UNIVERSIDADE FEDERAL DE UBERLÂNDIA
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PÓS-GRADUAÇÃO EM GENÉTICA E BIOQUÍMICA

Cinética de aparecimento e remoção de biomarcadores de lesão muscular, inflamação e estresse oxidativo após exercício combinado de alta intensidade

Aluno: Artur Luís Bessa de Oliveira

Orientador: Prof. Dr. Foued Salmen Espindola

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Foued Salmen Espindola

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SUMÁRIO

LISTA DE ABREVIATURAS

AMP - Adenosina monofosfato

AGEs - *Advanced glycation end product*

ADP - Adenosina difosfato

ATP - Adenosina trifosfato

CAT - Catalase

CML - Carboximetilisina

CuZn-SOD - Superóxido dismutase dependente de cobre e zinco

DAG - Diacilglicerol

DNA - Ácido desoxirribonucléico

eNOS - Óxido nítrico sintase endotelial

ERNs - Espécies reativas de nitrogênio

EROs - Espécies reativas de oxigênio

ET-1 - Endotelina-1

GADPH - Gliceraldeído-3-fosfato desidrogenase

GFAT - glutamina-frutose-6-fosfato amidotransferase

GLUT-4 -Transportador de glicose do tipo 4

GPDH- glicerol 3-fosfato desidrogenase

GR - Glutationa redutase

GSH - Glutationa reduzida

GSH-Px - Glutationa peroxidase

GSSG - Glutationa oxidada

H_2O_2 - Peróxido de hidrogênio

HbA1c - Hemoglobina glicada

HDL - Lipoproteínas de alta densidade

HNO_2 - Ácido nitroso

HOCl - Ácido hipocloroso

HSPs - *Heat shock proteins*

LV - Limiar ventilatório

MDA - Malonaldeído

Mn-SOD - Superóxido dismutase dependente de manganês

MPO - Mieloperoxidase

NADPH - Nicotinamida adenina dinucleótido fosfato reduzida

NF-kβ - Fator Nuclear kappa β

NO - Óxido nítrico

NOS - Oxido nítrico sintase

O_2 - Oxigênio molecular

$O_2^{\bullet-}$- Ânion radical superóxido

OGT - O-GlcNAc transferase

O-GlcNac - N-acetil glucosamina O ligado

$ONOO^-$ - Peroxinitrito

OPLA - *Onset of plasma lactate accumulation*

PARP - Poli (ADP-ribose) polimerase

PAI-1 - Inibidor do ativador do plasminogênio**-**1

PFGA - Produtos finais da glicação avançada

PKC - Proteína quinase C

PLA2 - Fosfolipase A2

pO_2 - Pressão parcial de oxigênio

RAGE - Receptor para PFGA

Se-GSH-Px - Glutationa peroxidase dependente de selênio

SOD - Superóxido dismutase

STZ - Estreptozotocina

Ig - Imunoglobulina

IMP - Inosina monofosfato

LAN - Limiar anaeróbio

LAN_{Amy} - Limiar da alfa amilas**eLDL** - Lipoproteínas de baixa densidade

LAN_{epi} - Limiar de epinefrina

LAN_{sa}- Limiar salivar

LL - Limiar de lactato

TGF-β - Fator de crescimento de transformação beta

TNFα - Fator de necrose tumoral-α

TBARS - Substâncias reativas ao ácido tiobabitúrico

UDP-GlcNAc - Uridina difosfato N-acetil glucosamina

VEGF - Fator de crescimento vascular derivado do endotélio

VO_2 - Consumo de oxigênio

XDH - Xantina desidrogenase

XO - Xantina oxidase

Apresentação

Essa tese está dividida em três capítulos. O capítulo 1 é uma revisão de literatura abordando os tópicos estudados em ambos os artigos derivados dessa tese. Os capítulos 2 e 3 são os artigos ciêntíficos já formatados de acordo com as revistas para qual serão enviados.

O capítulo 2 foi realizado na Universidade Federal de Uberlândia e teve como objetivo investigar os efeitos metabólicos de uma sessão de exercício agudo de alta intensidade em um grupo de ciclistas do sexo masculino, e mais especificamente, o aparecimento e remoção no soro de biomarcadores de lesão muscular, inflamação e estresse oxidativo após exercício combinado de alta intensidade.

O capítulo 3 foi realizado na Universidade de Toronto numa colaboração entre o Prof. Foued Espindola da UFU e os Prof. Scott Thomas e Marius Locke da U of T. Seu objetivo foi verificar se as adaptações metabólicas observadas após vários meses de treinamento crônico podem ser observadas após uma semana de treino. O foco desse trabalho foi a relação entre resposta imune e o exercício físico. Para tal foi analisado a expressão de proteínas de choque térmico e a ativação de fatores de trancrição em leucócitos após um treinamento de endurance de curta duração e alta intensidade.

Capítulo 1

Fundamentação Teórica

1 - Marcadores de lesão muscular

O uso de proteínas marcadoras de lesão muscular tem sido amplamente empregado na ciência bem como na clínica para identificar e quantificar lesões musculares, infarto agudo do miocárdio, hepatite, alcoolismo, entre outras entidades mórbidas (Hedges, 1995; Puoti, 2004; Harasymiw e Bean, 2007; Bessa, Nissembaum *et al.*, 2008). Classicamente, para tentar estimar a magnitude de uma lesão muscular usa-se um protocolo composto por enzimas presentes no citosol de células musculares: creatina quinase (EC 2.7.3.2; CK) e lactato desidrogenase (EC 1.1.1.27; LDH) (Gault e Geggie, 1969).

A CK é extensivamente usada como marcador de lesão muscular, tanto esquelética quanto cardíaca (Bassini-Cameron, Sweet *et al.*, 2007; Bassini-Cameron, Monteiro *et al.*, 2008) para o diagnóstico de infarto agudo do miocárdio (Hedges, 1995). Há três isoformas descritas de CK: MM-CK, MB-CK e BB-CK. A quantidade das isoformas de CK varia de acordo com o tecido, mas todas estão presentes em diversos tecidos e por isso a identificação somente dessa enzima não é conclusiva, fazendo-se necessário a análise de outros marcadores, como a LDH.

A monitoração dos níveis de LDH tem aplicações na ciência do exercício, onde recentemente foi demonstrado que a cinética do aparecimento de LDH no sangue após o exercício é mais rápida que a de CK (Bessa, Nissembaum *et al.*, 2008). A análise clínica da LDH, no sangue ou na urina, encontra utilidade no controle de doenças neuromusculares e do trato urinário (Gault e Geggie, 1969).

Tanto CK quanto LDH estão presentes tanto no músculo esquelético quanto no cardíaco, bem como em outros orgãos como fígado. Por isso a quantificação de marcadores de lesão nesses tecidos para determinação do sítio

primário de liberação por eliminação se faz necessária (Bessa, Nissembaum *et al.*, 2008). Os marcadores hepáticos clássicos são aspartato aminotransferase (EC 2.6.1.1; AST), alanino aminotransferase (EC 2.6.1.2; ALT) e gama glutamil transferase (EC 2.3.2.2; γGT) (Puoti, 2004; Muntoni e Reddel, 2005; Lin, Chang *et al.*, 2008; Solaro, Rosevear *et al.*, 2008). Estas enzimas, são transaminases encontradas em baixa quantidade nos músculos, mas presentes em abundância nos hepatócitos. Os níveis plasmáticos elevados desses marcadores são observados durante situações de injuria hepática (Tohidi, Harati *et al.*, 2008). Quando somadas às análises de CK e LDH nos dão uma idéia da localização e da extensão da lesão, mas não de forma precisa.

Para uma identificação ainda mais precisa, outros marcadores de lesão muscular devem ser utilizados. As troponinas (Tn), são proteínas regulatórias que controlam a interação actina-miosina mediada por cálcio, produzindo a contração (Lim, Qushmaq *et al.*, 2006). As lesões em células do músculo (esquelético ou cardíaco) resultam na liberação de Tn para o sangue, que podem ser detectadas por imuno-ensaio, cujo teste é amplamente utilizado como marcador de infarto do miocárdio mas ainda não foi utilizado como marcador de lesão muscular esquelética produzida pelo exercício físico.

Embora alguns destes marcadores venham sendo utilizados há bastante tempo pela ciência do exercício para monitorar a extensão da lesão muscular e indiretamente a intensidade do treinamento ao qual um atleta foi submetido, ainda há controvérsias em relação a cinética de aparecimento dos mesmos na corrente sanguínea após o exercício.

2 - Espécies reativas de oxigênio e exercício

Estresse oxidativo é definido como um desequilíbrio entre a produção de espécies reativas de oxigênio (EROs) e as defesas antioxidantes (Lamb e Goldstein, 2008), levando a alterações em diversas vias de sinalização e potencialmente a lesão tecidual (Halliwell, 1995).

O exercício físico é um notório produtor de EROs (Powers e Jackson, 2008; Ristow, Zarse *et al.*, 2009). Durante a atividade física a captação de oxigênio pode aumentar em até 20 vezes quando comparado ao repouso, e o fluxo de elétrons para as mitocôndrias de músculos esqueléticos é elevado de 100 a 200 vezes, o que inevitavelmente aumentará a formação de EROS (Chambers, Moylan *et al.*, 2009; Hattori, Hayashi *et al.*, 2009).

Os efeitos das EROs são dependentes da sua concentração (Chambers, Moylan *et al.*, 2009) e são amplamente estudados, havendo relatos de efeitos tanto prejudiciais a saúde (Halliwell, 1995; Lamb e Goldstein, 2008) quanto promotores da mesma (Chambers, Moylan *et al.*, 2009; Ristow, Zarse *et al.*, 2009).

O papel das EROs durante o exercício é convencionalmente proposto como consequência da atividade contrátil, potencialmente atrasando a recuperação tecidual. No entanto, novas evidências apontam que essas moléculas exercem funções na sinalização celular e na regulação da síntese proteíca (Scheele, Nielsen *et al.*, 2009).

No tratamento de algumas doenças, o consumo de antioxidantes tem sido eficiente para diminuir a formação de EROs e a consequente pertubação na hormonesis que é característica de algumas doenças, conseguindo diminuir ou neutralizar seus efeitos prejudiciais (Forstermann, 2010; Gobe e Crane, 2010) . O

consumo de antioxidantes associado a prática do exercício com o intuito de diminuir a formação de EROs também teve sua eficiência para tal propósito observada (Chambers, Moylan *et al.*, 2009). Por outro lado, os efeitos benéficos creditados às EROs produzidas pelo exercício podem ser bloqueados pelo consumo de antioxidantes (Ristow, Zarse *et al.*, 2009). Baseado nisso, o desequilíbrio momentâneo entre a formação de EROs e as defesas antioxidantes durante o exercício podem ser benéficos.

Além disso, o exercício é um notório modulador da resposta imune (Febbraio, 2007) e o crescente interesse no estresse oxidativo produzido pelo exercício leva a uma nova corrente que tem como objetivo investigar a resposta imune modulada pelas EROs produzidas pelo exercício (Akerstrom, Steensberg *et al.*, 2005; Jackson, 2005; Gleeson, 2007; Scheele, Nielsen *et al.*, 2009).

3 - EROS e a resposta imune ao exercício

As EROs podem induzir fatores de transcrição, notoriamente AP-1 e NFκβ, resultando na ativação de citocinas inflamatórias, isso pode explicar porque EROs e inflamação estão relacionados às doenças crônicas (Scheele, Nielsen *et al.*, 2009). No entanto, durante o exercício, algumas citocinas produzidas e secretadas pelos músculos esqueléticos parecem ter efeitos anti-inflamatórios e alteram o metabolismo celular. Desse moso, foi proposto que o efeito anti-inflamatório do exercício regular protegeria contra inflamações sistêmicas sub-clínicas (Pedersen, Steensberg *et al.*, 2003; Nielsen e Pedersen, 2007; Scheele, Nielsen *et al.*, 2009).

As EROs provavelmente regulam a transcrição de citocinas anti- e pró-inflamatórias, exercendo efeito dependente do contexto e da concentração das

mesmas; além disso, ambas possuem funções como moléculas sinalizadoras na adaptação do músculo esquelético ao exercício (Scheele, Nielsen *et al.*, 2009).

Uma sessão de exercício resulta na produção de EROs que poderão iniciar mecanismos de sinalização celular e induzir alterações na expressão gênica (Ristow, Zarse *et al.*, 2009) por meios de modificações diretas nas proteínas alvo ou por alterações no potencial redox intracelular (Jackson, 2005). Essa elevação na concentração de EROS é momentânea e será neutralizada pelas defesas antioxidantes endógenas.

A produção aguda de EROs durante o exercício físico pode ser benéfica, ao contrário da visão que as EROs suprimem a recuperação muscular. Níveis elevados de EROs produzidas pelo exercício podem contribuir para as alterações metabólicas promotoras de saúde que ocorrem durante o pós exercício (Scheele, Nielsen *et al.*, 2009).

Ainda assim, a afirmação de que EROs podem promover adaptação muscular ao exercício é controversa. Enquanto alguns pesquisadores acreditam em um papel estimulador das mesmas na adaptação ao exercício (Ristow, Zarse *et al.*, 2009; Scheele, Nielsen *et al.*, 2009), outros defendem que as EROs tem efeito inibitório na recuperação muscular e sugerem tratamento antioxidante (Reid, 2008).

Essas afirmações são embasadas nas seguintes evidências: EROs foram apontadas como mediadores endógenos de fadiga por diminuírem a sensibilidade ao cálcio (Reid, 2008); EROs podem causar danos ao DNA, peroxidação lipídica e carbonilação de proteínas (Moylan e Reid, 2007); EROs somadas as citocinas próinflamatórias podem induzir a expressão de proteassomos pela ativação do

fator nuclear kappa B (NFκβ), resultando em catabolismo muscular durante doenças crônicas (Moylan e Reid, 2007).

Há ainda uma corrente intermediária que acredita que baixos níveis de EROs são requeridos no metabolismo muscular, enquanto altas concentrações podem levar a disfunção contrátil, fraqueza muscular e fadiga (Powers e Jackson, 2008). Estudos em humanos suportam a idéia que as EROs podem estimular a produção de citocinas pelo músculo esquelético em resposta ao exercício (Vassilakopoulos, Karatza *et al.*, 2003; Fischer, Hiscock *et al.*, 2004). Por exemplo, a elevação dos níveis de interleucina 6 (IL-6) induzidas pelo exercício foi diminuída durante o tratamento simultâneo com antioxidantes (vitaminas A, C e E) (Vassilakopoulos, Karatza *et al.*, 2003), um resultado que indica a produção de IL-6 dependente de EROS em resposta ao exercício (Ristow, Zarse *et al.*, 2009; Scheele, Nielsen *et al.*, 2009).

4 - Miocinas

Atualmente é amplamente aceito que o conceito de miocina, ou seja, citocinas produzidas e liberadas pelo músculo esquelético podem regular a hipertrofia, *angiogenesis* e sensibilidade a insulina e possivelmente, outros mecanismos ainda não descobertos (Scheele, Nielsen *et al.*, 2009). A primeira miocina identificada foi a IL-6, produzida no músculo esquelético por uma via metabólica dependente de cálcio (Weigert, Dufer *et al.*, 2007) e que age via AMPK (Kelly, Keller *et al.*, 2004; Glund, Deshmukh *et al.*, 2007). A IL-6 é uma citocina pleiotrópica liberada pela contração muscular (Ostrowski, Rohde *et al.*, 1998) que estimula tanto o metabolismo de glicose quanto o de lipídios (Carey, Steinberg *et al.*, 2006; Glund, Deshmukh *et al.*, 2007). Também promove

hipertrofia muscular estimulando a proliferação de células satélites via STAT3 (Serrano, Baeza-Raja *et al.*, 2008).

Outra citocina, IL-15, foi definida como um fator anabólico, estando altamente expressa no músculo esquelético (Grabstein, Eisenman *et al.*, 1994) e é elevada no músculo após uma sessão de treinamento de força (Nielsen, Mounier *et al.*, 2007). Esses achados indicam que miocinas são potentes sinalizadores que estimulam a hipertrofia muscular em resposta ao exercício. Os efeitos da IL-6 e IL-15 parecem ocorrer proeminentemente durante o treinamento de força embora a ação de outras miocinas podem mediar as adaptações promovidas pelo treinamento de resistência (Bloor, 2005). Outras miocinas como a IL-8, previamente classificada como um fator angiogênico durante o desenvolvimento câncer (Brat, Bellail *et al.*, 2005), tem seus níveis elevados no músculo esquelético em resposta ao exercício (Akerstrom, Steensberg *et al.*, 2005).

Desta forma, o conceito de miocina representa uma mudança de paradigma, implicando que o músculo esquelético tenha um papel central em regular o seu próprio metabolismo. O ponto intrigante desse conceito é que entre as miocinas estão representadas citocinas pró-inflamatórias. Entretanto, parece que o exercício promove uma resposta de citocinas por um mecanismo independente de TNF alfa e NFκβ, e que ambas as vias de sinalização (*upstream* e *downstream*) para IL-6 diferem marcadamente entre células imunes e células musculares (Scheele, Nielsen *et al.*, 2009). Consequentemente, a resposta via citocinas ao exercício parece ter importantes papéis anti-inflamatórios, metabólicos e fisiológicos.

Neste aspecto, uma visão provocativa é que as EROs e a indução à resposta imune por citocinas representam mecanismos importantes para a adaptação ao treinamento, dando suporte a isso há o fato de antioxidantes atenuarem algumas das respostas fisiológicas normais ao exercício (Ristow, Zarse *et al.*, 2009; Scheele, Nielsen *et al.*, 2009). Em vista disso sugere-se um novo paradigma: o músculo esquelético tem função endócrina produzindo e liberando miocinas em resposta ao exercício e influenciando o metabolismo em outros tecidos e orgãos (Scheele, Nielsen *et al.*, 2009).

5 - Remodelamento muscular e ROS

A inflamação é necessária para a cicatrização e para a neutralização de infecções pela infiltração de leucócitos em tecidos lesados ou infeccionados, a descoberta que o exercício provoca um aumento na produção de citocinas estabelece uma potencial ligação entre o exercício e a resposta imune (Scheele, Nielsen *et al.*, 2009).

Diversos estudos contribuiram para a compreensão de um quadro complexo onde células inflamatórias podem promover tanto lesões quanto reparo tecidual através da ação combinada de radicais livres, fatores de crescimento e citocinas (Tidball, 2005). No caso de lesões musculares, a idéia geral é que neutrófilos e macrófagos dominam a resposta inflamatória (Tidball, 2005).

Trabalhos anteriores mostraram os neutrófilos como promotores de dano tecidual rapidamente após a lesão muscular, e não há evidências diretas de que os mesmos possam desempenhar um papel benéfico no reparo e na regeneração muscular (Tidball, 2005).

Macrófagos também são capazes de promover lesão muscular tanto *in vivo* quanto *in vitro* pela da liberação de radicais livres, embora outros achados indicam que eles também possam atuar no reparo e na regeneração sinalizada por fatores de crescimento e citocinas (Tidball, 2005).

A ligação entre remodelamento muscular e resposta imune ja foi descrita (Scheele, Nielsen *et al.*, 2009), tal como a relação entre resposta imune e EROs (Moylan e Reid, 2007; Scheele, Nielsen *et al.*, 2009). Além disso, a atividade física, o estresse oxidativo e a resposta inflamatória modulam a expressão de proteínas de choque térmico e de fatores de transcrição de ação rápida (De Maio, 1999; Gilmore, 2006).

6 - Proteínas de choque térmico e NFκβ

Um mecanismo primitivo de proteção cellular contra o estresse, envolve a expressão de uma familia de proteínas de choque térmico (HSPs). Algumas dessas HSPs, presentes nas células, atuam no empacotamento e translocação de polipeptídeos através de membranas, e por isso são chamadas de chaperones moleculares (De Maio, 1999).

Os membros dessa familia podem ser induzidos em resposta a varios estresses celulares, incluindo choque térmico subletal, radiação, isquemia, estresse oxidativo e outros estímulos capazes de ativar fatores de transcrição de choque térmico como NFκβ (Mosser e Morimoto, 2004; Place e Hofmann, 2005).

NFκβ é um complexo proteico que controla a transcrição de DNA em quase todos os tipos celulares de animais e está envolvido na resposta a estresses celulares (Gilmore, 2006), pertencendo a categoria dos fatores de transcrição de ação rápida, estando presentes nas células em estado inativo e por isso não

requerem síntese proteica para serem ativados. Isso confere ao NFκβ um papel como primeira resposta a um estímulo celular (Chandel, Trzyna *et al.*, 2000).

A relação entre exercício físico e HSPs pode ser direta, considerando o exercício como uma forma de estresse energético e estrutural. Mas se considerarmos que o exercício pode provocar respostas metabolicas, induzindo a expressão e/ou liberação de HSPs, como estresse oxidativo e inflamação (Kukreja, Kontos *et al.*, 1994; Locke e Noble, 1995), essa relação poderia ser também indireta.

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Capítulo 2

The influence of exercise intensity and recovery on biomarkers of injury, inflammation and oxidative stress

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ABSTRACT

We investigated biomarkers of injury, inflammation and oxidative stress in the blood after high intensity training. Nineteen male athletes performed a combination of high-intensity aerobic and anaerobic training. Samples were acquired immediately before and from 3 h to 72 h after the exercise. CK was elevated 200% at 3 h post-exercise, reaching a 300% peak increase at 12 h and returning to pre-exercise levels within 48 h. LDH activity was 25% higher 3 h after exercise, increasing to 56% higher 6 h after exercise and returning to pre-exercise levels within 12 h. Leukocyte levels were 50% higher and neutrophil levels were 70% higher 3 h after exercise than at baseline, while lymphocyte levels increased by up to 55% after 12 h. MCP-1 was elevated by 40% after 6 h and decreased by 37% 72 h after exercise. TNF-alpha levels were lower in all post-exercise samples. IL-6 and CRP levels remained stable throughout the entire recovery period. The levels of oxidative stress markers remained stable during the experiment. CK and LDH blood appearance and clearance are faster than classic described, coaches and physicians must respect these windows to accurately estimate muscle damage. The neutrophil/lymphocyte ratio summarizes the mobilization of two leukocyte subpopulations in a single marker and may be used to predict the end of the post-exercise recovery period. Further analysis of the immune response using serum cytokines indicated that the high-intensity exercise performed by highly trained athletes only generates inflammation localized to the skeletal muscle.

Key words: Sports training; Exercise biochemistry; ROS; Muscle damage.

A. INTRODUCTION

The ability to accurately gauge the physiological impact of an exercise session on the human body and the recovery time required before a second stimulus is a fundamental part of a training program, as it allows coaches to better decide the timing and loading of subsequent training sessions. The current understanding of exercise recovery regarding biomarkers is minimal, and because of this limitation, physical tests that evaluate strength (36), peak power output and fatigue (9) remain the primary tools for this purpose, even though physiological tests requires a few weeks to present noticeable alterations. In the other hand, the use of blood biomarkers represents a faster and more accurate method to evaluate muscle damage and inflammation caused by an exercise session. Despite the high accuracy of these markers, there is still no consensus about the window of their appearance in the blood after exercise of different types and intensities (5-7).

Technological advancement has made it possible to use biomarkers of muscle damage and inflammation (5) as measures of exertion. Many studies have assessed the appearance of muscle proteins in the blood after exercise to provide indirect evidence of muscle damage. The muscle enzymes lactate dehydrogenase (LDH), aspartate aminotransferase, carbonic anhydrase isoenzyme II, and creatine kinase (CK) have been assessed. Although all of these have been shown to increase after damage-inducing exercise, CK has received the most attention, perhaps because the magnitude of increase is so great relative to other proteins, and the cost of the assay is comparatively modest..

Muscle damage typically initiates a rapid and sequential invasion of muscle by inflammatory cell populations that can persist for days to weeks, while muscle repair occur. This relationship between inflammation and muscle repair has suggested that they may be mechanistically related and provides the basis for teleological arguments that muscle inflammation after muscle damage use is a functionally beneficial response (35).

In the quest for new biomarkers to monitor exercise recovery, we took into consideration the fact that inflammation is a fundamental part of muscle repair (35). Therefore, we hypothesized that the mobilization of Leukocytes subpopulations and cytokines in the blood stream could be used as biomarkers for recovery. The differences in the migration times of Leukocytes reflect their specificity in tissue repair. Neutrophils are normally the fastest migrating subpopulation, and lymphocytes are the slowest. The neutrophil/lymphocyte ratio (NLR) is a marker of the systemic inflammatory response that can easily be measured and may indicate the severity of muscle damage (39).

Even though inflammation is necessary for muscle repair, it also has deleterious effects on exercise performance, especially resulting from the increased production of reactive oxygen species (ROS) (29), which may lead to oxidative stress. The relationship between oxidative stress and exercise has been the focus of much research over the past decade. Despite the initial view that ROS could potentially damage cells, it now seems possible that these substances have important roles in the regulation of cell signaling (31), although the idea that ROS can promote muscle adaptation remains controversial (29).

A better understanding of previously described biomarkers of muscle damage and inflammation, and further elucidation of the outcomes of ROS production and oxidative stress among athletes is fundamental to our understanding of post-exercise recovery. To this end, we used Combined Training (CT) instead of only aerobic or anaerobic training. CT is a fundamental part of training of most sports modalities, including ones that are predominantly aerobic or anaerobic as many athletic events rely on multiple energy systems to generate ATP for exercise performance(38). The relevance of studying the recovery period after a training stimulus lies in the fact that it is during this period that the adaptations that will result in performance improvements take place. Further, overtraining can be avoided by having a clear model of the physiological recovery process as most athletes are training 1-2 times per 24-hour period, and up to 12 or more training sessions per week.

Therefore, the purpose of this study was twofold: (1) to investigate the appearance and clearance of biomarkers in the blood for both muscle damage and inflammation over a time course from 3 h to 72 h after a combined training exercise; and (2) to monitor oxidative damage and antioxidant enzyme activity and to investigate whether the increased ROS production during high-intensity exercise leads to oxidative damage in elite athletes.

B. METHODS

Experimental Approach to the Problem

Biomarkers of muscle injury, inflammation and oxidative stress are known to respond to high intensity exercise. However, the time course and the magnitude of this response remain unclear, as it is dependable of the intensity and the type of exercise, as well as of the fitness level of the athletes. The independent variable in the experimental design was a single bout of high intensity combined exercise performed by one group of amateur male cyclists. Individual assessments as dependent variables were conducted immediately before and 3 h, 6 h, 12 h, 24 h, 48 h and 72 h after the exercise and included the evaluation of biomarkers of muscle injury, inflamation and oxidative stress.

Subjects

Written informed consent was obtained from all subjects. This study was approved by the ethics committee for human research of the Universidade Federal de Uberlândia and conformed to the requirements for carrying out research in human subjects (Health National Council, Brazil, 1996). The authors inform that this study also complies with the ACSM statement regarding the use of human subjects and informed consent.

Nineteen top-level amateur male cyclists (28 ± 2.5 years old; 81.3 ± 2.9 kg body mass; height 184.6 ± 2.5 cm; VO2Peak 61.2 ± 1.7 mL O2/kg/min; HRmax 178 ± 4.6 beats/min; mean ± SE) enrolled voluntarily in this study. The subjects initially submitted to a laboratory analysis and denied using ergogenic substances.

Physical tests

Gas analysis was performed two weeks before the exercise protocol with a commercially available system (FITMATE, COSMED, Italy) to measure the plateau in oxygen consumption (VO2Peak) During the test, the heart rate was measured continually with a cardiac monitor (Polar Electro Oy,Kempele, Finland), and blood samples (25 μL) were obtained from the earlobe at the end of each stage to determine the lactate threshold. The test began with a 25-W load, which was increased by 25 W every 2 minutes until exhaustion. The one-maximum repetition (1RM) was used to determine maximum muscle strength the deep squat and for the bench press one week before the exercise protocol. Subjects warmed up by performing two sets of 10 repetitions of each exercise using light loads (20% of the expected 1RM as reported by the athlete) 5 min before the test to avoid injuries. Three minutes of rest were allowed between sets, and all participants successfully completed the test within three attempts. Upper extremity strength was assessed by handgrip strength. This was measured using a Jamar Hydraulic Hand Dynamometer (Sammons Preston, Bolingbrook, IL), which measures isometric grip force. Subjects were instructed to exert maximum effort with the dominant hand for three seconds during two trials, each separated by a 1-min rest. The maximum result was used for analysis.

Exercise protocol

After refraining from any kind of physical exercise for one week, all subjects cycled for 1 h at 85% of their VO2Peak immediately after performing a strength training session that consisted of six deep squat sets of maximum repetitions at 85% of 1RM alternating with six bench press sets at 85% of 1RM. The athletes rested for

5 min between each set. Physical exercise was also forbidden for 72 h after the exercise protocol, during which time recovery blood samples were collected.

Blood sample collection, handling and storage

We collected blood samples immediately before and 3 h, 6 h, 12 h, 24 h, 48 h and 72 h after the exercise protocol. The blood was collected via venipuncture performed by a certified phlebotomist into 10 mL EDTA, sodium heparin, and serum separator vacuum tubes (Vacutainer). All serum samples were allowed to clot and were then separated into serum by centrifugation at 4°C for 15 min at 2,000 g. After removal of the plasma and buffy coat layers, the erythrocytes were washed three times with two volumes of isotonic saline. Erythrocytes were lysed with cold distilled water. Blood serum or plasma was aliquoted and stored at −80°C for later analysis. All subject samples were analyzed in duplicate, and the results were averaged.

Hematological parameters, injury markers and white cell count

Biochemical analysis was performed using commercial kits in an automatic device (Autolab 18 - Boehringer Mannheim) for CK, LDH, cardiac troponin T (cTnT), □-glutamyltransferase (□GT) and C-reactive protein (CRP). Hematological analysis was performed immediately after collection by automated analysis (KX-21N, Sysmex) using blood collected into tubes containing EDTA. Total and differential white cell counts were performed, and red blood cells and platelets were also counted.

Cytokines

The levels of IL-6, MCP-1 and TNF-alpha in the serum were determined by an enzyme-linked immunosorbent assay (ELISA) with specific monoclonal antibody (MAb) pairs. Microplates (Nunc, Roskilde, Denmark) were sensitized overnight with Capture Antibody Purified Anti-Human IL-6, Capture Antibody Purified Anti-Human MCP-1 or Capture Antibody Purified Anti-Human TNF-alpha. Nonspecific binding was prevented by incubating the plates with 2% bovine serum albumin (Sigma, St. Louis, Mo.) in phosphate-buffered saline (PBS). The plates were incubated overnight with 100 µl of a 1:2 dilution of serum samples in PBS, 1% bovine serum albumin, and standard cytokines. The plates were then washed four times with 0.05% Tween in PBS and incubated with Detection Antibody Biotin Anti-Human IL-6, MCP-1 or TNF-alpha for 2 h. The plates were washed and incubated for 2 h with Enzyme Reagent Streptavidin-horseradish peroxidase conjugate (SAv-HRP). Finally, the plates were washed five times and incubated with p-nitrophenyl phosphate (BD). The A450-A630 was read in a microplate reader. BD OptEIA™ ELISA Sets were used for all analyses.

Oxidative stress parameters

We assayed erythrocytes for antioxidant enzyme activities and plasmatic total antioxidant status. Catalase (CAT) activity was assayed spectrophotometrically by monitoring hydrogen peroxide decomposition at 240 nm (1). Superoxide dismutase (SOD) and glutathione peroxidase (GSH-Px) activities and total antioxidant status (TAS) were assessed using a TEAC commercial kit (Randox NX2332, Crumlin, UK).

The levels of plasma lipid peroxidation products in plasma were measured by determining the thiobarbituric acid-reacting substance (TBARS) levels with a commercially available kit (Cayman Chemical, Ann Arbor, MI, USA). Lipid peroxide concentrations were expressed in terms of the malonaldehyde (MDA) concentration (□M). The levels of plasma protein-bound sulfhydryls (PBSH) were determined using 5,5-dithiobis(2-nitrobenzoic acid) (DTNB) as previously described by Faure and Lafond (12).

Statistics

All data were normalized to pre-exercise values and are expressed as means ± SE. Statistical significance was calculated by analysis of variance (One-way ANOVA) followed by Tukey's post-hoc test after the Kolmogorov-Smirnov test was carried out to assess the normality of the variable distribution. All tests were 2-tailed, and a P value < .05 was considered statistically significant.

C. RESULTS

Nineteen subjects completed the protocol. The hematological parameters showed no alterations after the high-intensity exercise protocol performed by the athletes (Table 1), allowing us to verify that the alterations of the injury marker, immune response and oxidative stress parameters were not due hemoconcentration.

Strength Test

Upper extremity strength dropped 14% ($p<0.02$) 3 h after exercise and returned to pre-exercise levels from 12 h to 24 h (Figure 4).

Injury Markers

We observed differing appearance and clearance kinetics of skeletal muscle injury markers in the blood during recovery. CK was elevated 200% ($p<0.01$) at 3 h post-exercise, reaching a peak increase of 300% ($p<0.01$) at 12 h, remaining higher than pre-exercise levels through 24 h and returning to pre-exercise levels within 48 h. LDH activity was 25% higher ($p<0.02$) 3 h and 56% higher ($p<0.02$) 6 h after exercise, returning to pre-exercise levels at 12 h post- exercise (Figure 1).

The investigated markers of myocardial and hepatic damage, cTnT and □GT, respectively, remained stable through the experiment, with levels of 0.01 ± 0.1 ng/mL and 20.5 ± 3.3 U/L, respectively.

Immune Response

Leukocytes levels were 50% higher ($p<0.05$) 3 h after exercise and remained higher than pre-exercise levels ($p<0.05$) until 12 h post-exercise, returning to pre-exercise values at 24 h (Figure 2A).

To assess the extent of the influence of exercise on leukocytes, we also did a differential count to measure leukocyte sub-populations. Monocytes presented no alterations in the post-exercise period (Figure 2B). Neutrophils were the major contributors to the leukocyte increase 3 h after exercise, presenting a 70% increase ($p<0.05$) and returning to pre-exercise levels after 6 h (Figure 2C). Lymphocytes increased by up to 55% ($p<0.05$) after 12 h and returned to pre-exercise levels within 24 h (Figure 2D).

To better understand the white blood cell migration in response to exercise, we also measured the levels of pro- and anti-inflammatory cytokines. MCP-1 was elevated 40% after 6 h and decreased 37% 72 h after exercise (Figure 3A). TNF-alpha was lower than baseline in all post-exercise samples, reaching an almost 100% drop 72 h after exercise (Figure 3B). IL-6 and CRP levels remained stable for the duration of the monitored post-exercise recovery period (Figure 3C and 3D).

The NLR was 60% higher 3 h after exercise, returned to pre-exercise levels 6 h after and then dropped 36% from baseline 24 h after exercise. The NLR stabilized at pre-exercise levels within 48 h post-exercise (Figure 4).

Oxidative Stress

Regarding antioxidant defenses, SOD, GSH-Px and TAS levels remained stable during all time points. Only CAT activity was altered in response to exercise, presenting a 35% ($p<0.05$) increase at 3 h and 24 h after exercise (Table 2). TBARS and PBSH levels were not statistically different when compared to pre-exercise levels (Table 2).

D. DISCUSSION

This study investigated the appearance and clearance kinetics of injury, inflammation and oxidative stress markers in the blood after high-intensity combined training. The exercise protocol was designed to simulate an athlete's typical training session, combining endurance and strength training. We assessed a training session in our protocol due to the fact that the majority of previous research that investigated biomarker kinetics and oxidative stress did so after real or simulated competition (5, 20). Even though understanding metabolism during a competition is fundamental, it is during the many training sessions that precede the competition that the adaptations that lead to performance improvements occur. Furthermore, we also aimed to identify biomarkers that could be easily measured and routinely used by elite athletes and coaches to track exercise intensity and recovery.

Injury Markers

The CK level was highly elevated from 12 h to 24 h after exercise and returned to pre-exercise values within 48 h. This behavior makes the measurement of the activity of this enzyme in the serum ideal for determining the intensity of an exercise performed one day before measurement. LDH had even faster appearance and clearance kinetics than CK. Its appearance window in the blood of 3 h to 6 h post-exercise makes LDH a good indicator of the intensity of a training session that took place only a few hours before its measurement. The higher increase in CK compared to LDH makes CK the best choice to monitor the intensity of a medium-intensity exercise session. On the other hand, the fast clearance kinetics of both CK and LDH make them a poor choice for monitoring

the intensity of a period of training longer than one day, for example to avoid overreaching (19) and overtraining (27).

In our experiment, CK and LDH had blood appearance and clearance kinetics that were faster than typically described in the literature (6). Previous studies have reported appearance windows of 24 h to 72 h (7), and even though more recent publications have reported faster appearance kinetics (5), to our knowledge, elevation 3 h after exercise for both CK and LDH has never been demonstrated.

Another novelty of this paper is the use of cTnT as a cardiac muscle-specific marker. The absence of detectable levels of cTnT in the blood stream after exercise shows that there was no significant myocardial damage (15) after the high-intensity combined training that our subjects completed. Taking the cTnT results together with the γGT (26), hematocrit and platelet levels, we believe that the increases in the blood concentrations of CK and LDH were caused only by skeletal muscle damage.

Immune Response

Leukocyte levels were elevated in the blood stream 3 h after exercise and returned to pre-exercise levels within 24 h. At 12 h after exercise, the average leukocyte count was still 30% higher than the pre-exercise level, even though the difference was not statistically significant.

This leukocyte appearance and clearance behavior was directly influenced by two cell subpopulations. Neutrophils were the fastest subpopulation to appear in the

blood stream and also the fastest to clear it. This behavior is in accordance with the literature (4, 13), as neutrophils are important to the removal of cellular debris (18, 35), but their long-term presence in the damaged tissue may delay its repair since they are notable ROS producers (25, 33). Lymphocytes were the slowest components of the immune response, only becoming elevated 12 h after exercise.

The difference in the migration time of neutrophils and lymphocytes indicates different stages of muscle repair (35) and therefore can be used to monitor recovery time. The NLR (39) is marker of systemic inflammatory responses that summarizes the mobilization of these two leukocyte subpopulations in a single marker that can easily be measured. The change in the NLR was inversely correlated with supercompensation and with the upper extremity strength measured in this experiment. Thus, NLR can be an alternative to for physical tests that evaluate strength (17, 36) as a tool to predict the end of the post-exercise recovery period.

The main advantage of using NLR instead of tests like 1RM or isokinetic dynamometers is that these tests normally involve many sets of maximal repetitions and are both time consuming and physically demanding for the athlete, limiting the use of these tests on a regular basis because they can increase the recovery period or even cause injuries.

The measurement of cytokine levels allows a deeper understanding of the post-exercise immune responses. The kinetic behavior of MCP-1 shows that the apparently steady blood level of monocytes is actually a dynamic equilibrium

between bone marrow secretion and damaged tissue mobilization. The elevated levels of MCP-1 6 h after the exercise indicate the moment when macrophages are required at the damaged tissue to help with tissue repairs (3, 34), and the immunosuppression 72 h after exercise, supported by both the MCP-1 and the TNF-alpha results, indicates that inflammation is no longer necessary at this time (21) and that muscle recovery may be complete.

Taking the MCP-1 and TNF-alpha results together with the levels of inflammation markers IL-6 (28) and CRP (16), we concluded that the high-intensity exercise protocol we used generated localized inflammation of skeletal muscle but not systemic inflammation.

Oxidative Stress

We did not observe any significant alterations of the levels of the antioxidant enzymes SOD, GSH-Px and CAT on the TAS or the oxidative stress damage markers TBARS and PBSH. This is in accordance with the fact that the antioxidant enzymes are regulated by redox status and not by exercise itself (14). Because the subjects had high basal levels of antioxidant enzymes, the possible increase in ROS production during exercise was not sufficient to alter the redox status enough to trigger the up-regulation of these antioxidant enzymes.

The exercise protocol used in this research did not generate oxidative stress even though it has previously been stated that intense exercise is a powerful stimulator of ROS production (24). The high basal levels of antioxidant enzymes that our subjects had, a fact that is widely described in the literature regarding athletes (10,

11), were probably sufficient to counterbalance the elevation in ROS production and to avoid oxidative stress after exercise in these well-trained subjects.

Previous studies that used well-trained volunteers (22, 30) presented similar reports showing no oxidative stress following intense exercise, but these reports have been criticized (14) for the following two reasons: (1) the samples were acquired only immediately after exercise (2, 32, 37), and (2) no alterations were observed in injury markers following the exercise protocol, suggesting that the exercise performed was of low intensity (23, 30). Our results address both of these issues. The elevation of the CK and LDH levels proves that the intensity of our exercise protocol was indeed high, and samples were acquired up to 72 h after exercise.

Conclusion

We used combined training to simulate a regular training session. CK and LDH blood appearance and clearance are faster than classic described. The NLR summarizes the mobilization of two leukocyte subpopulations in a single marker and may be used to predict the end of the post-exercise recovery period. Further analysis of the immune response using serum cytokines indicated that the high-intensity exercise performed by highly trained athletes only generates inflammation localized to the skeletal muscle.

E. PRACTICAL APPLICATIONS

Biomarkers results after training vary drastically depending on the type and intensity of exercise and on the biological Individuality of the athletes been trained.

The best way to use biomarkers to monitor athletes is to do a screening test like this experiment, using the type and intensity of exercise commonly used and performed by the athletes that will be trained. This way, the appearance and clearance window of the selected biomarkers will be accurately identified and can help coaches to identify the reasons why some athletes respond well to a type of training and others do not. The use of biomarkers does not substitute performance tests, but the deeper metabolic understanding achieved by evaluating biomarkers can, among other reasons, improve the coaches ability to determine the recovery period after an exercise session and the intensity of the following ones.

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Table 1 - Hematological parameters

	Basal (absolute value)	3 h (% of basal)	6 h (% of basal)	12 h (% of basal)	24 h (% of basal)	48 h % (of basal)	72 h % (of basal)
RBC (10^6/mm^3)	5.15 (0.12)	99.11 (0.64)	95.26 (0.88)	93.84 (1.57)	97.78 (1.36)	97.58 (1.35)	98.77 (1.35)
Hemoglobin (g%)	16.91(0.31)	97.41 (0.66)	94.57 (0.65)	93.90 (1.03)	96.18 (1.13)	96.38 (1.21)	97.86 (1.10)
Hematocrit (%)	49.28 (0.86)	98.82 (0.68)	96.10 (0.75)	94.26 (1.41)	98.58 (1.31)	98.30 (1.26)	98.90 (1.23)
Platelets (10^9/L)	265.22 (10.55)	98.24 (4.23)	93.28 (3.45)	101.56 (3.54)	85.82 (3.61)	88.09 (3.71)	96.87 (4.08)

Data are expressed as the mean (SE); P<0.05 for statistical significance; all post-exercise values are data normalized to basal values; RBC - red blood cell.

Table 2 - Oxidative stress marker levels before and after high-intensity exercise

	Basal (absolute value)	3h (% of basal)	6h (% of basal)	12h (% of basal)	24h (% of basal)	48h (% of basal)	72h (% of basal)
GPX (U/gHb)	37.07 (0.96)	102.59 (1.71)	100.44 (3.54)	102.94 (2.29)	94.28 (2.53)	98.57 (2.91)	95.94 (1.84)
SOD (U/gHb)	7023 (404.30)	152.65 (36.07)	143.53 (43.49)	167.88 (53.22)	166.54 (52.65)	115.41 (26.15)	155.86 (34.57)
CAT (k/gHb)	1.15 (0.07)	127.99 (8.08)	108.33 (5.58	111.44 (20.66)	129. 63 (11.49)	107.44 (7.52)	96.37 (5.43)
TAS (mM)	2.40 (0.11)	103.87 (4.01)	105.87 (9.53)	128.02 (30.37)	100.92 (3.88)	113.86 (6.37)	104.80 (7.20)
TBARS (mM MDA)	6.88 (0.98)	113.64 (37.11)	125.14 (19.98)	88.66 (11.21)	103.06 (18.17)	133.18 (26.25)	155.16 (34.41)
PBSH (mmol/g protein)	11.40 (1.16)	109.28 (7.52)	112.73 (12.38)	118.28 (5.42)	123.04 (9.85)	108.41 (8.90)	112.63(12 .63)

Data are expressed as the mean (SE); no statistically significant were detected ($p>0.05$); all post-exercise values are data normalized to basal values; PBSH - protein-bound sulfhydryl groups.

FIGURE LEGENDS

Figure 1. Muscle injury markers. Data are the blood creatine kinase (■, CK) and lactate dehydrogenase (●, LDH) levels plotted against time. Data are shown as the mean ± SE of the increase in the normalized activity relative to pre-exercise values (100%). * $p<0.05$ compared to pre-exercise.

Figure 2. White blood cells. (A) Leukocytes, (B) monocytes, (C) neutrophils and (D) lymphocytes. Data are shown as the mean ± SE of the increase in the normalized concentration relative to pre-exercise values (100%).* $p<0.05$ compared to pre-exercise.

Figure 3. Cytokines. (A) MCP-1, (B) TNF-alpha, (C) IL-6 and (D) CRP. Data are shown as the mean ± SE of the increase in the normalized concentration relative to pre-exercise values (100%).* $p<0.05$ compared to pre-exercise.

Figure 4. Neutrophil/lymphocyte ratio and upper extremity strength. The upper extremity strength (■) and the neutrophil/lymphocyte ratio (●) are plotted against time. Data are shown as the mean ± SE of the increase in the normalized activity relative to pre-exercise values (100%).* $p<0.05$ compared to pre-exercise.

FIGURE 1

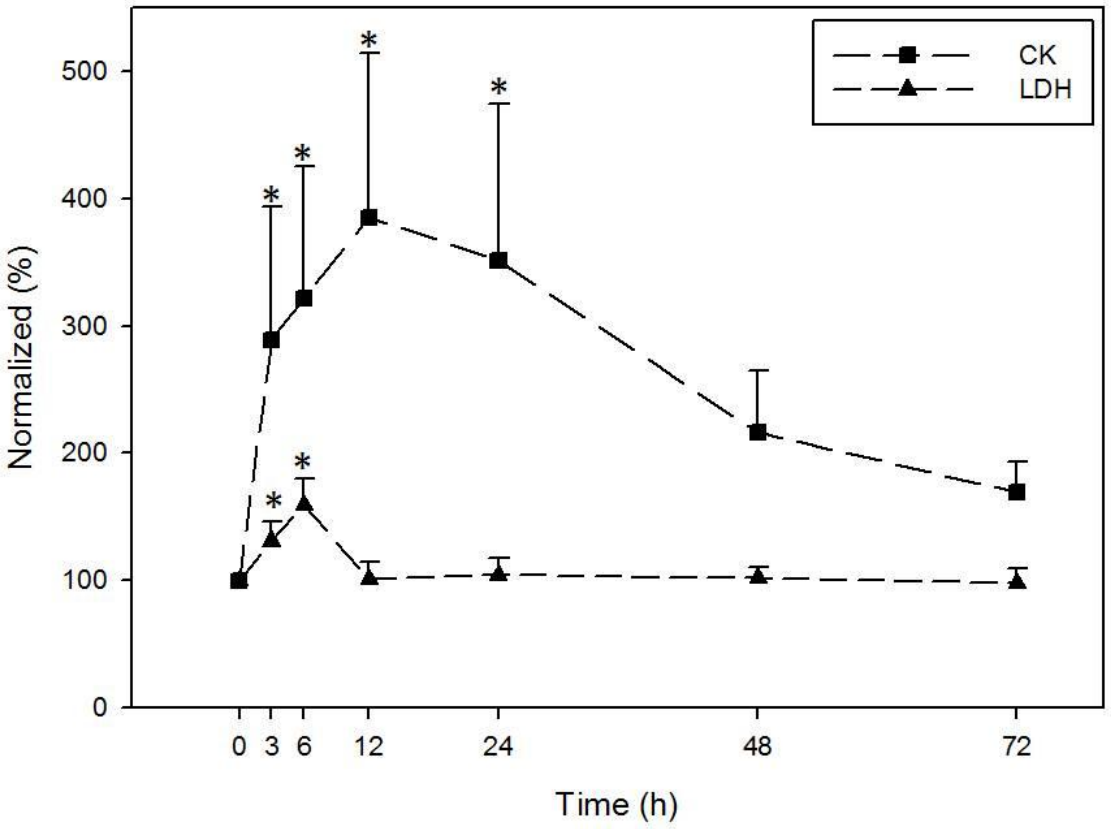


FIGURE 2

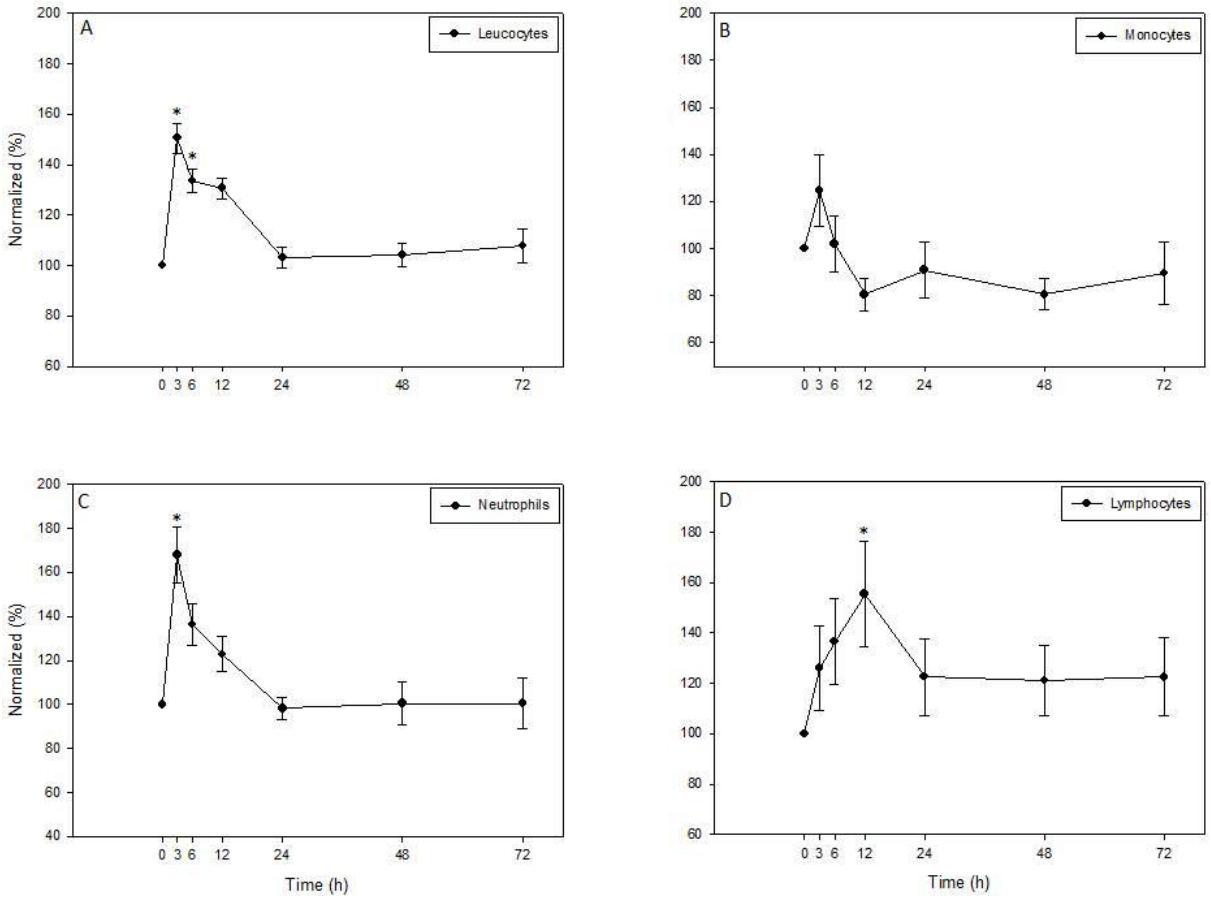

A
Leucocytes
B
Monocytes
C
Neutrophils
D
Lymphocytes
Normalized (%)
Time (h)
0 3 6 12 24 48 72

FIGURE 3

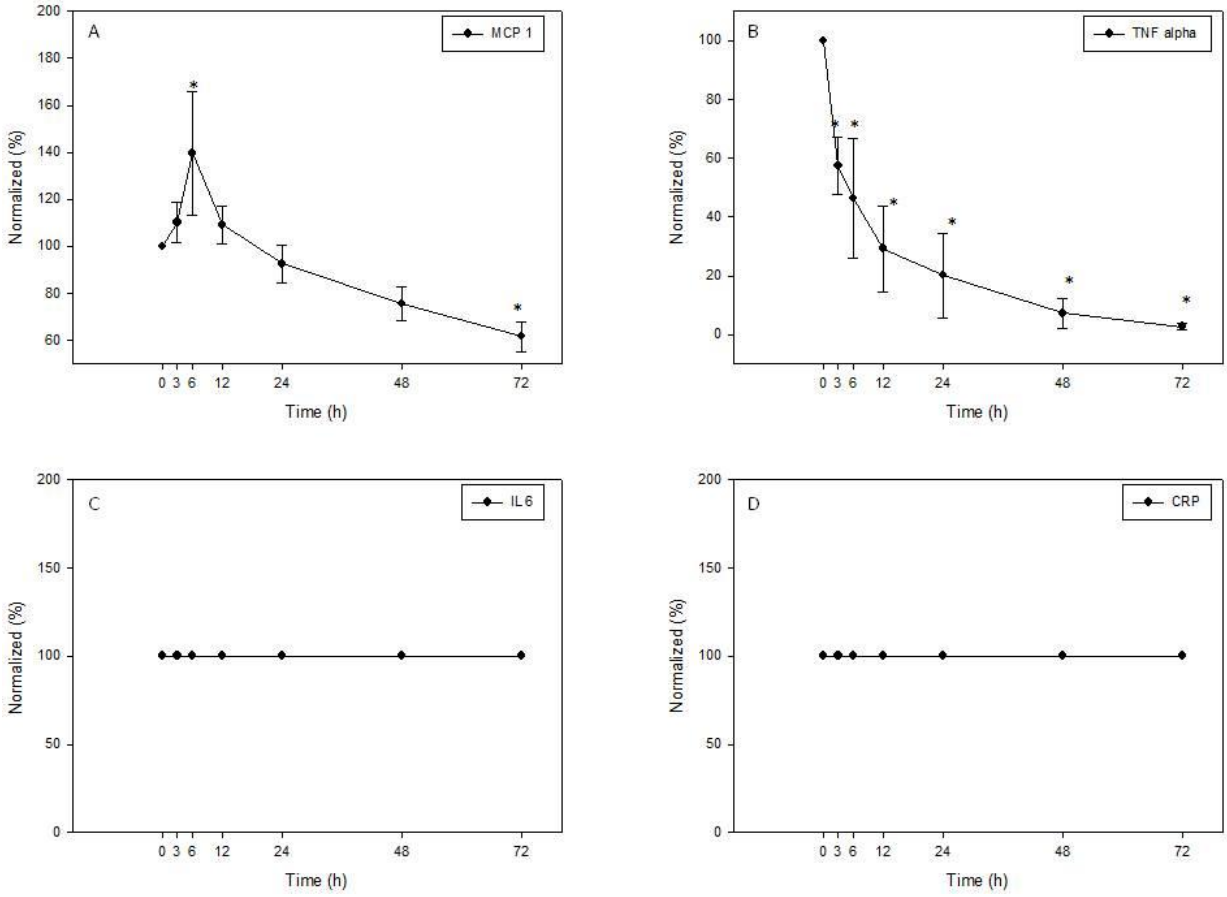
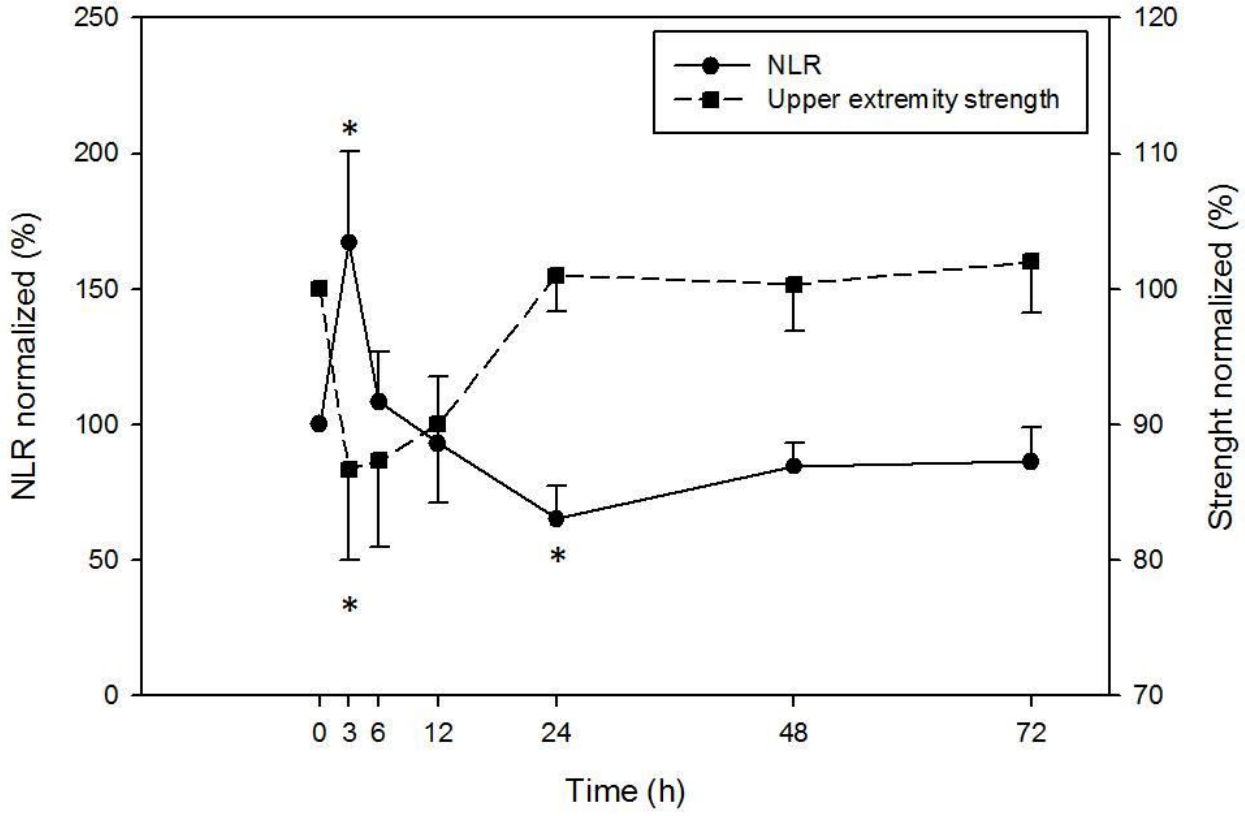

A
MCP 1
Normalized (%)
Time (h)
B
TNF alpha
Normalized (%)
Time (h)
C
IL6
Normalized (%)
Time (h)
D
CRP
Normalized (%)
Time (h)

FIGURE 4


NLR
Upper extremity strength
NLR normalized (%)
Strenght normalized (%)
Time (h)
250
200
150
100
50
0
120
110
100
90
80
70
0
3
6
12
24
48
72
*
*
*

Capítulo 3

A short-term model of endurance exercise training can lower inflammatory response

Autores: Artur Bessa; Foued Espindola; Marius Locke

1 - INTRODUCTION

The most primitive mechanism of cellular protection involves the expression of a polypeptide family named heat shock or stress proteins (HSPs). Some of these HSPs are present in unstressed cells and play an important role in the folding and translocation of polypeptides across membranes. Thus, they have been termed molecular chaperones. HSPs are well known to be expressed in response to a range of cellular stresses such as oxidative stress[4] and physical exercise[5]. Due to this variety of physiological stressors that induce HSPs expression, they are also commonly termed ‘stress proteins[6]. All of the mentioned stresses have in common that they disturb the tertiary structure of proteins and have adverse effects on cellular metabolism[7].

The human Hsp70 family contains 11 distinct genes located on several chromosomes, including both constitutive and inducible isoforms[8]. Hsp72 is the primary Hsp70 family responder and Hsp70B’ is a secondary responder[8].To date, knowledge about Hsp70B’ is limited. Unlike Hsp72, Hsp70B’ is strictly inducible, having no detectable basal level of expression in most cells[9].

NFκβ is a protein complex that controls the DNA transcription found in almost all animal cell types and is involved in cellular stress responses[10]. It belongs to the category of rapid-acting primary transcription factors that are present in cells in an inactive state and do not require new protein synthesis to be activated, allowing NFKB to be a first responder to harmful cellular stimuli[11].

The relationship between physical exercise and HSPs can be consider direct, as exercise is a stress itself, and indirect, as exercise triggers of metabolic responses

known to modulate HSPs expression, such oxidative stress[7] and inflammation[12].

Examining the effects of short-term training in humans is relevant because it can provide important information regarding the mechanisms and time courses of the adaptations observed with long-term training. Structural modifications are one type of adaptation observed with long-term training but appear unlikely to occur with short-term training. It is proposed that changes occurring over a short period of time are attributed to modifications in the physiological functioning of the system. Changes in metabolic function with short-term training include reductions in inosine monophosphate, Cr, P_i and lactate, conservation of phosphocreatine, and improvements in muscle oxidative capacity as indicated by increases in cytochrome c oxidase (COX) and COX subunits II and IV protein contet[13, 14]. While these studies do not provide insight into inflammatory responses to short-term training, they do provide important information about the design and implementation of the training models.

The goal of this study is to assess the effects of a short-term model of endurance exercise training on HSPs expression and transcription factors activation in healthy young males.

2 - METHODS

Subjects

Nine healthy young males (18-31 years) were recruited from the University of Toronto and surrounding area. All subjects participated in less than two hours of

low to moderate physical activity a week and were non-smokers, non-obese, normotensive, and had no prior history of cardiovascular, pulmonary, or other metabolic or musculo-skeletal diseases. All procedures were reviewed and approved by the University of Toronto Research Ethics Board, conforming to the Helsinki Declaration on the use of human subjects and written informed consent was obtained from all subjects prior to participation.

Exercise Test

A graded exercise test to exhaustion was performed on a cycle ergometer 48h before training before and 48h after training (Monark Ergomedic 828 E, Monark Exercise AB; Sweden). Following a brief warm up and familiarization with the testing procedure, participants started cycling for two minutes at a resistance of 0.8 kilopond (kp) (~48-60 watts), with pedaling frequency self-selected between 60-80 rpm. After the first two minutes, workload was increased by 0.3 kp (~18-22 watts) every minute until voluntary exhaustion. Expired gas was analyzed using a semi-automated metabolic cart following a two-point calibration (HRTrak II Heart Rate Tracker, Equilibrated Bio Systems Inc; New York, USA) and VO_2peak was determined from breath-by-breath samples averaged over 20 seconds.

Blood Sampling

Resting blood samples were taken 24h before the exercise training and 24h after and collected via venipuncture performed by a certified phlebotomist into 10 mL EDTA vacuum tubes (Vacutainer).

Short-Term Endurance Exercise Training Program

A short-term training program was utilized similar to previous six-day training models[15-17]. All exercise was performed in a temperature controlled room (22-26˚C, 30-60% humidity). Briefly, subjects cycled for two hours on six consecutive days at a power output (watts) equivalent to 65% of their pre-training VO_2peak. Subjects who were unable to perform continuous exercise at their given intensity were permitted to take short breaks (5 minutes), however they had to complete two hours of exercise during each training session. Water was provided ad libitum and water intake (ml), heart rate (bpm), tympanic temperature (˚C), and rating of perceived exertion (6-20, Borg Scale) were recorded after every 30 minutes of exercise.

Statistical Analysis

All data were normalized to pre-exercise values and are expressed as means ± SE. Statistical significance was calculated using paired t-tests after the Kolmogorov-Smirnov test was carried out to assess the normality of the variable distribution. The level of significance was set at $p < 0.05$.

EMSAs

To determine NFκβ and AP-1 activation, Electrophoretic Mobility Shift Assays (EMSAs) were performed on Leukocyte extracts as previously described[12]. Protein extracts (25 ug) were incubated with approximately 0.1 ng (50,000 cpm) of 32P-labeled NF-κB (5'-AGT TGA GGG GAC TTT CCC AGG C-3' Promega) or AP-1 oligonucleotide (5´-CGC TTG ATG AGT CAG CCG GAA-3´; E3201, Promega) in binding buffer (10% glycerol, 50 mM NaCl, 1.0 mM EDTA 28 [pH, 8.0], 20 mM Tris

[pH, 8.0], 1.0mM DTT, and 0.3mg/ml BSA) and 3.0 ug poly (dI dC) (Pharmacia Fine Chemicals, Piscataway, NJ, USA) for 30 minutes at room temperature. Samples were loaded into 10 or 15 wells per gel of a polymerized, 4% acrylamide gel (5x gel running buffer [pH 8.5], 30% acrylamide, 2% bis-acrylamide, 50% glycerol, 100 ul of 30% APS, and 34 ul of Temed) and electrophoresed at 200 V for ~2 hours. Gels were placed on filter paper and dried using a BioRad Slab dryer (Model 433) for approximately 60 minutes. Once dry, gels were placed in cassettes equipped with intensifying screens and exposed to radiographic film (Bioflex MSI Film. CLMS1417) for 1-4 days at -70°C. Films were developed and scanned using Canon Image J scanner and spot densitometry to quantify mean pixel density for each band was performed using NIH Image J software.

SDS-PAGE and Western Blotting

Following electrophoretic separation, proteins were transferred to nitrocellulose membranes (0.22 um pore size, Bio-Rad Laboratories, Mississauga, Canada, and modified to the Bio-Rad mini-protean II gel transfer system. Briefly, gels were equilibrated in transfer buffer (192 mM glycine, 25 mM Tris-Cl (pH 8.3), 0.1% SDS and 20% methanol) for 10 min, removed and placed into a sandwich consisting of a Brillo pad, 3 pieces of filter paper (Fisher Scientific, Nepean, Ontario, Canada), the nitrocellulose membrane, the gel, 3 more pieces of filter paper, and a second Brillo pad. All components of the sandwich were immersed in transfer buffer prior to, and during assembly. Sandwiches and an ice pack were placed in the gel transfer system. The proteins were transferred to the nitrocellulose membrane at a constant 50 V for 3 h, with an ice pack change at 1.5 h. Following protein transfer, the nitrocellulose membranes were reacted with a polyclonal antibody specific for

HSP72 (CAT# SPA-812, Assay Designs, Plymouth Meeting, PA), HSP60 (CAT# SPA-805, Assay Designs, Plymouth Meeting, PA) or TLR4 (CAT# sc-16240, Santa Cruz Biotechnology, California, USA) diluted 1:1000 in TTBS with 2% nonfat dried skim milk powder and left for 4 hours. Blots were washed three times in TTBS for 5 min each time. Blots were incubated for 1 hour at room temperature in a 1:1000 dilution of goat antirabbit secondary antibody conjugated to alkaline phosphatase (Bio-Rad Laboratories, Hercules, CA, CAT# 170-6515) in TTBS with 2% nonfat dried skim milk powder. Blots were washed twice in TTBS, once in TBS, and developed by immersing in a carbonate buffer (100 mM Na2CO3, 1 mM MgC12, pH 9.8) containing 1 ml of 3% (wt/vol) p-nitro-blue-tetrazolium chloride p-toluidine salt in 70% dimethyl-formamide (DMF) and 1 ml of 15% (wt/vol) 5-bromo-4-chloro30 3-indolyl phosphate in 100% DMF. After development, blots were washed with distilled H2O, dried and scanned using a Canon Image J scanner. For TLR4, spot densitometry was used for the quantification of bands with NIH Image J software (version 1.43).

3 - RESULTS

After a short-term endurance exercise training program the leukocyte HSP72 expression significantly decreased 30% but no alterations were observed on HSP70B' expression. NFκβ activation significantly decreased 16% and no changes were observed on AP1 activation (figure 1).

By the sixth day of training there were significant reductions in the amount of breaks taken (22 $\pm$ 11 minutes *vs*. 12 $\pm$ 6 minutes, p=0.025) and ratings of

perceived exertion (16 ± 1 *vs*. 15 ± 2, p=0.03). Baseline characteristics and VO_2peak data are presented in Table 1. There were no changes in height, weight, BMI or BP following six days of training (p>0.05). There was a PV expansion of 7.8% after six days of exercise (p=0.005). Following training, there were significant increases in the exercise test duration (p=0.001) and the peak power output achieved (p=0.001) during the post-training test; however heart rate at VO_2peak was significantly lower (p=0.001). Consequently, post-training VO_2peaks were determined by linear extrapolation of heart rate-VO_2peak graphs. The predicted post-training values demonstrated a significant increase in VO_2peak following six days of training (p=0.02). There was a trend towards a lower heart rate at anaerobic threshold after training (p=0.063) (table 1).

4 - DISCUSSION

This study investigated the effects of a short-term model of endurance exercise training on Inflammatory function in healthy young males. HSP72 decreased expression showed that the leukocytes were in a lesser stressed state after the exercise program[5]. As HSP70B' is a secondary responder to stress, the lack of alterations showed after the exercise program suggests that the exercise program proposed was intense enough to generate a first response but not enough to trigger a second response[8].

To further evaluate the inflammatory response after the short term endurance exercise training , the transcription factors NFκβ and AP-1 were also assessed. NFκβ activation decrease corroborate with the interpretation that these cells were

in a lesser stressed state after the exercise training, as NFκβ activation is known to increase in response to a variety of stress stimuli[18] . Visual assessment showed that AP-1 activation was detected in cells from both groups but no significant differences were observed when comparing pre and post exercise samples, suggesting that AP-1 transcription is not significantly altered after a short term exercise program.

It has been proposed that regular exercise has an anti inflammatory effect protecting against low magnitude systemic inflations[2, 3]. In conclusion, the short-term endurance exercise proposed can lower inflammatory response by decreasing HSP72 expression and NFκβ activation in leukocytes, both known fast responders to stress[6, 10].

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Table 1 Subject Characteristics and Peak Oxygen Consumption

Variable	Pre-	Post-
Body mass (kg)	79.1 ± 10.8	79.4 ±10.7
BMI (kg/m^2)	24 ± 3	24.1 ± 3.2
Brachial Systolic BP (mmHg)	123 ± 12	122 ± 12
Brachial Diastolic BP (mmHg)	70 ± 6	69 ± 5
Relative VO_2peak ($ml \cdot kg^{-1} \cdot min^{-1}$)	42.8 ± 6	44.1 ± 5.5 ‡
Respiratory Exchange Ratio	1.26 ± 0.06	1.24 ± 0.08
Peak HR (bpm)	187 ± 11	182 ± 10
AT ($ml \cdot kg^{-1} \cdot min^{-1}$)	27.7 ± 5.0	28.3 ± 3.9
HR at AT (bpm)	151 ± 20	142 ± 12
Power Output at AT (watts)	162 ± 38	166 ± 34
Exercise Duration (s)	677 ± 100	730 ± 100 ‡
Peak Power Output (watts)	250 ± 39	270 ± 34 ‡

Values are mean ± SD. HR, heart rate; AT, anaerobic threshold.
‡$p<0.001$ for pre vs. post.

FIGURE

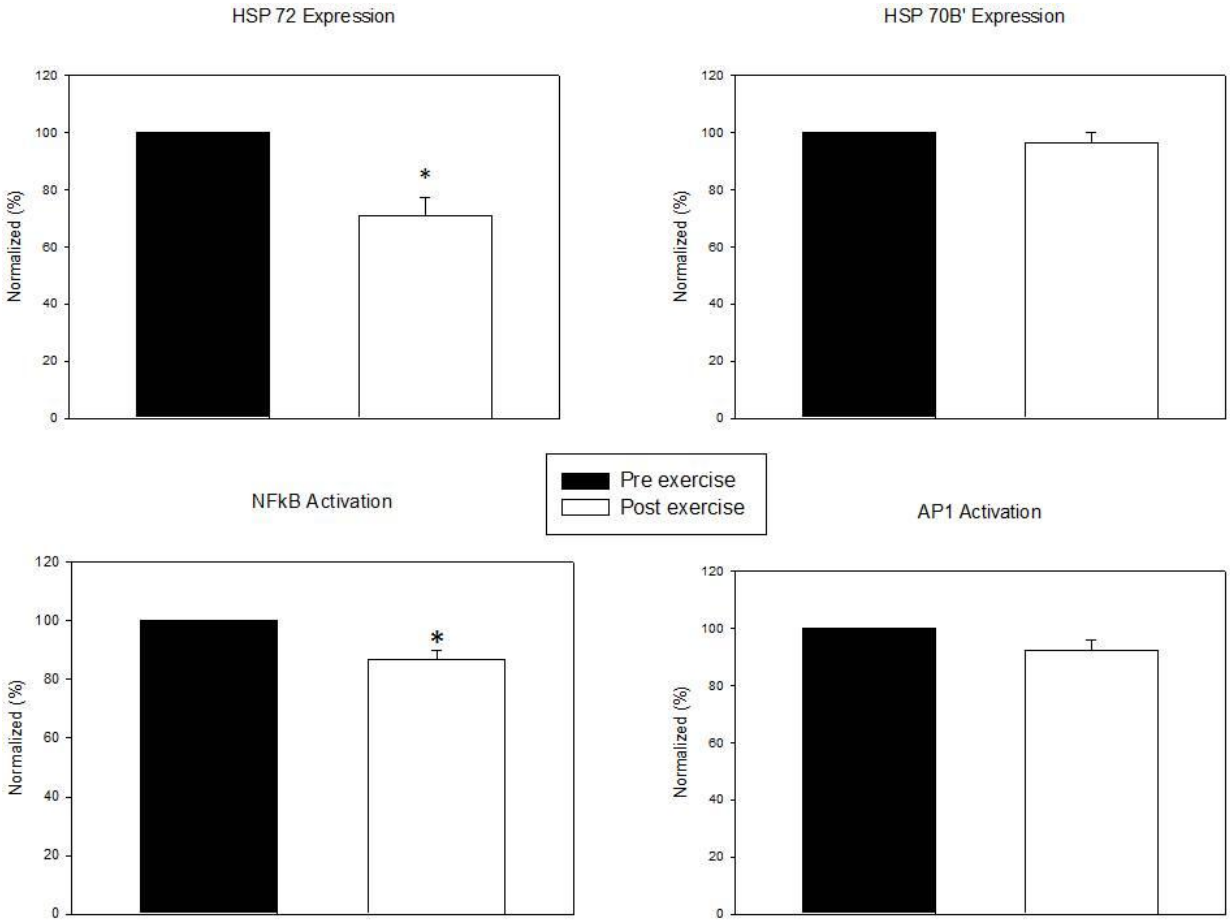

Figure 1 – HSP72 expression and NFKB expression are reduced after a short term endurance exercise program