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THÁSSIA CASADO LIMA FRANÇA

ANÁLISE ESPORTÔMICA NO METABOLISMO DE AMINOÁCIDOS E PRODUÇÃO DE AMÔNIA EM JOGADORES DE FUTEBOL

MACEIÓ- AL,

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Orientador: Prof. Dr. Antonio Euzebio Goulart Santana

Coorientadores: Prof. Dr. Eduardo Seixas Prado

Prof. Dr. Henrique Fonseca Goulart

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“*Sometimes it takes a long time to learn how to play yourself*”
Miles Davis

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RESUMO

A esportômica é a aplicação da metabolômica no exercício e, tem sido utilizada para investigar as alterações metabólicas induzidas pelo exercício no esporte. Durante o exercício, a produção elevada de amônia pode causar fadiga central. Acredita-se que a hiperamonemia pode ter um impacto significativo durante o exercício por promover prejuízos no desempenho cognitivo. No entanto, há pouca informação disponível sobre o papel do metabolismo de aminoácidos na descoberta de biomarcadores que possam ajudar a elucidar alterações de desempenho durante o exercício. O objetivo da tese foi realizar uma análise esportômica no metabolismo de aminoácidos e produção de amônia em jogadores de futebol. Anteriormente, foi avaliado em corredores o efeito agudo do salbutamol inalado na amônia sanguínea, contagem de glóbulos brancos e desempenho cognitivo-motor após exercício prolongado sob condições de calor. Os resultados mostraram que a inalação aguda de salbutamol pode diminuir a síntese de ureia e subsequentemente aumentar as concentrações de amônia no sangue. Além disso, o salbutamol inalatório pode induzir uma redução na contagem de linfócitos, mas não afeta o desempenho cognitivo-motor durante exercícios prolongados no calor. Em seguida, um novo estudo foi realizado e baseado na esportômica para investigar mudanças no metabolismo durante uma partida de futebol em 30 jogadores de futebol profissionais juniores do sexo masculino. Amostras de urina foram coletadas antes e depois dos 25 minutos da partida e analisadas por cromatografia líquida e espectrometria de massa. Os resultados mostraram 26 alterações significativas no metabolismo da tirosina. O exercício causou uma regulação negativa dos metabólitos homogentisato 4-maleylacetoacetato e succinilacetona para ($p = 4{,}69E\text{-}5$) e 16% ($p = 4{,}25E\text{-}14$), respectivamente. Descobriu-se que o 4-hidroxifenilpiruvato, um precursor do homogentisato, está regulado positivamente em 29-26% ($p = 7{,}20E\text{-}3$). A concentração de hawkinsina e seu metabólito 4-hidroxiciclohexila acetato aumentou ~6 vezes ($p = 1{,}49E\text{-}6$ e $p = 9{,}81E\text{-}6$, respectivamente). Diferentes vias do metabolismo da DOPA também foram afetadas pelo exercício. DOPA e dopaquinona aumentaram de quatro a seis vezes ($p = 32\ 5{,}62E\text{-}14$ e $p = 4{,}98E\text{-}13$, respectivamente). 3-metoxitirosina, indol-5,6-quinona e melanina foram regulados negativamente de 1 a 25%, assim como a dopamina e a tiramina (diminuindo para até 5% ou 80%; $p = 34\ 5{,}62E\text{-}14$ e $p = 2{,}47E\ \text{-}2$, respectivamente). O TCO_2 sanguíneo diminuiu, bem como a glutationa urinária e o glutamato (40% e 10% respectivamente) associados a um aumento de duas vezes no piroglutamato. Além disso, observou-se alterações no metabolismo da arginina e compostos guanidínicos em resposta a uma partida de futebol. Os metabólitos N-alfa-acetil-L-arginina, D-arginina, gama glutamil ornitina, agmatina, ácido argininíco e espermina, aumentaram após a partida de futebol. Entre as análises do metabolismo de aminoácidos e produção de amônia, não foram encontradas vias para a produção de amônia através da abordagem esportômica. No entanto, foi identificado a produção de hawkinsina e ácido argininíco, sugerindo a descoberta de novos marcadores com potencial de promover danos ao desempenho.

Palavras-chave: Neurometabolismo. Metabolômica. Hiperamonemia. Metabolismo de aminoácidos. Vias metabólicas. Ciência do esporte.

ABSTRACT

Sportomics is application of exercise metabolomics in sports science to investigate the metabolic changes induced by exercise in sport. During exercise, increased ammonia production can cause central fatigue. It is postulated that hyperammonemia leads a significant impact during exercise by promoting impairments in cognitive performance. However, studies in sports remains limited on the role of amino acid metabolism in discovering biomarkers that can help elucidate performance changes during exercise. The present study is to investigate, through a sportomics analysis, the metabolism of amino acids and ammonia production in football players. Previously, the acute effect of inhaled salbutamol on blood ammonia, white blood cell count and cognitive-motor performance after prolonged exercise under hot conditions was evaluated in runners. The results showed that acute inhalation of salbutamol can decrease urea synthesis and subsequently increase blood ammonia concentrations. Furthermore, inhaled salbutamol may induce a reduction in lymphocyte counts, but does not affect cognitive-motor performance during prolonged exercise in the heat. Then, a new study based on sportomics aimed to investigate changes in metabolism during a soccer match in 30 male junior professional football players. Urine samples were collected before and after 25 minutes of the match and analyzed by liquid chromatography and mass spectrometry. The results showed 26 significant changes in tyrosine metabolismo. Exercise caused a downregulation of the homogentisate metabolites 4-maleylacetoacetate and succinylacetone to (p = 4.69E-5) and 16% (p = 4.25E-14), respectively. 4-Hydroxyphenylpyruvate, a homogentisate precursor, was found to be upregulated by 26% (p = 7.20E-3). The concentration of hawkinsin and its metabolite 4-hydroxycyclohexyl acetate increased ~6-fold (p = 1.49E-6 and p = 9.81E-6, respectively). Different DOPA metabolism pathways were also affected by exercise. DOPA and dopaquinone increased four- to six-fold (p = 5.62E-14 and p = 4.98E-13, respectively). 3-Methoxytyrosine, indole-5,6-quinone, and melanin were downregulated from 1 to 25%, as were dopamine and tyramine (decreasing to up to 5% or 80%; p = 5.62E-14 and p = 2.47E-2, respectively). Blood TCO2 decreased as well as urinary glutathione and glutamate (40% and 10% respectively) associated with a two-fold increase in pyroglutamate. Furthermore, changes in the metabolism of arginine and guanidine compounds were observed in response to a soccer match. N-alpha-acetyl-L-arginine, D-arginine, gamma glutamyl ornithine, agmatine, argininic acid and spermine increased after the football match. Among the analyzes of amino acid metabolism and ammonia production, no pathways for ammonia production were found through the sportomic approach. However, the production of hawkinsine and argininic acid was identified, suggesting the discovery of new markers with the potential to promote damage to performance.

Keywords: Neurometabolism. Metabolomics. Hyperammonemia. Amino acids metabolism. Metabolic pathways. Sports science.

SUMÁRIO

1. INTRODUÇÃO GERAL

O termo -ômica refere-se ao campo das ciências biológicas que caracterizam grandes conjuntos de moléculas, incluindo DNA, RNA, proteínas e metabólitos (YAN etal., 2015). Na década de 1990, essa nova tecnologia denominada “Ciências Ômicas” surgiu através do Projeto Genoma Humano, criado com o objetivo de sequenciar os pares de bases do DNA humano. Além disso, projetos de sequenciamento de outros seres vivos também estavam em andamento, como *Drosophila melanogaster, Escherichia coli,Arabdopiss thaliana* e *Mus musculus*, modelos comumente utilizados em pesquisas científicas (COLLINS et al., 1998; CRAIG VENTER et al., 2001; MORAES; GÓES, 2016).

Um ponto de virada significativo na história da pesquisa genética, foi o lançamento em 2003 da sequência completa do genoma humano pelo Consórcio Internacional de Sequenciamento do Genoma Humano. Essa publicação abriu o caminho para a pesquisa “genoma” e “genômica” e iniciou a chamada era pós-genômica na pesquisa biomédica (YAN et al., 2015).

Com os avanços tecnológicos a partir do projeto Genoma Humano, tecnologias ômicas adicionais foram criadas, referindo-se ao estudo completo das funções e interações de vários tipos de moléculas nas células de um organismo (OLIVIER et al., 2019). Isso inclui ciências como a própria Genômica, Proteômica, Transcriptômica e Metabolômica.

A Gênomica examina o código genético de um determinado organismo para entender a sua biologia (WARD; FRASER, 2005). A Transcriptômica é o estudo de todos os transcritos de RNA produzidos por um determinado tecido ou célula (MOORE;SEIBOLD, 2022). Essa tecnologia possibilita a comparação de tecidos ou células sob determinadas condições biológicas ou estados de doença para determinar mudanças na expressão gênica (CHAMBERS et al., 2019; VON REUMONT, 2018).

Já a Proteômica, corresponde a identificação e quantificação de proteínas em uma amostra biológica. Adicionalmente, possibilita a análise das modificações e interações de proteínas em um determinado evento celular (ASLAM et al., 2017; CIFANI; KENTSIS, 2017).

Comparada com as outras “ômicas”, a metabolômica foi reconhecida recentemente como um tópico científico separado (os primeiros estudos publicados na literatura são do final dos anos 1990 e início dos anos 2000). No entanto, a metabolômica se desenvolveu e alcançou relevância científica, como evidenciado pelo aumento exponencial do número de publicações e pelo fato de estar sendo empregada em uma ampla variedade de aplicações em todo o mundo

(MONTEIRO et al., 2013).

A Metabolômica é o estudo quantitativo e qualitativo de todos os metabólitos (moléculas de baixo peso molecular < 1500 Da) presentes em uma amostra biológica. Nadécada de 1990, essa ciência foi definida para complementar todas as tecnologias "- ômicas" para mensurar e identificar o metaboloma presente em tecidos, células e fluidos,como sangue, urina, suor e saliva.

Ressonância magnética nuclear e espectrometria de massas são as técnicas comumente utilizadas na metabolômica (BEGER et al., 2016; MONTEIRO et al., 2013;NICHOLSON; LINDON; HOLMES, 2008).

A ressonância magnética nuclear (RMN) é um método não invasivo que detecta compostos orgânicos em amostras biológicas (ETTL et al., 1994; MUNZ; JAKOB; BORISJUK, 2016). A técnica de RMN consiste em um método espectroscópio utilizadopara informar em uma molécula o número de átomos magneticamente distintos do isótopoestudado. Vários núcleos podem ser estudados pela técnica, entre eles estão ^{1}H, ^{13}C, ^{15}N,^{19}F e ^{31}P.

O fundamento do RMN é baseado no fato de que um próton possui dois estados de spin de mesma energia com números quânticos +1/2 e -1/2, quando aplicado um campomagnético esses spins passam a ter energias diferentes podendo absorver radiaçãoeletromagnética. Sendo assim, o espectro de RMN depende da absorção de energia quando o núcleo de um átomo é excitado de seu estado de spin de energia mais baixa aopróximo de energia mais alta (PAVIA et al., 2010; SILVERSTEIN et al., 2007).

Por outro lado, a espectrometria de massas (do inglês, mass spectrometry - MS) éuma técnica analítica que identifica e quantifica compostos químicos a partir do seu pesomolecular em uma diversidade de amostras (BUCHBERGER et al., 2018a; UNSIHUAY; MESA SANCHEZ; LASKIN, 2021). A MS, é uma ferramenta poderosa que tem sido utilizada em diversas áreas das ciências "ômicas", como proteômica e metabolômica (TAKÁTS; WISEMAN; COOKS, 2005).

Desde as primeiras pesquisas de Joseph John Thomson, a MS vem acompanhandoa evolução tecnológica e tem sido aperfeiçoada para facilitar a análise de analitos em níveis de traços e de misturas complexas (HOFFMANN; STROOBANT, 2007).

O espectrofotômetro de massas compreende a "fonte de íons", em que a amostra previamente preparada é ionizada e separada com base nas diferentes razões massa/carga(m / z) e, o "detector de íons" no qual os íons são detectados e identificados com base deum banco de dados, o qual resulta no espectro de massas correspondente (BUCHBERGER et al., 2018;

SAUER; KLIEM, 2010).

A amostra pode ser analisada após ser inserida diretamente no espectrômetro de massas, ou acoplando o aparelho a um sistema de separação, como o sistema de cromatografia líquida de alta eficiência acoplado à espectrometria de massas (do inglês, ultra-high-performance liquid chromatography mass spectrometry – UPLC-MS) (CHIARADIA; COLLINS; JARDIM, 2008; ZHANG et al., 2012).

A metabolômica pode usar estratégias direcionadas e não direcionadas, assim como as outras abordagens “ômicas”. As análises direcionadas se aplicam na identificação e quantificação de metabólitos associados a processos biológicos pré- conhecidos e esperados. Essa abordagem, dimui a possibilidade de erros falsos positivos, que podem levar a uma interpretação errada da via metabólica de interesse. Em contraste,a análise não direcionada não se limita, pois essa técnica, identifica todos os metabólitospresentes na amostra biológica, possibilitando a descoberta de novos biomarcadores (GRIFFITHS et al., 2010; HEANEY; DEIGHTON; SUZUKI, 2019; RIBBENSTEDT; ZIARRUSTA; BENSKIN, 2018).

O uso da análise por espectrometria de massa não direcionada (EMND) é considerada uma abordagem campeã para compreender, holisticamente, uma avaliação in vivo das alterações proteômicas e metabolômicas induzidas pelo exercício. A EMND possibilita uma nova era de pesquisa metabólica induzida por exercício de alto rendimento, proporcionando uma melhor compreensão da integração de processos biológicos e estendendo o conhecimento do exercício e os sistemas biológicos (ATHERTON; PHILLIPS; WILKINSON, 2015; FENG et al., 2008; RA et al., 2014).

A abordagem esportômica, que é a aplicação da metabolômica no esporte, tem sido utilizada para investigar os efeitos da amônia. A amônia é um metabólito tóxico, e em altas concentrações sanguíneas pode levar a danos na função cerebral, por alterar a neurotransmissão glutamatérgica (HERMENEGILDO et al., 1998; MONFORT et al., 2002; FELIPO et al., 2012a; FELIPO et al., 2012b). Pacientes com encefalopatia hepática (EH) apresentam vários sintomas neuropsiquiátricos, incluindo comprometimento do ciclo sono-vigília, função cognitiva e atividade de coordenação motora, bem como alterações na personalidade e na consciência que podem piorar progressivamente (FELIPO 2013). O desenvolvimento de EH grave está associado a um maior grau de mortalidade (GARCIA-MARTINEZ 2011).

Por outro lado, sabe-se também que o exercício promove um conjunto de ajustes metabólicos para permitir que o músculo esquelético ativo atenda as suas necessidades energéticas (GIMENEZ et al., 2013). Entre as alterações impostas sobre o metabolismo pelo exercício, destaca-se a hiperamonemia e o aumento sanguíneo de metabólitosrelacionados (uréia e urato, entre outros), especialmente, quando associada à dietareduzida de carboidratos (CARVALHO-PEIXOTO; ALVES; CAMERON, 2007). Postula-se que a hiperamonemia induzida pelo exercício também pode afetar funções intelectuais, de personalidade e coordenação neuromuscular em vários graus, tal como naEH (FELIPO et al., 2012a; FELIPO et al., 2012b; STEWART; CERHAN, 2005; MONFORT et al., 2009).

Durante o exercício, a produção elevada da amonemia ocorre pelo catabolismo de aminoácidos e desaminação do monofosfato de adenosina (AMP), sendo ativadas de modo dependente da sua intensidade e duração (WILKINSON; SMEETON; WATT, 2010). Sugere-se que um aumento exacerbado da amonemia durante o exercício pode causar fadiga central por promover alterações das funções cerebrais (ataxia, letargia e estupor), similares aos sintomas da EH (BANISTER; CAMERON, 1990). Acredita-se que a hiperamonemia pode ter um impacto significativo durante o exercício por promoverprejuízos no desempenho cognitivo (aqui denominado de desempenho cognitivo-motor)(OTT; VILSTRUP, 2014; FELIPO, 2013).

Baseado na abordagem esportômica, esse trabalho apresenta dois artigos derivados da tese. O primeiro focado no metabolismo da tirosina e o segundo (em construção) focado no metabolismo da arginina. Além disso, como trabalho preparatório,foi realizado uma análise com corredores usando a abordagem esportômica com utilização de um β2 agonista. Portanto, o objetivo da presente tese é realizar uma análise esportômica no metabolismo de aminoácidos e produção de amônia em jogadores de futebol.

CAPÍTULO 1 - A SPORTOMICS SOCCER INVESTIGATION UNVEILS AN EXERCISE-INDUCED SHIFT IN TYROSINE METABOLISM LEADING TO HAWKINSINURIA.

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A sportomics soccer investigation unveils an exercise-induced shift in tyrosine metabolism leading to hawkinsinuria

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Tyrosine metabolism has an intense role in the synthesis of neurotransmitters. Our study used an untargeted, sportomics-based analysis of urine samples to investigate changes in metabolism during a soccer match in 30 male junior professional soccer players. Samples were collected before and after the match and analyzed using liquid chromatography and mass spectrometry. Results showed significant changes in tyrosine metabolism. Exercise caused a downregulation of the homogentisate metabolites 4-maleylacetoacetate and succinylacetone to (p=4.69E−5) and 16% (p=4.25E−14), respectively. 4-Hydroxyphenylpyruvate, a homogentisate precursor, was found to be upregulated by 26% (p=7.20E−3). The concentration of hawkinsin and its metabolite 4-hydroxycyclohexyl acetate increased ~six-fold (p=1.49E−6 and p=9.81E−6, respectively). Different DOPA metabolism pathways were also affected by exercise. DOPA and dopaquinone increased four-to six-fold (p=5.62E−14 and p=4.98E−13, respectively). 3-Methoxytyrosine, indole-5,6-quinone, and melanin were downregulated from 1 to 25%, as were dopamine and tyramine (decreasing to up to 5% or 80%; p=5.62E−14 and p=2.47E−2, respectively). Blood TCO_2 decreased as well as urinary glutathione and glutamate (40% and 10% respectively) associated with a two-fold increase in pyroglutamate. Our study found unexpected similarities between exercise-induced changes in metabolism and the inherited disorder hawkinsinuria, suggesting a possible transient condition called exercise-induced hawkinsinuria (EIh). Additionally, our research suggests changes in DOPA pathways may be involved. Our findings suggest that soccer exercise could be used as a model to search for potential countermeasures in hawkinsinuria and other tyrosine metabolism disorders.

KEYWORDS

neurometabolism, amino acids metabolism, exercise metabolome, metabolomics, biochemistry of exercise, metabolic pathways, sports science

Q7 Q8

1. Introduction

Exercise can potentially impact the human metabolome (1, 2). The application of "-Omics" sciences in the field of play of various sports was conceived as sportomics, aiming to investigate the real challenges athletes face, using sports as a model of metabolic stress (3). Sportomics, like all "-Omics" sciences, depends on analytical methods with high processing power to analyze massive volumes of data and is a promising field of investigation (4). Although the number of metabolomic studies in sports remains limited, it continues to grow, supporting investigations in translational sports medicine (3, 5).

We have used sportomics to understand exercise-induced metabolic alterations in sports, including soccer (6). Several recent investigations of amino acid metabolism have focused on the production of ammonia, a toxic metabolite (7, 8). And, we have been studying metabolic changes in response to exercise, especially related to peripheral and central fatigue, such as exercise-induced hyperammonemia (1). There needs to be more information available about the role of amino acid metabolism in biomarker discovery that may help elucidate performance changes during exercise and physiological and physiopathological conditions.

Analysis through a noninvasive sample, such as urine, supports the understanding of the metabolic responses induced by exercise and can lead us to investigate future practical interventions. Under these conditions, we hypothesized that a Sportomics approach might reveal new markers that influence performance during exercise. We previously performed a sportomics investigation of semiprofessional soccer players during a match by mass spectrometry nontargeted analysis (NTA). We were able to identify 3,500+ metabolites in urine. We previously focused on purine metabolism Prado et al. (6). In this study, other data and metabolic pathways were reanalyzed. Through our data, we have identified an exercise-induced metabolic deviation in tyrosine metabolism, resulting in various changes. In this sense, we evaluate these metabolic alterations, pinpointing potential key analytes that warrant further investigation to enhance our understanding of tyrosine metabolism during metabolic challenges.

2. Materials and methods

2.1. Participants

Thirty male soccer players (19.2 ± 0.2 years old; 71.5 ± 2.2 kg; 1.78 ± 0.01 m) from a junior professional team, playing in the main soccer national league and affiliated with the Confederação Brasileira de Futebol (CBF, Brazilian Soccer Confederation), participated in this study as volunteers. The players were healthy and did not have detectable diseases. The athletes underwent routine medical evaluations, including bimonthly clinical exams and regular assessments of general physiological parameters twice a week. Pre-participation exams were also conducted regularly by the medical department of the soccer club. Participants were instructed not to ingest any supplement or medication in the 10 days prior to the experiment. The athletes were in the training ground canteen, during which their dietary habits were carefully monitored and controlled. Overall, the athletes were following a similar diet, although there may have been some individual variations in taste preferences. The participants were informed previously about the study, and written informed consent was obtained from each subject. All procedures were performed according to the ethical standards of the Ethics Committee for Human Research at the Federal University of the State of Rio de Janeiro (117/2007, renewed in 2011) and met the requirements for regulating research on human participants (Health National Council, Brazil, 1996). The participants were tested during the same match ($n = 30$).

2.2. Experimental design, sample collection, and preparation

Two different urinary samples were collected, one immediately prior to and another one immediately after a soccer match (PRE vs. POST).

Urine samples were immediately transferred to a dry ice cooler and transported to the laboratory. Later, the samples were stored in an ultralow temperature freezer (−80°C) until they were prepared for liquid chromatography, followed by alternating low-and high-energy multiplexed MS/MS (UPLC–MSE) injections. Mass spectrometry analyses were further performed at a starting volume of 700 μL. Raw urine samples were then centrifuged at 10,000 × g for 30 min at 4°C, and the supernatants were perfused through a dialysis membrane with a 3,000 Da molecular weight cutoff (MWCO) (Amicon, Merck Millipore, Germany). The filtrate was desalted using a solid phase hydrophilic–lipophilic-balanced extraction cartridge (Oasis® HLB, Waters Corporation, United States). The samples were concentrated using a SpeedVac Plus (Model: SC110A, ThermoSavant, United States) and reconstituted in solvent solution containing 3% acetonitrile and 0.1% formic acid in Milli-Q pure water. Finally, the samples were transferred to a UPLC autosampler vial (Waters Corporation, United States).

2.2.1. UPLC–MSE method

UPLC–MSE data were acquired in an ultrahigh-performance liquid chromatography system (Acquity UPLC I-Class, Waters, United States) coupled to an ESI (+) Qq-oaTOF mass spectrometer (Xevo G2-S Q-Tof, Waters, United Kingdom). A total of 10 μL of each sample was injected, and the separation was performed on an ACQUITY UPLC CSH C18 column, 130 Å, 1.7 μm, 2.1 mm × 50 mm conditioned at 40°C. The mobile phases were 0.1% formic acid (pump A) and 0.1% formic acid in acetonitrile (pump B), and the flow rate was 900 μL min^{-1}.

The gradient method was programmed to achieve maximum separation performance as follows: initial condition 3% B (pump B), 2.37 min 35% B, 4.37 min 85% B, 5.37 min 85% B, and 6.37 min 3% B with a total run time of 8.37 min and a calculated percent B/column volume (Cv) factor of 2.6%B/Cv. The sample tray temperature was defined at 8°C. The mass spectrometry method and conditions were adjusted, including alternating the continuous ion current with low-and high-energy multiplexed MS/MS mode (MSE) that could achieve a collision energy ramp set to 10^{-30} Ev.

MS was controlled by the MassLynx V4.1 software package (Waters Corporation, United Kingdom). The scanning mass range, quadrupole profile, and instrument calibration were set to transmit the ion current from m/z 50 to 1,000. The source conditions were tuned as follows: capillary at 3 kV, sampling cone (skimmer) set to 15 V, step wave source offset of 30 V, cone gas flow (curtain gas) set to 50 Lh^{-1}, and desolvation temperature set to 550°C. All runs were acquired with a detector voltage of 2,450 V with the following hybrid analog-to-digital converter (ADC) parameters: an amplitude threshold of 2 V, an ion area threshold of 3 V, and an ion area offset of 15 V. Instrument calibration was achieved with an automatic Intellistart application included in the MassLynx software package (Waters, United Kingdom), and the acquisition was conducted with a solution of 0.1% formic acid:0.1 M NaOH:acetonitrile at a ratio of 1:1:8 to achieve less than 1 ppm across 14 monoisotopic masses, such as [M+H]+. The LockSpray setup was also performed prior to acquisition with leucine enkephalin (leu-enk) [M+H]+ = 556.2771, and DRE lenses were automatically adjusted to allow for maximum transmission with a solution at 1 ng uL^{-1} and an infusion rate of 5 $\mu Lmin^{-1}$. An average ion area of 32 was also obtained from the detector setup with leu-enk.

2.2.2. UPLC–MSE data processing with progenesis QI

Raw UPLC–MSE data files were processed and grouped by conditions as described previously (9). The identification and relative quantification based on ion accounting of putative metabolites were performed (default parameters) *via* Progenesis QI v.2.0 (Nonlinear Dynamics, Waters, United Kingdom). The metabolites were identified "on the fly" with the use of precursor ion exact mass, isotopologue distribution match, and fragment mass ion matching with the Human Metabolome Database (HMDB) and filtered with the urine metabolites database.

2.2.3. Blood sampling and total carbon dioxide (TCO_2) measurement

Accredited phlebotomists performed venipuncture, and samples of venous blood were taken from players ($n = 27$) at PRE and POST. Drops of blood were inserted into MetLyte 8 to measure TCO_2 using a Piccolo Xpress (Abaxis, CA, United States).

2.3. Data analysis

Raw data are available in a previous report Prado et al. (6). Urine samples were normalized by specific gravity to ensure comparisons. Tyrosine metabolic pathways were investigated using the KEGG database. Data were filtered based on metabolite replication over individual analytical data acquisition, and data are presented as either up-, unchanged or down-regulated. After testing for normality (Shapiro–Wilk), the changes (PRE and POST) were analyzed using a paired Student's *t*-test. Significance was set as $p < 0.05$.

3. Results

3.1. Soccer-induced hawkinsinuria

The homogentisate metabolites 4-maleylacetoacetate and succinylacetone were downregulated to 20 or 16% (i.e., 80 or 84% from the prematch), respectively, in response to the exercise protocol. 1,4-Benzoquinone acetate, a postulated homogentisate metabolite, also decreased to 10% of this original concentration. 4-Hydroxyphenylpyruvate, a precursor of homogentisate, was found to be upregulated by 26%. The concentration of hawkinsin and its metabolite 4-hydroxycyclohexyl acetate (4-HCCA) increased ~six-fold (Table 1). The 3-(4-hydroxyphenyl) lactate and 4-hydroxyphenylacetate metabolites of 4-hydroxyphenylpyruvate decreased to ~30 and ~60%, respectively (Table 1).

3.2. Tyrosine neurotransmitters changed pathways

The DOPA urinary concentration after the match increased four-fold from the pre-match, while dopaquinone increased by ~500% and 3-methoxytyramine increased ~30%. The sulfated forms of dopamine and tyramine, the predominant form of the compounds in blood, decreased to up to 5% or 80% compared to the pre-match concentrations. Other metabolites of DOPA significantly decreased in response to exercise. 3-methoxytyrosine, indole-5,6-quinone, and melanin were downregulated from 1% to 25% of PRE. Otherwise, homovanillin increased by 26% (Table 1).

3.3. Exercise changes In pH and REDOX

Exercise is well known for decreasing pH and the buffering reserve inside muscle cells and blood. We measured a decrease in TCO_2 as well as glutathione and glutamate (reaching 40% and 10% compared to pre-match) associated with a twofold increase in pyroglutamate in urine. Carnosine, a dipeptide with an essential role in pH maintenance, was downregulated to less than 2% compared to pre-exercise (Table 1 and Figure 1).

4. Discussion

We previously performed a sportomics investigation of soccer players during a match focused on purine metabolism Prado et al. (6). Here, some results were reexamined and reanalyzed on amino acid metabolism, leading us to tyrosine metabolism.

Mass spectrometry NTA can analyze a large number of metabolites present in human matrices (e.g., blood, urine, and saliva) and has potential applications in sports and exercise science (10). Urine is a convenient matrix due to its easy accessibility. Due to the systemic character of the urine, our data need to be interpreted as reflecting the sum of filtered blood metabolites.

Here, we show for the first time the upregulation of 4-hydroxyphenylpyruvate, hawkinsin, and 4-HCCA urinary excretion after exercise. We demonstrated an exercise-induced increase in hawkinsin in urine (which we propose to call exercise-induced hawkinsinuria; EIh). These unexpected findings are similar to those found in the inherited disorder hawkinsinuria (ORPHA:2118) (11, 12). The physiopathology of hawkinsinuria is not entirely understood, and the current knowledge regarding the role of hawkinsin in metabolism is limited to investigations of Hawkinsinuria. Patients with hawkinsinuria can develop cognitive impairments, ataxia, and degradation in visual sensation, manifestations widely recognized as impairing sports performance (13).

TABLE 1 Changes in metabolites related to tyrosine metabolism in the urine of athletes in response to a soccer match.

Compound	Relative concentration	Log (POST/PRE)	*t*-test (*p*)	HMDB ID
1,4-Benzoquinone acetate	DOWN	−1.0	3.00E−4	HMDB0002334
Carnosine	DOWN	−1.9	6.33E−13	HMDB0000033
DOPA	UP	0.6	5.62E−14	HMDB0000181
Dopamine 3-O-sulfate	DOWN	−1.2	2.21E−6	HMDB0006275
Dopaquinone	UP	0.8	4.98E−13	HMDB0001229
Glutamate	DOWN	−1.0	6.09E−9	HMDB0000148
Glutathione	DOWN	−0.4	2.17E−2	HMDB0000125
Hawkinsin	UP	0.8	1.49E−6	HMDB0002354
Homovanillin	UP	0.1	3.58E−2	HMDB0005175
4-Hydroxycyclohexyl acetate	UP	0.8	9.81E−6	HMDB0000451
4-Hydroxyphenylacetate	DOWN	−0.2	9.00E−4	HMDB0000020
3-(4-Hydroxyphenyl)lactate	DOWN	−0.5	4.55E−6	HMDB0000755
4-Hydroxyphenylpyruvate	UP	0.1	7.20E−3	HMDB0000707
Indole-5,6-quinone	DOWN	−1.0	9.66E−11	HMDB0006779
4-Maleylacetoacetate	DOWN	−0.7	4.69E−5	HMDB0002052
Melanin	DOWN	−2.1	4.81E−12	HMDB0004068
3-Methoxytyramine	UP	0.1	6.30E−3	HMDB0000022
3-Methoxytyrosine	DOWN	−0.6	1.75E−5	HMDB0001434
p-Octopamine	UP	0.1	5.24E−3	HMDB0004825
Pyroglutamate	UP	0.3	4.31E−2	HMDB0000267
Succinylacetone	DOWN	−0.8	4.25E−14	HMDB0000635
Tyramine O-sulfate	DOWN	−0.1	2.47E−2	HMDB0006409
Vanillactate	UNCHANGED	−0.1	6.12E−1	HMDB0000913

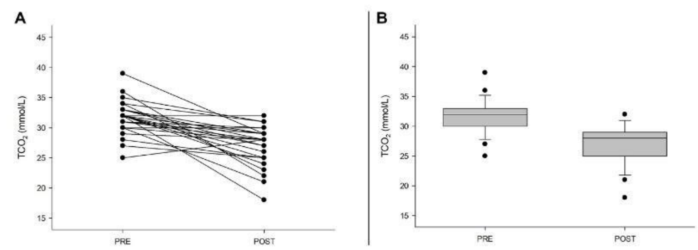

FIGURE 1
Exercise-induced decrease in blood TCO_2. **(A)** Individual blood changes in TCO_2 ($p<0.001$). **(B)** Box plot showing the changes in TCO_2 before (PRE) and after the match (POST).

Hawkinsinuria is caused by a mutation of the 4-hydroxyphenylpyruvate dioxygenase (4-HPPD, EC 1.13.11.27) gene, located in the chromosomal locus 12q24-qter, and is an autosomal dominant rare inborn error of metabolism characterized by acidosis and underdevelopment (14). It was previously described that the impairment of 4-HPPD leads to an increase in urinary hawkinsin in hawkinsinuria (15). Our data revealed a significant increase in both hawkinsin and 4-HCCA in urine. The metabolism of 4-hydroxyphenylpyruvate remains to be elucidated and seems to be related to exercise (16). Under normal conditions, 4-HPPD catalyzes 4-hydroxyphenylpyruvate oxidation, decarboxylation, and final rearrangement to homogentisate. We measured a decrease in 3-(4-hydroxyphenyl)lactate and 4-hydroxyphenylacetate, together with a 10–20% urinary decrease in the metabolites succinylacetone, 4-maleylacetoacetate and 1,4-benzoquinone acetate as well as an increase in 4-hydroxyphenylpyruvate (~25%). These data may suggest an impairment of 4-HPPD activity in EIh. The decrease in 4-hydroxyphenylacetate was recently described in another soccer study (17).

In hawkinsinuria, the 4-HPPD activity is impaired, hindering its rearrangement of an intermediate compound, generating a reactive

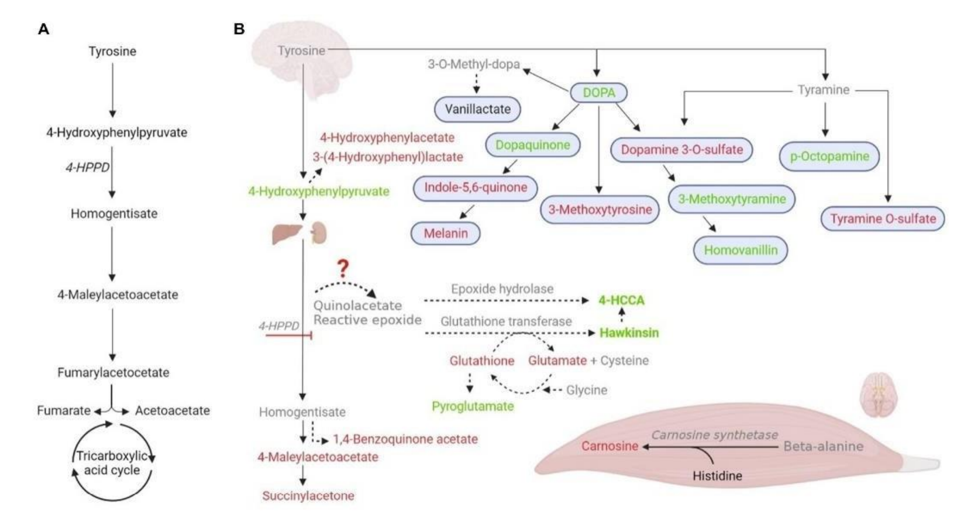

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FIGURE 2
Postulated tyrosine metabolism changes during exercise, leading to an increase in hawkinsinuria. **(A)** Normal tyrosine metabolism and 4-hydroxyphenylpyruvate dioxygenase (4-HPPD; EC 1.13.11.27). **(B)** Metabolic measured changes in our study in response to exercise and their possible tissue specificity. Tyrosine metabolic-intermediate changes are shown in red (downregulation); green (upregulation); black (unchanged), and gray (not found). Solid arrows are established pathways, while dashed lines represent accessory pathways. Organs in the figure indicate the major metabolic contributors.

epoxide. The epoxide can dissociate from 4-HPPD, ultimately producing quinoloacetate (18). Quinoloacetate can be drained to produce 4-HCCA or hawkinsin (19). In our study, we observed that a decrease in glutamate and glutathione is accompanied by an increase in pyroglutamate. Hawkinsinuria-related acidosis is not clearly understood, but it is believed to occur by pyroglutamate accumulation secondary to glutathione depletion (13). Children presenting hawkinsinuria showed an increased urinary excretion of pyroglutamate during acidotic phases (19). Decreased glutathione levels can modify the y-glutamyl cycle, increasing the formation of pyroglutamate and saturating 5-oxoprolinase (EC 3.5.2.9).

The genesis of muscle H+ and subsequent blood acidosis seems to be related to massive ATP hydrolysis during muscle exercise (20). We measured different biomarkers, such as TCO_2, glutathione, and carnosine, that are affected by a decrease in pH buffering reserve. Carnosine buffering may be used sooner in exercise to counteract acid production, being estimated to be approximately 7% of total muscle buffering (21–23). It is challenging to express causality during an intense exercise, such as a soccer match. In addition, carnosine deficiency may impair skeletal muscle metabolism (24).

In our study, the soccer match produced an upregulation of DOPA and dopaquinone concentrations in urine. The impact of exercise on tyrosine metabolism was briefly postulated (17). Both DOPA and tyramine metabolism seemed to be altered in our protocol. Tyrosinase is a busy enzyme in DOPA metabolism. The synthesis of indole-5,6-quinone and melanin seems to be impaired in our protocol, while DOPA and dopaquinone were up-regulated. Oxidation of DOPA and dopamine generates dopaquinone and dopaminoquinone, which are both neuron-cytotoxic molecules. Tyrosinase may rapidly oxidize excess amounts of cytosolic dopamine and DOPA in the brain, maintaining DOPA levels (25). The enzyme is present in a wide range of normal human organs, and some of its catalytic reactions seemed to be affected in our study (26). Additionally, in our study, the presence of tyramine metabolites was affected by the soccer match, suggesting possible changed routes for DOPA pathways in the central nervous system.

A 4-HPPD transient defect in newborns was already published (27). The transient defect in the newborn is probably caused by retarded maturation of 4-HPPD, together with impaired ascorbate ingestion associated with the high ingestion of protein, leading to an increase in hawkinsin formation (28). However, the impact of these findings on athletic performance must be further investigated to exploit the magnitude and reproducibility of these findings across sex, age, ethnic groups, and other important potentially modifying factors. We believe that EIh (which data we summarized in Figure 2) can be a transient condition of exercise metabolism, such as exercise-induced hyperammonemia (9), and exercise can be used as a model for the understanding of hawkinsinuria and perhaps other tyrosine metabolism disorders.

5. Conclusion

The results suggest that under these conditions, exercise induces changes on amino acids metabolism, such as tyrosine metabolism. Combined, our results show that physical exercise can impair 4-HPPD activity, damaging the proper conversion of

4-hydroxyphenylpyruvate to homogentisate in normal tyrosine metabolism, which leads to hawkinsin urinary excretion. Therefore, the sportomics approach may be a suitable model for hawkinsinuria investigation. To the best of our knowledge, this is the first study to report physical exercise metabolism mimicking a rare genetic disorder. A future targeted replication and quantitative study will be necessary for a better understanding of the findings and showing how exercise stress could be used to study hawkinsinuria and other metabolic disorders.

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Data availability statement

The original contributions presented in the study are included in the article/Supplementary material, further inquiries can be directed to the corresponding authors.

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Ethics statement

The studies involving human participants were reviewed and approved by Ethics Committee for Human Research at the Federal University of the State of Rio de Janeiro (117/2007, renewed in 2011). The patients/participants provided their written informed consent to participate in this study.

Q13

Author contributions

MA, ABo, ABa, and LC: conceptualization. TF and RM-S: visualization. AS and LC: funding acquisition. EP and LC: supervision. TF, RM-S, EP, and LC: writing—original draft and writing—review and editing. All authors contributed to the article and approved the submitted version.

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Q14

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Conflict of interest

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Q16

MA was employed by Former Waters Corporation, Currently at SpectraMass.

The remaining authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's note

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CAPÍTULO 2 – CAN ARGININIC ACID IMPAIR PERFORMANCE? (ARTIGOEM CONSTRUÇÃO)

Methods

Subjects

Thirty male soccer players (18-20 years old) from a team affiliated with the Confederação Brasileira de Futebol (CBF, Brazilian Soccer Confederation) participated in this study as volunteers. The players were healthy and did not have detectable diseases.They were clinically evaluated twice per year. Participants were instructed not to ingest any supplement or medication in the 10 days prior to the experiment. The subjects were informed previously about the study, and written informed consent was obtained from each subject. All procedures were performed according to the ethical standards of the Ethics Committee for Human Research at the Federal University of the State of Rio de Janeiro (117/2007, renewed in 2011) and met the requirements for regulating research onhuman subjects (Health National Council, Brazil, 1996). The subjects were tested duringthe same match (n = 30).

Experimental design, sample collection, and preparation

Two different urinary samples were collected, one immediately prior to and another one immediately after a soccer match (PRE vs. POST).

Urine samples were immediately transferred to a dry ice cooler and transported tothe laboratory. Later, the samples were stored in an ultralow temperature freezer (-80 °C)until they were prepared for liquid chromatography, followed by alternating low- and high-energy multiplexed MS/MS (UPLC–MSE) injections. Mass spectrometry analyses were further performed at a starting volume of 700 µL. Raw urine samples were then centrifuged at 10,000 x g for 30 min at 4 °C, and the supernatants were perfused througha dialysis membrane with a 3,000 Da molecular weight cutoff (MWCO) (Amicon, MerckMillipore, Germany). The filtrate was desalted using a solid phase hydrophilic-lipophilic-balanced extraction cartridge (Oasis® HLB, Waters Corporation, USA). The samples were concentrated using a SpeedVac Plus (Model: SC110A, ThermoSavant, USA) and reconstituted in solvent solution containing 3% acetonitrile and 0.1% formic acid in Milli-Q pure water. Finally, the samples were transferred to a UPLC autosampler vial (WatersCorporation, USA).

UPLC–MS^E method

UPLC–MSE data were acquired in an ultrahigh-performance liquid chromatography system (Acquity UPLC I-Class, Waters, USA) coupled to an ESI (+) Qq-oaTOF mass spectrometer (Xevo G2-S Q-Tof, Waters, UK). A total of 10 µL of eachsample was injected, and the separation was performed on an ACQUITY UPLC CSH C18 column, 130 Å, 1.7 µm, 2.1 mm × 50 mm conditioned at 40 °C. The mobile phaseswere 0.1% formic acid (pump A) and 0.1% formic acid in acetonitrile (pump B), and theflow rate was 900 µL min-1.

The gradient method was programmed to achieve maximum separationperformance as follows: initial condition 3% B (pump B), 2.37 min 35% B, 4.37 min 85%B, 5.37 min 85% B, and 6.37 min 3% B with a total run time of 8.37 min and a calculatedpercent B/column volume (Cv) factor of 2.6%B/Cv. The sample tray temperature was defined at 8 °C. The mass spectrometry method and conditions were adjusted, includingalternating the continuous ion current with low- and high-energy multiplexed MS/MS mode (MSE) that could achieve a collision energy ramp set to 10-30 Ev.

MS was controlled by the MassLynx V4.1 software package (Waters Corporation,UK). The scanning mass range, quadrupole profile, and instrument calibration were set to transmit the ion current from m/z 50 to 1000. The source conditions were tuned as follows: capillary at 3 kV, sampling cone (skimmer) set to 15 V, step wave source offsetof 30 V, cone gas flow (curtain gas) set to 50 L h -1, and desolvation temperature set to 550 °C. All runs were acquired with a detector voltage of 2450 V with the following hybrid analog-to-digital converter (ADC) parameters: an amplitude threshold of 2 V, an ion area threshold of 3 V, and an ion area offset of 15 V. Instrument calibration was achieved with an automatic Intellistart application included in the MassLynx software package (Waters, UK), and the acquisition was conducted with a solution of 0.1% formicacid:0.1 M NaOH:acetonitrile at a ratio of 1:1:8 to achieve less than 1 ppm across 14 monoisotopic masses, such as [M + H]+. The LockSpray setup was also performed priorto acquisition with leucine enkephalin (leu-enk) [M + H]+ = 556.2771, and DRE lenses were automatically adjusted to allow for maximum transmission with a solution at 1 ng uL−1 and an infusion rate of 5 µLmin−1. An average ion area of 32 was also obtained from the detector setup with leu-enk.

UPLC–MSE data processing with progenesis QI

Raw UPLC–MSE data files were processed and grouped by conditions as described previously. 1 The identification and relative quantification based on ionaccounting of putative metabolites were performed (default parameters) via Progenesis QI v.2.0 (Nonlinear Dynamics, Waters, UK). The metabolites were identified "on the fly"with the use of precursor ion exact mass, isotopologue distribution match, and fragment mass ion matching with the Human Metabolome Database (HMDB) and filtered with theurine metabolites database.

Blood sampling and total carbon dioxide (TCO2) measurement

Venipuncture was performed by accredited phlebotomists, and samples of venousblood were taken from players (n = 27) at PRE and POST. Drops of blood were insertedinto MetLyte 8 to measure TCO2 using a Piccolo Xpress (Abaxis, CA, USA).

Data analysis

Raw data are available in a previous report. [2] For this study, only tyrosine metabolites were used in all samples measured. Tyrosine metabolic pathways were investigated using the KEGG database. Data were filtered based on metabolite replicationover individual analytical data acquisition, and data are presented as either up-, unchanged or downregulated. After the normality test, significance was examined using ANOVA based on data distribution. ANOVA was performed comparing PRE and POSTspectra areas for each urinary metabolite. In addition, after testing for normality (Shapiro–Wilk), the changes in TCO_2 (PRE and POST) were analyzed using a paired Student's t test. For all outcomes, *P*<.05 was considered statistically significant.

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RESULTS

Table 1. Changes in arginine metabolism in response to a soccer match.

Compound	Relative Concentration	Log (Post/Pre)	ANOVA (*p*)	HMDB ID	Percentual (%)
2- oxoarginine	DOWN	-0.4	5.81E-07	HMDB04225	-60
N-alpha-Acetyl-L-arginine	UP	0.3	0.007250161	HMDB04620	99
4-guanidino butanoic acid		0.0	0.348628232	HMDB03464	0
Homo-L-arginine	DOWN	-0.1	0.419095775	HMDB00670	-21
D-arginine	UP	0.1	0.017614067	HMDB03416	26
Gamma glutamyl ornithine	UP	0.2	0.010271651	HMDB02248	58
N-Acetylornithine	DOWN	-0.4	0.18911517	HMDB03357	-60
Agmatine	UP	0.4	0.002005994	HMDB01432	151
Argininic acid	UP	0.9	1.78E-09	HMDB03148	694

CAPÍTULO 3 - INHALED SALBUTAMOL AND ITS EFFECTS ON AMMONIA METABOLISM, WHITE BLOOD CELL COUNTS AND PERFORMANCE AFTER PROLONGED EXERCISE UNDER HEAT STRESS CONDITIONS USING A SPORTOMICS APPROACH

INHALED SALBUTAMOL AND ITS EFFECTS ON AMMONIA METABOLISM, WHITE BLOOD CELL COUNTS AND PERFORMANCE AFTER PROLONGED EXERCISE UNDER HEAT STRESS CONDITIONS USING A SPORTOMICS APPROACH

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Abstract

This study evaluated the acute effect of inhaled salbutamol on blood ammonia, white blood cell counts, and cognitive-motor performance after prolonged exercise under heat conditions using a Sportomics approach. Male endurance runners were divided into two groups: inhaled salbutamol (SEx) and placebo (PEx). The runners performed a half- marathon race (21.1 km), and the metabolic status, white blood cell counts, and cognitive- motor performance were assessed before (PreR) and after (PostR) the race using the absolute (Δ) and percentage (Δ %) changes. An increase of ~ 340% in blood ammonia concentration was observed in the Δ % SEx group but not in the Δ % PEx group (~ 200%).The Δ % PEx group had a smaller increase in blood urea (~ 26%) and blood glucose (~ 11%) concentrations after exercise than the Δ % PEx group (~ 35% and ~ 34%,respectively). The white blood cell counts indicated a significant decrease in the lymphocyte count after the race in the SEx group. We suggest that acute inhaled salbutamol might increase blood ammonia concentrations and induce decreased lymphocyte counts but does not affect cognitive-motor performance during prolonged exercise in the heat.

Keywords: metabolism; exercise; heat stress; white blood cell counts; ergogenic effect;β2-agonist

Introduction

Salbutamol is a β2-agonist and a widely used bronchodilator [1,2]. However, thedrug is known to influence other physiological and metabolic functions [3]. It has been shown that salbutamol can improve metabolism and provide ergogenic benefits to athletes[4,5]. Presently, several β-agonists are prohibited by the World Anti-Doping Agency [6], although inhaled salbutamol can be administered at a maximum of 800 μg over 12 h. Eliteathletes need to fulfill the WADA's Therapeutic Use Exemption (TUE) and provide a prescription and demonstrate the medical need for this type of drug [3,6,7]. Nevertheless,inhaled salbutamol has been a contentious topic in sports for 45 years, and that trend continues [8].

Several studies have examined the use of salbutamol at different doses and diverse administration methods (inhaled or oral), and they identified its effects on athletic performance, such as strength and psychomotor performance, as well as its effects on physiologic and metabolic changes [9–14]. Regarding metabolic changes, the therapeutic inhalation of salbutamol produced a rise in plasma glucose [15–18]. In addition, an inhaled β2-agonist (400 μg) was shown to decrease blood ammonia concentrations duringsubmaximal exercise under thermoneutral conditions [19]. Ammonemia occurrenceduring exercise depends on the carbohydrate metabolic availability [20–22]. Therefore, the increase in blood glucose concentration caused by inhaled salbutamol can be important during prolonged exercise, which decreases both amino acid catabolism and adenosine monophosphate (AMP) deamination and subsequently hinders the release of ammonia into the bloodstream [23].

The increase in ammonemia during exercise has been implicated in central fatigueby altering cerebral function and impairing cognitive performance (termed cognitive- motor performance) [24,25]. Thus, controlling the increase in ammonemia is believed to improve exercise performance [26]. However, exercise is performed in heat can promote metabolic alterations that are exacerbated by an increase in body temperature or dehydration, which impair exercise performance. These previous conditions appear to aggravate the exercise-induced increase in ammonemia [27–32].

Furthermore, hyperammonemia is linked to an increase in ammonia metabolites,urea and urate in blood [33]. We previously described that decreases in urea synthesis with caffeine use could be due to the decrease in urea cycle amino acid concentrations inthe blood due to impairment of glutamine transport of ammonia [34]. The acute effects of inhaled salbutamol on blood ammonia and its metabolites and the cognitive-motor performance response to exercise are poorly understood. A previous study demonstratedthat oral salbutamol (12 mg/day for three weeks) decreased blood urea during exercise inrecreational athletes [35]. Additionally, the prolonged administration of salbutamolreduced both ketogenic and urea cycle amino acid concentrations in blood, while drug withdrawal promoted a decrease in glycogenic amino acids in an animal model [36]. To the best of our knowledge, only one study has explored the acute effect of inhaled salbutamol on ammonemia during exercise [19].

We previously demonstrated that exercise is an excellent tool to study the interactions between ammonia metabolism and white blood cell response [37,38]. We proposed that increased lymphocyte counts could be related to changes in ammonia metabolism and a possible signal of muscle injury during exercise [38–40]. Although themajor action of salbutamol is the relaxation of airway smooth muscle, this drug has several other outcomes, such as an anti-inflammatory effect by inhibiting the release of mediators from eosinophils, macrophages, T-lymphocytes, and neutrophils [41,42] However, little information is available on the acute effect of inhaled salbutamol on white blood cell counts and muscle injury marker responses to prolonged exercise.

Studies of athletes engaged in exercise in the field that mimic both the real challenges and conditions that are faced during sports situations (such as hot environmental conditions), such as in the Sportomics approach, can better reflect the realmetabolic changes during exercise [43]. The Sportomics approach can be helpful to better understand the effect of inhaled salbutamol on metabolism and performance following prolonged exercise under high-heat stress conditions.

Based on our previous findings, we hypothesized that inhaled salbutamol might affect both ammonia genesis and metabolism, white blood cell counts and cognitive- motor performance in response to exercise. Consequently, we evaluated the acute effect of inhaled salbutamol on these variables during a half-marathon race under heat stress conditions using a Sportomics approach.

Methods

Subjects

Male amateur endurance runners who were nonusers of salbutamol or other β2- agonists were enrolled in a half-marathon race (21.1 km), with heat stress, under naturalconditions. All subjects had at least three years of training and frequently participated in training and competitions under heat stress. Therefore, the runners were acclimatized to training and competitions in a hot environment. The subjects who volunteered for the study were divided into two groups, starting with nine runners each, as follows: salbutamol experimental group – SEx, (39.8 ± 3.1 years; 74.9 ± 2.5 kg; 1.75 ± 0.01 m) and control group – PEx, (41.4 ± 3.2 years; 75.8 ± 3.1 kg; 1.71 ± 0.02 m). Both groups showed similar levels of maximal oxygen consumption (VO_{2max}) (45.3 ± 1.9 $mL.kg^{-1}.min^{-1}$ and 44.3 ± 2.0 mL.kg-1.min^{-1}, SEx and PEx, respectively). Diseases (respiratory tract, recognized asthma, or allergy), regular use of tobacco, or the use of ergogenic aids wereexclusion criteria. The subjects were informed about the study before participation, and written informed consent was obtained. All procedures were performed in accordance with the guidelines dictated by the Declaration of Helsinki and the ethical standards of the Ethics Committee for Human Research at the Federal University of Alagoas, Brazil (017640/2011-61).

Study design and protocols

The runners were subjected to an anthropometric assessment, and dietary intake was determined one week before the half-marathon race. After the evaluation of dietary intake, all runners were screened for medical history (such as pulmonary function). All participants received an individualized diet plan, where the recommended energy intakewas 15% from proteins, 20% from lipids and 65% from carbohydrates. The subjects were asked to start the proposed diet two days before the race. Diet adherence was verified viaketonuria analysis by qualitative reagent strips for urinalysis (Biocolor/Bioeasy®, MinasGerais, Brazil). The absence of ketonuria was used for the assessment of adequate carbohydrate ingestion before the race.

The runners were also asked to avoid their normaltraining schedule until two days before the race day, maintain a fluid intake of ~ 3 $L.d^{-1}$ and avoid beverages containing caffeine and other xanthines. VO_{2max} was determined using a motorized treadmill (Inbramed®, Porto Alegre, Brazil), as described previously [44]. The subjects were familiarized and exhaustively trained in the appropriate use of inhalers before the experimental protocol ensured the effective delivery of inhaledsalbutamol.

One week after the preparation procedures, the subjects performed a half- marathon race (the race started at 8:00 AM). One hour before the race, the SEx group received four puffs (400 µg) of inhaled salbutamol (Aerolin® 100 µg; GlaxoSmithKline,Rio de Janeiro, Brazil) using a spacer device, and the PEx group (placebo) received the same number of puffs using propellant only. Both treatments were provided using the same inhaler type in a randomized double-blind manner. Immediately after the administration of inhaled salbutamol or placebo, the following assessments (in order) were performed before the race (PreR) and repeated after the race

(PostR): Hydration status was assessed by evaluating the percentage change in body mass(Δ % BM), urine color, and specific gravity (USG). Urine color was evaluated accordingto a previous study [45], and USG was measured using a manual refractometer (Biobrix®,São Paulo, Brazil). Hydration status was classified according to a previous study [46], where at least two of the three markers indicating the same state of hydration served as atiebreaker.

Immediately after the hydration status tests, blood samples were obtained from the median antecubital vein. The blood samples were immediately centrifuged (3000 x *g*,4 °C) to avoid the loss of volatile compounds used for biochemical analyses. Serum andplasma were divided into aliquots and stored at 4 °C. To prevent loss, plasma ammonia was measured immediately, and the other biochemical analyses (serum) were performed within 24 h. Glucose, lactate, urate, urea, creatine kinase (CK), lactate dehydrogenase (LDH), aspartate aminotransferase (AST), and alanine aminotransferase (ALT) were measured using commercial spectrophotometric assays (Labtest®, Minas Gerais, Brazil).Ammonia was measured using an enzymatic UV method (Randox, Crumlin, UK). All biochemical analyses were performed using a dade model dimension RXL automated chemistry analyzer (Dade Behring®, Eschborn, Germany). The samples were measured in duplicate, and the coefficient of variation for analysis was < 5%. Additionally, blood samples were collected in an EDTA vacuum tube to determine the white blood cell count,which was performed in a hematology analyzer (Human®, Hessen, Germany).

Following blood collection, the runners were tested to evaluate their cognitive- motor performances based on their immediate memory, motor coordination (by the finger-to-nose test), and simple reaction time as previously described [44].

Subsequently, the runners started the half-marathon race. The subjects were instructed to maintain their typical exercise intensity and allowed to drink water ad libitum during the race. Water intake was measured and evaluated as previously described(Monteiro et al 2022). Heart rate (HR) was recorded at the end of the race using a heart rate monitor (Polar® FT1, Kempele, Finland). At the same time, race time and the subjective perception of effort (SPE) was evaluated [47].

Before and after the race, the runners' body temperature was measured using a tympanic thermometer (GeniusTM 2®, Minnesota, USA). The tympanic temperature values were used to calculate an equivalent rectal temperature (ERT) [48]. Additionally,the ambient temperature, relative humidity, air movement, and solar radiation (Instrutemp®, São Paulo, Brazil) were measured and used to calculate the wet-bulbe globe temperature (WBGT) Index. A WBGT > 25.7 °C was considered a high-heat stressenvironment [49].

Statistical analysis

All values are expressed as the mean ± SEM. After testing for normality (Shapiro-Wilk) and equal variance (Brown-Forsythe), a 2 x 2 repeated-measures ANOVA was used to test for differences in condition (SEx and PEx) and time (PreR vs PostR). To isolate which group(s) differed from the others, multiple-comparison procedures were conducted with Bonferroni t-tests. An unpaired t-test was used to analyze changes in thefollowing variables: Δ % BM, Δ Urea/ammonia ratio, HR, SPE, fluid replacement and race time. For all of the measurements, $P < 0.05$ was considered statistically significant. Metabolic status was also shown by evaluating the absolute (PreR and PostR) and normalized for PreR value (Δ %) changes.

Results

We conducted a study during a half-marathon under environmental conditions that produced high thermal stress and evaluated the parameters in the PreR (WBGT, 27.5 °C)and PostR (WBGT, 30.9 °C) periods to determine some of the acute effects of inhaled salbutamol on metabolism and cognitive-motor performance. The race times were similarin both groups (SEx: 120.1 ± 3.4 min and PEx: 118.1 ± 2.3 min). The runners reached similar intensity (SEx, HR: 147.0 ± 2.8 beats/min and PEx, HR: 141.6 ± 5.0 beats/min; SEx, SPE: 14.8 ± 0.9 and PEx, SPE: 14.9 ± 1.4) and fluid replacement during the race (SEx: 444.4 ± 47.5 mL and PEx: 450.0 ± 44.9 mL). The ketonuria analysis revealed thatrunners were not in ketosis before the race. No adverse events were reported.

The ERT values increased above baseline levels in response to similar high heat stress exercise in both conditions without a significant difference between the groups. After the race, the runners were hypohydrated with similar Δ % BM in both groups. Theurine color and USG values were elevated prior to the start of the race and showed that the runners were equally hypohydrated between minimal and significant (Table 1).

To investigate the effect of inhaled salbutamol on ammonia metabolism, we measured the concentrations of ammonia and some related metabolites in blood. Ammonemia increased in both groups in response to exercise and increased in response to salbutamol and exercise in all runners (Figure 1). There was a 3.4-fold significant increase in blood ammonia concentration in the SEx group, whereas a twofold increase was observed in the PEx group (Table 2).

To indirectly determine whether ammonia was produced by amino acid deamination or by AMP deamination, we measured the blood urea and urate concentrations. The blood urea and urate concentrations increased above baseline levelsafter the race in both groups. Exercise induced a smaller increase in blood urea concentrations in the runners (Figure 2). The salbutamol group showed a smaller increase(~ 26%) in blood urea concentrations after exercise compared with the PEx group (~ 35%). Furthermore, there was a significant decrease in the Δurea/ammonia ratio in the SEx group compared with the PEx group. Blood urate showed a similar increase in bothgroups (Table 2).

To understand the role of inhaled salbutamol in glycemia, we measured the bloodglucose concentrations during the half-marathon race. The changes in glycemia analyzedby group did not show significant differences due to the variance. When studied individually, we verified that the normalized glycemia had a smaller increase in the SExgroup than the PEx group (Table 2 and Figure 3). We also evaluated blood lactate concentration as an indicator of glucose metabolism during exercise. The blood lactate concentration significantly increased after the race in both groups (Table 2).

To investigate the role of salbutamol in muscle performance, we investigated enzyme biomarkers of muscle damage and white blood cell count responses. Both groupsshowed an early significant increase in blood CK and AST after the race compared withthe baseline values, and differences were not observed between groups. LDH in the bloodsignificantly increased after the race in the SEx group but not in the PEx group. No significant difference in blood ALT was found after the race and/or between the groups (Table 3). The leukocyte counts equally increased by almost twofold in both groups, which was mainly due to the neutrophil and monocyte counts. The lymphocyte count significantly decreased after the race in the SEx group but not in the PEx group (Table 4).

To evaluate the half-marathon race in the heat as a model for studying the effectsof hyperammonemia on cognition, we employed different cognitive-motor performance tests. We did not find differences in the finger-to-nose test, simple reaction time, orimmediate memory before and/or after the half-marathon race (data not shown).

Discussion

Previous studies have shown that elevated blood ammonia, urea, and urate concentrations can be reduced through the use of amino acids or carbohydrate supplementation during exercise under thermoneutral conditions [50,51]. However, onlyone study has explored the acute effect of inhalation of salbutamol on blood glucose, lactate, ammonia, and urea during exercise under thermoneutral conditions [19].

This study aimed to evaluate the acute effects of inhaled salbutamol on ammonia metabolism, white blood cell counts, and cognitive-motor performance after prolonged exercise under high heat stress conditions using a Sportomics approach. Here, we showedthat inhalation of salbutamol increased the ammonemia response and decreased the bloodurea concentration after prolonged exercise in heat. Additionally, inhaled salbutamol induced a reduction in the lymphocyte response to exercise but did not induce differencesin enzyme biomarkers of muscle damage, which were elevated after the race. Moreover, we did not observe significant differences in the runners cognitive-motor performance after inhaling salbutamol.

Hyperammonemia has been suggested to promote neurological alterations, including impairment in cognitive-motor functions, such as in patients with clinical hepatic encephalopathy [25]. Ammonemia accumulation during exercise leads to impaired activity of neurotransmitters that are involved in cognitive-motor disturbances [43,52]. Therefore, induced hyperammonemia may also affect cognitive-motor performance [24,53]. Environmental heat stress is known to cause severe metabolic disorders during exercise, including hyperammonemia [53]. In the present study, we usedprolonged exercise in the heat to decrease the availability of glycogen and increase both amino acid catabolism and AMP deamination, which led to the release of more ammoniainto the bloodstream [20,30,54].

Matthys et al. demonstrated that the blood glucose concentration was maintainedand that the blood lactate concentration was slightly but significantly increased after salbutamol inhalation during exercise, and these results suggest higher carbohydrate availability [19]. Our results showed that the salbutamol group demonstrated a much smaller percentage change in blood glucose concentration. Our findings are inconsistentwith that of Goubault et al., who demonstrated no significant enhancement of metabolism(glucose and lactate) in highly trained nonasthmatic cyclists with different doses of inhaled salbutamol (200 μg or 800 μg) [12]. Dickinson et al. demonstrated that there was no significant difference in blood lactate concentrations following the inhalation of up to1600 μg of salbutamol in nonasthmatic athletes in temperate (18 °C) and hot (30 °C) environments, both with 40% relative humidity [55].

These contradictory results are difficult to explain, and precisely how the acute effect of inhaled salbutamol promotes metabolic changes is unclear, especially during heat exercise. Inhalation of salbutamol appears to be associated with a more marked localeffect on pulmonary β2-adrenoceptors than on peripheral skeletal muscle adrenoceptors [3,4,56]. Moreover, the metabolic actions of acute oral salbutamol intake have been reported to include the stimulation of glycogenolysis and glycolysis with increased lactaterelease from tissues such as muscle [3]. However, short-term oral salbutamol intake during submaximal exercise may also decrease blood glucose concentrations [57]. Several studies of orally administered β2-agonists have investigated a greater number ofparameters than inhalation studies [3]. Therefore, additional studies are needed to elucidate the effect of salbutamol (inhaled and oral) on blood glucose concentrations under exercise in hot environments.

Matthys et al. demonstrated that inhaled salbutamol (400 µg) decreased blood ammonia concentrations during submaximal exercise and concluded that exercise- induced ammonemia is regulated by type β2 receptors [19]. In the present study (similar inhaled salbutamol, 400 µg), we showed that the salbutamol group had a much higher percentage and greater absolute changes in the increase in blood ammonia concentrationsafter exercise in the heat compared with the placebo group. As previously mentioned, ammonemia during exercise depends on carbohydrate metabolic availability, and we showed that salbutamol and exercise induced a smaller increase in blood glucose concentrations in runners.

In our study, blood urea concentrations showed a smaller increase after exercise in the salbutamol group than the placebo group. Hyperammonemia can occur during exercise via both amino acid and AMP deamination, in which urea and urate are the finalmetabolites, respectively [54]. The primary pathway for blood ammonia removal in a healthy individual is via ureagenesis [58]. Thus, the smaller increase in blood urea concentrations in the salbutamol group compared with the results for the blood ammoniaconcentrations may appear to contradict the measured blood urea decrease. However, a reduction in urea cycle amino acids was observed after salbutamol administration (2 $mg.kg^{-1}$ diet. day-1) for 38 days in animals (lambs), although such a change was not apparentin animals after salbutamol administration for 33 days [36]. Similarly, the blood urea concentrations were significantly decreased after oral salbutamol (12 mg/day for 3 weeks)treatment during resting and exercise [11]. Therefore, we suggest that the urea synthesisdelay induced by inhaled salbutamol could be explained by a reduction in urea cycle activity. Similarly, Bassini et al. observed that caffeine might decrease systemic urea by decreasing the glutamine serum concentration, which decreases the transportation of ammonia to the liver and thus decreases urea synthesis[34].

Strenuous exercise is known to result in increased oxidative stress, which leads tomuscle damage, as evidenced by the release of biomarkers such as CK, LDH, AST, and ALT [59–61]. The elevation of these enzyme biomarkers of muscle damage is generallyobserved when measured hours or days after exercise [62]. However, the results of thepresent study showed that CK and AST were higher in both groups immediatelyfollowing the race. Previous studies have shown changes in injury biomarkers immediately after high-intensity endurance exercise [38,63]. An adventure sprint raceincreased the leukocyte, neutrophil, and monocyte counts and increased the AST but notthe ALT, which is consistent with our study [63]. In addition, limited information isavailable on the influence of inhaled salbutamol on CK, and such effects may be a rarephenomenon [64]. Although the most likely explanation of such changes could beprolonged exercise in the heat, markers of muscle damage were elevated immediatelyafter a soccer match under high-heat stress as well as under thermoneutral conditions [65]. Physical exercise is known to induce an increase in leukocyte counts and their subpopulations (especially neutrophil, lymphocyte, and monocyte counts) [66]. In thisstudy, we observed an increase in white blood cell counts, with leukocytosis, neutrophilia, and monocytosis in both groups. We also showed that inhaled salbutamol caused asmaller lymphocyte count increase and led to higher ammonemia. Salbutamol has beenshown to decrease acute inflammation in rats, and stimulation of β-2 adrenergic receptorsmay be the underlying mechanism responsible for the observed anti-inflammatory effects[67]. The most likely explanation for the contradictory results is that inhaled salbutamol exerts anti-inflammatory effects, which could at least partially explain the mechanism underlying the reduction in lymphocyte count.

As mentioned above, we observed a reduction in lymphocyte counts with inhaled salbutamol and exercise. Leukocytosis may be involved in the regenerative inflammatory response to exercise, such as in muscle recovery and repair [63]. In particular, lymphocytosis can be associated with markers of muscle damage [38]. Our findings reinforce that inhaled salbutamol exerted anti-inflammatory effects, which could at least partially explain the mechanism underlying the reduction in lymphocyte counts.

In our study, we did not identify changes in the tested cognitive-motor performance. Goubault et al. also demonstrated a lack of significant enhancement of psychomotor performance (such as on the simple reaction test) [12]. Thus, the results of the present experiment are consistent with these previous studies according to a physicalperformance perspective [68]. An acute dose of up to 4000 µg of inhaled salbutamol likely would not improve the cycling time to exhaustion or oxygen kinetics [69]. In addition, Dickinson et al. demonstrated that chronic daily accumulated doses of 1600 µgdid not improve endurance, strength, or power. Compared with inhaled β2-agonists, oraladministration of salbutamol seems to be able to improve muscle strength and enduranceperformance [70]. Thus, prohibiting inhaled β2-agonists does not appear to be justified according to the ergogenic effects [5].

We used a Sportomics approach, and it is necessary to perform a preamble. Compared with studies carried out in the laboratory under extremely controlled conditions, during Sportomics experiments, the samples are collected in the field and anuncontrolled environment to reproduce the challenges presented in real training and competition [38,39,71]. Therefore, sportomics seeks to avoid the use of the trial-error method applied in personalized medicine because of the importance of the role of variability of the metabolic response between individuals. Usually, the investigative standards used to examine athletes' performances are based on empirical information collected by coaches and laboratory tests and clinical data obtained by scientists. In manycases, subjects that maintain the same training, diet, and recovery routines present completely different metabolic responses according to their systemic response samples [72].

Study limitations

Due to laboratory limitations, we were not able to measure the achieved concentrations of salbutamol in the blood. Previous studies have shown that salbutamol is 100% absorbed, with its peak in blood achieved within 5-10 min and its levels maintained for up to 4 h [73–75]. According to these results on the pharmacokinetics andpharmacodynamics of salbutamol, we may have designed the study using the peak of thedrug in the blood. Additionally, we needed to perform tympanic temperature measurements that are not considered the gold standard method for temperature studies due to nonscientific factors [76].

Conclusions

To the best of our knowledge, this study is the first to evaluate the acute effect of inhalation of salbutamol on metabolism ammonia, white blood cell counts, and cognitive-motor performance after prolonged exercise under high heat stress conditions. Wesuggest that acute inhaled salbutamol might decrease urea synthesis and subsequently increase blood ammonia concentrations. Additionally, inhaled salbutamol can induce a reduction in lymphocyte counts but does not affect cognitive-motor performance during prolonged heat exercise.

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Declaration of interest statement

The authors declare that they have no conflicts of interest.

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Authors' contributions

Thássia Casado Lima França, Eduardo Seixas Prado: conceived and designed research. Thássia Casado Lima França, Edla de Azevedo Herculano, Natally Monteiro de Oliveira, Mayara Roberta Fernandes Vieira, Saulo Rodrigo Alves e Silva Camerino, Eduardo Seixas Prado: performed experiments. Thássia Casado Lima França, Eduardo Seixas Prado, L. C. Cameron, Aníbal M. de Magalhães-Neto, Marcos Guilherme de Sousa, Alexandre Magno-França, Euzébio Goulart Santana: data analysis and interpretation. Thássia Casado Lima França, Edla de Azevedo Herculano, Natally Monteiro de Oliveira, Mayara Roberta Fernandes Vieira, Eduardo Seixas Prado, L. C. Cameron: drafted the manuscript and all authors commented on previous versions of the manuscript. All authors have read and approved the manuscript.

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Figure Legends

Figure 1: Salbutamol increased ammonemia in response to exercise in most individuals. Athletes exercised for 21.1 km under heat stress after inhalation of either salbutamol (SEx; ●) or placebo (PEx, ○). Data are expressed as absolute values, before (PreR) and after (PostR) the race (A and B) and normalized according to the subject pre-exercise concentration (C and D).

Figure 2: Salbutamol decreased the total blood urea raise due to exercise. Athletes exercised for 21.1 km under heat stress after inhalation of either salbutamol (SEx; ●) or placebo (PEx, ○). Data are expressed as absolute values, before (PreR) and after (PostR)the race (A and B) and normalized according to the subject pre-exercise concentration (Cand D).

Figure 3: Salbutamol diminished the raise in glycemia due to exercise in most individuals. Athletes exercised for 21.1 km under heat stress after inhalation of either salbutamol (SEx; ●) or placebo (PEx, ○). Data are expressed as absolute values, before (PreR) and after (PostR) the race (A and B) and normalized according to the subject pre-exercise concentration (C and D).

Table Legends

Table 1: Body temperature and hydration markers in the Sex and PEx groups before (PreR) and after (PostR) the race. Values are expressed as the mean ± SEM. ERT: equivalent rectal temperature. Δ% BM: body mass change percentage. USG: urinespecific gravity. * Significant changes between PreR and PostR, within the groups (P < 0.05).

Table 2: Biochemical parameters in the SEx and PEx groups before (PreR) and after (PostR) the race. Values are expressed as the mean ± SEM. * Significant changes betweenpre- and post-race, within the groups (P < 0.05). † Significant changes between SEx PostR - SEx PreR *vs* PEx PostR - PEx PreR (Δ SEx *vs* Δ PEx) (P < 0.05). # Significant changesbetween Δ urea/ammonia ratio SEx *vs* Δ Urea/Ammonia ratio PEx (P < 0.05).

Table 3: Enzyme biomarkers of muscle damage in the SEx and PEx groups before (PreR)and after (PostR) the race. Values are expressed as the mean ± SEM. * Significant changesbetween PreR and PostR, within the groups (P < 0.05).

Table 4: White blood cell counts in the SEx and PEx groups before (PreR) and after (PostR) the race. Values are expressed as the mean ± SEM. * Significant changes betweenPreR and PostR, within the groups (P < 0.05). $ Significant changes between SEx PostRand PEx PostR (P < 0.05).

Table 1 Body temperature and hydration markers in the SEx and PEx groups before (PreR) and after (PostR) the race

	SEx		PEx	
	PreR	PostR	PreR	PostR
ERT (°C)	37.0 ± 0.2	37.6 ± 0.1*	37. 2 ± 0.2	37.8 ± 0.1*
Δ% BM	-	- 1.84 ± 0.77	-	- 2.48 ± 0.65
Urine color	4.7 ± 0.4	6.2 ± 0.4	4.8 ± 0.6	5.4 ± 0.9
USG	1020.7 ± 2.5	1022.1 ± 2.1	1019.2 ± 1.7	1019.5 ± 2.6

Values are expressed as the mean ± SEM. ERT: equivalent rectal temperature. Δ% BM: body mass change percentage. USG: urine specific gravity. * Significant changes between PreR and PostR, within the groups (P < 0.05)

Table 2 Biochemical parameters in the SEx and PEx groups before (PreR) and after (PostR) the race

	SEx		PEx	
	PreR	PostR	PreR	PostR
Ammonia(µmol/L)	33.5 ± 7.0	113.2 ± 7.6*†	54.2 ± 8.2	110.6 ± 7.2*†
Urea (mmol/L)	3.8 ± 0.3	4.8 ± 0.5*	4.0 ± 0.4	5.4 ± 0.4*
Δ Urea/ammonia ratio	-	0.012 ± 0.004	-	0.031 ± 0.007#
Urate (µmol/L)	288.9 ± 27.3	354. 3 ± 30.3*	259.1 ± 30.9	343. 2 ± 29.3*
Glucose (mmol/L)	4.4 ± 0.3	4.9 ± 0.4	4.7 ± 0.3	6.3 ± 1.0
Lactate (mmol/L)	2.2 ± 0.4	5.1 ± 0.5*	2.4 ± 0.3	5.1 ± 0.8*

Values are expressed as the mean ± SEM. * Significant changes between pre- and post-race, within the groups (P < 0.05). † Significant changes between SEx PostR - SEx PreR *vs* PEx PostR - PEx PreR (Δ SEx *vs* Δ PEx) (P < 0.05). # Significant changes between Δ urea/ammonia ratio SEx *vs* Δ Urea/Ammonia ratio PEx (P < 0.05)

Table 3 Enzyme biomarkers of muscle damage in the SEx and PEx groups before (PreR) and after (PostR) the race

	SEx		PEx	
	PreR	PostR	PreR	PostR
CK (µkat/L)	2.6 ± 0.3	3.9 ± 0.3*	3.2 ± 0.5	4.5 ± 0.7*
LDH (µkat/L)	5.1 ± 1.2	6.7 ± 1.6*	5.9 ± 1.3	7.1 ± 1.6
AST (µkat/L)	0.4 ± 0.8	0.5 ± 0.7*	0.4 ± 0.1	0.6 ± 0.1*
ALT (µkat/L)	0.3 ± 0.8	0.3 ± 0.8	0.3 ± 0.1	0.3 ± 0.1

Values are expressed as the mean ± SEM. * Significant changes between PreR and PostR, within the groups (P < 0.05)

Table 4 White blood cell counts in the SEx and PEx groups before (PreR) and after (PostR) the race

	SEx		PEx	
	PreR	PostR	PreR	PostR
Leukocytes ($x10^9$/L)	6.75 ± 0.76	13.00 ± 2.18*	5.85 ± 0.41	11.35 ± 1.75*
Neutrophils	2.92 ± 0.60	9.25 ± 1.69*	2.72 ± 0.34	7.84 ± 1.02*
Eosinophils	0.24 ± 0.06	0.23 ± 0.03	0.16 ± 0.03	0.21 ± 0.04
Basophils	0.03 ± 0.02	0.08 ± 0.05	0.04 ± 0.02	0.09 ± 0.02
Lymphocytes	2.91 ± 0.36	2.55 ± 0.27$	3.13 ± 0.43	3.28 ± 0.19
Monocytes	0.32 ± 0.07	0.66 ± 0.11*	0.26 ± 0.07	0.55 ± 0.14*

Values are expressed as the mean ± SEM. * Significant changes between PreR and PostR, within the groups (P < 0.05). $ Significant changes between SEx PostR and PExPostR (P < 0.05).

CONCLUSÃO

Entre as análises do metabolismo de aminoácidos e produção de amônia, nãoforam encontradas vias para a produção de amônia através da abordagem esportomica. No entanto, foi identificado a produção de hawkinsina e ácido argininico, sugerindo adescoberta de novos marcadores com potencial de promover danos ao desempenho.