UNIVERSIDADE FEDERAL DE UBERLÂNDIA
INSTITUTO DE GENÉTICA E BIOQUÍMICA
PÓS-GRADUAÇÃO EM GENÉTICA E BIOQUÍMICA

USO DE CETOANÁLOGOS PARA REDUÇÃO DA AMONEMIA EM RATOS SUBMETIDOS A EXERCÍCIOS FÍSICOS

Rosemeire Dantas de Almeida

UBERLÂNDIA – MG
2010

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Rosemeire Dantas de Almeida

Tese apresentada à Universidade Federal de Uberlândia como parte dos requisitos para obtenção do Título de Doutor em Genética e Bioquímica (Área Bioquímica)

Orientador: Prof. Dr. Luiz Claudio Cameron

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_____

Prof. Dr. Luiz Claudio Cameron

DEDICATÓRIA

A Deus; Pai fiel, sempre fiel e eternamente fiel, meu Pastor, Salvador, meu Pai! Ao meu esposo Eduardo por toda força e apoio nos momentos difíceis e a minha filha Maria Eduarda, maior presente de Deus nesta vida.

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Aos colegas do LBP, vocês são especiais.

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LISTA DE ABREVIAÇÕES

[Ca^{2+}] - Cálcio

1RM – uma repetição máxima

ADP-adenosina difosfato

AL- arginossuccinato liase

Ala - alanina

AMP- adenosina monofosfato

AMPD – adenosina de monofosfato desidrogenase

AO - oxaloacetato

AS - argininosuccinato sintase

ASP- aspartato

ATP- adenosina trifosfato

BCAAs- aminoácidos de cadeia ramificada

BCAT – BCAA aminotransferase

BCKA - αcetoácido de cadeia ramificada

BCKDH – complexo enzimático αcetoácido de cadeia lateral ramificada desidrogenase

BCOA- α-cetoácido análogo dos aminoácidos de cadeia ramificada

CHO- carboidrato

CP- creatina fosfato

CSF- fluido cerebro espinhal

EH – encefalopatia hepática

GDH- glutamato desidrogenase

Gln - glutamina

Glu - glutamato

GS – glutamina sintetase

GTP- trifosfato de guanosina

H^+ - íon hidrogênio

IMP- inosina monofosfato

KAAA – cetoanálogos e aminoácidos

MK - mioquinase

NAD - nicotinamida adenina dinucleotídeo oxidada

NADH - nicotinamida adenina dinucleotídeo reduzida

NH_3 - amônia

$NH_3 + NH_4^+$ - amônia

NH_4^+ - íon amônio

NMDA- receptores N-methil-D-aspartato

OCT- ornitina transcarbamilase

OG – 2-oxoglutarato

OTC- ornitina transcarbamilase periféricas

PFK- fosfofrutoquinase

Pi – fosfato inorgânico

PNC- ciclo das purinas nucleotídeos

Pyr - piruvato

SNC- sistema nervoso central

TCA- ciclo do ácido tricarboxílico

VO_2máx – consumo máximo de oxigênio

APRESENTAÇÃO

O exercício altera o metabolismo energético e induz a utilização de reservas e a produção de inúmeros metabólitos que podem ou não ser nocivos ao próprio organismo. Entre estes metabólitos a amônia vem sendo cada vez mais estudada. As causas da toxicidade da amônia não estão bem elucidadas, mas sabe-se que quando a concentração desta é elevada, inúmeros são os efeitos deletérios no organismo, principalmente no SNC, ocasionando o desenvolvimento clínico de doenças como a encefalopatia hepática.

Exercícios de alta intensidade têm sido implicados com o desenvolvimento de fadiga e exaustão física pelo aumento nas concentrações da amônia plasmática. Assim, o laboratório de bioquímica de proteínas tem se dedicado a pesquisa com amônia e alguns estudos têm usado suplementação com objetivo de diminuir a concentração desta durante os exercícios, promovendo melhor desempenho.

Desta maneira a suplementação com cetoanálogos associada ao exercício físico, pode retardar ou superar o efeito tóxico da elevação da amônia. Este estudo investiga a possível proteção causada pelo cetoanálogos à elevação da amônia em ratos durante modelo de exercício.

Assim, o estudo foi compartimentalizado objetivando melhor entendimento:

No capítulo I encontra-se a fundamentação teórica onde são descritas considerações gerais sobre a amônia, origem, fontes de geração, amônia e exercício e efeitos fisiopatológicos, entre outros.

O capítulo II tem por objetivo investigar como exercícios de força e resistência em ratos, modificam o metabolismo de amônia associada à suplementação cetoanáloga.

O capítulo III compara as implicações da suplementação de cetoanálogos agregados com aminoácidos sobre a amonemia durante o exercício propondo investigar e comparar os resultados entre modelos humanos e animais.

O capítulo IV descreve o efeito da suplementação de cafeína sobre a amonemia durante o exercício prolongado em ciclistas.

CAPÍTULO I: Fundamentação Teórica

RESUMO

A amônia vem sendo estudada como indicador de diversas funções metabólicas durante o exercício e deve ser considerada em exercícios prolongados e exaustivos como um marcador de estresse energético. A amônia elevada pode ser tóxica para a célula muscular e alterar o metabolismo cerebral, induzindo fadiga periférica e central. Exercícios físicos intensos podem promover falência na ressíntese de ATP, desencadeando maior deaminação da AMP, e conseqüente aumento de amônia. Alta concentração de amônia é tóxica e prejudica o desempenho físico. Desta maneira a suplementação com cetoanálogos associada ao exercício físico, pode retardar ou superar o efeito tóxico da elevação da amônia. Este estudo investiga a possível proteção causada pelo cetoanálogos a elevação da amônia em ratos durante modelo de exercício. O uso de cetoanálogos tem sido proposto para captar compostos nitrogenados do sangue, tais como amônia, transformando-se em aminoácidos correspondentes. Este estudo verificou o efeito protetor da suplementação aguda de cetoanálogos contra compostos nitrogenados e desempenho físico, em ratos submetidos a exercício de força e resistência.

Palavras-chave: amônia, exercício, cetoácidos.

ABSTRACT

Concentration of ammonia in blood increases during endurance exercise and can be toxic for muscle cells and brain metabolism, potentially leading to both peripheral and central fatigue. Intense exercise can promote failure in replace ATP leading to greater deamination of AMP and consequent increase in blood ammonia. High concentration of ammonia is toxic and harms the performance. Keto acids has been proposed to capture the blood nitrogen compounds. Here we describe the protective effects of acute and chronic keto acids supplementation on nitrogen metabolism and physical performance in male rats submitted to a resistance training protocol. This study investigated the protective effect of supplementation keto analogues against acute nitrogen compounds and physical performance in rats submitted to exercise.

Keywords: ammonia, exercise, ketoacids.

Introdução

1.Amônia

1.1.Considerações gerais, conceito e origem

A amônia (NH_3+NH_4^+) é um produto de degradação de compostos nitrogenados e é considerado um indicador de diversas funções metabólicas. Refere-se a soma total de amônia livre (NH_3) e íons amônio (NH_4^+), já que a amônia na forma de base livre é gasosa e hidrofílica difundindo-se facilmente pelas células através das membranas. O íon amônio NH_4^+ é formado a partir de NH_3 na reação equilíbrio $NH_3 + H^+ \rightarrow NH_4^+$ e não é difundido com facilidade, necessitando de mecanismos de transporte mediados (COOPER e PLUM, 1987; COOPER, 2001).

Este metabólito dependendo do pH pode se apresentar como NH_4^+ ou como gás (NH_3). Como o pK da amônia a 37º C é de 9,15, cerca de 98% desta se encontra na forma ionizada nos fluídos fisiológicos (FELIPO e BUTTERWORTH, 2002b; BOSOI e ROSE, 2009).

A amônia é encontrada em quantidades vestigiais na atmosfera, sendo produzido a partir da putrefação dos animais nitrogenados e matéria vegetal. Amônia e sais de amônio também são encontrados em pequenas quantidades na água de chuva, enquanto no organismo humano a amônia é absorvida a partir da via entérica originaria de produção da microbia e produzida pelas reações de desaminação dos aminoácidos e da adenosina de monofosfato (AMP) (HELLSTEN et al., 1999).

Seu excesso é extremamente tóxico para os mamíferos que a excretam sob forma de uréia, onde os níveis normais de amônia são fundamentais para o bom funcionamento do Sistema Nervoso Central (SNC). Sendo um metabólito excretado em sua maior parte pelo ciclo da uréia, este regula a concentração de amônia na circulação sistêmica.

1.2.Transporte e metabolismo de amônia

A amônia é um produto metabólico importante final e intermediário de várias vias metabólicas no corpo, sua aparência na circulação sistêmica decorre de fontes como: intestino, músculo, rim e cérebro (OLDE DAMINK et al., 2002). Em condições fisiológicas, a maior parte da amônia sistêmica é liberada a partir do intestino ou sistema gastrointestinal (SUMMERSKILL e WOLPERT, 1970; ROMERO-GÓMEZ et al., 2009). Proveniente de compostos nitrogenados da dieta, desaminação da glutamina pela glutaminase e metabolismo de substâncias nitrogenadas da flora do cólon intestinal, a maioria da amônia é metabolizada em uréia no fígado. A atividade bacteriana libera grandes quantidades de amônia, que através de uma combinação de difusão passiva e mecanismos de transporte ativo, como recentemente identificado, são transportados através da mucosa epitelial para a circulação portal hepática. Tão pouco como 1% da amônia permanece no trato gastrointestinal a ser excretada na matéria fecal (WILKINSON, 2010) (Figura 1).

Derivações porto-sistêmicas e insuficiência hepática causam aumento da amônia no sangue que podem afetar a função cerebral, induzindo vários distúrbios em astrócitos, que podem prejudicar o tráfico de glutamina mitocondrial e do glutamato entre os neurônios e astrócitos (MUTCH e BANISTER, 1983; CÓRDOBA e MÍNGUEZ, 2008). Grandes quantidades de amônia circulam no organismo a partir da veia porta vindo do sistema gastrointestinal, contudo, a concentração desta molécula se mantém baixa em virtude de um eficiente mecanismo hepático de remoção (FELIPO e BUTTERWORTH, 2002b; BUTTERWORTH, 2002) (Figura 2).

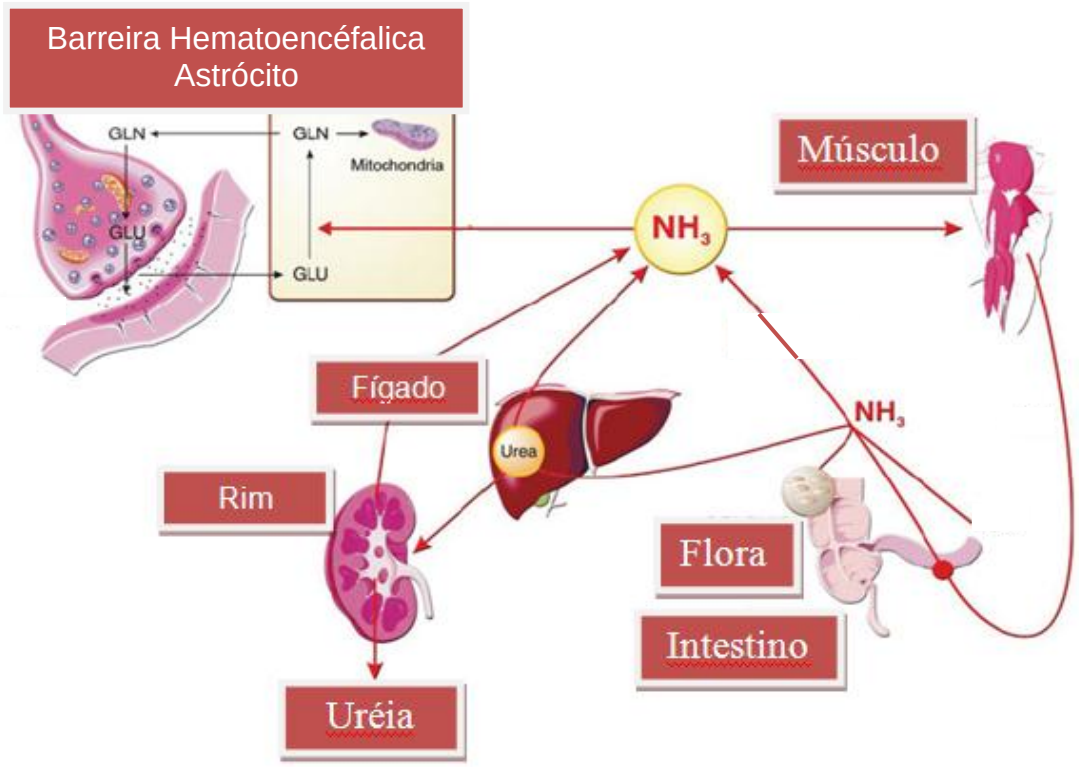

Figura 1: Transporte e metabolismo de amônia inter-órgãos. Extraído e Adaptado de CÓRDOBA e MÍNGUEZ, 2008.

A homeostasia do metabolismo de aminoácidos entre o fígado, músculo esquelético e intestino maximiza a disponibilidade de glutamina para amoniogênese renal. A amônia gerada do catabolismo dos aminoácidos intestinal ou entra no sistema portal ou é utilizada localmente para a síntese de uréia. A presença de um ciclo da uréia funcional serve de primeira linha de defesa contra a toxicidade da amônia em mamíferos (WU, 2009). Um complexo sistema de órgãos em intercâmbio com os rins e trato gastrointestinal, promove eliminação para manter o equilíbrio ácido-base e balanço de nitrogênio (WILKINSON, 2010). Este eficiente sistema de desintoxicação pelo intercâmbio de órgãos garante que concentrações plasmáticas de amônia sejam mantidas dentro de um baixo intervalo de não mais que 100 µmol/L (FELIPO e BUTTERWORTH, 2002). Além destes, outros tecidos e órgãos, tais como o cérebro e o músculo esquelético também contribuem para metabolismo e regulação da amônia (OLDE DAMINK et al., 2002; OLDE DAMINK et al., 2009). O músculo esquelético representa cerca de 40% do total de massa corporal e tem um grande potencial de produção, absorção e metabolismo da amônia. Alguns estudos

estimam que aproximadamente 50% da amônia pode ser metabolizada no músculo para formar glutamina, através da reação da glutamina sintetase (GS) (WILKINSON, 2010; WAGENMAKERS, 1998).

Metabolismo de amônia em repouso é um processo cíclico, com entrada e vários pontos de saída. Mesmo as menores alterações, em qualquer parte desse processo podem afetar a homeostase, levando à necessidade de mudanças no sistema para lidar com estas perturbações. A disfunção hepática e o exercício podem provocar mudanças, quer por diminuição da capacidade de remoção ou aumento na produção de amônia. Disfunção neurológica é bem documentada em doenças do fígado (WILKINSON, 2010) e intimamente associada às alterações no metabolismo da amônia (FELIPO e BUTTERWORTH, 2002).

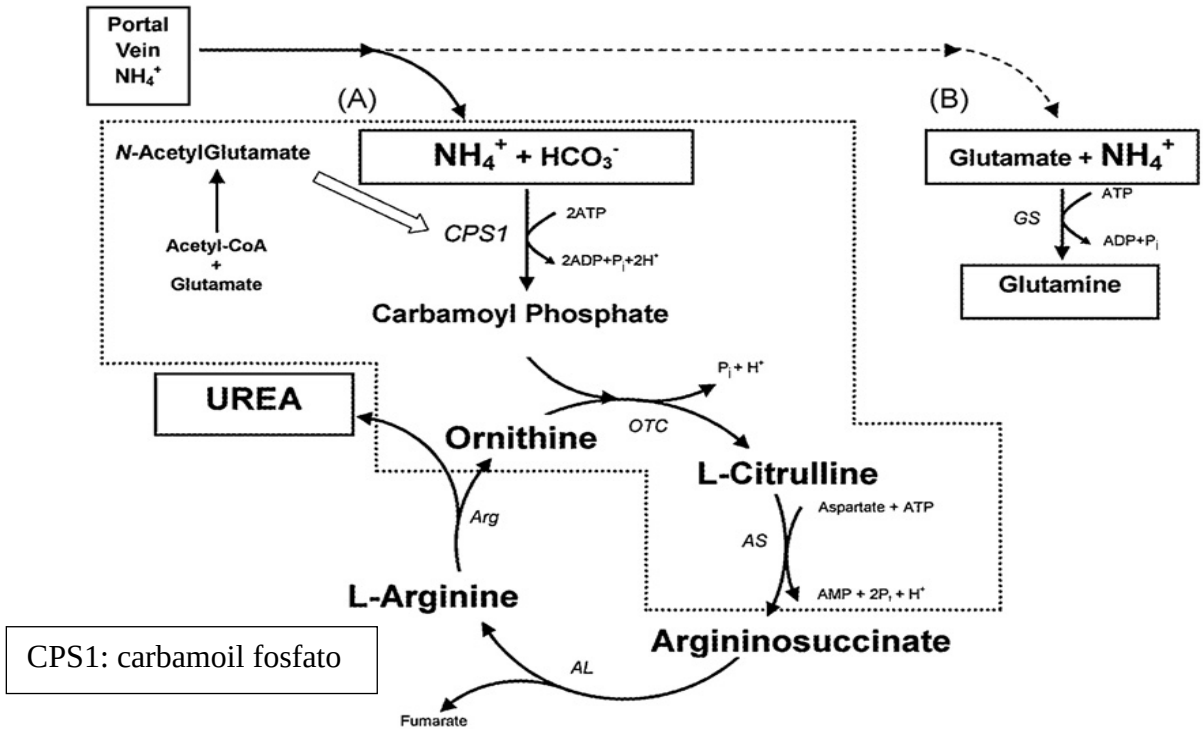

Figura 2: Caminhos de detoxificação da amônia no fígado. Extraído e Adaptado de WILKINSON, 2010.

1.3.Catabolismo de aminoácidos, ciclo das purinas nucleotídeos e amônia durante o exercício

Os aminoácidos livres são substratos para síntese protéica, para a anaplerose e gligoneogênese. Durante o metabolismo os aminoácidos são desaminados ou transaminados em reações que podem resultar na formação de cetoácidos (cetoanálogos) e amônia (SCHLOERB, 1966; FÜRST, 1989; KELLY e STANLEY, 2001, COOPER, 2001) (Figura 3).

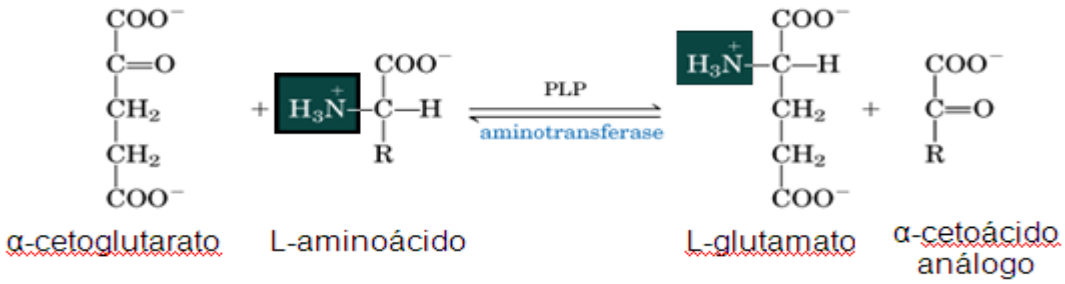

Figura 3. Reação de transaminação. PLP: piridoxal fosfato como co-fator; grupo amino na cor verde. Extraído e adaptado de Nelson e Cox, 2005.

O exercício é considerado indutor de estresse metabólico e o acompanhamento do “pool” de aminoácidos no sangue pode nos fornecer informações sobre como o metabolismo esta sendo modificado (MacLEAN et al., 1996). Aminoácidos na célula muscular podem ceder o grupamento amina a um cetoácido ou glutamato gerando alanina ou glutamina (respectivamente), que são enviadas pela corrente sanguínea e novamente desaminadas no fígado, onde sua cadeia lateral pode servir como agente neoglicogênico e seu grupamento amina para formação de uréia (GIBALA et al., 1997; GIBALA et al., 1998; BLOMSTRAND e SALTIN, 1999).

Durante o exercício o músculo é a principal fonte de produção de amônia. A elevação da amônia induzida pelo exercício é freqüentemente associada a um maior estresse energético. A descoberta de produção de amônia pelo músculo foi relatado por Parnas e colaboradores no final de 1920 (WILKINSON, 2010; MUTCH e

BANISTER, 1983). Foi descoberto que a reação, controlada através da enzima AMP deaminase (AMPD), é responsável pela produção de amônia observada no músculo. Esta reação foi mais tarde identificada como parte de um processo chamado de ciclo das purinas (PNC), que envolve três reações interligadas controlada pelas enzimas AMPD, adenilossuccinato sintetase (AS) e adenilossuccinato liase (AL). Durante exercício intenso, quando a produção de adenosina de monofosfato (AMP) e desaminação são elevados, a adenosina de difosfato (ADP) aumenta os níveis também como o uso de adenosina de trifosfato (ATP) excede a refosforilação, o pH muscular diminui significativamente (WILKINSON, 2010).

Um aumento nas concentrações de ADP livre no citosol associado com redução na sua refosforilação resulta em estímulo da mioquinase (MK), o que provoca um aumento de AMP livre com reação catalisada pela adenilato quinase. Se as concentrações tornarem-se excessivas o AMP é desaminado a inosina de monofosfato (IMP), hipoxantina e amônia (Figura 4), pelo aumento da atividade do PNC (SAHLIN, 1994). O PNC é ativado pelo aumento das concentrações de AMP, ADP, H^+ e inibido por concentrações normais de ATP, GTP e aumento na concentração de Pi, eventos que estão associados com a velocidade de desaminação do AMP (GRAHAM e MacLEAN, 1992; ZIELIJSKI, 2009).

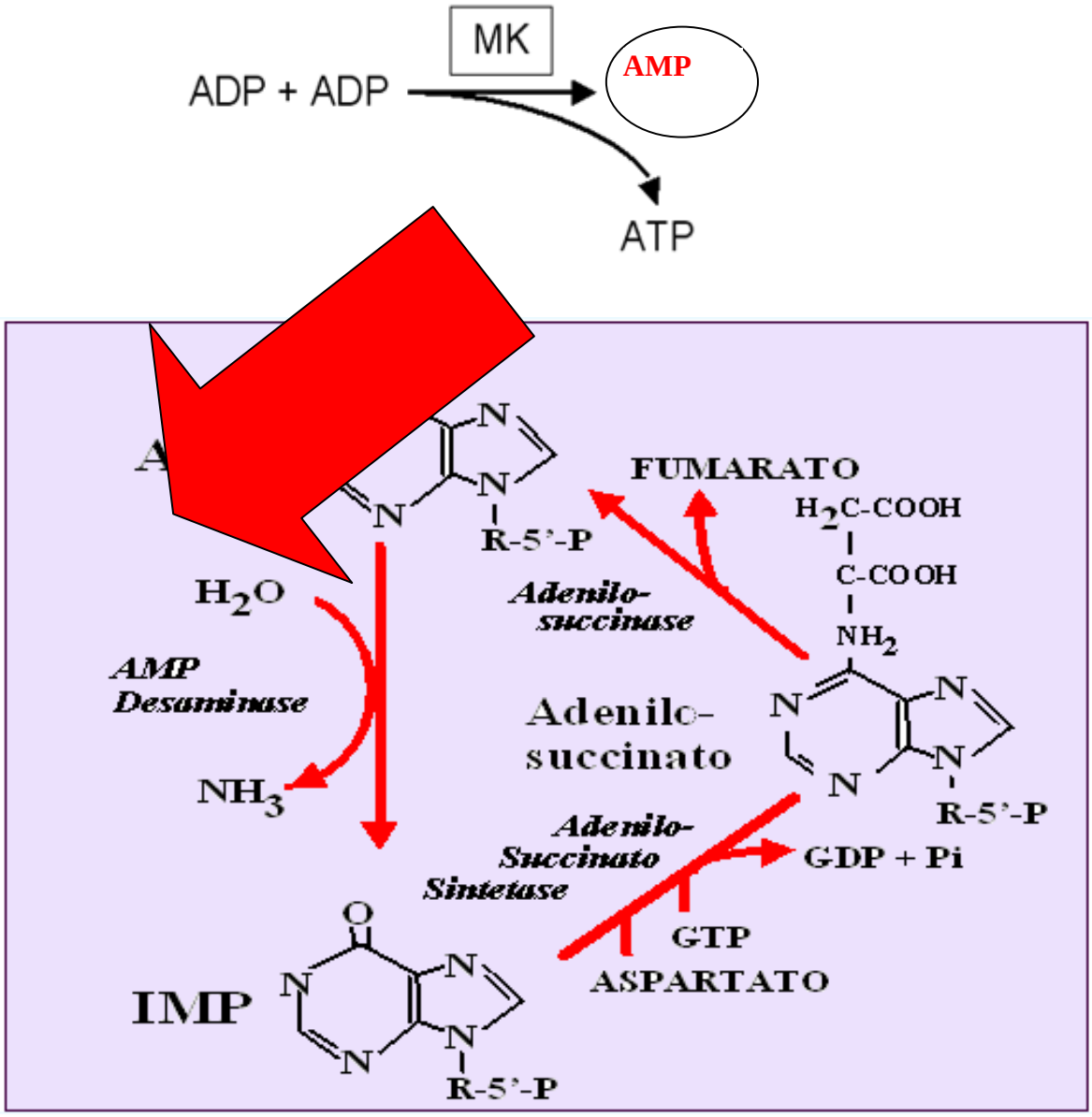

Figura 4: Gênese da amônia a partir da degradação de adenosina. A ação da mioquinase transfere um fosfato do ADP para outro com produção de ATP e AMP. Reações do PNC e a produção de amônia pela AMP deaminase. Extraído e adaptado de SAHLIN, 1994.

O PNC provê o caminho para a deaminação dos aminoácidos, a conversão de aspartato para fumarato e amônia através do PNC, que consiste em reações

catalisadas pela adenilato deaminase (ABERNETHY e WEHR, 1997), adenilossuccinato sintetase e pela adenilssuccinase. O PNC possui outras funções, como: manutenção da alta taxa ATP/ADP e reabastecimento dos intermediários do Ciclo de Krebs (SAHLIN, 1994).

Exercícios até exaustão também podem levar a aumentos na concentração de amônia no organismo (GUEZENNEC et al., 1998). A elevação da amônia pode ocorrer durante um exercício intenso além da atividade aumentada da AMPD, enzima que é grandemente ativada durante o exercício, também por desaminação da glutamina (TULLSON, ARABADJIS et al., 1996). O urato resultante é filtrado pelos glomérulos e reabsorvido em seguida pelos túbulos em proporção aproximada de 90%. Ele representa o produto final do metabolismo das purinas, ácidos nucléicos e nucleoproteínas. O teor de urato plasmático é influenciado por fatores renais e extra-renais (SAHLIN, 1994).

Acredita-se que a recuperação de *pool* de adenina nucleotídeos depois de um exercício intenso é alcançada primariamente pela reaminação do IMP a AMP através de reações do PNC. A AMPD é uma enzima controlada e relativamente inativa nos músculos em repouso tornando-se intensamente ativa durante as contrações musculares e quando há deficiência energética (SAHLIN, et al., 1999; CZARNOWSKI, et al., 1995; SAHLIN, 1994).

A taxa de amônia produzida pelo músculo depende da composição da fibra muscular, intensidade e duração do exercício. Dependendo da intensidade e duração do esforço, o tecido muscular pode difundir amônia para o plasma e outros órgãos. O fornecimento de energia nos músculos ativos promove aumento de amônia, sugerindo a ativação da via MK (SAHLIN, 1994).

O aumento da produção, a acumulação e distribuição de amônia durante o exercício exaustivo, podem levar a distúrbios na função do SNC e contribuem para o aparecimento da fadiga central e periférica e incapacidade de sustentar o desempenho ou exercício máximo. Durante o exercício prolongado, a percepção de esforço está relacionada com a acumulação de amônia no SNC, onde é sustentado pela depleção de glicogênio nos astrócitos o que limita a capacidade do cérebro para

acelerar o seu metabolismo (BANISTER e CAMERON, 1990; NYBO et al., 2005; WILKINSON, 2010).

A deficiência de energia periférica aumenta a produção de amônia tanto pelo PNC como pelo catabolismo de aminoácidos de cadeia ramificada (BCAAs), alanina e glutamina (TULLSON,1999; WAGENMAKERS, 1999), visto que no exercício intenso, o fígado parece não aumentar a extração da amônia, o que pode deixar o organismo exposto a uma potencial elevado deste metabólito (ERIKSSON, et al.,1985; BUTTERWORTH, 2001).

Em exercícios de alta intensidade (90% $VO_{2máx}$), a maior via para a ressíntese de ATP é a hidrólise de creatina fosfato (CP), a degradação de glicogênio muscular para lactato e o PNC (HARGREAVES, MCKENNA et al., 1998). As unidades motoras ativas promovem um aumento das demandas energéticas e da taxa de utilização de ATP muscular quando comparada com a taxa de ressíntese, o que leva à diminuição do ATP e ao acúmulo de ADP e AMP, associado com a formação de IMP e amônia. Isto é estabelecido pelas mudanças nas concentrações de substratos (adenina nucleotídeos), produtos da reação (IMP e NH3) muscular e a baixa capacidade do músculo encontrar uma demanda energética (TULLSON, ARABADJIS et al., 1996). (Figura 4).

Aumento dos níveis de amônia também são observados em exercícios intermitentes, tais como o futebol (KELLIS, KATIS, VRABAS, 2006; BASSINI-CAMERON et al., 2008).

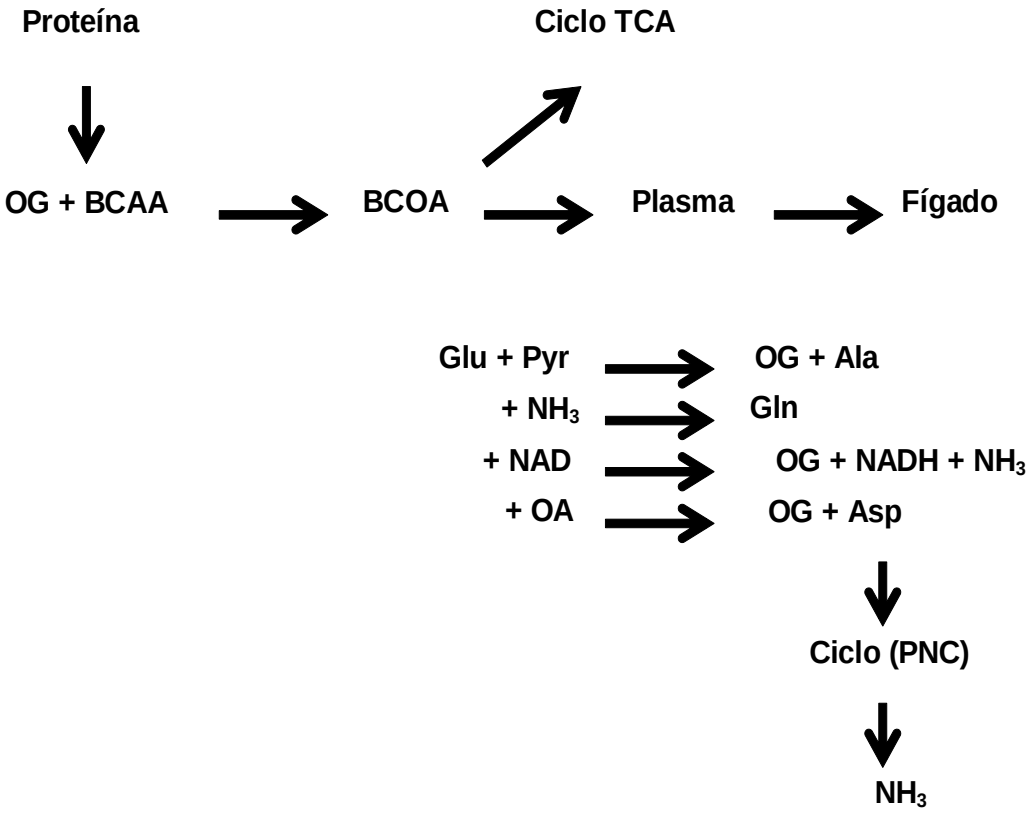

Figura 5: Possíveis vias dos aminoácidos de cadeia ramificada (BCAA). TCA (ácido tricarboxílico), OG (2-oxoglutarato), BCAA (aminoácido de cadeia ramificada), BCOA (oxoácido de cadeia ramificada), GLU (glutamato), Pyr (piruvato), Ala (alanina), NH3 (amônia), Gln (glutamina), NAD e NADH (adenina nicotinamida dinucleotídeo oxidada e reduzida), AO (oxaloacetato) ASP (aspartato) e PNC (ciclo das purinas nucleotídeos). Extraído e adaptado de GRAHAM e MACLEAN, 1992.

A amônia pode atravessar a barreira hematoencefálica sob a influência de gradientes de pH (BANISTER e CAMERON, 1990). Nesta condição pode ocorrer depleção de ATP em regiões críticas do encéfalo, já que durante o exercício exaustivo sustentado, a capacidade de detoxificação de órgãos periféricos pode ficar saturada e provocar elevação da amônia (SAHLIN, 1994). A amônia aumentada no SNC pode interferir com a concentração de metabólitos chave do ciclo de Krebs e do

conjunto malato-aspartato que transporta equivalentes reduzidos do citossol para cadeia transportadora de elétrons na mitocôndria, o que provoca um desequilíbrio na produção de neurotransmissores (MONFORT et al., 2002).

Outro fator que pode alterar as respostas metabólicas durante o exercício é a exposição ao calor que tem como resposta aguda uma redistribuição do fluxo sanguíneo levando a redução deste para o músculo o que pode promover aumentos nas concentrações intramusculares dos metabólitos e influenciar no metabolismo e na concentração da amônia muscular, lactato e IMP (NIELSEN, SAVARD et al., 1990; FEBBRAIO, SNOW et al., 2000; FEBBRAIO, CAREY et al., 1996; LINNANE, BRACKEN et al., 2004; SAHLIN, TONKONOGI et al., 1999).

A taxa de formação de amônia sanguínea durante o exercício isométrico até a exaustão é mais elevada durante a ação muscular isométrica quando comparada com exercício dinâmico intenso e o repouso, isso devido ao processo de restrição sanguínea que ocorre na isometria durante a contração muscular (KATZ, BROBERG et al., 1986; ITOH e OHKUWA, 1991; SAHLIN e KATZ, 1989).

O metabolismo de amônia no SNC está diretamente ligado ao ciclo glutamina/glutamato, essencial para os neurônios glutamatérgicos. Este ciclo envolve a síntese de glutamato no terminal pré-sináptico pela reação de desaminação oxidativa catalisada pela glutamato desidrogenase, glutamato este liberado na fenda sináptica para transmissão do impulso nervoso (KELLY e STANLEY, 2001; FELIPO e BUTTERWORTH, 2002b).

A captação do glutamato se dá principalmente pelos astrócitos através de transportadores. Assim que é captado é convertido a glutamina pela enzima glutamina sintetase e liberado para o interstício onde o neurônio pré-sináptico captura-o novamente (SUÁREZ et al., 2002) .

A atividade da GS é fundamental não só para o controle da concentração de amônia como também para manutenção da concentração de glutamato intersticial, prevenindo assim a excitotoxidade (SUÁREZ et al., 2002). A concentração arterial e cerebral de amônia deve ser mantida em condições normais, assim sendo, as células gliais devem estar continuamente captando glutamato e gerando glutamina para que

os efeitos tóxicos da amônia não sejam evidenciados (COOPER, 2001; BUTTERWORTH, 2002).

A produção de amônia no músculo sugere uma relação dependente da intensidade do exercício. Estudos em ratos e seres humanos relataram maior taxa de amônia em exercícios de alta intensidade com valores entre (70-110% $VO_{2máx}$). Já em intensidades abaixo de 50% $VO_{2máx}$, pouca ou nenhuma acumulação é observada (KATZ et al., 1986). Devido a esta relação, pesquisadores acreditavam que durante muitos anos foi a desaminação do AMP o único caminho através do qual a amônia era formada durante o exercício. Esta interpretação foi provavelmente devido a metodologias limitadas utilizadas para investigar esse fenômeno (WILKINSON, 2010). Muitos negligenciaram outras fontes potenciais de produção de amônia no músculo, ou seja, como a desaminação dos BCAA (GRAHAM et al., 1997, 1995a).

Os aminoácidos de cadeia ramificada, leucina, isoleucina e valina, compõem aproximadamente 40% dos aminoácidos essenciais da dieta e desempenham um papel vital devido à sua hidrofobicidade forte. Ao contrário de outros aminoácidos, BCAA são metabolizados em tecidos extra-hepáticos, como a maioria desta ocorrendo nas mitocôndrias do músculo esquelético (BROSNAN e BROSNAN, 2006). Através deste processo, os BCAAs são divididos por duas reações, aminotransferase dos aminoácidos de cadeia ramificada (BCAT) e complexo enzimatico αcetoácido de cadeia lateral ramificada (BCKDH) e α cetoácido de cadeia ramificada (BCKA), para formar compostos que podem ser utilizados no TCA para a produção de energia oxidativa (SHIMOMURA et al., 2006). Na reação BCAT primário, o grupo amino do BCAA é utilizada para formar glutamato a partir de 2 - oxoglutarato, após o que este glutamato pode então formar glutamina via GS ou alanina através da combinação com piruvato. Em alguns casos, o glutamato pode reagir com o co-fator NAD^+ através da reação glutamato desidrogenase (GDH), levando à formação de amônia, identificando que AMPD não é o único caminho no músculo que a geração de amônia pode ocorrer (WAGENMAKERS et al, 1990).

A opinião consensual é que a produção de amônia durante o exercício ocorre por meio de uma combinação de ambos, desaminação do AMP e

metabolismo de BCAA, que são ativadas em intensidade e duração de forma dependente (HELLSTEN et al., 1999; DALSGAARD et al., 2004; NYBO et al., 2005; MOHR et al., 2006).

1.4.Efeitos Fisiopatológicos da Amônia

Níveis elevados de amônia têm origem congênita ou adquirida. Em sua forma congênita está normalmente relacionada à falhas no ciclo da uréia, mas marcadamente na ornitina transcarbamilase (OTC ou ornitina carbamoil transferase) responsável pela etapa de conversão de ornitina em citrulina. Já a forma adquirida está relacionada à falência hepática provocada por ingestão de toxinas como exemplo o etanol, infecções virais ou doenças auto-imunes (FELIPO e BUTTERWORTH, 2002b).

A uréia constitui a fração de nitrogênio não protéico mais importante na maioria dos líquidos biológicos. Ela é proveniente da conversão da amônia na mitocôndria dos hepatócitos (MOOREN e VOLKER, 2004).

Em mamíferos a amônia é excretada sob a forma de uréia, cuja vantagem evolutiva é o uso de um metabólito menos tóxico na corrente sanguínea. Este processo acontece durante o ciclo da uréia, executado unicamente no hepatócito. O ciclo da uréia inicia-se no interior das mitocôndria dos hepatócitos, porém três de seus passos ocorrem no citosol, abrangendo o ciclo os dois compartimentos celulares. A liberação da uréia se dá no passo de regeneração da ornitina a partir da arginina.

Este grupo amina é utilizado na síntese de carbamoil fosfato junto com o HCO_3^- produzido pela respiração mitocondrial. O segundo grupo é fornecido pelo aspartato gerado na mitocôndria por transaminação e transportado para o citossol. Antes que o ciclo possa prosseguir, a citrulina formada deve ser transferida ao citossol e passada diretamente para o sítio ativo de uma molécula da argininossuccinato sintetase. Isto indica que as enzimas mitocondriais e citosólicas envolvidas no ciclo estão agregadas. Apenas a uréia é liberada na solução geral no

interior citosólico. O custo final é bastante alto, já que são necessários quatro ATPs, produzindo apenas uma molécula de uréia (Figura 6).

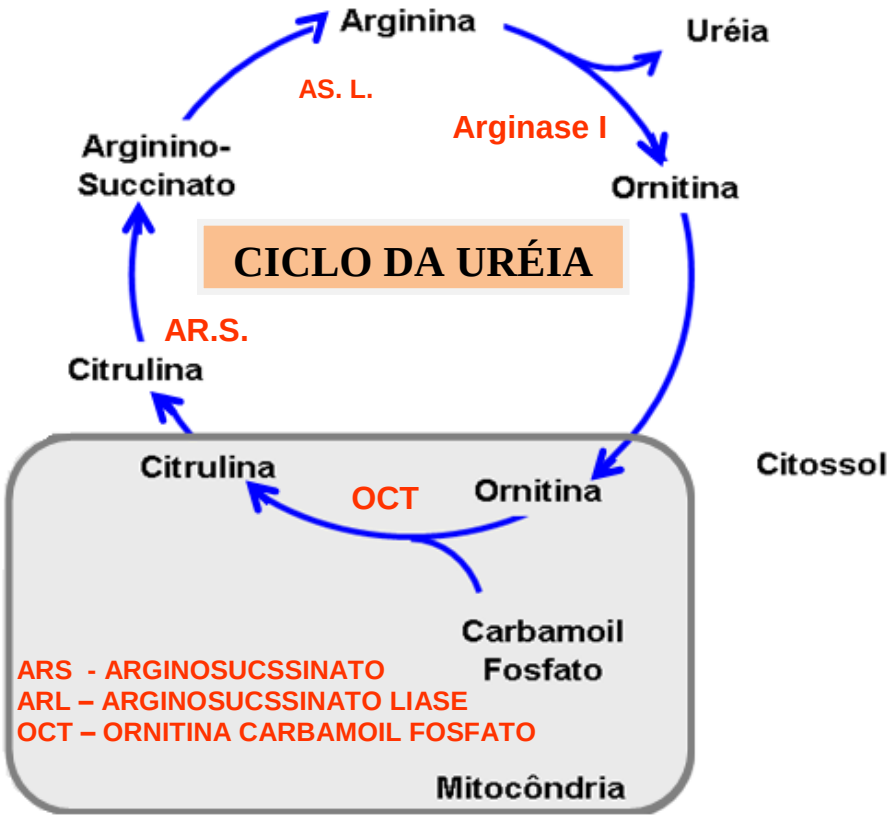

Figura 6: Etapas do ciclo da uréia. Consiste em quarto passos. (1) Formação de citrulina a partir de ornitina e carbamoil fosfato; a citrulina passa para o citossol (2) formação de argininosuccinato (3) fomação de arginina a partir de arininosuccinato e (4) formação de uréia. Extraído e Adaptado de NELSON e COX, 2005.

Podemos ainda classificar a elevação da amônia em aguda, geralmente associada à rápida morte de pacientes ou animais, e crônica, associada a alterações na função cerebral como incoordenação neuromuscular, alterações na cognição e

torpor entre outros (MONFORT et al, 2000; ROSE, 2006; CÓRDOBA e MÍNGUEZ, 2008).

Assim, a amonemia deve ser mantida no intervalo 50-100 µmol/L e a redução na capacidade hepática para a sua remoção, pode provocar alterações encefálicas e, conseqüentemente, um espectro neuropsiquiátrico e neurológico, sintomas que incluem a memória prejudicada, reduzida capacidade de atenção, inversões do ciclo sono-vigília, edema cerebral, hipertensão intracraniana, convulsões, ataxia e coma. Durante insuficiência hepática, tem sido demonstrado em humanos que concentração de amônia no cérebro pode atingir 1-5 mM provocando morte (CLEMMESEN et al. 1999) e o mesmo é observado em estudos com animais (ROSE et al., 1999; BOSOI e ROSE, 2009). É fato que a amônia tem um papel importante na neurotoxicidade. A evidência substancial apóia a idéia de que os astrócitos constituem o alvo principal deste processo, levando a proposta de que ela representa uma “Gliopatia” (NOREMBERG et al., 2009).

O aumento das concentrações plasmáticas de amônia tem efeitos deletérios no SNC, e apresenta um importante papel no desenvolvimento da encefalopatia hepática (EH) com alterações no metabolismo que afetam regulamente atividades de enzimas importantes (SCHLIESS, GÖRG e HÄUSSINGER, 2009; TIMMERMANN et al., 2005; BOSOI e ROSE, 2009).

A toxicidade provocada por uma elevação da amônia no plasma resulta em desequilíbrio na reação da glutamato desidrogenase em direção ao glutamato, o que reduz intermediários para o ciclo de Krebs, como o 2-oxoglutarato e diminuição da síntese de ATP, deixando o cérebro mais vulnerável à elevação da amônia (MUTCH e BANISTER, 1983; NYBO et al., 2005).

EH oriunda da elevação da amônia define um amplo espectro de sintomas neuropsiquiátricos associada à insuficiência hepática aguda e crônica, onde todos são potencialmente reversíveis (SCHLIESS, GÖRG e HÄUSSINGER, 2009; HAUSSINGER e SCHLIESS 2008, HÄUSSINGER, 2009). A associação entre a neurotoxicidade da amônia e EH foi sugerido por estudos em cães submetidos a anastomose porto-cava e desenvolvimento de manifestações neurológicas quando alimentados com carne (CÓRDOBA e MÍNGUEZ, 2008).

1.5. Amônia e Suplementação

A utilização de manipulações dietéticas e o consumo de substâncias com o propósito de aumentar a performance são um fenômeno que cresce a cada dia, e com a cafeína não poderia ser diferente já que esta, tem sido utilizada no sentido de favorecer alterações orgânicas e melhorar o desempenho (SINCLAIR e GEIGER, 2000; SPRIET,1995).

Estratégias nutricionais são sugeridas para reduzir a depleção energética e a elevação da amonemia aguda e transitória, induzida pelo exercício, por meio da manipulação de dietas e suplementos, com o objetivo de aumentar a carga energética celular e, portanto minimizar o aumento da amônia (LANGFORT et al., 2004; ANDREWS et al., 2003; MOURTZAKIS e GRAHAM, 2003; BRUCE et al., 2001; MADSEN et al., 1996).

Estudos mostram que indivíduos treinados apresentam mecanismo de detoxificação para amônia mais eficiente e, portanto menor elevação da amônia quando comparados com não treinados, fato explicado possivelmente, pela redução da atividade das enzimas AMP desaminase e glutamato desidrogenase, relacionadas com a produção direta de amônia no músculo e com o provável aumento na formação de alanina e glutamina e de enzimas ligadas a transaminação e oxidação de aminoácidos (GRAHAM et al., 1997; WAGENMAKERS, 1999; SAHLIN, 1994).

Dados obtidos em nosso laboratório demonstraram que a suplementação de carboidrato (CnH2nOn) e glutamina, isolados ou combinados, têm um efeito semelhante na inibição da elevação da amonemia em corredores após 60 min de exercício (CARVALHO-PEIXOTO et al., 2007).

A cafeína é um dos agentes farmacológicos mais consumidos no mundo, podendo ser encontrado facilmente em vários produtos, seja na forma medicamentosa ou alimentar como: chás, chocolate, refrigerante e café. Nas últimas décadas, o uso de cafeína tem crescido provavelmente devido ao aumento do consumo de bebidas do tipo refrigerantes.

Classificado como um alcalóide, pertencente ao grupo das drogas denominada metilxantina (1,3,7- trimetilxantina), que possui propriedades psico-

estimulante, e é considerado um ergogênico. A xantina é encontrada na forma natural em diversas plantas, sendo suas fontes as mais diversas torna-se difícil quantificar seu consumo (COSTIL et al, 1978). Pode ser ingerida e administrada de diferentes maneiras, dentre as quais se destacam a via oral, intraperitoneal, subcutânea ou intramuscular. A abstinência da cafeína por um longo período provoca sintomas desagradáveis, como: dor de cabeça, sonolência, mau humor, fadiga, diminuição da energia, da atenção, dificuldade de concentração e irritabilidade. Além disso, apresenta os sintomas pseudogripais como, náuseas, dores musculares e vômitos.

Para se discutir sobre os efeitos da cafeína se faz necessário um conhecimento sobre os mecanismos de ação a nível celular. No exercício físico a presença de cafeína induz liberação de cálcio [Ca^{2+}] para o reticulo sarcoplasmático ativando os receptores de rianodina, aumentando a freqüência e duração dos canais de [Ca^{2+}] abertos, reduzindo o tempo de relaxamento e aumentando o tempo de contração do músculo esquelético (MAGKOS e KAVOURAS, 2005; FRYER e STEPHENSON, 1996).

A cafeína é um antagonista dos receptores A_1 e A_2 de adenosina que ultrapassa rapidamente a barreira hematoencefálica, agindo como estimulante (STEPHERNSON, 1997; EVANS, 1999; DAGER, 1999; VARANI, 2000). A suplementação de cafeína durante o esforço moderado eleva a atividade do eixo hipotálamico-hipofisário-adrenal e o sistema nervoso autonômico (ADLER, 2000), diminuindo o tempo de reação ao estímulo (KRUK, 2001) e reduzindo a percepção ao esforço (DENADAI e DENADAI, 1998). Estudos têm demonstrado a atividade ergogênica positiva da cafeína no exercício de resistência (TARNOPOLSKY, 1994, 2000; GREER, 2000), causada provavelmente pelo retardo do aparecimento da fadiga e aumento do poder contrátil dos músculos esqueléticos e cardíaco (APPLEGATE, 1999; CLARKSON, 1996).

Os achados de Laurente et al. (2000) sugerem que a cafeína somada ao exercício estimula a liberação mais rápida das β-endorfinas e cortisol. Outros estudos demonstraram que a sensação de dor muscular diminuiu nos sujeitos que ingeriram

cafeína (MOTL, 2003; 2004). Spriet (1995) observou a ação da cafeína no SNC afetando a percepção subjetiva ao esforço e/ou a propagação de sinais neurais entre o cérebro e a junção neuromuscular, aumentando a liberação de catecolaminas, particularmente a epinefrina.

Ramanaviciene (2004) demonstrou que a cafeína inicia um importante papel protetor na resposta immune. A liberação de epinefrina modulada pela cafeína induz a leucocitose, linfocitose e neutrofilia.

2.Cetoácidos

Os aminoácidos livres são substratos para síntese protéica, para a anaplerose e gligoneogênese. Durante o metabolismo, os aminoácidos são deaminados ou transaminados em reações que podem resultar na formação de cetoácidos e a amônia (SCHLOERB, 1966; FÜRST, 1989; KELLY e STANLEY, 2001, COOPER, 2001). Os aminoácidos são um importante combustível energético para ressíntese de ATP, principalmente durante o exercício físico intenso (BLOMSTRAND e SALTIN, 1999; GRAHAM e ADAMO, 1999).

O músculo tem uma grande capacidade de produção de amônia durante o exercício, essa produção é acompanhada pela gênese concomitante de alanina, glutamina e pela grande captação de glutamato (Figura 7) (GRAHAM e MacLEAN, 1992).

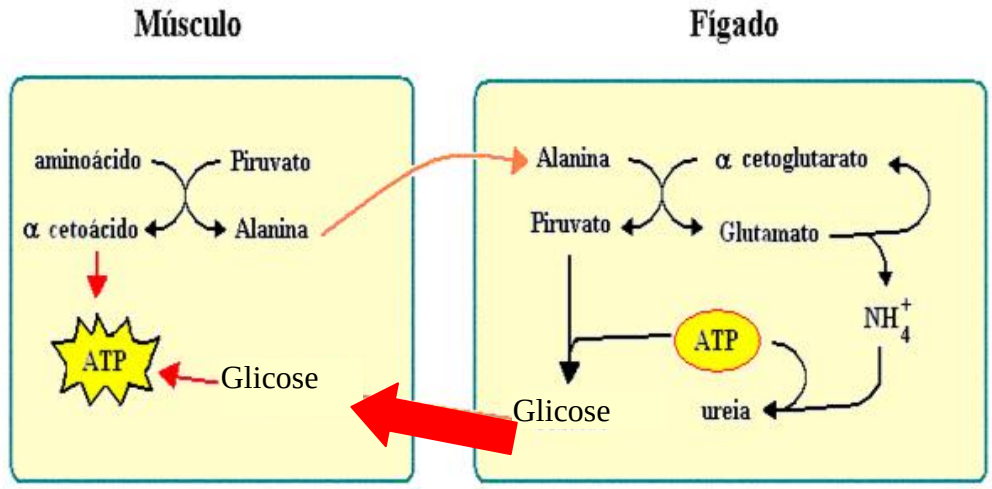

Figura 7: Ciclo alanina/ glicose.

Os cetoácidos são usados como substituição de seus respectivos aminoácidos para manter o balanço de nitrogênio, onde, ao mesmo tempo em que supre as necessidades de aminoácidos essenciais do organismo, o uso de cetoácidos promove diminuição da disponibilidade de nitrogênio, reduzindo assim a formação de compostos nitrogenados tóxicos resultantes do metabolismo (Figura 8).

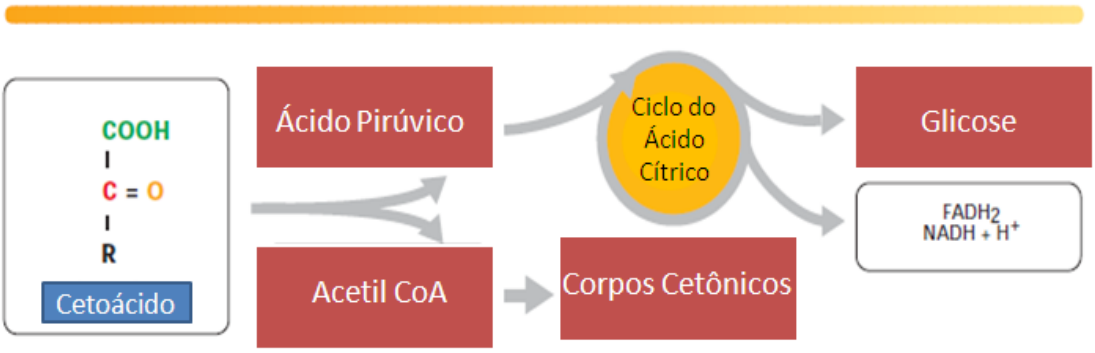

Figura 8: Destino do cetoácido formado por desaminação oxidativa

Entre os possíveis benefícios do uso de cetoácidos associada a restrição protéica convencional, estão à diminuição mais acentuada de sintomas urêmicos, da

acidose metabólica, da hiperfosfatemia e da resistência insulínica (LOFBERG et al., 1997; TEPLAN et al., 2000).

Durante a atividade física, vários aminoácidos são recrutados em maior velocidade, para obtenção de energia e manutenção da glicemia. Sempre que um aminoácido deve ser utilizado para gerar energia ou em neoglicogênese, este é desaminado para a sua entrada nas diversas vias metabólicas. A genêse da amônia está intimamente correlacionada com o tipo de atividade física, treinamento prévio, alimentação e herança genética. É sabido que a produção de amônia está relacionada ao sexo, sendo sua produção maior em homens, provavelmente devido a sua maior massa muscular (DERAVE et al., 1997).

O incremento da excreção de amônia está correlacionado ao aumento da concentração de lactato no sangue (AMENT et al., 1999). A baixa produção de amônia, assim como a de lactato, tem sido considerada um fator de contribuição ao melhor condicionamento físico (SALTIN et al., 1995). Além disso, a elevação da amônia sanguínea com conseqüente elevação da concentração de glutamato leva ao aumento da freqüência respiratória, acarretando uma maior demanda energética. O aumento progressivo da amonemia observado durante a atividade física intensa é um marcador do stress muscular correlacionado a utilização de aminoácidos metabolicamente (SNOW et al., 2000). A diminuição da amoniogênese é assim uma das preocupações a ser estabelecida por profissionais que orientam atletas.

Atualmente, novas modalidades terapêuticas são estudadas com o objetivo de atenuar alterações inerentes à complicações causadas pela elevação da amonemia tais como a EH, doença renal e no esporte com o objetivo de reduzir a fadiga central e periférica. No Brasil esse suplemento já está disponível no mercado, embora limitado devido ao alto custo, o Ketosteril ®,Fresenius Kabi, onde é descrito sua composição (Tabela 1).

Keto analogue or amino acid	(mg)
Calcium 3-methyl-2-oxovaleric acid (a-ketoanalogue of isoleucine)	335
Calcium-methyl-2-oxovaleric acid (a-ketoanalogue of leucine)	505
Calcium-2-oxo-3-phenylpropionic acid (a-ketoanalogue of phenylalanine)	430
Calcium-3-methyl-2-oxobutyric acid (a-ketoanalogue of valine)	340
Calcium-DL-2-hydroxy-4-(methylthio)-butyric acid (a-hydroxyanalogue of methionine)	295
L-lysine acetate (= L-lysine 75 mg)	525
L-threonine	265
L-tryptophan	115
L-histidine	190
L-tyrosine	150
Total nitrogen content per tablet	180

Tabela 1: Composição do Ketosteril ®,Fresenius Kabi.

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CAPÍTULO II: Acute supplementation with keto analogues and amino acids in rats during resistance exercise.

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Acute supplementation with keto analogues and amino acids in rats during resistance exercise

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During exercise, ammonia levels are related to the appearance of both central and peripheral fatigue. Therefore, controlling the increase in ammonia levels is an important strategy in ameliorating the metabolic response to exercise and in improving athletic performance. Free amino acids can be used as substrates for ATP synthesis that produces ammonia as a side product. Keto analogues act in an opposite way, being used to synthesise amino acids whilst decreasing free ammonia in the blood. Adult male rats were divided into four groups based on receiving either keto analogues associated with amino acids (KAAA) or a placebo and resistance exercise or no exercise. There was an approximately 40 % increase in ammonaemia due to KAAA supplementation in resting animals. Exercise increased ammonia levels twofold with respect to the control, with a smaller increase (about 20 %) in ammonia levels due to exercise. Exercise itself causes a significant increase in blood urea levels (17 %). However, KAAA reduced blood urea levels to 75 % of the pre-exercise values. Blood urate levels increased 28 % in the KAAA group, independent of exercise. Supplementation increased glucose levels by 10 % compared with control animals. Exercise did not change glucose levels in either the control or supplemented groups. Exercise promoted a 57 % increase in lactate levels in the control group. Supplementation promoted a twofold exercise-induced increase in blood lactate levels. The present results suggest that an acute supplementation of KAAA can decrease hyperammonaemia induced by exercise.

Ammonia: Urate: Urea: Resistance exercise

Ammonia ($NH_3 + NH_4^+$) is a toxic metabolite with deleterious effects on the central nervous system[1]. Exercise can be used as a model to study ammonia metabolism in an intensity-dependent way[2–5]. During prolonged exercise, ammonia is mainly produced by the catabolism of amino acids[6]. On the other hand, during high-intensity exercise, the largest source of ammonia production is from AMP deamination[7]. Ammonia levels are related to the appearance of both central and peripheral fatigue[8]. Therefore, controlling increases in ammonia is an important strategy in ameliorating the metabolic response to exercise and in improving athletic performance[9,10].

The combination of keto analogues with amino acids has been used to treat patients with chronic kidney disease (CKD), portal systemic encephalopathy and hyperammonaemia[11,12]. Free amino acids can be used as substrates for ATP synthesis, which produces ammonia as a side product[13]. In an opposite manner, the use of keto analogues associated with amino acids (KAAA) has been proposed as a way to synthesise amino acids whilst decreasing free ammonaemia[14,15]. During metabolism, amino acids are deaminated or transaminated to form keto acids via release of the amino group[16]. These reactions are reversible, and the use of keto analogues could reduce the blood ammonia concentration, resulting in the production of amino acids[17]. Thus, keto analogues may serve as nutritional supplements to synthesise amino acids of high biological value, especially in CKD patients. Furthermore, it is acknowledged that resistance and aerobic exercise programmes may serve important roles in the approach to the treatment, prevention and slowed progression of CKD[18].

Although KAAA supplementation is effective in the treatment of CKD, particularly for postponing the necessity for dialysis, the use of KAAA is not popular due to its cost and

Abbreviations: CKD, chronic kidney disease; Ctl group, control group (neither keto analogues associated with amino acids nor exercise); Ex group, exercise-only group; KA group, keto analogues associated with amino acids-only group; KAAA, keto analogues associated with amino acids; KAEx group, keto analogues associated with amino acids and exercise group.

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the requirement for a low protein intake[19]. Thus, there has been a lack of interest in studies involving KAAA due to the lack of cost effectiveness in its use as a therapeutic agent. This lack of interest has limited the number of new papers published on the mechanism of action of KAAA. In the present study, we evaluated the effect of KAAA supplementation on ammonia production and blood urea levels during resistance exercise, showing metabolic effects that can enhance performance and post-exercise recovery.

Materials and methods

Male Wistar rats (12 weeks of age and body mass ranging from 280 to 350 g) were divided into four groups of twelve animals each. The group that received only KAAA (KA group) and the group that received KAAA and exercise (KAEx group) received 0·1 g Ketosteril® (Fresenius Kabi, Bad Homburg, Germany) in 0·5 ml water (0·3 g/kg). The composition of the KAAA mixture per tablet was as follows: α-keto analogues of isoleucine, 335 mg; leucine, 505 mg; phenylalanine, 430 mg; valine, 340 mg; α-hydroxy analogue of methionine, 295 mg; L-lysine acetate, 75 mg; L-threonine, 265 mg; L-tryptophan, 115 mg; L-histidine, 190 mg; L-tyrosine, 150 mg. The group that received neither KAAA nor exercise (control (Ctl) group) and the group that received only exercise (Ex group) received 0·5 ml of 0·9 % NaCl, 1 h before exercise by oral administration. The animals were maintained in collective cages (four per cage) at 22 ± 2°C with a photoperiod of 12 h and fed *ad libitum* (diet and water). The study was approved by the Ethics Committee in Research of the University of Tiradentes, and followed the Guiding Principles for Research Involving Animals and Human Beings.

Resistance exercise was performed according to a previous study[20] after familiarisation and determination of the load that was to be applied according to the one repetition maximum (1RM) test. Familiarisation consisted of attaching the animal to the exercise device daily without stimulating the animals to exercise, starting 6 d before the experiment. The 1RM test was performed 1 d before resistance exercise and determined the heaviest weight that could be lifted. On the day of the experiment, fifty repetitions were performed with a load equal to 75 % of 1RM. The animals were stimulated to perform the repetitions through sticker electrodes (Axelgaard ValuTrode CF3200; Axelgaard Manufacturing Co. Ltd, Fallbrook, CA, USA) placed in the tail and connected to an electrostimulator (4 mA to 15 mA at 1 Hz for 1 s; Quark Dualpex 961; Quark Medical Products, São Paulo, Brazil).

Blood was collected through cardiac puncture before exercise (Ctl and KA groups) or immediately after exercise (Ex and KAEx groups). The blood samples were immediately centrifuged to obtain sera, which was subsequently frozen and stored at −70°C for future biochemical analysis. Biochemical analyses of glucose, urea, urate and creatinine concentrations were performed using commercially available spectrophotometric assays (Labtest, Minas Gerais, Brazil). Lactate and ammonia were measured using an enzymic UV method (Randox, Crumlin, Co. Antrim, UK) on a Dade Model Dimension RXL Automated Chemistry Analyzer (Dade Behring, Eschborn, Germany), and haematological parameters were analysed using a Sysmex SE-9500 Automated Hematology Analyzer (TOA Medical Electronics, Kobe, Japan). Standard curves were taken at a minimum r value of 0·98 and the experimental points were always within the calibration curve and at least 20 % above the lower limit of detection.

Statistical significance was evaluated by one-way ANOVA. Significances ($P<0·05$) were confirmed using the Tukey test as a *post hoc* analysis. Data are reported as mean values with their standard errors.

Results

We used a weight-lifting exercise to evaluate the effect of KAAA on blood ammonia concentration after resistance

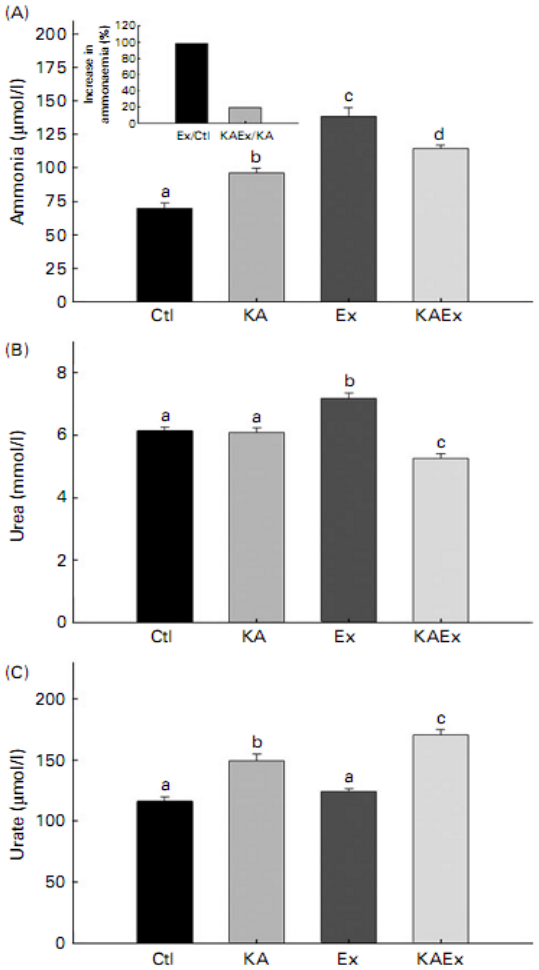

Fig. 1. Acute supplementation of keto analogues associated with amino acids (KAAA) affects ammonia (A), urea (B) and urate (C) metabolism. Ctl, control group (neither KAAA nor exercise); KA, KAAA-only group; Ex, exercise-only group; KAEx, KAAA and exercise group. Values are means, with standard errors represented by vertical bars. ^{a,b,c,d}Mean values with unlike letters were significantly different ($P<0·05$). Inset of Fig. 1(A) shows the increase in ammonia levels in experimental and control groups when normalised against rest values.

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exercise. There was an increase of about 40 % in ammonia in resting animals due to KAAA supplementation. Compared with unsupplemented controls, exercise resulted in a twofold increase in ammonia levels in the animals. However, the supplemented group had a much smaller increase (about 20 %) in ammonia levels after exercise (Fig. 1(A)). Since KAAA is proposed to decrease blood urea levels, we evaluated the response of blood urea to acute KAAA supplementation during exercise. We did not measure the effect of supplementation on blood urea at rest. With no supplementation, exercise significantly increased blood urea levels by 17 % compared with the levels in the Ctl group. However, with KAAA supplementation, blood urea was reduced to 75 % of the pre-exercise values (Fig. 1(B)).

To differentiate the ammonia production derived from AMP deamination from that derived from amino acid deamination, we measured urate, the end metabolite of inosine monophosphate. Blood urate levels increased 28 % in the group supplemented with KAAA, independent of exercise. This effect was enhanced in the supplemented group after exercise. There was no change in blood urate levels in the control group in response to exercise (Fig. 1(C)).

KAAA supplementation has been shown to increase creatinine clearance. Our study model did not detect a change in blood creatinine in response to exercise. However, blood creatinine decreased by 40 % in the groups supplemented with KAAA, independent of exercise (Table 1).

To understand the effect of KAAA on glucose maintenance, we measured glucose levels after exercise. Supplementation increased glucose levels in resting animals by 10 %. Exercise did not change glucose levels in either the Ctl or KAEx groups (Table 1).

Blood lactate is an indicator of glucose utilisation during exercise. Exercise promoted a 57 % increase in blood lactate in the Ctl group. The supplementation promoted a twofold exercise-induced increase in blood lactate (Table 1).

Discussion

It is widely reported that ammonia production increases during exercise and that ammonia could be a deleterious metabolite that promotes fatigue[8,20,21]. The production of ammonia can lead to significantly elevated systemic ammonia levels to levels between 90 and > 200 μmol/l. Patients with either liver or kidney disease also show sharp increases in ammonia levels that may range from 70 to 300 μmol/l in liver disorder patients[1,22]. Patients with CKD have lower peak levels of ammonia during exercise, but experience ammonia increases of about 30–60 % compared with resting values[1,23,24]. KAAA has been widely used as a supplement to treat patients with kidney failure and as a therapeutic agent for liver failure and encephalopathy[12]. Additionally, regular physical activity and close clinical and dietary monitoring, including the use of keto analogues, should be recommended in patients with CKD[25].

One of the problems associated with human studies has been ensuring that subjects have adhered to the recommended diet and have properly taken the supplements. Here, we used a previously described resistance exercise animal method[19] to investigate a possible ammonia-chelating effect of KAAA during exercise in rats. The production of ammonia during exercise occurs via both AMP deamination and branched-chain amino acid metabolism[1].

The use of KAAA increased ammonaemia during the resting state, demonstrating that amino acid metabolism during exercise is associated with anaplerosis of Krebs cycle intermediates[26,27]. Increases in ammonia levels in response to exercise can be managed through the use of amino acids or carbohydrates that interfere with ammonia metabolism[28]. It is possible to propose that the amino acids in the supplement are being using either as carbon skeleton donors to obtain energy or as gluconeogenic precursors. Even with an increase in ammonia levels at rest, KAAA supplementation was able to reduce the exercise-induced increase in blood ammonia by 80 %. When compared with the non-supplemented exercise group, the absolute decrease was 20 %. Previous data in our laboratory showed that there is a habituation of basal ammonia levels in response to amino acid supplementation, since the resting ammonia level decreases with an increase in basal blood urea levels correlated to supplementation time[3]. On the basis of these data, we postulate that the effect of KAAA supplementation on basal ammonia levels can be diminished by chronic KAAA use.

Our exercise model increased ammonia and urea levels in animals without any changes in urate levels. It has been pointed out that excess ammonia is metabolised to urea by the liver for excretion to minimise toxicity[2]. During exercise, KAAA was able to decrease the blood urea concentration to 75 % of the resting urea level. This finding is related to the widely described therapeutic effect of KAAA (for a review, see Savica *et al.*[13]). Urate appears more quickly in blood in response to exercise compared with urea[29]. KAAA supplementation increased resting urate levels. However, we detected changes in blood urate in the supplemented exercise group when compared with the non-supplemented exercise group after resistance exercise. It is known that during

Table 1. Creatinine, glucose and lactate as obtained from the four supplementation protocols
(Mean values with their standard errors)

Group...	Ctl		KA		Ex		KAEx	
Parameter	Mean	SE	Mean	SE	Mean	SE	Mean	SE
Creatinine (mmol/l)	47·66[a]	0·91	28·89[b]	0·71	47·49[a]	0·76	27·76[b]	0·76
Glucose (mmol/l)	6·11[a]	0·12	6·64[b]	0·20	6·06[a]	0·12	6·81[b]	0·16
Lactate (mmol/l)	2·66[a]	0·08	2·67[a]	0·07	4·18[b]	0·09	5·44[c]	0·09

Ctl, control (no keto analogues associated with amino acids or exercise); KA, keto analogues associated with amino acids only; Ex, exercise only; KAEx, keto analogues associated with amino acids and exercise.
[a,b,c]Mean values within a row with unlike superscript letters were significantly different ($P<0·05$; ANOVA).

high-intensity exercise such as resistance exercise, the largest quantity of urate is produced when the ATP:ADP ratio decreases which leads to increases in both AMP deamination and urate synthesis[8]. It is possible that our resistance exercise model with fifty repetitions activates pathways associated with resistance and prolonged exercise. KAAA supplementation promoted an increase in creatinine clearance. This is a well-described effect of this supplement in chronically ill patients[12]. Taking these results together, we postulate that the majority of ammonia production results from the deamination of amino acids instead of AMP[7,8,30].

Some studies have shown that amino acid supplementation increased the pool of Krebs cycle intermediates during exercise[5,31]. KAAA supplementation produced a 10 % increase in resting glucose levels that were maintained even after exercise. Since KAAA is a mixture of ketogenic and glucogenic keto analogues and amino acids, we postulate that KAAA provide glucose for exercise. It is important to state that the use of the amino acids from KAAA as carbon skeleton donors augments the net ammonia release. On the other hand, the anaplerosis using the keto analogues does not increase ammonia release. However, both situations increase ATP synthesis, leading to a decelerating ammonia production due to AMP deamination.

The results of the present study showed that KAAA supplementation exacerbated blood lactate levels after exercise. It is known that lactate is formed during glycolysis in active skeletal muscles and many conditions can attenuate lactate levels during exercise, such as muscle glycogen depletion[32]. Thus, such alterations in the present study may be explained by KAAA providing glucose for exercise through gluconeogenesis. Since the central nervous system has no effective urea cycle and depends on the synthesis of glutamine for removal of the excess ammonia[9,33], high levels of blood ammonia have been proposed to be related to the development of both local and central fatigue[9,21,34]. Here, we describe for the first time that acute supplementation of KAAA can be used to reduce the increase in ammonia levels caused by resistance exercise. The practical significance of these findings may be important for the individual exerciser and merits further research to examine the efficacy of chronic KAAA intake. Therefore, we believe that the present study contributes important data to our understanding of metabolism and that these findings could be helpful for the development of future therapies.

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R. D. A., E. S. P. and L.-C. C. were responsible for the study design. All authors contributed to data collection and interpretation, and manuscript writing.

The authors have no conflicts of interest to declare.

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CAPÍTULO III: Keto analogues and amino acids supplementation changes the ammonemia response during exercise under ketogenic conditions.

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Short Communication

Keto analogue and amino acid supplementation affects the ammonaemia response during exercise under ketogenic conditions

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Abstract

Hyperammonaemia is related to both central and peripheral fatigue during exercise. Hyperammonaemia in response to exercise can be reduced through supplementation with either amino acids or combined keto analogues and amino acids (KAAA). In the present study, we determined the effect of short-term KAAA supplementation on ammonia production in subjects eating a low-carbohydrate diet who exercise. A total of thirteen male cyclists eating a ketogenic diet for 3 d were divided into two groups receiving either KAAA (KEx) or lactose (control group; LEx) supplements. Athletes cycled indoors for 2 h, and blood samples were obtained at rest, during exercise and over the course of 1 h during the recovery period. Exercise-induced ammonaemia increased to a maximum of 35 % in the control group, but no significant increase was observed in the supplemented group. Both groups had a significant increase (approximately 35 %) in uraemia in response to exercise. The resting urate levels of the two groups were equivalent and remained statistically unchanged in the KEx group after 90 min of exercise; an earlier increase was observed in the LEx group. Glucose levels did not change, either during the trial time or between the groups. An increase in lactate levels was observed during the first 30 min of exercise in both groups, but there was no difference between the groups. The present results suggest that the acute use of KAAA diminishes exercise-induced hyperammonaemia.

Key words: **Ammonia: Uric acid: Ketogenic diet: Endurance**

Ammonia (used here as a synonym for the sum of NH_3 and NH_4^+) is highly toxic to humans and can cross the blood–brain barrier, which leads to a decrease in cerebral function, neuropsychiatric disorders and death[1,2]. Ammonia-mediated excitotoxicity has been implicated in the mediation of central nervous system damage[3,4].

Data obtained from exercise studies have been used to elucidate the effects of hyperammonaemia. Several investigations have demonstrated that increased ammonaemia occurs during various types of exercise[5–8]. During prolonged submaximal exercise, an increase in ammonaemia (>160 μmol/l) has been observed in various studies[8,9]. The consensus view is that the production of ammonia during exercise occurs via a combination of both AMP deamination and catabolism of amino acids, processes that are activated in an intensity- and duration-dependent manner[2]. It has been suggested that ammonia promotes both central and peripheral fatigue and that better control of ammonia production will improve exercise performance[4,10].

Abbreviation: KAAA, keto analogue and amino acid.

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The increase in the ammonia levels in response to exercise can be managed through the use of amino acids or carbohydrates that interfere with ammonia metabolism[7]. During metabolism, amino acids are deaminated or transaminated to form keto acids via release of the amino group[11]. These reactions are reversible, and the use of keto analogues could reduce blood ammonia concentration, resulting in the production of amino acids[12]. The long-term use of keto analogues associated with amino acids (KAAA) to provide amino acid supplementation has been described previously[13]; to our knowledge, the acute use of KAAA has never been studied.

Although ammonia has been shown to be produced by branched chain amino acid catabolism, independent of glycogen availability[14], various studies have linked ammonia formation to carbohydrate availability[4,7]. Adopting a low-carbohydrate diet (termed a ketogenic diet) combined with physical exercise can reduce glycogen stores before exercise and induce hyperammonaemia[15,16]. We used this metabolic effect of a ketogenic diet to enhance the effect of exercise on ammonia production.

In the present study, we evaluated the acute effect of KAAA supplementation on ammonia production during prolonged exercise. We hypothesised that acute KAAA supplementation can prevent the increase in ammonaemia during exercise owing to the function of keto analogues as energetic substrates or as ammonia-chelating agents.

Materials and methods

A total of thirteen male endurance-trained cyclists (28·6 (SEM 1·6) years; 68·8 (SEM 2·3) kg; 1·77 (SEM 0·01) m) with similar exercise training levels (VO_{2max} 52·7 (SEM 2·8) ml/kg per min and maximum heart rate 191·4 (SEM 1·6) beats/min) participated in the study voluntarily. All subjects had similar physical capabilities and had a minimum of 3 years of training. They had not used ergogenic substances or any other drugs. The present study was conducted according to the guidelines laid down in the Declaration of Helsinki, and all procedures involving human subjects were approved by the Ethics Committee for Human Research at the Federal University of the State of Rio de Janeiro; ethics number: 117/2007. Written informed consent was obtained from all subjects.

Athletes reported to the laboratory 3 d before the start of the experiment to become familiar with the cycle ergometer and to optimise the power output. Subjects received an individualised ketogenic diet (Table 1; 12·9 (SEM 0·4) MJ; 35 % of the recommended energy intake from protein, 3·8 (SEM 0·1) g/kg; 55 % from lipids, 2·7 (SEM 0·1) g/kg; and less than 10 % from carbohydrates, 1·1 (SEM 0·1) g/kg; see Westman *et al.*[17] for a review of the effects of a low-carbohydrate diet on metabolism) for 2 d before the experiment and during the day of the experiment.

Subjects were asked to maintain their normal training schedule (approximately 70 km/d) and to follow the ketogenic diet up to 48 h (normal training and a ketogenic diet were used to reduce muscle glycogen stores and to induce a higher increase in ammonaemia) before the day of the experiment. On the day of the experimental period, the subjects reported to the laboratory in a fasting state and received breakfast and a light lunch. At 1 h after lunch, the subjects received either five tablets of a KAAA mixture (experimental group (KEx), *n* 6; Ketosteril®; Fresenius, Bad Homburg, Germany) or five 200 mg tablets of lactose (control group (LEx), *n* 7; Via Farma, São Paulo, Brazil) in a randomised double-blind manner. The composition of the KAAA mixture/tablet was as follows: α-keto analogues of isoleucine (335 mg), leucine (505 mg), phenylalanine (430 mg) and valine (340 mg); α-hydroxy analogue of methionine (295 mg); L-lysine acetate (75 mg L-lysine); L-threonine (265 mg); L-tryptophan (115 mg); L-histidine (190 mg); L-tyrosine (150 mg). Both supplements were provided in indistinguishable capsules.

Table 1. Metabolisable energy from a ketogenic diet*

	Size	Carbohydrates	Lipids	Proteins
Meat	400	0	42·0	135·4
Cheese	300	17·2	83·2	28·2
Ham	300	2·2	22·7	48·3
Egg	400	2·5	39·1	53·2
Tangerine	200	19·6	0·2	1·6
Orange	200	17·9	0·2	2·0
Watermelon	200	16·6	0·1	1·8
Total		76·0	187·5	270·5

*Values are expressed in g from prepared meals.

Before the experimental trial, initial stretching was followed by a warm-up of 10 min at 50 % of the maximum heart rate. The experiment began 1 h after supplementation, and athletes cycled indoors for 2 h at 80 rpm according to a metronome in a room with constant temperature and relative humidity (23 ± 2°C and 60 ± 5 %, respectively). The subjects' heart rates were recorded continuously throughout the exercise period using a heart rate monitor (Polar CS200, Kempele, Finland). The power output was modified for each individual every 5 min so that the athletes maintained 75–85 % of their estimated maximum heart rate (approximately 156·0 (SEM 2·8) beats/min with a work of 180·0 (SEM 1·4) W, respectively).

A catheter was placed into the median cubital vein. At 1 h after the supplementation, blood samples were obtained at rest and at 30 min intervals throughout the exercise period. Finally, blood samples were collected during a 1 h recovery period at 30 min intervals. Athletes received water *ad libitum* during the trial.

Blood samples were analysed after collection. To avoid the loss of volatile compounds, blood samples were immediately centrifuged, and the serum was separated, frozen in liquid N_2 and stored at −70°C for subsequent biochemical analysis in a 24 h period. Biochemical determination of glucose, urea and urate concentrations was performed in serum using commercially available

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spectrophotometric assays (Labtest, Minas Gerais, Brazil). Lactate and ammonia concentrations were measured using an enzymatic UV method (Randox, Crumlin, UK) on a Dade Model Dimension RXL Automated Chemistry Analyzer (Dade Behring, Eschborn, Germany).

Statistical analyses were performed using SigmaStat version 3.5 for Windows (Systat Software Inc., San Jose, CA, USA). To decrease individual variability, data were normalised to 0 min values. After testing for normality (Kolmogorov–Smirnov) and equality test variance (Levene median), the changes in the variables between time points were analysed by a one-way ANOVA, and the group changes were evaluated by a two-way ANOVA for repeated measures. Significance ($P<0{\cdot}05$) was confirmed using the Tukey *post hoc* test. Data are presented as means with their standard errors. The area under the curve for blood ammonia data for each individual in each treatment was determined using the following equation:

$$\text{AUC} = A_i(T_i + 1 - T_i) + 0{\cdot}5(A_i + 1 - A_i)(T_i + 1 - T_i),$$

assuming that the ammonia level at baseline corresponds to the resting ammonia level and where AUC is the area under the curve, A is ammonia and T is time.

Results

A 120 min cycling session was employed to evaluate the effect of KAAA on blood ammonia concentration. The resting ammonaemia, before exercise and after supplementation, was elevated (approximately 90 μmol/l) in both groups, and ammonia concentration increased up to a maximum of 35 % above baseline levels in response to exercise in the LEx group (normalised values) at 60, 90 and 120 min ($P<0{\cdot}001$). In contrast, ammonia concentration was not increased (approximately 17 %) in the KEx group at 60, 90 or 120 min. The KEx group actually experienced an approximately 18 % decrease in ammonia concentration at 60 min ($P=0{\cdot}040$) and 90 min ($P=0{\cdot}049$) compared with the LEx group. The area under the curve of the KEx group was 12 % smaller than that of the LEx group. Ammonia concentrations in the LEx group returned to baseline levels at 150 min (30 min into the recovery period). The KEx group demonstrated a greater decrease in ammonaemia (approximately 20 %) compared with the LEx group at 150 min, reaching levels significantly lower than those of the control after 1 h of recovery ($P=0{\cdot}022$; Fig. 1(a)).

To evaluate the effect of KAAA on urea synthesis, we measured the blood urea concentration. Both groups had a significant increase in blood urea levels in response to exercise (approximately 35 % at 120 min; $P<0{\cdot}001$). Even after a 60 min recovery period, the levels of urea remained constant (Fig. 1(b)).

To differentiate the ammonia produced by AMP deamination from that produced by amino acid deamination, we measured blood urate levels. Resting urate levels were equivalent in both groups and remained statistically unchanged in the KEx group up to 90 min. In contrast, the blood urate levels increased by approximately 16 % at 90 and 120 min in the LEx group. Urate levels remained constant from the end of the exercise period throughout the recovery period (Fig. 1(c)).

To understand the role of KAAA in gluconeogenesis, we measured the blood glucose level during the exercise and recovery periods. No observable difference in glucose levels was found during the trial time between the different groups. An increase in the lactate level was observed during the first 30 min of exercise in both groups, with no difference between the groups (Fig. 1(d)).

Discussion

For several years, it has been accepted that KAAA are able to prevent nephrotoxicity, delaying the necessity for dialysis in patients with chronic nephropathies[13]. Therefore, we investigated the effect of KAAA on exercise-induced ammonia production.

Ammonia production in muscle may be due to the depletion of glycogen stores and the deamination of both AMP and amino acids[15,18]. A low-carbohydrate diet increases the production of ammonia during exercise[19]. Furthermore, it is acknowledged that excessive protein intake leads to increased ammonaemia, and ammonia is metabolised to urea by the liver[1,20]. In the present study, we used a ketogenic diet (with 10 % of energy from carbohydrates and 35 % from protein) to decrease the availability of glycogen in the liver and muscle and to increase the availability of amino acids to supply energy. Exercise intensity is the key point in this type of metabolism because increases in exercise intensity increase the rate of AMP deamination, leading to the release of more ammonia into the bloodstream.

Although both studied groups were ammonaemic at baseline due to the ketogenic diet (approximately 90 μmol/l), and although we know that ammonia is critical to the pathogenesis of hepatic encephalopathy and brain oedema, clinical observations have not shown a consistent correlation between the concentration of ammonia in the blood and symptoms of hepatic encephalopathy[20,21].

NH_3 concentration increased in response to exercise in the LEx group, and this effect was reduced by the administration of KAAA. The supplement also kept the blood ammonia level lower during the recovery period. These effects may be due to the anaplerotic action of KAAA entering directly into the Krebs cycle as intermediates or due to the chelation of ammonia by the keto analogues. Additionally, KAAA may increase glucose availability via gluconeogenesis. In the present study, we did not detect any changes in glucose levels during exercise or as a result of KAAA supplementation. In addition, blood lactate concentration fluctuated similarly in both groups. These data may suggest that ammonia production

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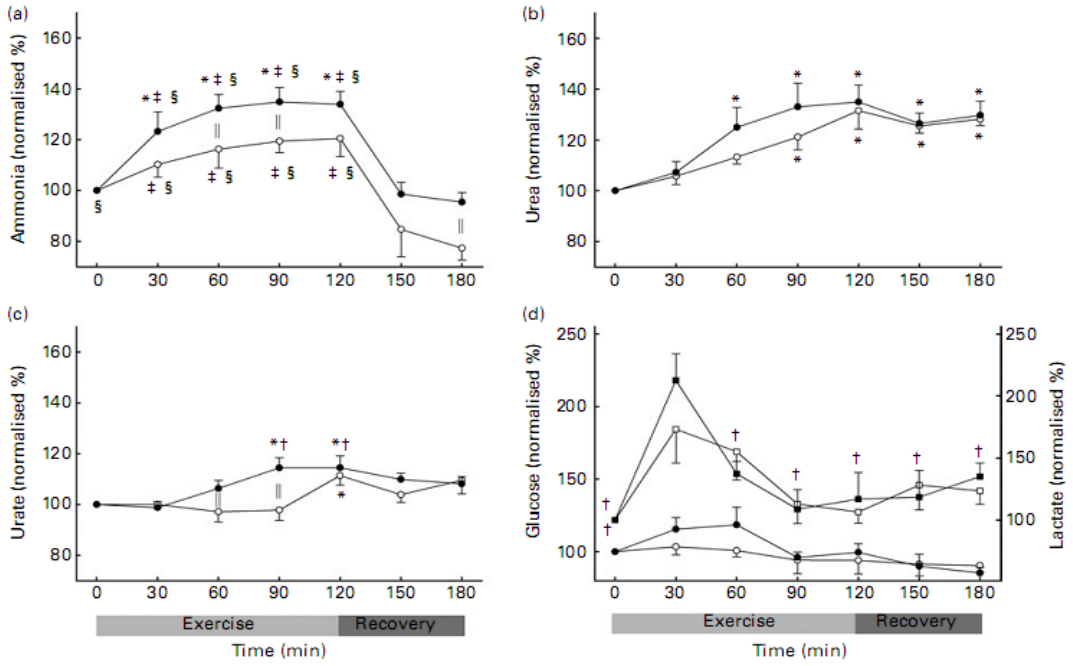

Fig. 1. Acute keto analogue and amino acid (KAAA) supplementation affects the ammonia and urate levels but not the urea level, the glucose level or lactate metabolism. Athletes exercised for 2 h after KAAA supplementation (experimental group (KEx), ○) or control supplementation (LEx, ●). Note that the lactate values were KEx (□) or LEx (■). (a) Ammonia: resting values were KEx 94·31 (SEM 6·86) μmol/l and LEx 84·36 (SEM 9·06) μmol/l; (b) urea: resting values were KEx 17·25 (SEM 1·94) mmol/l and LEx 16·47 (SEM 1·79) mmol/l; (c) urate: resting values were KEx 295·51 (SEM 20·47) μmol/l and LEx 293·25 (SEM 19·24) μmol/l; (d) glucose: resting values were KEx 4·72 (SEM 0·17) mmol/l and LEx 5·24 (SEM 0·18) mmol/l; lactate: resting values were KEx 1·70 (SEM 0·08) mmol/l and LEx 1·92 (SEM 0·15) mmol/l. The 0 min values for the five metabolites did not differ between the two groups. Values are means, with standard errors represented by vertical bars; data were normalised to individual 0 min values (100 %). * Mean values were significantly different from 0 min within the group. † Mean values were significantly different from 30 min within the group. ‡ Mean values were significantly different from 150 min within the group. § Mean values were significantly different from 180 min within the group; || Mean values were significantly different between treatments ($P<0·05$).

related to the ingestion of KAAA is not decreased by gluconeogenesis.

Previous studies have shown that KAAA supplementation can effectively decrease blood urea levels after long-term usage[13,22]. In the present study, in which acute supplementation of KAAA was used in the presence of a high basal concentration of urea, exercise increased blood urea levels similarly in both groups. During a low-energetic state, the ATP:ADP ratio decreases in muscle, and myokinase is activated to synthesise ATP. This process leads to an increase in both AMP deamination and urate synthesis rates[23]. However, while AMP deamination appears to be inhibited during the initial phase of intense exercise, it is pronounced during the recovery period[24]. In the present study, acute KAAA supplementation delayed the increase in blood urate concentration during prolonged exercise. Our data suggest that the effect of KAAA during exercise is not primarily due to ammonia removal via the synthesis of urea but rather to the carbon bodies used to produce ATP.

The KAAA supplement is a mixture of glucogenic and ketogenic amino acids. Thus, this supplement may promote anaplerosis via different Krebs cycle intermediates. In addition, it has been described previously that KAAA supplementation does not increase the insulin response, an important goal in exercise supplementation[25].

In a recent study, we employed KAAA as a supplement to modify amino acid metabolism and ammonia biogenesis during resistance exercise in an animal model[26]. In the present study, we confirmed that when using a ketogenic diet to promote metabolic stress, the acute use of a mixture of amino acids and keto acids in acute supplementation can diminish the increase in ammonaemia caused by endurance exercise in humans. Due to the high amount of amino acids in the diet used in the present study, this effect seems to be due more to the presence of keto acids in the supplement than to a contribution from an acute intake of additional amino acids.

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G. D. d. M., R. D. d. A. and L.-C. C. were involved in the data collection and interpretation; E. S. P., R. D. d. A., J. M. d. R. N. and L.-C. C were involved in the manuscript writing. The authors have no conflicts of interest to declare.

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CAPÍTULO IV: Caffeine affects the ammonemia response in athletes during prolonged exercise under ketogenic conditions.

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Abstract: Both exercise and ketogenic diet favoring glycogen depletion and increased ammonemia. High ammonia levels can impair physical performance. Caffeine has been used as a resource to improve performance in exercise situations. The aim of this study was to evaluate the effect of caffeine supplementation on ammonemia cyclists under ketogenic diet and prolonged exercise. Thirteen athletes male cyclists (18-36 years) followed a ketogenic diet (35% protein, 55% fat and 10% carbohydrate) before and during the experimental trial experiment and were divided into groups: caffeine (CEx, n = 7) and placebo (LEx, n = 7). The athletes performed cycling indoors for 120 minutes (min) with an intensity of 75% to 85% HRmax estimated and blood samples were obtained at rest, during exercise, and throughout 60 min during the recovery period. Ammonemia rest was high (~ 90 mol/L) in both groups and increased up to 35% in response to exercise in LEx. The CEx group showed a significant decrease ammonemia at 60 min (P=0.001), 90 min (P=0.013), 120 min (P=0.004) and 180 min (P=0.045) compared with group LEx. Both groups showed a significant increase (~ 35%) of blood urea levels in response to exercise after 120 min of exercise, but with a higher concentration of blood urea in the LEx group. Blood urate levels were equivalent in both groups at rest and during exercise. Our results suggest that the use of caffeine may attenuate the increased ammonemia that occurs during exercise and ketogenic diet because it prevents glycogen depletion.

Abstract

Both exercise and ketogenic diet favoring glycogen depletion and increased ammonemia. High ammonia levels can impair physical performance. Caffeine has been used as a resource to improve performance in exercise situations. The aim of this study was to evaluate the effect of caffeine supplementation on ammonemia cyclists under ketogenic diet and prolonged exercise. Thirteen athletes male cyclists (18-36 years) followed a ketogenic diet (35% protein, 55% fat and 10% carbohydrate) before and during the experimental trial experiment and were divided into groups: caffeine (CEx, n = 7) and placebo (LEx, n = 7). The athletes performed cycling indoors for 120 minutes (min) with an intensity of 75% to 85% HR_{max} estimated and blood samples were obtained at rest, during exercise, and throughout 60 min during the recovery period. Ammonemia rest was high (~ 90 μmol/L) in both groups and increased up to 35% in response to exercise in LEx. The CEx group showed a significant decrease ammonemia at 60 min (P=0.001), 90 min (P=0.013), 120 min (P=0.004) and 180 min (P=0.045) compared with group LEx. Both groups showed a significant increase (~ 35%) of blood urea levels in response to exercise after 120 min of exercise, but with a higher concentration of blood urea in the LEx group. Blood urate levels were equivalent in both groups at rest and during exercise. Our results suggest that the use of caffeine may attenuate the increased ammonemia that occurs during exercise and ketogenic diet because it prevents glycogen depletion.

Keywords: ammonia; xanthine; ketogenic diet; endurance.

Introduction

Caffeine is a 1,3,7-trimethylxanthine metabolised in the liver and the primary metabolic pathways involve demethylation reactions to form three dimethylxanthines: paraxanthine, theobromine, and theophylline [1]. The xanthine is a psychoactive drug and probably one of the most commonly used stimulants in sports because of its low cost and minimal side effects [2,3]. The performance improvement occurs with administration of oral doses ranging 3-9 $mg.Kg^{-1}$, especially in prolonged exercise, which can be explained, for various organic mechanisms [4,5]. The most physiologically relevant mechanism of action is probably the blockade of adenosine receptors, but evidence suggests that, at least under certain conditions, other biochemical mechanisms may also be operational, such as: mobilization of free fatty acids (FFA) from adipose tissue, resulting in higher rates of fat oxidation and sparing of muscle glycogen [1,5].

Ammonia (used here as a synonym for the sum of NH_3 and NH_4^+) is highly toxic to humans and can cross the blood–brain barrier, which leads to a decrease in cerebral function, neuropsychiatric disorders, and death [6,7]. Ammonia-mediated excitotoxicity has been implicated in the mediation of central nervous system damage [8]

Data obtained from exercise studies have been used to elucidate the effects of hyperammonaemia. Several investigations have demonstrated that increased ammonaemia production occurs during various types of exercise [9-12]. During prolonged sub-maximal exercise a rise in ammonaemia concentration (>160 µmol/L) was observed in various studies [13,12] The consensus view is that the production of ammonia during exercise occurs via a combination of both AMP deamination and catabolism of amino acids, which are activated in an intensity and duration dependent manner [7] It has been suggested that ammonia promotes both central and peripheral fatigue and that better control of ammonia production will improve exercise performance [14]. The relationship between the effects of caffeine on performance and amonemia not been studied.

Furthermore, although ammonia has been shown to be produced by BCAA catabolism, independent of glycogen availability [15] various studies have linked ammonia formation to

carbohydrate availability [9,16] A low carbohydrate diet (termed a ketogenic diet) combined with physical exercise can reduce glycogen stores and induce hyperammonaemia [17,18] We used this metabolic effect of a ketogenic diet to enhance the effect of exercise on ammonia production.

In the present study, we evaluated the effect of caffeine supplementation on ammonia production during prolonged exercise.

Materials and methods

Thirteen male endurance-trained cyclists aged 18 to 36 years (27.8 ± 2.3 years; 70.42 ± 1.9 kg; 1.77 ± 0.01 m; VO_{2max} 54.6 ± 1.8 mL/kg^{-1}/min^{-1} and maximum heart rate (HR_{max}) 192.14 ± 2.35; mean ± SE) participated in the study voluntarily. All subjects had similar physical capabilities and a minimum of three years of training. They had not used ergogenic substances or any other drugs. This study was approved by the Ethics Committee for Human Research at the Federal University of the State of Rio de Janeiro.

Athletes reported to the laboratory three days before the experimental trial (D-3) to become habituated with the cycle ergometer and to optimize the power output. Subjects received an individualized ketogenic diet (35% of the recommended energy intake from proteins; 55% from lipids; and less than 10% from carbohydrates; see [19] for a review on the effects of a low carbohydrate diet on metabolism) for two days before (D-2; D-1) and during the experimental day (D0) .

Subjects were asked to maintain their normal training schedule (~70 km per day) and to follow the ketogenic diet up to 48 h (normal training and ketogenic diet was used to reduce muscle glycogen stores and induce ammonaemia) before the experimental day. On the experimental day, the subjects reported to the laboratory in a fasting state and received breakfast (T-360) and a light lunch (T-120). One hour after lunch (T-60), the subjects received either 5mg.Kg^{-1} body weight of caffeine (CEx; n = 7) or lactose (LEx; n=7) in a random double-blind manner. Each of the two types of supplement was provided in indistinguishable capsules (Figure 1).

Before the experimental trial, initial stretching was followed by a warm-up of 10 minutes at 50% of the HRmax. The experiment began one hour after supplementation and athletes cycled indoors for two hours at 80 revolutions per minute (rpm) according to a metronome in a room with a constant temperature and relative humidity (23 ± 2 °C and 60 ± 5%, respectively). HR was recorded continuously throughout the exercise using a HR monitor (Polar CS200, Kempele, Finland). The power output was modified for each individual every 5

min so that the athletes maintained 75% to 85% of their estimated HR_{max} (~180.15 ± 1.13 W; ~157.35 ± 2.41 beats.min^{-1}; mean ± SE, respectively).

A catheter was placed into the medial cubital vein. Blood samples were obtained at rest (T0) and at thirty-minute intervals throughout the exercise period (T30, T60, T90, T120). Finally, blood samples were collected during one hour of recovery period every thirty-minute intervals (T150, T180). Athletes received water *ad libitum* during the trial.

Blood samples were analyzed after collection. To avoid the loss of volatile compounds, a portion of the blood samples were immediately centrifuged, and the sera was separated, frozen in liquid nitrogen, and stored at -70°C for subsequent biochemical analysis. The remaining blood sample was stored for hematological analysis. Biochemical analyses of glucose, urea and urate concentration were performed using commercially available spectrophotometric assays (Labtest, Minas Gerais, Brazil). Lactate and ammonia were measured using enzymatic UV method (Randox, Crumlin, United Kingdom) on a Dade Model Dimension RXL Automated Chemistry Analyzer, and hematologicals were analyzed using a Sysmex SE-9500 Automated Hematology Analyzer.

To decrease individual variability, the data were normalised to the 0 min values. The data changes between time points were analyzed by one-way ANOVA. Group changes were evaluated by two-way ANOVA for repeated measures. Significance ($P<0.05$) was confirmed using the Tukey post hoc test. The data are presented as the mean ± SE. The area under the curve (AUC) for the blood ammonia data for each individual in each treatment was determined using the following equation:

$$AUC = Ai\,(Ti+1 - Ti) + 0.5(Ai+1 - Ai)\,(Ti+1 - Ti)$$

assuming resting ammonia data as the baseline, and where A is ammonia and T is time.

Results

A 120-min cycling session was employed to evaluate the effect of caffeine on blood ammonia concentration. Ammonemia rest was high (~90 µmol/) in both groups and increased up to 35% in response to exercise in LEx. No significant increase in ammonemia was detected in the CEx group. The CEx group showed a significant decrease ammonemia 60 min ($P = 0.001$), 90 min ($P = 0.013$), 120 min ($P = 0.004$) and 180 min ($P = 0.045$) compared with group LEx. The AUC from the supplemented group was 14% smaller than the control group. The ammonia concentrations in the LEx group returned to the baseline levels at 150 min. The CEx group demonstrated a significantly greater decrease in ammonemia (~20%) compared to LEx at 150 min, reaching levels significantly lower after 1 h of recovery (Figure 2A).

To evaluate the effect of caffeine on urea synthesis, we measured the blood urea concentration. Both groups showed a significant increase (~ 35%) of blood urea levels in response to exercise after 120 min of exercise, but with a higher concentration of blood urea LEx apparent in the group, with 60-90 min of exercise (Figure 2B).

To differentiate the ammonia produced by AMP deamination from amino acid deamination, we measured blood urate levels. Resting urate levels were equivalent in both groups and remained statistically unchanged in the KEx group throughout of the exercise period (Figure 2C).

To understand the effect of caffeine on glycemia maintenance, we measured glycemia during the exercise and recovery periods. Glucose was not altered either during exercise or between groups (Figure 3A). An increase was observed in blood lactate during the first 30 min of exercise in both groups, but group CEx showed higher lactate production during exercise, especially after 60 min of exercise ($P = 0.026$) (Figure 3B).

Discussion

The benefits of caffeine on endurance performance are well established in human models, even under adverse conditions [20,21]. Of the mechanisms purported to explain the beneficial effects of caffeine supplementation, recent findings support a CNS response mediated by antagonism of adenosine receptors, but other biochemical mechanisms may also be operational [1,5]. On the other hand, it is widely reported that ammonia production increases during exercise and that ammonia could be a deleterious metabolite that promotes fatigue [14,7]. Further, a low carbohydrate diet increases the production of ammonia [22]. Therefore, we investigated the effect of caffeine on exercise-induced ammonia production.

The relationship between ammonia formation and carbohydrate availability has been investigated [17]. Ammonia production in the muscle may be due to the depletion of glycogen stores and the deamination of AMP [17,23]. Furthermore, it is acknowledged that excessive protein intake leads to increased generation of ammonaemia, and ammonia is metabolized to urea by the liver [6]. In this study, we used a ketogenic diet (with 10% from carbohydrates and 35% from proteins) to decrease the availability of glycogen in the liver and muscle and increase the availability of amino acids to supply energy. The coingestion of caffeine (5.3 $mg.kg^{-1}$) with carbohydrate during exercise enhanced time trial performance lasting approximately 45 min by 4.6% compared with only carbohydrate and 9.0% compared with water placebo. However, caffeine did not influence exogenous carbohydrate oxidation or glucose kinetics during 105-min steady-state cycling at 62% VO_{2max}[24].

Although basal ammonaemia was high in both studied groups due to the ketogenic diet (~90 µmol/L) and we know that ammonia is critical to the pathogenesis of hepatic encephalopathy and brain edema, clinical observations did not show a consistent correlation between the concentration of ammonia in the blood and symptoms of hepatic encephalopathy [25]. The levels are higher than levels commonly seen in healthy subjects at rest (20-80 µmol/L) [15].

The ammonia concentration increased in response to exercise in the control group, and this effect was reduced by administration of caffeine. The supplement also increased ammonia clearance in the blood during the recovery period. These effects may be due to the

mobilization of free fatty acids from adipose tissue, resulting in higher rates of fat oxidation and sparing of muscle glycogen.

In the present study, we did not detect any changes in glycemia during exercise or as a result of caffeine supplementation. In addition, the blood lactate concentration, fluctuated similarly in both groups but caffeine group showed higher lactate production during exercise. These data demonstrate that caffeine can promotes higher rates of sparing of muscle glycogen and and consequently decreases ammonemia during exercise.

Although there is very little evidence to support the hypothesis that caffeine has effects as a result of enhanced fat oxidation [26], it is has been commonly proposed to enhance exercise capacity, among others, by promoting fat oxidation and inhibiting carbohydrate oxidation. This action has been suggested to result in a reduced dependence on muscle glycogen stores, and the glycogen sparing then promotes increased endurance (5,20).

It seems that caffeine promotes an inhibition of conversion of glycogen phosphorylase b (inactive form) to glycogen phosphorylase a (active form), resulting in less degradation of glycogen in both muscle and in liver glycogen resulting in higher rates of sparing of muscle glycogen. Furthermore, caffeine appears to inhibit the activity of phosphodiesterase isoenzymes, producing an accumulation of intracellular cAMP and thereby potentiating the stimulation of adenylyl cyclase. This action, stimulate protein kinase A to promote cellular responses such as fat oxidation[5].

In the study, even in the presence of a high basal concentration of urea, exercise increased blood urea levels similarly in both groups. This result elucidates the source of ammonia production because urea is the primary route of ammonia excretion in humans. An increase en urea synthesis has been found in rats administered large doses of caffeine, but this increase appeared only after some days of caffeine treatment [27,28]..

Our data suggest that the effect of caffeine as a ergogenic aid during exercise may be due to reduction amonemia and consequent reduction in central fatigue and / or peripheral.

Acknowledgements

This study was supported in part by the Institute of Technology and Research - Tiradentes University. Design: ESP and LCC; Data collection and interpretation: ESP, JMRN, MGDM, RDA and LCC; Manuscript writing: ESP, RDA, JMRN and LCC.

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Figure 1. Experimental design. Cyclists were subjected to habituation and received an individualized dietary prescription (D-3). Two days before the experimental day (D-2; D-1), the subjects followed a ketogenic diet and normal training. On the experimental day (D0), cyclists received breakfast (T-360) and lunch (T-120) in accordance with the ketogenic diet. Supplement (CEx or LEx) was administered 60 min before the trial (T-60), and the athletes exercised for two hours (T0 to T120) and recovered for 60 min (T150 and T180). Blood was collected before, during, and after exercise.

Figure 2. Acute caffeine supplementation affects ammonia, urea and urate metabolism. Athletes exercised for two hours after caffeine supplementation (CEx, ○) or control supplementation (LEx, ●). Note that graph values were normalized (mean ± SE) to the pre-exercise values (0 min; 100%). (A) Ammonia: resting values were CEx = 96.33 ± 9.30 µmol/L and LEx = 84.36 ± 9.06 µmol/L; (B) Urea: resting values were CEx = 18.46 ± 0.83 mmol/L and LEx = 16.47 ± 1.79 mmol/L; (C) Urate: resting values were CEx = 293.25 ± 24.65 µmol/L and LEx = 293.25 ± 19.24 µmol/L. 0 min values for the three metabolites did not differ between the two groups. * denotes a difference from 0 min within the group; † denotes a difference from 30 min within the group; ‡ denotes a difference from 150 min within the group; § denotes a difference from 180 min within the group; || denotes a difference between treatments ($P<0.05$).

Figure 3. Acute caffeine supplementation affects glucose and lactate metabolism. Athletes exercised for two hours after caffeine supplementation (CEx, ○) or control supplementation (LEx, ●). Note that lactate values were CEx (□) or LEx (■) and graph values were normalized (mean ± SE) to the pre-exercise values (0 min). (A) Glucose: resting values were CEx = 5.16 ± 0.25 mmol/L and LEx = 5.24 ± 0.18 mmol/L; and (B) Lactate: resting values were CEx = 1.97 ± 0.14 mmol/L and LEx = 1.92 ± 0.15 mmol/L. 0 min values for the two metabolites did not differ between the two groups. * denotes a difference from 0 min within the group; † denotes a difference from 30 min within the group; ‡ denotes a difference from 150 min within the group; § denotes a difference from 180 min within the group; || denotes a difference between treatments ($P<0.05$).

***Title page (including all author details and affiliations)**

Caffeine affects the ammonemia response in athletes during prolonged exercise under ketogenic conditions

Running head: **Caffeine reduce ammonemia in exercise**

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Cover Letter

Professor Gregory Kolt

Editor-in-Chief

Journal of Science and Medicine in Sport

Dear Editor,

Please find enclosed the manuscript entitled "**Caffeine affects the ammonemia response in athletes during prolonged exercise under ketogenic conditions**" by Prado et al. for publication in Journal of Science and Medicine in Sport. This manuscript contains original material that has not been previously published and is not currently under consideration elsewhere. In addition, the manuscript will not be submitted elsewhere until a final decision has been made by the journal.

We studied a low-carbohydrate ketogenic diet and exercise as a hyperammonaemia model in order to understand the role of the association of caffeine supplementation in ammonia metabolism.

Previous results from our laboratory showed that both glutamine and carbohydrates protect against an increase in ammonia levels in the blood (Carvalho-Peixoto et al., 2007 - Appl Physiol Nutr Metab), and that glutamine and alanine function distinctly in metabolism in an exercise intensity-dependent way (Bassini-Cameron et al., 2008 - Br J Sports Med). In parallel, we attempted to clarify the kinetics of muscle injury and its relation to white blood cells (Bassini-Cameron et al., 2007 and Bessa et al., 2008 – Br J Sports Med; Lazarim et al., 2007 - J Sci Med Sport; Gonçalves et al., 2010 – Amino Acids - submitted). Indeed two of our papers were recently quoted in a Wilkinson and colleagues review (Ammonia metabolism, the brain and fatigue; revisiting the link. Wilkinson DJ, Smeeton NJ, Watt PW. Prog Neurobiol. 2010).

> "Since the publication of the original ideas by Banister and colleagues, few researchers have attempted to fully substantiate this theory. Of the studies which have been performed, the majority have either investigated the production and metabolism of ammonia within the periphery (Spodaryk et al., 1990; Graham et al., 1993, 1995b; Rush et al., 1995; Esbjornsson-Liljedahl and Jansson, 1999; Snow et al., 2000; Mohr et al., 2006), or investigated the link between attenuation of ammonia production during exercise and performance (Denis et al., 1991; Eto et al., 1994; **Carvalho-Peixoto et al., 2007; Bassini-Cameron et al., 2008**). In light of this, it is unsurprising that the ammonia fatigue theory has not remained at the forefront of research interest. However, the findings presented in some more recent literature may warrant the link between ammonia, the brain and fatigue to be revisited."

Here, we attempted to elucidate caffeine supplementation and the protection it confers in ammonia production. Since ammonia is toxic to the nervous system, studying protection against increases in ammonia due to exercise can identify ways to improve athletes' performance and facilitate the elucidation of therapeutic targets. Therefore, we believe that the

present study contributes important data to our understanding of metabolism, and that these findings could be helpful for the development of future therapies. This knowledge could also be used for caffeine supplementation during hyperammonaemic states.

The present research was conducted with written consent from the subjects, who were instructed about the nature of and the procedures used in this study, thus meeting the requirements for carrying out research in human subjects (National Health Council, Brazil, 1996), and was approved by the Human Research Committee of the Universidade Castelo Branco. The manuscript meets all points of the Journal's guidelines and has not conflict of interest. In addition, we declare that the manuscript has not been submitted to any other journal and that all authors have read and approved the final version of the manuscript and made significant contributions to the study.

We sincerely believe that our work is in accordance with the scope of Journal of Science and Medicine in Sport and that it is acceptable for publication as a original research papers due to the importance of these findings.

Sincerely yours,

L. C. Cameron, Ph.D.
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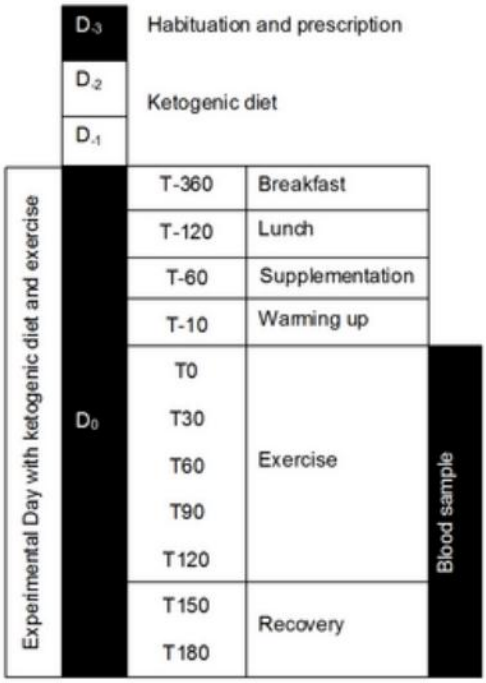

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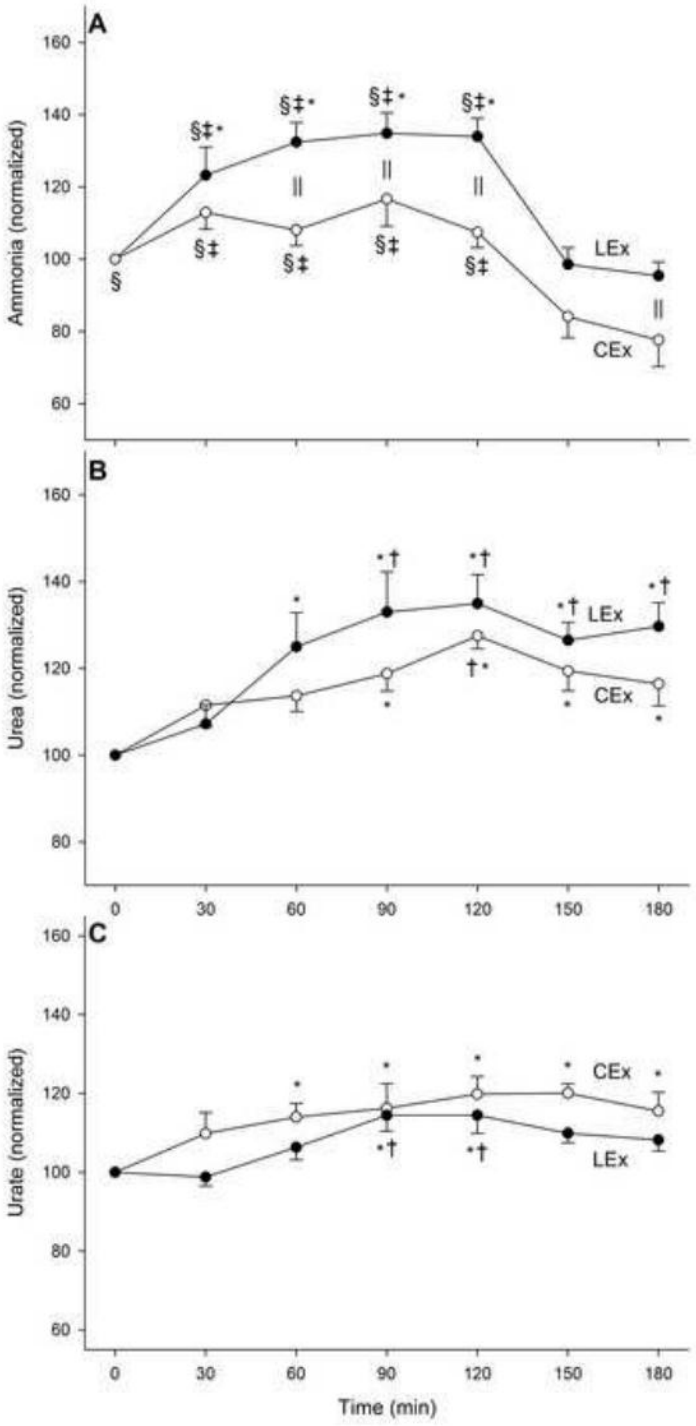

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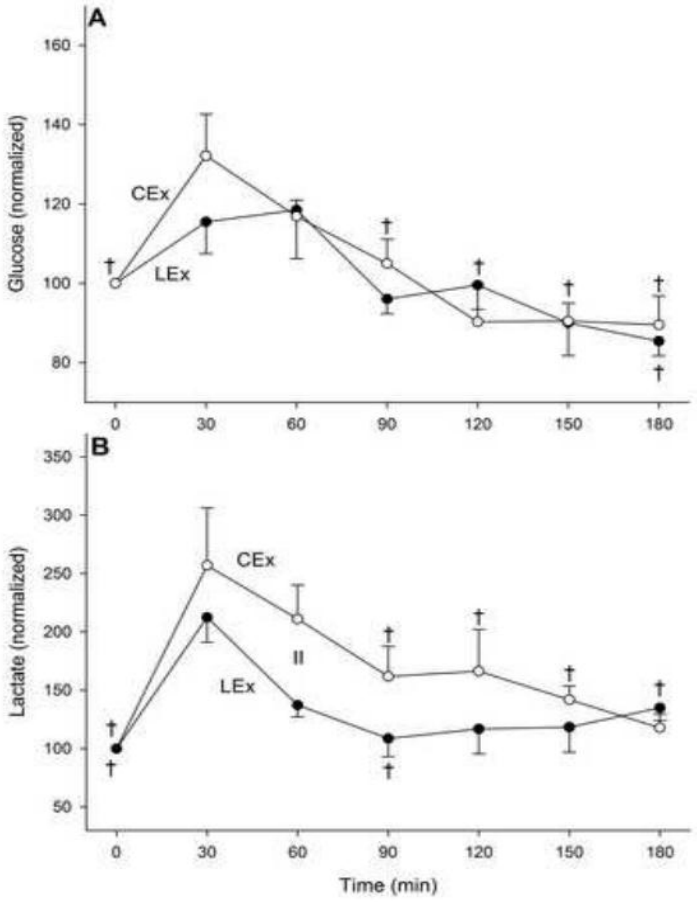

Considerações Finais

Os achados encontrados neste estudo, demostraram que a suplementação de cetoanálogos, interfere na amoniogênese reduzindo a velocidade de aparecimento da amônia no exercício de força e resistência. Portanto o aumento da síntese de ATP, promovendo maior carga energética celular minimizando os riscos de desencadeamento de fadiga periférica e central. Além disso, alterou as concentrações de metabólitos associados a amônia, sugerindo novas perspectivas de estudos para alvos terapêuticos.