamento do sistema nervoso central (SNC) (Wyss e Kaddurah-Daouk, 2000). Uma diminuição na atividade da CK acarreta na redução dos níveis de creatina, reduzindo a eficiência da rede e a capacidade antioxidante da célula (Lawler et al., 2002). O sistema da AK cataliza a transferência reversível de grupos fosfato entre as moléculas de ATP e AMP da mitocôndria para o núcleo e citosol da célula, sendo assim a principal responsável pela rede de fosforiltransferência (Dzeja e Terzic, 2003; Price et al., 1975). A PK é uma importante enzima do metabolismo da glicose, catalisa a conversão irreversível do fosfoenolpiruvato (PEP) a piruvato, dando origem à molécula de ATP a partir do ADP. A deficiência de piruvato em consequência da inibição da atividade da PK pode induzir

a morte celular (Valentini et al., 2000). A GAPDH oxida o gliceraldeído-3-fosfato em 1,3-bifosfoglicerato, alterações na atividade desta enzima estão envolvidas em diversas doenças neurodegenerativas e neoplásicas, como no câncer de próstata, e nas doenças de Alzheimer, Huntington e Parkinson (Perrota et al., 2014). Os grupos fosfatos de alta energia gerados pelas enzimas glicolíticas, PK, HK e GAPDH podem ser usados para suprir algumas funções especificas na célula, como na manutenção do gradiente iônico de membrana e em processos nucleares (Ottaway e Mowbray, 1977; Masters et al., 1987).

O déficit no metabolismo energético do SNC tem um papel crítico na progressão de doenças neurológicas (Beal, 2000; Chaturvedi, 2008). Portanto, em ambos os trabalhos, observamos que todas as enzimas tiveram suas atividades diminuídas após a administração crônica de leucina no córtex e no hipocampo, exceto pela atividade da PK no córtex que não foi alterada no primeiro trabalho. Além disso, o tratamento com creatina e piruvato e o tratamento com taurina foram capazes de prevenir a inibição da atividade destas enzimas.

Quando a atividade de uma enzima da rede de fosforiltransferência diminui, esta pode ser compensada pelo aumento na atividade das outras enzimas para que não ocorra prejuízo energético à célula. Como exemplo, quando ocorre a inibição no transporte de grupos fosfatos de alta energia pelo sistema da AK, outra via alternativa é ativada, como o sistema da CK (Dzeja e Terzic, 2003; Wallimann et al., 1992; Neumann et al., 2003) ou quando ocorre um desequilíbrio em um local específico da cadeia glicolítica, este pode ser compensado em outro compartimento da célula (Goldbeter e Nicolis, 1976; Mair e Muller, 1996). No entanto, quando ocorre a diminuição na atividade de duas ou mais enzimas desta rede pode ocorrer uma alteração significativa na transferência de grupos fosfatos de alta energia entre os locais de geração e consumo de ATP (Dzeja et

al., 2000; Saks et al., 2006). Estas cinases contem um grupamento tiólico na sua estrutura, o que favorece o ataque de radicais livres levando à sua inativação. A atividade da CK pode ser inativada pelo ânion superóxido (Wendt et al., 2003) e a atividade da PK pode ser inativada pelo peróxido de hidrogênio (Halliwell e Gutteridge 2007). O acúmulo de outros aminoácidos podem induzir estresse oxidativo e levar a inibição destas enzimas pela oxidação dos grupamentos sulfidrilas, isto ocorre em modelos animais de erros inatos do metabolismo, como na cistinose (Feksa et al., 2004; Figueiredo et al., 2009), fenilcetonúria (Feksa et al., 2003; Costabeber et al., 2003), tirosinemia (de Andrade et al., 2012), beta-alaninemia (Gemelli et al., 2013), histidinemia (Rojas et al., 2012).

Os dados também foram analisados pela correlação de Spearman e encontramos os seguintes resultados: no primeiro trabalho houve uma correlação significativa entre a formação de espécies reativas através da oxidação do DCFH e a inibição das enzimas CK mit e CK cit, AK e PK no córtex e hipocampo, exceto para PK no córtex. Além disso, encontramos uma correlação entre o conteúdo de sulfidrilas totais e a inibição destas enzimas. Sendo assim, os resultados sugerem que a inibição na atividade das enzimas pode ocorrer através da ação das espécies reativas sobre o grupamento tiólico das enzimas. Portanto, é possível afirmar que a indução do estresse oxidativo, e a consequente alteração do equilíbrio energético, podem contribuir significativamente para o dano cerebral observado em pacientes com a DXB. E no segundo trabalho observamos uma correlação significativa entre a oxidação de DCFH, sulfidrilas totais e conteúdo de GSH, sugerindo que o aumento no conteúdo de sulfidrilas e de GSH pode ser uma resposta celular devido à indução do estresse oxidativo. Além disso, houve correlação significativa entre o conteúdo de sulfidrilas totais e as enzimas da rede de fosforiltransferência, sugerindo que a inibição da atividade destas enzimas pode ocorrer

devido a alteração no grupamento tiólico das enzimas causado pela ação dos radicais livres.

Antioxidantes são substâncias que em baixas concentrações atrasam, previnem ou removem o dano oxidativo, competindo com substratos oxidáveis e inibindo a sua oxidação (Halliwell e Gutteridge, 2010). Creatina, piruvato e taurina são importantes antioxidantes. A creatina além de possuir capacidade antioxidante direta (Lawler et al., 2002), também possui outros mecanismos de ação, atua como scavengers de radicais livres, faz a quelação de metais (Halliwell, 2001) e aumenta o conteúdo energético da célula (Persky e Brazeau., 2001). A suplementação de creatina tem sido eficaz em diversos modelos animais e modelos *in vitro* de doenças neurodegenerativas, como as doenças de Alzheimer, Parkinson e Huntington (Klein e Ferrante, 2007). O piruvato oferece proteção contra o estresse oxidativo e o dano mitocondrial, servindo de substrato para produção de ATP, uma vez que atua na síntese de acetil-coenzima A no ciclo dos ácidos tricarboxílicos (TCA) e possui propriedade antioxidantes como scavengers de H_2O_2, protegendo as células do declínio energético e da apoptose (Andrae et al., 1985; Mukherjee et al., 1997, Vlassenko et al., 2006). A associação de creatina e piruvato tem mostrado efeito protetor contra o estresse oxidativo e previne e déficit energético em diversos trabalhos do nosso grupo de pesquisa (de Franceschi et al, 2012; Berti et al., 2012; de Andrade et al, 2015, 2017; Bortoluzzi et al., 2014). A taurina possui muitas funções, além de antioxidante, faz a detoxificação, modulação de neurotransmissores, estabilização de membrana, osmorregulação e modulação dos níveis de cálcio atuando na neuromodulação e neuroproteção, fazendo com que sirva de agente terapêutico para muitas doenças neurodegenerativas. A taurina atua como sequestradora de espécies reativas de oxigênio e nitrogênio (Oliveira et al., 2010, Cunningham et al., 1998, Kocak-Tocker et al., 2005). A sua interação com o óxido

nítrico previne a geração de ânions superóxido na mitocôndria, o que evita a nitração e inativação de enzimas como a GAPDH e evita o dano a metaloproteínas (Halliwell e Gutteridge, 2007).

Através da utilização de um modelo crônico de indução química da Doença do Xarope do Bordo em ratos Wistar, os resultados obtidos neste trabalho corroboram com os dados anteriores da literatura demonstrando que o estresse oxidativo contribui para a disfunção neurológica observada na doença (Bridi et al., 2005; Scaini et al., 2012; Mescka et al., 2013). Creatina, piruvato e taurina atuaram de forma eficiente desempenhando a função de antioxidante/sequestradora neste modelo animal, prevenindo a ação de radicais livres que causam a oxidação de lipídios e a inativação de importantes enzimas da rede de fosforiltransferência e de enzimas detoxificadoras de espécies reativas. Estes resultados mostram que creatina, piruvato e taurina podem desempenhar um papel neuroprotetor como adjuvante na terapia de pacientes afetados pela DXB. Além disso, estes dados contribuem para a compreensão dos mecanismos responsáveis pela neurotoxicidade decorrente do acúmulo dos metabólitos envolvidos na doença.

III.2 CONCLUSÕES

1 – Em ambos os trabalhos, a administração crônica de leucina induziu estresse oxidativo e diminuiu a atividade das enzimas da rede de fosforiltransferência, CK citosólica, CK mitocondrial, AK, PK, GAPDH e HK no córtex e no hipocampo, exceto pela atividade da PK no córtex que não foi alterada no primeiro trabalho. Os resultados indicam que a administração crônica de leucina pode estimular o estresse oxidativo e alterar o metabolismo energético em córtex cerebral e hipocampo de ratos;

2 – A coadministração de creatina e piruvato e de taurina foi eficaz na prevenção das alterações provocadas pela administração de leucina sobre o estresse oxidativo e as enzimas da rede de fosforiltransferência, sugerindo que estes antioxidantes podem ter um papel protetor no cérebro.

Portanto, através deste estudo, é possível concluir que o estresse oxidativo e a consequente inibição da atividade das enzimas da rede de fosforiltransferência podem contribuir para a fisiopatologia da disfunção cerebral encontrada na DXB. Os dados apresentados dão suporte à função de antioxidante/scavenger de creatina, piruvato e taurina, indicando um possível papel neuroprotetor e sugerindo que possam ser usados como adjuvantes na terapia dos pacientes com DXB. Estes resultados contribuem para o entendimento do mecanismo de ação dos efeitos citotóxicos da leucina acumulada na DXB e os efeitos dos antioxidantes neste processo.

III.3 PERSPECTIVAS

1. Avaliar parâmetros de estresse oxidativo no plasma de ratos Wistar;

2. Avaliar parâmetros inflamatórios;

3. Avaliar o possível efeito protetor da norleucina;

4. Avaliar o quanto as alterações enzimáticas induzidas pela leucina se devem à inibição e o quanto se devem à alteração na síntese das mesmas.

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