UNIVERSIDADE CASTELO BRANCO
PROGRAMA DE PÓS-GRADUAÇÃO STRICTU SENSU EM CIÊNCIA DA MOTRICIDADE HUMANA

EXERCÍCIO DE ALTA INTENSIDADE E ULTRAENDURANCE: SEUS EFEITOS NO METABOLISMO E INTEGRIDADE MUSCULAR

Marcelo Nissenbaum

Rio de Janeiro
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Por

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Dissertação de Mestrado apresentada a Universidade Castelo Branco, como requisito à obtenção do título de Mestre em Ciência da Motricidade Humana.

Orientador: Luiz-Cláudio Cameron, Ph. D.
Co-orientador: Adriana Bassini, Ph. D.

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6. Acetaminofen

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RESUMO

EXERCÍCIO DE ALTA INTENSIDADE E ULTRAENDURANCE: SEUS EFEITOS NO METABOLISMO E INTEGRIDADE MUSCULAR

Por

Marcelo Nissenbaum

UNIVERSIDADE CASTELO BRANCO

PROCIMH / Laboratório de Bioquímica de Proteínas (LBP)

2009

Orientador: Luiz-Cláudio Cameron Número de Palavras: 214

O exercício de “ultraendurance” (HIU) acarreta elevado estresse metabólico, observado pelo aumento na freqüência e potência do processo de contração-excitação, induzindo modificação nas vias metabólicas na tentativa de manter o estado energético celular. Estas contrações musculares intensas provocam maior entrada de aminoácidos no ciclo do ácido cítrico com consequente produção de amônia. Acredita-se que este estado transitório de hiperamonemia modifica a captação de glutamato na fenda sináptica levando a fadiga central. Além deste efeito neurotóxico há desequilíbrio na homeostase mitocondrial e celular com possível instalação de fadiga periférica. Dessa forma, a amônia tem sido sugerida como indicador metabólico em situações de injúria há duas décadas. Avaliamos quatro triatletas amadores e os submetemos ao HIU para mimetizar situações de estresse celular. Após extensa avaliação clínica, antropométrica, bioquímica e hematológica, acompanhamos o treinamento durante duas semanas associada à prova de ciclismo por 800km. Medimos a injúria através dos níveis de marcadores de lesão muscular e hepática como controles interno do experimento. Nesta

dissertação, induzimos a regeneração hepática via N-acetil-cisteína, metionina e cisteína em um dos atletas que se automedicava com acetaminofen durante o período de treinamento, estes resultados estão apresentados no artigo 1. Em adição, no artigo 2 comparamos a cinética enzimática dos marcadores de lesão muscular e hepática com os compostos nitrogenados durante o HIU.

ABSTRACT

EXERCÍCIO DE ALTA INTENSIDADE E ULTRAENDURANCE: SEUS EFEITOS NO METABOLISMO E INTEGRIDADE MUSCULAR

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The exercise ultraendurance (HIU) leads high metabolic stress, observed by the increase in frequency and power in the process of contraction-excitation, inducing changes in the metabolic pathways in order to maintain the cellular energy state. These muscle contractions intense entry of amino acids in the citric acid cycle with production of ammonia. It is believed that this transient state of hyperammonemia alters the uptake of glutamate in the synaptic cleft leading to central fatigue. In this neurotoxic effect is an imbalance in mitochondrial homeostasis and cell with possible inducing of peripheral fatigue. Thus, ammonia has been suggested as metabolic indicator in cases of injury for two decades. We use four amateur triathletes and submitted them to the HIU to mimic situations of cellular stress. After extensive clinical, anthropometric, biochemical and hematological accompany the training for two weeks and competition ultraendurance by cycling 800km. We measure the injury through the levels of injury markers of muscle damage, and liver as internal control of the experiment. In this thesis we induced liver regeneration via N-acetyl-

cysteine and methionine in one of the athletes who self-medicated with acetaminophen during training process. The results are described in Article 1. In addition, Article 2 compared the enzymatic markers of muscle damage and liver with nitrogen components during the HIU.

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2	High-intensity ultraendurance promotes early release of muscle injury markers	A Bessa, M Nissenbaum, A Monteiro, P G Gandra, L S Nunes, A Bassini-Cameron, J P S Werneck-de-Castro, D Vaz de Macedo, L C Cameron	British Journal of Sports Medicine	2.126

DEFINIÇÃO DE TERMOS E ABREVIATURAS

AAs – aminoácidos

ADP – adenosina difosfato

Ala – alanina

ALP – fosfatase alcalina

ALT = TGP – alanina aminotransferase

AMP – adenosina monofosfato

AMPK – miokinase

APAP – acetaminofen

Arg – arginina

Asp – aspartato

AST = TGO – aspartato aminotransferase

ATP – adenosina trifosfato

BCAA – aminoácidos de cadeia ramificada

CK – creatina kinase

CrP – creatina fosfato

Cys – cisteína

FAD – flavina adenina dinucleotídeo

$FADH_2$ – flavina adenina dinucleotídeo fosfato reduzida

FFA – ácidos graxos livres

GABA – ácido gama amino-butírico

GGT – gama glutamil transferase

Gln – glutamina

Glu – glutamato

GSH – glutationa reduzida

GTP – guanosina trifosfato

H^+ – íon hidrogênio

HCO_3^- - bicarbonato

HIU – exercício de ultraendurance

IMP – inosina monofosfato

LDH – lactato desidrogenase

Met – metionina

NAC – N-acetil cisteína

NADH – nicotinamida adenina dinucleotídeo reduzida

NADPH – nicotinamida adenina dinucleotídeo fosfato reduzida

NH_3 – amônia

NH_4^+ – íon amônio

NMDA – n-metil d-aspartato

NO – óxido nítrico

PCR – protein C reativa

PFK – fosfofrutokinase 1

PNC – ciclo das purinas nucleotídeos

SNC – sistema nervoso central

TAN – total de adenina nucleotídeos

TCA – ciclo do ácido tricarboxílico

CAPÍTULO 1

CIRCUNSTÂNCIAS DO ESTUDO

1.1. INTRODUÇÃO

Exercícios de muita longa duração, dentre eles os de ultraendurance (HIU) são considerados exercícios maiores que as provas clássicas de maratonas (Wu, Chen et al. 2004).

É importante notar que os eventos metabólicos causados pelo HIU devem ser elucidados e comparados com estados patológicos sub-clínicos, pois estes induzem também um estresse similar como descrito em recente artigo publicado pelo Laboratório de Bioquímica de Proteína (LBP-UNIRIO).

Devido ao longo tempo durante a prova e a intensidade do HIU é comum encontrarmos nos sujeitos praticantes a perda de massa gorda e magra, desidratação, fadiga central e periférica e principalmente lesão do tecido músculo esquelético (Milias, Nomikos et al. 2005).

Usaremos nesta dissertação parâmetros hematológicos e bioquímicos para a melhor compreensão deste tipo de estresse, como serão descrito nos artigos 1 e 2.

1.2. OBJETIVO

1.2.1. Objetivo geral

Utilizar o HIU como agente modificador do metabolismo nitrogenado.

1.2.2. Objetivo específico

1. Avaliar o perfil hematológico e bioquímico de triatletas amadores pré, per e pós competição de ciclismo;

2. Verificar a cinética para corrente sanguínea de compostos nitrogenados e enzimas marcadoras de lesão durante o HIU;

3. Utilizar N-acetilcisteina associada à metionina no processo regenerativo do hepatócito.

1.3. PROCEDIMENTOS DO ESTUDO

1.3.1. Para responder o objetivo 1: Avaliar o perfil hematológico e bioquímico de triatletas amadores pré, per e pós competição de ciclismo.

Para a investigação foram feitas análise bioquímicas, antropométricas, hematológicas e clínicas iniciais. Este tipo de anamnese nos permite verificar o status alimentar e de treinamento; metabolismo de carboidratos, lipídeos e proteínas, capacidade de transporte de oxigênio; equilíbrio hídrico; integridade hepática e renal dentre outros. Nesta dissertação possibilitamos diagnósticos metabólicos que excluem sujeitos com disfunções clínicas ou subclínicas que pudesse interferir nos resultados ou dificultar as interpretações dos resultados duas semanas antes do teste. Os resultados das análises mostraram que o grupo era antropometricamente homogêneo, saudável, e estatisticamente equivalentes em D0.

Durante o HIU dosamos enzimas marcadoras de lesão hepática, muscular esquelética e cardíaca para compreendermos as respostas metabólicas agudas advindas da injúria.

Controlamos a resposta muscular via setor branco que reage rapidamente à fase aguda da inflamação. Devido ao processo inflamatório houve incremento do número de plaquetas 20-30% comparado com o pré-exercício, leucócitos 210% durante a competição e neutrófilos 300% parecendo este ser um dos maiores contribuinte à resposta inflamatória. Este fenômeno é transitório e depende da intensidade e duração do exercício. Conclui-se que o exercício induz a síntese de proteína C reativa (PCR) e outros compostos às respostas inflamatórias.

1.3.2. Para responder o objetivo 2: Verificar a cinética para corrente sanguínea de compostos nitrogenados e enzimas marcadoras de lesão durante o HIU.

O exercício extenuante mimetiza estados de hiperamonemia e situações de injúria. Medimos aspartato aminotransferase (AST), alanina aminotransferase (ALT), amilase, proteína C-reativa (PCR), glutamiltransferase (GGT), creatina kinase (CK), lactato desidrogenase (LDH), fosfatase alcalina (ALP) pré, per e pós competição e observamos que as enzimas responderam agudamente ao estresse do exercício.

Para endereçar este objetivo medimos a cinética de aparecimento de marcadores de microlesão muscular e correlacionamos com a intensidade do exercício. Mostramos pela primeira vez a cinética de CK e LDH em função da intensidade (r^2=98), onde CK teve aumento de 40% e LDH de 300% nas primeiras 6-8h.

Por supormos que o HIU induzisse a injúria muscular e consequentemente resultados falso positivos, monitoramos AST, ALT e GGT. Os resultados nos mostraram que a saída destas enzimas não é hepática e possivelmente muscular.

Propomos que a diferença de 260% entre CK e LDH seja pelo seu peso molecular (CK~86Da e LDH~140Da).

Acompanhamos o clearance de amônia através de uréia e urato. O urato aumentou cerca de 130-140% nos primeiros 50km, seguido pela uréia a partir dos 150km. Na primeira metade da prova observou-se que a enzima AMP deaminase estava estimulada promovendo prioritariamente a liberação de amônia na primeira metade da competição. Na segunda metade da competição postulamos maior velocidade no ciclo da uréia pelo aumento deste metabólito.

1.3.3. Para responder o objetivo 3: Utilizar N-acetil cisteína e metionina como intermediários na regeneração do hepatócito.

Um dos atletas encontrava-se lesionado com dores musculares e utilizando acetaminofen (APAP) sem orientações.

APAP é uma substância metabolizada pelo fígado no citocromo P450, no sistema glutationa com consequente síntese de óxido nítrico (NO), aumentando sua hepatotoxidade, e aproximadamente 90% é conjugado com o ácido glicurônico ou sulfato na bile ou no sangue. Supõe que a injúria hepática é sinérgica ao APAP causando severo estresse oxidativo mitocondrial, resultando em inflamação.

A N-acetil cisteína (NAC) evita a depleção da glutationa. A glutationa ao ser desacetilada produz glutationa reduzida (GSH), um dos mais potentes agentes citoprotetores. GSH neutraliza a toxicidade causada pela formação de íons peróxidos.

Com base nestes dados pudemos observar que o uso terapêutico de NAC associado a metionina (Met) e cisteína (Cys) diminuiu a concentração de GGT séricas e outros marcadores de lesão.

Portanto, a triagem inicial nos permitiu viabilizar o estudo e prescrever o uso terapêutico de NAC por 15 dias que antecederam a competição.

O atleta depois da suplementação e recomendações apresentava valores enzimáticos similares ao da equipe e menor grau de dor. Baseado neste histórico recente, observamos que a suplementação diminuiu o estresse oxidativo e a hepatotoxidade causada por APAP.

CAPÍTULO 2

REFERENCIAL TEÓRICO

2.1. INTRODUÇÃO

O exercício de ultraendurance (HIU) não é um tipo de exercício estudado com tanta freqüência pela falta de indivíduos e cinética das competições. Este tipo de exercício parece ser um bom modelo para o estudo do metabolismo durante situações de injúria muscular (Neumayr, Pfister et al. 2002; Knechtle, Wirth et al. 2009).

É sabido que este tipo de atividade exaustiva pode causar distúrbios momentâneos ao metabolismo celular afetando as principais vias energéticas: síntese e degradação dos aminoácidos, Ciclo de Krebs, fosforilação oxidativa, glicólise, gliconeogênese dentre outras (Borsheim, Cree et al. 2004).

Para compreender os eventos metabólicos e mecânicos do exercício de alta intensidade necessitamos analisar as possíveis vias energéticas e os substratos selecionados. A regulação da seleção de substrato é influenciadoa pelas reservas de glicogênio no pré-exercício, nível de condicionamento dentre outras (Kemppainen, Aalto et al. 2005). Considerando o início do HIU, o estoque hepático e muscular de glicogênio, e a disponibilidade rápida de aminoácidos são os principais fatores contribuintes a regeneração de adenosina trifosfato (ATP) e fornecimento de energia para a contração muscular (Forslund, Hambraeus et al. 2000).

Graham (2009) sugere que durante a fase final do exercício intenso e prolongado, a depleção de glicogênio é responsável pela maior utilização destes aminoácidos na manutenção da glicemia.

Um déficit da via glicolítica ocasionado pela diminuição do glicogênio na célula muscular aumentaria as concentrações de amônia plasmática, lactato, e inosina monofosfato (IMP) alterando os intermediários do ciclo do ácido tricarboxílico (TCA) inibindo a via (van Loon, Greenhaff et al. 2001). Durante este processo em busca do equilíbrio ocorre a síntese de alanina (Ala) e glutamina (Gln) que são poderosos aminoácidos gliconeogênicos para manter o fluxo da via glicolítica e estado energético mitocondrial refazendo a relação adenosina trifosfato/adenosina difosfato (ATP/ADP) seja mantida (Jeukendrup, Moseley et al. 2006).

Portanto, uma alteração da via glicolítica por acidose celular pode inibir a fosfofrutokinase 1 (PFK) acarretando diminuição da regeneração ATP (Borsheim, Cree et al. 2004). Neste caso, aumentos da concentração de ADP podem gerar aumento no fluxo de aminoácidos (AAs) ao TCA que através da desaminação oxidativa produzindo amônia e cetoácidos. No hepatócito esta via auxilia a manutenção da neoglicogênese e nos músculos esqueléticos na manutenção do potencial redox (Gibala, MacLean et al. 1998; Gibala and McGee 2008).

Estudos ressaltaram que esta via catabólica pode contribuir com 10% da energia requerida durante o exercício de endurance (Blomstrand and Saltin 1999). Portanto, a oxidação dos AAs no TCA têm função anaplerótica auxiliando a produção de NADH e $FADH_2$ com consequente produção de amônia (Gibala, MacLean et al. 1998; Graham, Sgro et al. 2000; Blomstrand and Saltin 2001).

No início da atividade e de “sprints” a amônia também pode ser produzida durante a síntese de ATP a partir de ADP via miokinase (AMPK) e estimulação da creatina kinase (CK) diminuindo creatina fosfato (CrP), ambas para manutenção do balanço energético da célula (Franco and Canela 1984).

Nesta dissertação avaliaremos o efeito induzido pelo HIU na produção de compostos nitrogenados e sua possível relação com a lesão muscular.

2.2. GÊNESE DA AMÔNIA

Nesta dissertação chamaremos de amônia a soma das formas NH_3 e NH_4^+.

Em humanos, a formação da amônia ocorre durante a síntese de ATP a partir de ADP (via mioquinase); durante a utilização de AAs como doadores de esqueletos de carbono (desaminação); e principalmete pela microbia no período pós-prandial (Hellsten, Richter et al. 1999; Zhao, Snow et al. 2000).

O ciclo purina nucleotídeos (PNC) é importante para a conservação do equilíbrio da energia intramuscular. O PNC é regulado negativamente pelas concentrações de ATP e guanosina trifosfato (GTP) o aumento das concentrações de adenosina monofosfato (AMP), ADP e H^+ estimulam a velocidade de descida da via (Starritt, Angus et al. 1999), como está demonstrado na Figura 1.

O surgimento da amônia está relacionado com o incremento do PNC e com o catabolismo de aminoácidos. O PNC libera AMP que é um importante modulador alostérico estimulando enzimas que catabolizam o glicogênio muscular e consequentemente a glicólise (de Lange, Moreno et al. 2007).

Desta forma o ciclo trabalha para aumentar a razão ATP/ADP na regulação da célula (Gibala, MacLean et al. 1998). Propõe-se que o equilíbrio de adenina nucleotídeos no PNC seja regulado pela capacidade de reaminação do IMP a AMP. Em baixa demanda energética o IMP é reaminado através da entrada de aspartato e GTP, que é catalisado pela enzima adenilsuccinato sintase. Concomitante, adenilsuccinato é degradado a fumarato alimentando o TCA possibilitando um suprimento energético extra para o ciclo (Maughan, Greenhaff et al. 1997).

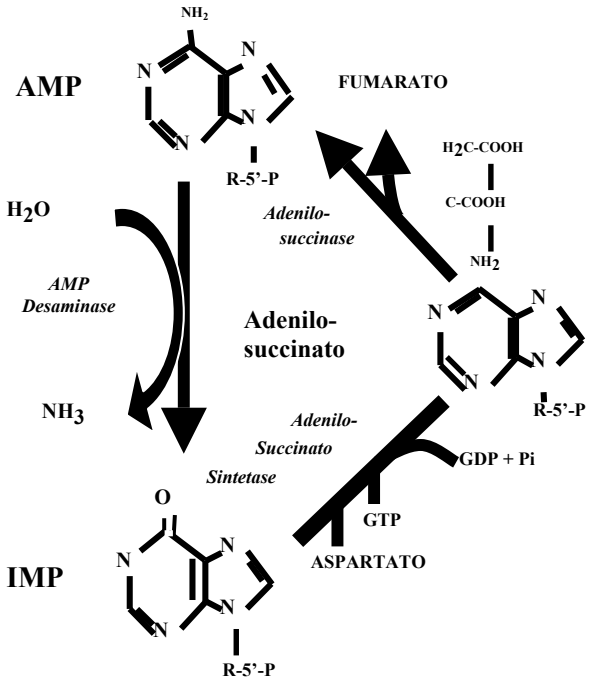

Figura 1: Reações do ciclo Purina nucleotídeos. Reaminação do IMP e formação de fumarato para o TCA. Adaptado: (Terjung 1999)

A concentração de amônia pode aumentar por diferentes fatores, dentre eles: a intensidade e a duração do exercício (Yuan, So et al. 2002). Durante a contração muscular no exercício de alta intensidade o PNC é estimulado pela alteração da relação ATP/ADP, ativando a enzima AMP deaminase formando IMP e amônia (Hellsten, Richter et al. 1999). O exercício físico de alta intensidade altera a homeostase do organismo acarretando inúmeras modificações, desvios e sobrecarga nas vias metabólicas (Fallon, Sivyer et al. 1999) como pode ser visto na Figura 2.

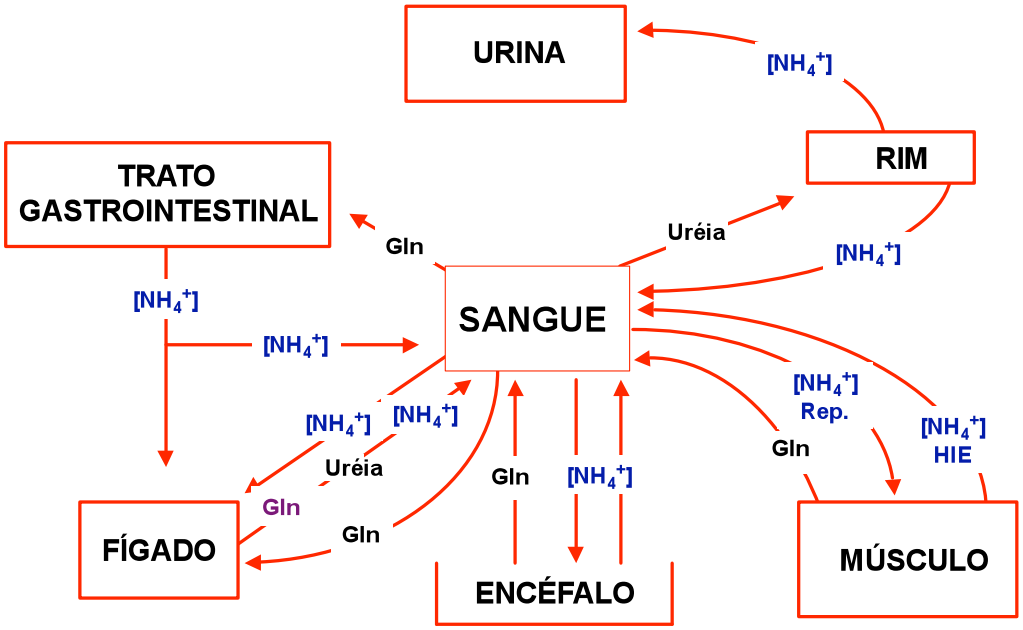

Figura 2 - Gênese da amônia. Órgãos e tecidos responsáveis pela formação, utilização e circulação e de compostos nitrogenados. Extraído e adaptado de Banister & Cameron, 1990.

A inosina proveniente do PNC pode ser escoada a hipoxantina, xantina no músculo e ser metabolizada a ácido úrico no fígado. Podemos sugerir através das concentrações séricas de urato a velocidade do ciclo e o utilizamos como marcador (Bangsbo, Krustrup et al. 2001). Recente artigo publicado por Bessa et al (2008) observou aumentos de urato séricos e a tentativa na manutenção do equilíbrio em relação a demanda energética exigida.

AAs também são substratos requeridos para os processos de transdução de energia durante exercício como ocorre no PNC. Deste modo a função anaplerótica dos aminoácidos pode ser proveniente de incrementos de ADP estimulando a deaminação oxidativa, e transaminação formando um cetoácido para o TCA (Gibala, MacLean et al. 1998).

Gln é o aminoácido livre mais abundante no tecido muscular. A Gln exerce funções muito importantes para os eventos metabólicos, manutenção do sistema imunológico, equilíbrio do balanço ácido/básico durante estado de acidose, possívelmente reguladora da síntese e da degradação de proteínas durante o extresse metabólico, remoção de nitrogênio e da amônia em órgãos e tecidos (Bruce, Constantin-Teodosiu et al. 2001; Bassini-Cameron, Sweet et

al. 2007). A Gln tem uma função específica de proteção no balanço ácido/ básico que é o pH sangüíneo que pode variar numa faixa entre 7.35 e 7.45 (Graham, Sgro et al. 2000; MacLean, Imadojemu et al. 2000).

2.3. DEPURAÇÃO DE AMÔNIA

A degradação da Gln nos túbulos distais é um dos caminhos para aumentar a quantidade de amônia renal. Um H^+ se junta a amônia formando um íon de amônium, que se une ao cloreto podendo ser excretado pela urina. A outra forma de clerance de amônia é o aumento na produção de íons bicarbonato pela oxidação dos carbonos das cadeias de Gln. O bicarbonato seria lançado para a corrente sangüínea e tamponaria o H^+ excedente (Bellinger, Bold et al. 2000; Blomstrand and Saltin 2001).

A síntese da uréia ocorre no fígado, a principal via de remoção da amônia. Um bloqueio, insuficiência, deficiência ou defeitos genéticos de enzimas deste complexo pode resultar em consequências severas para os seres humanos como danos hepáticos, hiperamonemia, encefalopatias e consequentemente efeitos deletérios no sistema nervoso central (SNC) (Van Hall, Saltin et al. 1999; Olde Damink, Dejong et al. 2006).

A amônia livre pode se combinar com o glutamato (Glu) e formar Gln e ser liberada do músculo. Neste caso, a amônia produzida é transportada pela Gln diminuindo o acúmulo no sangue protegendo-o de uma possível acidose (MacLean, Graham et al. 1996; Olde Damink, Dejong et al. 2006).

Portanto o ciclo da uréia também pode produzir, em uma das suas reações intermediárias o fumarato que poderá ser utilizado no TCA para a manutenção da energia. Estes dois mecanismos citados ajudam a equilibrar o complexo celular (Neumayr, Pfister et al. 2005) como pode ser observado na figura 3.

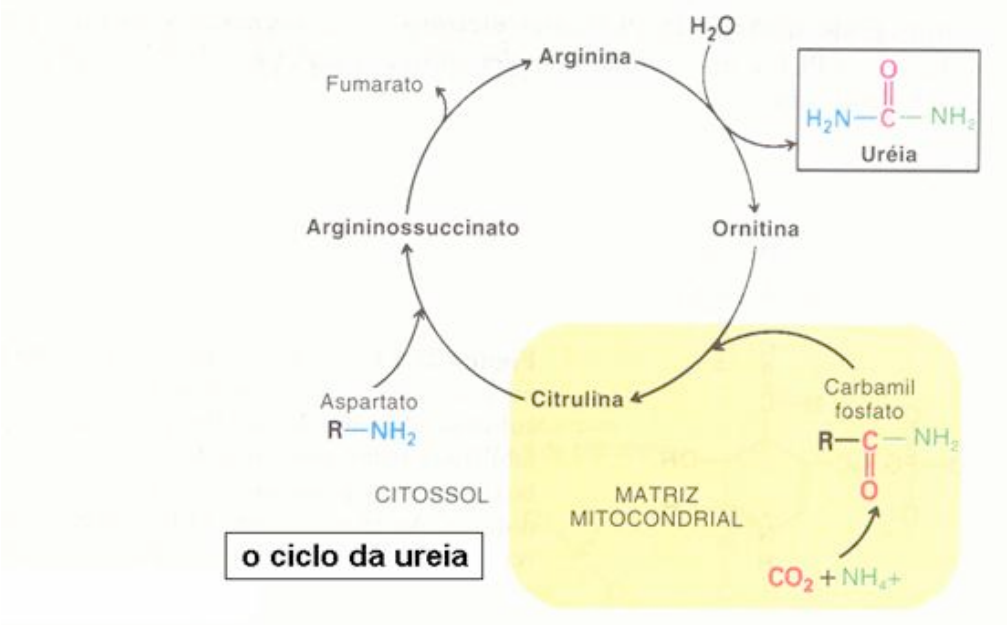

Figura 3 – Ciclo da Uréia. Manutenção do balanço nitrogenado no hepatócito. Ilustração do livro - Stryer, Bioquímica.

2.4. AMÔNIA E EXERCÍCIO

O trabalho mecânico aumenta a taxa de hidrólise de ATP excedendo sua velocidade de ressíntese, acarretando aumento na concentração intracelular de AMP e amônia (Hellsten, Richter et al. 1999). Deste modo uma alteração da relação ATP/ADP estimula a deaminação e transaminação dos aminoácidos formando amônia e cetoácidos. Propomos que estes estímulos induzam uma hiperamonemia transitória, modificando o metabolismo em responder às necessidades energéticas imediatas e de defesa aos processos inflamatórios pela injúria muscular em exercícios extenuantes.

Necessariamente o músculo esquelético em contração intensa, tem a velocidade da glicólise excedida em comparação com a do TCA (Robertson, Watt et al. 2004). Aumentos das concentrações de piruvato o direcionam a síntese de lactato. Desta forma, incrementos de Ala e Gln fornecem energia para a sustentação da via glicolítica (Kemppainen, Aalto et al. 2005). (Figura 4).

Um aumento da amônia induzido pelo exercício pode estar associado ao desequilíbrio energético, intensidade do exercício, duração do exercício, tipo de fibra, dieta e outros fatores (Zhao, Snow et al. 2000; Carvalho-Peixoto, Alves et

al. 2007). O exercício físico tem sido um bom modelo para se estudar a hiperamonemia sem a infusão de sais de amônia.

Estudos do Laboratório de Bioquímica de Proteína (LBP-UNIRIO) tem utilizado o HIU como modelo experimental para induzir um estado de hiperamonemia transitória para investigar os seus efeitos sobre o metabolismo (Franco and Canela 1984; Carvalho-Peixoto, Alves et al. 2007).

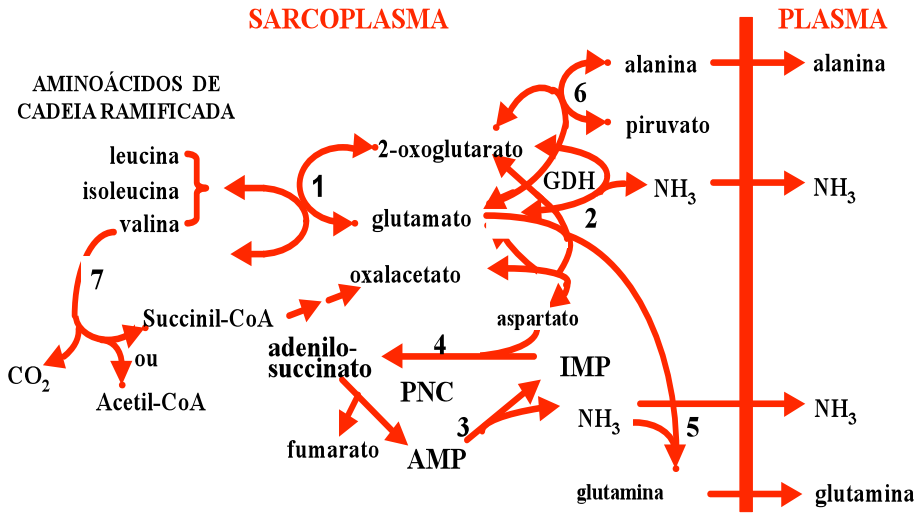

Figura 4 – Deaminação de aminoácidos. Formação, utilização, transporte de amônia e substratos neoglicogênicos para o plasma: 1- aspartato aminotranferase/ 2-glutamato desidrogenase/ 3- AMP deaminase/ 4- adenilsuccinato sintetase/ 5- glutamina sintetase/ 6- piruvato carboxilase e alanina aminotransferase. Extraído e adaptado de Terjung.

No exercício de alta intensidade e longa duração, postula-se maior catabolismo dos aminoácidos de cadeia ramificada (BCAA) produzindo amônia. A amônia é um dos produtos resultantes deste processo necessitando ser excretada. A amônia livre é tóxica e é preferencialmente transportada no sangue na forma de NH_4^+. A Gln no músculo é o principal precursor de amônia, liberando dois íons NH_4^+ e bicarbonato (HCO_3^-). Neste caso o ânion produzido ajuda no tamponamento e conseqüentemente balanço nitrogenado do metabolismo (Bruce, Constantin-Teodosiu et al. 2001; Steele, Chadwick et al. 2001; Nybo, Dalsgaard et al. 2005).

2.5. AMÔNIA E ALTERAÇÕES DO SNC

Estudos têm evidenciado que a alta concentração de amônia induzida pelo exercício físico pode gerar desequilíbrio momentâneo no funcionamento do SNC parecidos com aqueles encontrados em diversas doenças relacionadas à hiperamonemia e/ou doenças neuro-degenerativas (Suarez, Bodega et al. 1997; Saez, Llansola et al. 1999).

Em seres humanos a amônia pode ter variações entre 20 a 1000μM/L (Yuan, So et al. 2002). Em muitos casos como em doenças genéticas, estresse físico, pós-operatório e traumas gerais, a amônia pode alcançar níveis tóxicos ao organismo humano (Butterworth 2001; Felipo and Butterworth 2002). Uma variedade de mecanismos e diferentes teorias têm sido propostas para explicar os efeitos neurotóxicos da amônia nos últimos anos.

A hiperamonemia ou alta concentração de amônia está associada à alteração na regulação de neurotransmissores podendo ocasionar o desequilíbrio nos sistemas glutamatérgico, GABAérgico e serotoninérgico que pode ser suficiente para causar excitotoxidade neural e/ou morte (Brustovetsky, Brustovetsky et al. 2001; Butterworth 2001; Felipo and Butterworth 2002).

A toxicidade cerebral e fadiga muscular podem ser induzidas por altas concentrações de amônia, diminuindo a capacidade dos indivíduos em se exercitar (Hellsten, Richter et al. 1999)

Em muitos casos, doenças neuro-degenerativas ocorrem devido à incapacidade do fígado em responder rapidamente às mudanças sistêmicas dos níveis de amônia causados pelo exercício ou doenças do SNC (Olde Damink, Dejong et al. 2006). Neste caso, aumento da síntese de Gln nos astrócitos pode estar associado à resposta neurotóxica pela ação da amônia que ultrapassa a barreira hemato-encefálica (Nybo, Dalsgaard et al. 2005).

A hiperamonemia pode direcionar uma redução do Glu e aumento de lactato e Gln no SNC (Odland, Heigenhauser et al. 2000). A hiperamonemia aumenta o estresse oxidativo no cérebro o que pode induzir o desequilíbrio osmótico ocasionando efeitos deletérios (Cauli, Rodrigo et al. 2009; Monfort, Cauli et al. 2009).

Uma das principais alterações neurológicas como descrito acima é a condições de estresse metabólico por alterar as vias de transdução cerebral

associadas a diferentes tipos de receptores de Glu como os N-metil-D-aspartato (NMDA), causando excitotoxidade e/ou morte (Butterworth 2001). Antes destes eventos, as alterações do SNC podem causar ataxia, torpor, falta de coordenação, falta de concentração e diminuição da cognição (Nybo, Dalsgaard et al. 2005) (Figura 5).

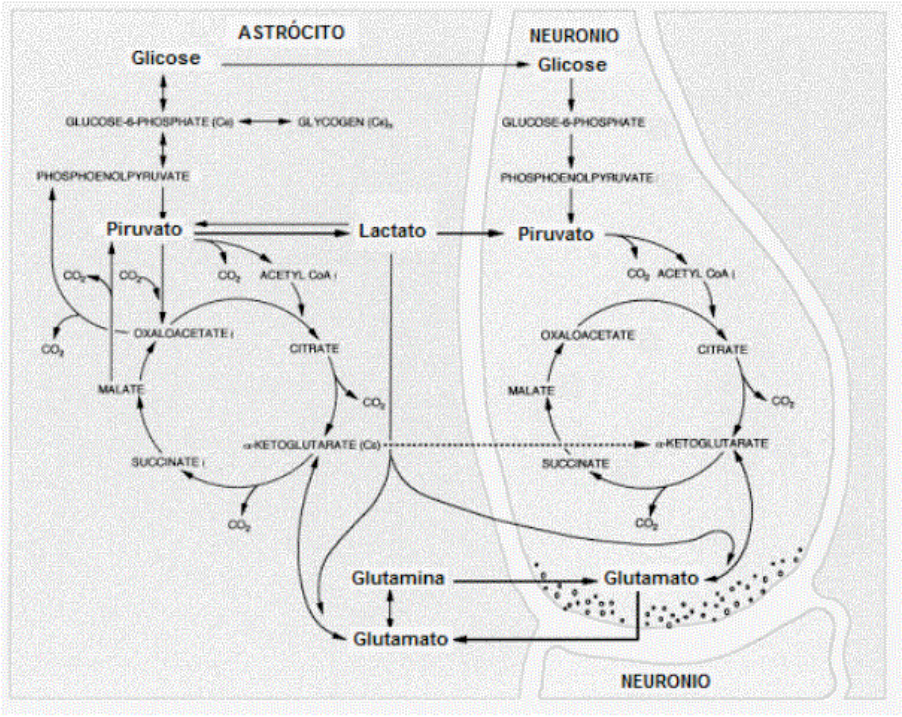

Figura 5 - Exercício, amônia e SNC. Formação de lactato a piruvato e liberação de glutamato no neurônio pós-sináptico. Extraído de Hertz et. al, 2000.

2.6. FÁRMACOS MODIFICADORES DO METABOLISMO

Acetaminofen (APAP) ou como conhecido comercialmente por Paracetamol é um fármaco com propriedades analgésicas, antipiréticas e sem propriedades antiinflamatórias atuando na inibição da síntese de prostaglandinas. O fígado responde rapidamente a droga podendo ocasionar hepatotoxicidade em pouco tempo dependendo da dose administrada (Chun, Tong et al. 2009). Como descrito no artigo Case Report, um dos atletas auto-administrava doses de APAP para diminuir um dor que foi causada por um acidente de bicicleta em treinamento.

Sabe-se que crianças com menos de 37kg tem a dose limite diária em 80mg . kg^{-1}. Em indivíduos adultos pode ocorrer toxicidade em doses únicas de 10-15g (150-250mg . kg^{-1}) (Liu, Govindarajan et al. 2004). Sem um tratamento durante a superdosagem de paracetamol pode ocasionar falência hepática e em casos extremos, morte em poucos dias (Imaeda, Watanabe et al. 2009). Os estudos laboratoriais podem demonstrar a evidência de necrose hepática com elevação da AST, AST, bilirrubina e tempo de coagulação aumentado. Geralmente a AST é um pouco superior à de ALT na hepatotoxicidade induzida pelo paracetamol (Saito, Zwingmann et al. 2009).

Como descrito, quanto mais cedo se administrar a NAC, maiores são chances e benefícios em reverter os efeitos ocorridos pela administração de APAP (Doyon and Klein-Schwartz 2009). Em nosso estudo concluímos que a administração de NAC teve efeito hepatoprotetor, reduzindo as enzimas marcadoras de lesão hepática (Figura 6).

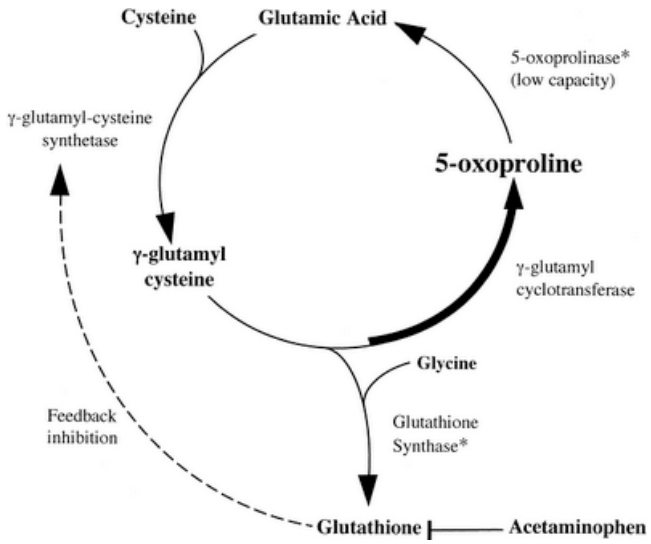

Figura 6- N-acetil cisteína precursor de glutationa. A glutationa como fator de proteção oxidativa e hepatoprotetora. Extraído: Humphreys et. al. 2005.

CAPÍTULO 3

PROCEDIMENTOS METODOLÓGICOS

3.1. MATERIAL E MÉTODOS

Usamos o HIU como modelo de estresse metabólico na tentativa de avaliar a cinética da amônia e pequenas enzimas para a corrente sanguínea em indivíduos saudáveis durante uma competição de ciclismo de 800 km com revezamento.

A equipe de ciclismo constituída por quatro triatletas amadores do sexo masculino, com as seguintes características antropométricas: 37 ± 2,7 anos; 82,5 ± 3,5 kg; 180,2 ± 2,2 cm; VO_{2max} = 47,6 ± 1,8 mL . Kg^{-1}. min^{-1}; FC_{max} = 170 ± 5 bpm, participaram voluntariamente no estudo. Os indivíduos foram inicialmente submetidos a uma avaliação antropométrica e laboratorial (D0) e não foi permitida a participação de sujeitos sob utilização de recursos ergogênicos.

Este estudo foi aprovado pelo comitê de ética para pesquisas com seres humanos da Universidade Castelo Branco e conforme os requisitos para a realização de pesquisas em seres humanos (National Health Conselho, Brasil, 1996) e o termo de consentimento por escrito foi assinado por todos os sujeitos.

Durante a analise dos exames de D0 foi diagnosticado um quadro de lesão silenciosa do fígado pela automedicação e com altas doses de

paracetamol (APAP) para aliviar a dor causada por um acidente de bicicleta durante os treinamentos. Foi prescrito N-acetilcisteina e metionina como descrito no artigo case report.

Os triatletas que participavam do campeonato internacional de ciclismo de revezamento, pedalaram numa rodovia pública por 800 km ultrapassando os limites do Estado de São Paulo, sem interrupção de trânsito e com um carro de segurança para protegê-los.

O Extradistance faz parte de um circuito internacional de competição de Ciclismo de revezamento. Para isso, foi necessário à logística do experimento três veículos de suporte: 1) chamado laboratório móvel, sendo estruturado para coleta, processamento e armazenamento do sangue, evitando a perda de compostos voláteis e a degradação de proteínas; 2) carro para o transporte da equipe responsável pela coleta de sangue; e 3) Microônibus adaptado para transporte das bicicletas e ciclistas.

Durante o revezamento, cada atleta separada e alternadamente pedalava cerca de 20-25 minutos até a exaustão, completando um total de 200 km (1/4 da corrida) e a prova foi completada em ~23h.

Entre cada ciclo, os atletas foram apanhados pelo carro de apoio onde, foram capazes de descansar até que o próximo ciclo de pedalada (em torno de 60 a 75 min). A cada início e fim de ciclo os atletas eram pesados e a perda de líquido era reposta através de água ou gatorate® para evitar a desidratação. Os indivíduos foram autorizados a comer e beber sob orientação ad libittum - gel de carboidrato, frutas, batata cozida e massas.

O sangue foi coletado de todos os atletas no início da prova e logo após o esforço máximo de cada um, na eminência de completar as seguintes distâncias 50, 100, 150 e 200 km individuais, que têm a seguintes correspondências: 1) Atletas na coleta e na eminência do quilômetro 50 estavam com 200 km do percurso percorrido; 2) Atletas na coleta e na eminência do quilômetro 100 estavam com 400 km do percurso percorrido; 3) Atletas na coleta e na eminência do quilômetro 150 estavam com 600 km do percurso percorrido; 4) Atletas na coleta e na eminência do quilômetro 200 estavam com 800 km do percurso percorrido.

O sangue foi coletado da veia antecubital em um intervalo máximo de 30 segundos entre parada da bicicleta e coleta. As amostras para o ensaio

bioquímico foram coletadas em tubos com gel de separação contendo intensificador de coágulo (Vacuette, Greiner Bio-One) e imediatamente centrifugado (3.000 x g, 10 min). O soro foi armazenado em nitrogênio líquido para posterior análise. As amostras foram enviadas para análise bioquímica e hematológica no Laboratório de Bioquímica do exercício (LABEX-UNICAMP).

A análise bioquímica foi realizada em um dispositivo automático (Autolab 18 - Boehringer Mannheim) de creatina kinase (CK), aspartato aminotransferase (AST), alanina aminotransferase (ALT), glutamiltransferase (GGT), lactato desidrogenase (LDH), fosfatase alcalina (ALP), glicose, uréia, creatinina, urato, colesterol, triglicerídios (TG), total proteínas, albumina, ferro sérico, bilirrubina, proteína C-reativa (PCR) e ácido alpha-1-glicoproteína.

As análises hematológicas foram realizada pelo analisador (KX-21N Sysmex) a partir do sangue coletado em tubos contendo EDTA e armazenado a 4 ºC. O total e contagem diferencial de leucócitos foram realizados, os glóbulos vermelhos e plaquetas também foram medidos. De acordo com o quadro abaixo:

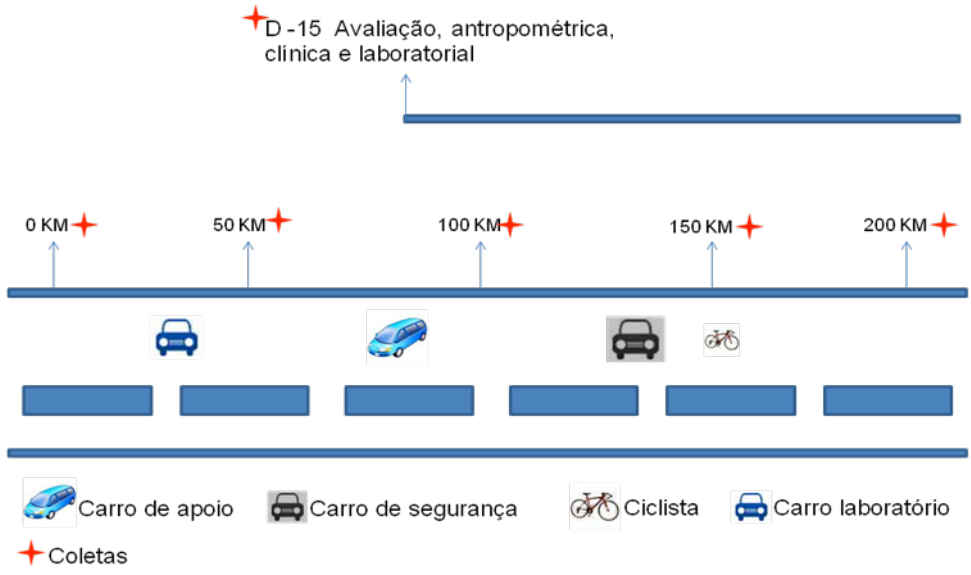

Figura 1: Linha temporal. Triatletas amadores em campeonato internacional de ciclismo de revezamento (800 km). Cada atleta pedala 20-25 minutos até a exaustão até completar aproximadamente 200 km.

3.1.1. Tratamento estatístico

Todos os dados foram normalizados para valores pré-corrida e foram expressos em média ± SE

A significância estatística foi calculada pela análise de variância (one-way-ANOVA) e o nível de significância foi estabelecido em $p<0,05$. A regressão linear foi feita por Correlação de Pearson.

Foi usada regressão não-linear para determinar a cinética de LDH e CK.

O aumento na atividade da curva LDH foi ajustado de acordo com dois parâmetros da equação hiperbólica: Os parâmetros calculados para os dados foram: a = 49,8 ± 1,8 e b = 30,1 ± 4,8.

$$Y= ax/b+x$$

aumento sigmoidal da curva de atividade CK foi ajustado de acordo à seguinte equação de três parâmetros:

$$Y = \frac{a}{1 + e^{-\frac{(-x - x0)}{b}}}$$

Os parâmetros calculados para os dados foram: a = 351,3 ± 23,8; b = 36,5 ±5,0 e x0 = 117,0 + 7,6.

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ANEXO 01

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Willem Meeuwisse
Editor
Clinical Journal of Sport Medicine.

Dear Dr. Meeuwisse,

I have enclosed a manuscript entitled "**Muscle Injury Followed by Acetaminophen (APAP) Hepatotoxicity: A Case Report**" by Bessa et al. for publication in the Clinical Journal of Sport Medicine.
We are trying to understand the impact of exercise on metabolism. Results from our lab showed that either glutamine or carbohydrate protects against a rise in ammonia levels in the blood (Carvalho-Peixoto et al., 2007 - Appl Physiol Nutr Metab; Bassini-Cameron et al., 2008 – Br J Sports Med) and that glutamine and alanine act distinctly in metabolism in an intensity-dependent way (Bassini-Cameron et al., 2008 - Br J Sports Med). In parallel we are attempting 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).
Here we present a muscle injury case that we treated successfully in a short time frame (15 days). The case involves an athlete who came to us with his team in search of metabolic advice for performance improvement. We studied his metabolic profile and assessed a liver and muscle injury evaluation. This led us to diagnose a muscle injury in conjunction with hepatotoxicity, which had probably been induced by the widely used analgesic and anti-inflammatory acetaminophen.
For diagnosis purpose we compare the athlete with three other athletes on the same team following the same training and nutritional program. We believe this case study is important because we were able to decrease the biochemical markers for both injuries using training, diet, and supplementation.
I would like to submit this report with a table and a figure. I believe that this information is important for readers to understand the text.
This case was approved by the Human Research Committee of the Universidade Castelo Branco and was conducted with written consent from the subjects after the nature and procedures of the study had been explained to them. Thus this study fulfilled the requirements for carrying out research on human subjects (National Health Council, Brazil, 1996). In addition, I would like to affirm that the manuscript has not yet been submitted to any other journal, and that all the authors made significant contributions to the study and have read and approved the final version of the manuscript.
We sincerely believe that our work is within the scope of the Clinical Journal of Sport Medicine and hope that it is found to be acceptable for publication.
Sincerely yours,

L. C. Cameron, Ph. D.
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Muscle Injury Followed by Acetaminophen (APAP) Hepatotoxicity: A Case Report

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INTRODUCTION

A team of triathletes came to our laboratory requesting a biochemical assessment. One athlete caught our attention because some of his blood markers were inconsistent with our expectations, suggesting that the athlete had suffered both muscle and liver injury.

CASE REPORT

We evaluated the VO_2 of the athletes, as well as their hematological, biochemical, and enzymatic profiles. They had been following the same training and nutritional program, and their performance was very similar based on ergospirometry (Fig. 1). After the initial analysis, one athlete showed unexpected levels of some blood proteins and biochemical indicators. The athlete presented much higher levels of muscle injury markers than his teammates. The athlete's value for creatine kinase (CK) level was almost 275% higher, the lactate dehydrogenase (LDH) level was approximately 25% greater, and C-reactive protein (CRP) was 140% greater than the average value of three teammates. We also noticed a higher level (36%) of aspartate aminotransferase (AST), a 184% increase in γ-glutamyltransferase (γGT), and a 350% increase in total bilirubin level, when compared with his teammates. In contrast, the athlete had levels of the liver injury markers alkaline phosphatase (ALP) and alanine aminotransferase (ALT) that were similar to the group. (Table 1).

After a careful anamnesis, we found that the athlete had experienced back pain in the past 96 h. He attributed this pain to a previous training session in which a bike drift occurred and he began to feel severe muscle pain. The athlete indicated that he used

~9 g of acetaminophen (APAP) per day to relieve muscle soreness. After ruling out diseases such as hepatitis and cirrhosis, we hypothesized that the athlete had suffered a muscle injury, after which his use of APAP resulted in liver injury.

Based on all these analyses, we suggested that the athlete decrease both training intensity and volume to 30-40% and increase rest and sleeping time. We did not ask the athlete to stop training completely, since he was scheduled to compete in two weeks. We also suspended the use of APAP and advised dietary changes. Our dietary plan called for high carbohydrates (≥ 6 g of carbohydrates/kg/day) and high protein (≥2 g of proteins/kg/day). We also recommended a high intake of dietary methionine (Met) and cysteine (Cys), and a daily dose of 3600-4200 mg of N-acetylcysteine (NAC).

After two weeks of following our training and dietary recommendations, the athlete reported that the pain had been relieved. This was corroborated by biochemical analysis, which showed that the amounts of CK and γGT in the blood were less than 20% and 70% of pre-treatment levels, respectively. We did not, however, detect a change in the level of total bilirubin in the blood; the athlete's level following treatment was still higher than the average value of his teammates. Nevertheless, we did detect an improvement in the bilirubin conjugation measured by the way of both direct and indirect bilirubin.

DISCUSSION

Endurance exercise is very stressful for the body [1] [2]. In fact, it has been proposed that endurance training can cause chronic liver injury [3]. Given the high CK values of this athlete, we initially thought that he might be suffering from overtraining syndrome (OTS) [4]. However, the athlete's VO_{2max} test analysis showed him to have similar performance

to his teammates. Since the VO_{2max} test is widely accepted as the gold standard for OTS diagnosis, we discarded this possibility.

The athlete reported having back pain after a bike drift. Due to the role of this muscle group in torso stabilization, we suspected that muscle injury was the principal cause of the high CK values. Consistent with this idea, the athlete had higher levels of the classical injury markers CK and LDH than in his teammates. In fact, the CK level was greater by almost 275% and the LDH by 25%, and these increases are consistent with our recent report of the kinetics of CK and LDH appearance in the blood [5]. Our explanation of muscle injury is further supported by the elevated CRP value, which indicates acute phase inflammatory response.

We previously showed that it is possible to separate muscle and liver injuries using alkaline phosphatase and γGT as hepatocyte integrity markers [6 7]. In the present case study, the athlete had a γGT level nearly twice as high as teammates following the same training schedule, and his AST level showed a slightly smaller increase. These findings support the hypothesis of liver injury.

Following the bike drift, the athlete had begun taking a large daily dose of APAP to reduce back pain. APAP is a widely used, over-the-counter painkiller. Excessive doses of APAP have been linked to hepatocyte injury [8 9]. Although we cannot ignore the reported influence of endurance training on chronic liver injury [3], we believe that the hepatotoxic effect of APAP is to blame in this case. Therefore we concluded that the athlete was suffering from acute back pain caused by muscle injury due to mechanical load, and that subsequent APAP overdose led to liver injury. We recommended that he use N-acetylcysteine (NAC), an APAP antidote widely used in clinical treatment. We

further recommended that he increase his dietary intake of naturally occurring Met and Cys, which have also been proposed to protect against APAP hepatotoxicity[8].

By the time a second evaluation was conducted 15 days after the first, the CK and CRP levels in the athlete had fallen within the range observed in his teammates. The level of γGT had decreased by 38% to level measured at the first evaluation, but it was still higher than in his teammates. We observed an increase in hepatic function based on measurement of the liver's ability to conjugate bilirubin. These results suggest that our diagnosis was correct, and that the interventions we developed may be effective for treating mild APAP intoxication in athletes.

KEY WORDS: N-acetylcysteine; creatine kinase; C-reactive protein; bilirubin; γ-glutamyltransferase

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FIGURE CAPTIONS

Figure 1. No difference was found in injured athlete's VO_{2max} when compared with his team average. Dashed line, injured athlete; straight line, health athletes' average.

Figure

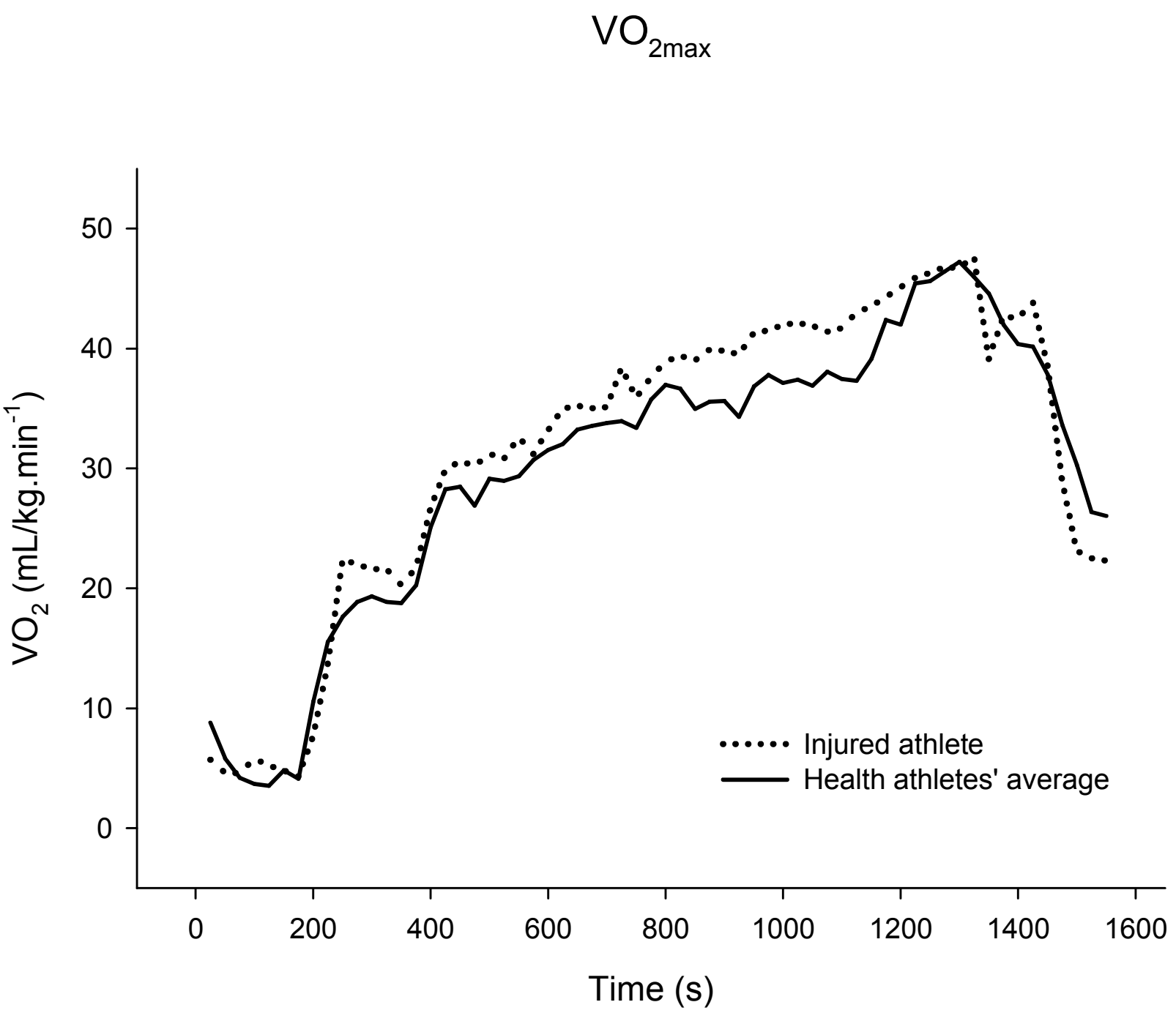
VO2max
VO2 (mL/kg.min-1)
50
40
30
20
10
0
0
200
400
600
800
1000
1200
1400
1600
Time (s)
Injured athlete
Health athletes' average

TABLE 1

Parameter	Injured athlete (1^{st} analysis)	Health athletes (1^{st} analysis)	Injured athlete (2^{nd} analysis)	Health athletes (2^{nd} analysis)
CK (UI/L)	868.0	182.7 ± 19.8	147.0	190.3 ± 20.8
LDH (UI/L)	383.0	308.0 ± 50.6	408.0	296.3 ± 53.0
AST (UI/L)	57.3	42.0 ± 3.1	55.6	46.9 ±11.4
ALT (UI/L)	27.7	25.3 ± 3.1	19.8	35.2 ±14.4
γGT (UI/L)	47.1	16.6 ± 2.7	33.9	15.3 ± 2.3
ALP (UI/L)	54.0	75.3 ± 11.3	44.0	81.3 ± 16.8
CRP (nmol/L)	62.5	25.9 ± 10.8	3.4	12.7 ± 5.1
Total bilirubin (μmol/L)	44.3	9.7 ± 1.6	40.0	18.1 ± 2.3
Direct bilirubin (μmol/L)	9.6	4.4 ± 0.7	18.8	13.7 ± 4.8
Indirect bilirubin (μmol/L)	34.7	5.3 ± 1.6	21.0	4.3 ± 2.5

Table title: Muscle and liver injury markers measured before and after treatment.

Column heads:

Parameter

Injured athlete (1st analysis)

Health athletes (1st analysis)

Injured athlete (2nd analysis)

Health athletes (2nd analysis)

Explanatory legends: Liver and muscle injury parameters measured before and after team counseling and athlete treatment. CK, creatine kinase; LDH, lactate dehydrogenase; AST, aspartate aminotransferase; ALT, alanine aminotransferase; γGT, γ-glutamyltransferase; ALP, alkaline phosphatase; CRP, C-reactive protein. Data for health athletes are average ± SE.

ANEXO 02

High-intensity ultraendurance promotes early release of muscle injury markers

A Bessa, M Nissenbaum, A Monteiro, P G Gandra, L S Nunes, A Bassini-Cameron, J P S Werneck-de-Castro, D Vaz de Macedo and L-C Cameron

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High-intensity ultraendurance promotes early release of muscle injury markers

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AB and MN contributed equally to this work

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ABSTRACT

Objective: To evaluate the impact of high-intensity ultraendurance (HIU) cycling, using it as a possible way to understand muscle injury kinetics and blood immune cells' release during high-intensity prolonged exercise

Design: Male amateur triathletes enrolled during a cycling race of the International Bike Championship 800 km cycling relay (~23 h). Each athlete alternately cycled 20–25 minutes until exhaustion and performed a total of approximately 200 km.

Results: Creatine kinase levels in blood reached a 300% rise in a sigmoidal pattern, while lactate dehydrogenase levels increased by 30–40% following a hyperbolic pattern. Aspartate aminotransferase and alanine aminotransferase levels increased by up to 250% and 140%, respectively. Liver injury markers such as alkaline phosphatase and γ-glutamyltransferase remained stable. Platelets increased by 20–30% from pre-exercise, and there was no change in haematocrit during the race. White blood cells rose by nearly 200%. Leucocytes rose 210% during the race, with a major component coming from neutrophils, which increased more than 300%. Triacylglycerol levels were decreased at the finish and total cholesterol levels remained unchanged. Urate increased (by up to 35%) during the first half of the race, and urea levels increased with a different pattern, increasing by 45% in the second half.

Conclusions: This study showed the blood appearance kinetics of muscle injury markers and some metabolites. It is suggested that the increase in these enzymes came primarily from muscle damage, rather than liver damage, and that white blood cells are selectively mobilised independently of haemoconcentration. The early appearance of muscle injury markers in this kind of exercise was also shown.

Endurance and ultraendurance exercise is an extreme challenge to human metabolism. High-intensity ultraendurance (HIU) exercise has been defined as repeated bouts of high-intensity exercise (>75% VO_{2max}) during an ultraendurance race with limited recovery.[1] Several studies have described metabolic changes during and after endurance exercise,[2] high-intensity exercise,[3] and ultraendurance exercise,[4] but very little is known about metabolism in HIU. This kind of activity exposes athletes to exercise intensities higher than in regular endurance events,[1 5 6] and is poorly studied, especially in field protocols that conserve psychological and environmental stress. The high-level physical demand during these events induces acute changes in metabolism, including muscle injury.[5] Some of these changes can be accessed by haematological and biochemical analysis of blood, powerful tools in understanding exercise intensity and metabolism in physical stress.[7–9]

Among all of the changes imposed on the metabolism by exercise, ammonia and its metabolites have been the focus of several recent studies; for review see.[10 11] Blood ammonia concentration increases during endurance exercise mainly due to myokinase activity and deamination by muscle,[4] and has been proposed as a cause for both peripheral and central fatigue.[10–12] Also, metabolites such as urate and urea increase during high-intensity exercise in response to the IMP and ammonia clearance demand.[2]

The strong muscle contractions during exercise may cause micro-tears in both muscle and the vascular endothelium, which increases the migration of white blood cells into the muscle, inducing acute-phase inflammatory reactions. We have shown that muscle injury markers appear in an early window with leucocytosis during intermittent exercise.[13] Since HIU exercise is high-intensity and intermittent, we decided to evaluate its impact on classical muscle injury markers, white blood inflammatory cells and muscle and liver metabolism.[14–19]

Here, we use HIU exercise to evaluate extreme physical stress on biochemical and haematological parameters. Since athletes were subjected to high intensities for a long period of exercise, we also hypothesised that HIU exercise is a good model for understanding part of nitrogen metabolism and the relationship between muscle injury marker kinetics and the white blood cell response. There are a couple of unique features of our study. First, this research took place on a highway with almost constant traffic (night and day, ~22 h), which adds a considerable amount of environmental stress. In addition, we also measured several biochemical and haematological parameters, making this a more accurate investigation of this subject.[1 5] Moreover, we here show the appearance of muscle injury markers within as short a time as 6 h of exercise.

MATERIALS AND METHODS

Subjects

An entire cycling team, consisting of four healthy male amateur triathletes (37 (SE 2.7) years old; 82.5 (SE 3.5) kg body mass; height 180.2 (SE 2.2) cm; VO_{2max} 47.6 (SE 1.8) ml O_2/kg/min; HR_{max} 170 (SE 5) beats/min) enrolled voluntarily in this study. The subjects were initially submitted to an anthropometric and laboratory analysis and denied using ergogenic resources or drugs. Written informed consent was obtained from all athletes.

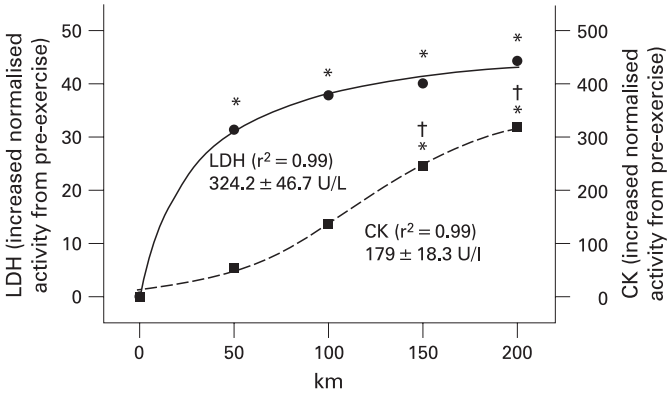

Figure 1 Muscle injury marker increase during high-intensity ultraendurance cycling. Data are plotted as blood creatine kinase (■, CK) and lactate dehydrogenase (•, LDH) against distance cycled. Lines show non-linear fitting curves using the parameters described under Materials and Methods. Data are shown as average (SE) of the increase in normalised activity from pre-exercise (0%). Absolute pre-exercise values are shown within the graphs. *$p<0.05$ compared with pre-exercise; †$p<0.05$ compared with 50 km.

This study was approved by the ethics committee for human research of the Universidade Castelo Branco and conformed to the requirements for carrying out research in human subjects (Health National Council, Brazil, 1996).

The race and blood sampling

We collected blood samples during one cycling race of the International Cycling Championship. Briefly, the race consisted of 800 km relay cycling (~23 h); each athlete alternately cycled 20–25 minutes until exhaustion and performed approximately a total of 200 km (1/4 of the race). The athletes cycled on a regular highway without transit interruption and with a safety car to protect them. After each cycle, the athletes were picked up by a support team car and were able to rest until the next ride set. We used two more vehicles: a mobile laboratory that had to be developed to collect and to pretreat blood samples in order to avoid the loss of volatile compounds and protein degradation, and another car to transport the blood collection team.

Blood samples were collected after the exercise set whenever the subject completed 50 km. In other words, we collected blood right after the maximum effort of each athlete at 50, 100, 150 and 200 km. We weighed the athletes immediately after each cycle for drink counselling regarding weight loss. While resting between the exercise sets (60–75 min), athletes were allowed to eat ad libitum and drink under our counselling. Water, sports drinks, carbohydrate gels, fruits and pasta were available in the support car.

Haematological and biochemical analysis

Venipuncture was performed by a certified phlebotomist before and during the race. After each running cycle, the athlete stopped and in a 30 sec window had his blood collected. Samples for biochemical assay were collected into tubes with coagulation enhancer and splitting gel (Vacuette, Greiner Bio-One) and immediately centrifuged (3000×g; 10 min). Blood serum or plasma was aliquoted and stored in liquid nitrogen for later analysis. Samples were analysed for muscle injury markers and biochemical variables. Biochemical analyses were performed in an automatic device (Autolab 18 – Boehringer Mannheim) for creatine kinase (CK), aspartate aminotransferase (AST), alanine aminotransferase (ALT), γ-glutamyltransferase (γGT), lactate dehydrogenase (LDH), alkaline phosphatase (ALP), glucose, urea, creatinine, urate, cholesterol, triacylglycerols (TG), total proteins, albumin, serum iron, bilirubin, C-reactive protein (CRP) and acid α-1-glycoprotein.

Haematological analysis was performed by automated analysis (KX-21N Sysmex) from blood collected into tubes containing EDTA and stored at 4°C. Total and differential white cell counts were performed, and red blood cells and platelets were also measured.

Statistics

All data were normalised to pre-race values and are expressed as means (SE); this is so that the changes between pre-race and post-race values are more clear. Statistical significance was calculated by analysis of variance (one-way ANOVA) and the level of significance was set at $p<0.05$. Linear regression was performed by Pearson's correlation.

Nonlinear regression was performed to determine LDH and CK kinetics.

The increase in LDH activity curve was fitted according to data following a two-parameter hyperbolic equation:

$$y = \frac{ax}{b+x}$$

The calculated parameters for the data were: $a = 49.8 \pm 1.8$ and $b = 30.1 \pm 4.8$.

The sigmoidal increase in CK activity curve was fitted according to data following a three-parameter equation:

$$y = \frac{a}{1 + e^{-\left(\frac{x - x0}{b}\right)}}$$

The calculated parameters for the data were: $a = 351.3 \pm 23.8$; $b = 36.5 \pm 5.0$ and $x_0 = 117.0 + 7.6$.

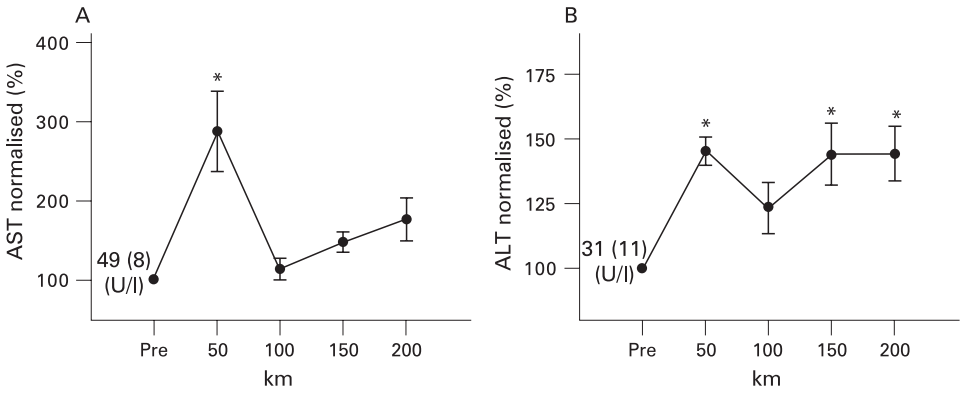

Figure 2 Blood presence of aminotransferases during HIU. (A) Aspartate aminotransferase (AST) and (B) alanine aminotransferase (ALT) measured in blood. Data are normalised (average (SE)) to pre-exercise values (100%). Absolute pre-exercise values are shown within the graphs (U/l).*$p<0.05$ compared with pre-exercise. HIU, high-intensity ultraendurance exercise.

Table 1 Haematological parameters measured during high-intensity ultraendurance cycling

	Pre (absolute value)	50 km (% of pre)	100 km (% of pre)	150 km (% of pre)	200 km (% of pre)
Haematocrit (%)	44.6 (0.9)	99.2 (1.6)	98.2 (2.2)	99.0 (2.1)	99.5 (2.0)
Haemoglobin (mmol/l)	2.32 (0.06)	100 (2.08)	99 (2.1)	99.2 (1.7)	100.3 (1.6)
MCV (fl)	88.7 (2.7)	99.5 (0.3)	99 (0.4)	97.2 (1.4)	98.7 (0.2)
MCH (pg)	29.7 (0.97)	100 (0.4)	100 (0.4)	98.2 (2.1)	100.3 (0.4)
MCHc (g/dl)	32.2 (1.3)	105.3 (4.6)	106 (4.7)	105.5 (4.6)	106.3 (4.9)
Platelets ($\times 10^9$/l)	251 (31)	115.0 (8.4)	122.3 (2.1)*	127 (7.1)*	121.0 (2.0)

Data are mean (SE). Statistical analyses were carried out using normalised values. *$p<0.05$ compared with pre-exercise.
MCH, mean corpuscular haemoglobin; MCHc, mean corpuscular haemoglobin concentration; MCV, mean corpuscular volume; pre, pre-exercise.

RESULTS

Injury markers

We observed different blood marker changes during the race. Creatine kinase (EC 2.7.3.2; CK) blood levels rose in the race, reaching a 300% increase in a sigmoidal pattern. Conversely, lactate dehydrogenase (EC 1.1.1.27; LDH) levels showed a hyperbolic increase of 30–40%, reaching most of their increase in the first 50 km (fig 1). Aspartate aminotransferase (EC 2.6.1.1; AST) and alanine aminotransferase (EC 2.6.1.2; ALT) had a clear increase up to 250% (fig 2A) and 140% (fig 2B) of pre-exercise values, respectively, at 50 km. ALT remained higher than before exercise at the end of the race.

Typical liver injury markers such as alkaline phosphatase (EC 3.1.3.1; ALP) and γ-glutamyltransferase (EC 2.3.2.2; γGT) remained stable during the race at 22.4 (SE 9.6) U/l and 75.5 (SE 26.7) U/l respectively, due to personal differences.

Blood cells

To understand the effect of high-intensity ultraendurance exercise on blood cells, we measured the amount of platelets and both red and white blood cells. There was no change in haematocrit, haemoglobin, MCV, MCH or MCHC during the race. Platelets increased by 20–30% from pre-exercise at 100 km, returning to pre-exercise levels (table 1).

Total white blood cell count (WBC) rose to nearly 200% with hyperbolic kinetics from pre-exercise over the whole race, without differences between the points. To assess the extent of exercise influence on WBC, we also performed a differential WBC count, measuring the leucocyte subpopulations. Lymphocytes increased by up to 210% during the race (fig 3A). The major contribution to the leucocyte increase came from neutrophils, which increased by more than 300% from the first measurement (50 km). Even with an increase from 300–740%, CRP was higher than pre-exercise only at 150 km (fig 3B). We plotted the erythrocyte counts together with leucocyte subpopulations to control for blood volume variations. The red blood cells remained at the same level during the competition.

Glucose, fat metabolism, urate and urea

Glucose levels fluctuated during the contest, as expected, due to carbohydrate intake (fig 4A). Triacylglycerol levels decreased at the end of the race to 87% compared with those at 50 km (fig 4B), and total cholesterol levels remained unchanged (data not shown). Urate increased (by up to 35%) during the first half of the race, and urea levels increased with a different pattern, reaching a 45% increase during the second half (fig 5A and 5B, respectively).

DISCUSSION

In contrast to usual ultraendurance competitions, athletes had time for partial metabolic recovery after each exercise bout. We believe that the interpretation of data in this study leads to the understanding of some metabolic responses in HIU and also gives some direction about the metabolic response during this kind of exercise.

Due to the high difficulty of data collection during the race we evaluated an apparently small number of athletes. This was possible due to previous biochemical and haematological analysis showing group similarity. During an 800 km relay cycling race we measured markers of inflammation, muscle injury and nitrogen metabolism. To our knowledge, this is the first study to examine haematological and biochemical responses in a non-staged field protocol of a HIU cycling race. It is important to remember that we used an in-field experimental design allowing us to keep all the race stress.

Injury markers

It is clear that exercise is a powerful inducer of muscle injury. In our study, LDH (~140 kDa) showed a burst with a hyperbolic increase, tending to saturation at 100 km. As expected, the

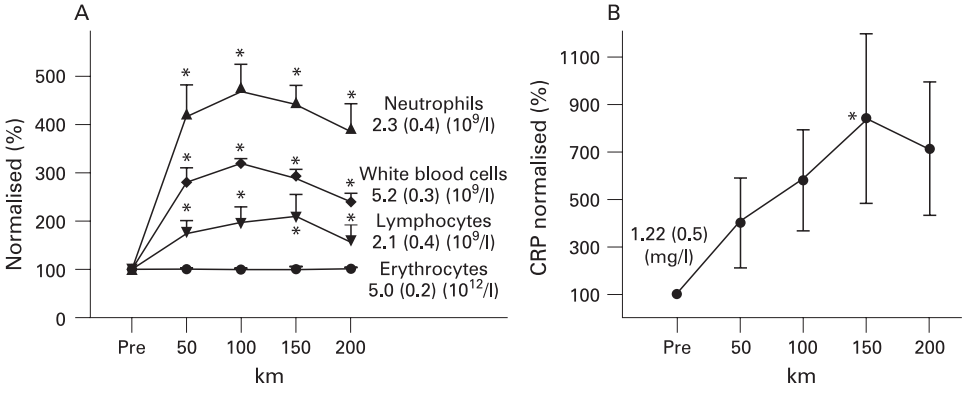

Figure 3 HIU effect on blood cells and CRP. (A) Both white blood cells and erythrocytes' presence are plotted in response to exercise; (B) C-reactive protein (CRP) is also shown during HIU cycling. Data are normalised to pre-exercise values (100%), and absolute pre-exercise values are shown within the graphs (U/l). Data are shown as average (SE). In erythrocyte counts SE bars are inside the symbols.*$p<0.05$ compared with pre-exercise. HIU, high-intensity ultraendurance exercise.

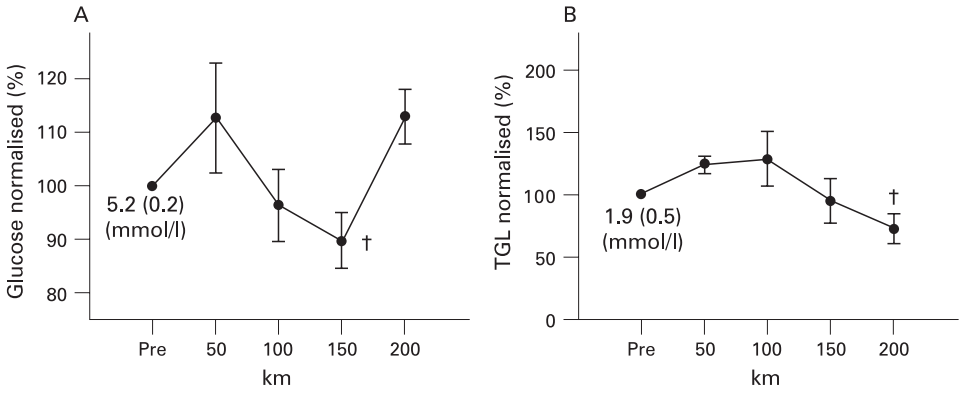

Figure 4 Glycaemia (A) and triacylglyceridaemia (B) during HIU exercise. Data are normalised (average (SE)) to pre-exercise values (100%), and absolute pre-exercise values are shown within the graphs (U/l).*$p<0.05$ compared with pre-exercise; †$p<0.05$ compared with 50 km. HIU, high-intensity ultraendurance exercise.

smaller protein CK (~86 kDa) showed a greater increase, reaching a 300% increase with a Hill number of ~1.8. This represents cooperative behaviour to its appearance in blood, probably because most CK is bound to the sarcomere M-line. Our data are in agreement with the changes in injury biomarkers being more pronounced during the second half of a 200 km ultramarathon race.[20]

An interesting finding in this study is that we were able to show an increase in the muscle injury markers CK and LDH much earlier than classically described,[21 22] a result also recently depicted by another group.[23] This could be due to the race design, whereby the athletes rested and were able to maintain high-intensity exercise, or simply because these early time frames were not exhaustively investigated previously.

Since injury markers such as LDH, ALT and AST increase following both liver and muscle injury, it is unclear whether hepatic damage occurs in ultraendurance exercise.[24] Furthermore, it has been proposed that basal values of these enzymes in ultraendurance athletes are high due to chronic liver damage following long-term strenuous exercise.[9] In the present study, we observed increased amounts of blood CK, LDH, AST and ALT. These increases could be due to muscle and/or liver damage. Our laboratory recently proposed that muscle damage can be distinguished from liver damage by using more specific hepatic injury markers such as ALP and γGT.[13] Taking these data together with maintenance of either ALP or γGT amount in blood, as found here, we suggest that the increases of these enzymes came primarily from muscle damage rather than liver damage.

Blood cells

Athletes' body weights were constant during the race (data not shown). During the experiment, there were no differences in haematocrit, haemoglobin, MCV, MCH or MCHC, which represents a lack of volaemic variation. On the other hand, there is a previously described inverse correlation between body weight and haematocrit after a marathon.[8] In addition, some studies reported increases in haematocrit, haemoglobin, MCV, and MCH, indicating haemoconcentration at the end of a 36 h continuous marathon.[25 26] Since the athletes were allowed to rest and to drink (water and isotonic beverages) during our study, this maintenance of volaemia can be explained by the relay race dynamics and drink counselling by our group. In accordance with our results, previous studies did not find differences in haematological parameters during a 24 h ultra-endurance marathon in relay runners or non-stop runners.[5 9]

White blood cell count increases after both endurance and ultraendurance exercise have been extensively reported.[8 9 25] We measured an important increase in total WBC, especially neutrophils and lymphocytes. This 280% increment in the total WBC population was already observed 6 h after the beginning of the race. More importantly, erythrocyte counts did not change at all over the race. Taken together, these results suggest that WBC mobilisation is due not to a non-specific exercise-induced spleen release, but rather to a specific signal. In addition, platelets also seem to be affected by exercise in a spleen-independent manner, since we observed thrombocytosis without any change in erythrocyte counts. These data suggest that leucocytosis and thrombocytosis could be induced by muscle injury, as proposed previously.[9 20]

Muscle lesions stimulate immune cell mobilisation to the bloodstream and migration to muscle tissue, which is consistent with WBC mobilisation due to muscle injury.[13] We show here that there was an increase in skeletal muscle injury markers in smaller time frames than classically reported. We were also able

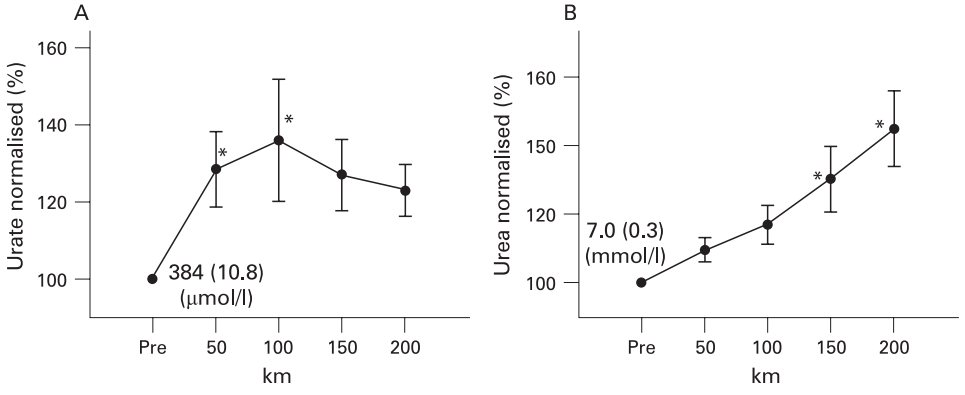

Figure 5 Blood urate (A) and urea (B) during race. Data are normalised (average (SE)) to pre-exercise values (100%) and absolute pre-exercise values are shown within the graphs (U/l).*$p<0.05$ compared with pre-exercise.

What is already known on this topic

HIU exercise is an enormous challenge to the body. Muscle damage is known to affect many athletes and its measurement has become an important tool to avoid injuries and performance decrease.[1 5] The analysis of muscle enzymes such as CK and LDH released into the blood stream has been used to quantify muscle injury extension with a blood appearance window of 24–72 h after exercise.[1 21 24]

What this study adds

The increase in WBC population after endurance, ultra-endurance and HIU exercise has been reported, but the kinetics has never been evaluated. Furthermore, we show an early appearance of several injury markers, such as CK, LDH, AST and ALT, blood cells and metabolic indicators during the HIU event. We also show that during HIU there is a higher metabolic demand in the first half of the race followed by a metabolic adaptation.

to measure the increase in WBC together with classical muscle injury markers, suggesting a possible signalling role from muscle injury to promote this early WBC migration. Hence, it is possible to propose that we can relate the WBC increase measured in this study to precocious muscle damage.

Metabolism

It is easily predictable that HIU exercise could promote high metabolic stress. To investigate this, we evaluated the impact of HIU exercise on indicators of exercise metabolism intensity.[3] Since the athletes had access to food, it is difficult to perform a metabolic interpretation of glucose levels. It is interesting to note that the levels of triacylglycerols decreased without changes in cholesterol. Since the athletes were not fed with a significant amount of lipids, these findings may reflect the use of fatty acids as fuels, as predicted in long-term exercise,[27] in addition to a smaller synthesis of triacylglycerols.

In high-intensity exercise some ammonia production comes from amino acid deamination, which contributes less than ammonia released due to adenosine monophosphate (AMP) conversion to inosine monophosphate (IMP). Despite a huge supply of carbohydrate during the race, both urate and urea increased by nearly 40–50%. Urate was significantly increased above baseline values in the first half of the race, and decreased after that. On the other hand, urea increased in the second half, with a linear progression ($r^2 = 0.98$). Some attempts have previously been made to predict the behaviour of metabolic pathways in whole organisms which is difficult, even without considering metabolite compartmentalisation in the cell.[28–30] We suppose that the blood urea increase in the second half of the race followed a slower ammonia metabolism than IMP degradation, probably due to the need for mitochondrial contribution. These findings lead us to believe that in the first part of the race the athletes had an energetic demand with high IMP production, which was partially equilibrated by the use of less entropic energetic substrates in the latter half of the race. Taking all these data together, our results lead us to suggest that the exercise demand of the first half of HIU is metabolically compensated after that, probably due to the consumption of fatty acids.

Competing interests: None.

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