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Juliano Marchi Vieira

POTENCIAIS EFEITOS DA CAFEÍNA SOBRE PARÂMETROS BIOQUÍMICOS E INFLAMATÓRIOS EM RATOS SUBMETIDOS AO TREINAMENTO INTERVALADO DE ALTA INTENSIDADE

Santa Maria, RS

2017

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Tese apresentada ao Curso de Doutorado do Programa de Pós-Graduação em Ciências Biológicas: Bioquímica Toxicológica do Centro De Ciências Naturais e Exatas, da Universidade Federal De Santa Maria (UFSM, RS), como requisito parcial para a obtenção do título de **Doutor em Ciências Biológicas: Bioquímica Toxicológica**.

Orientadora: Roselia Maria Spanevello

Santa Maria, RS
2017

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Dedico este trabalho a minha família.
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pela base, ensinamentos e valores que me foram proporcionados.
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“A nossa sorte está em nossas próprias mãos, como está nas mãos do escultor a matéria-prima que ele converterá em obra de arte. Com essa atividade artística acontece o mesmo que com todas as outras: simplesmente nascemos com o potencial de fazê-lo. A habilidade para moldar o material no objeto almejado deve ser aprendida e cultivada com empenho”.

– Johann Von Goethe, escritor e pensador alemão –

RESUMO

POTENCIAIS EFEITOS DA CAFEÍNA SOBRE PARÂMETROS BIOQUÍMICOS E INFLAMATÓRIOS EM RATOS SUBMETIDOS AO TREINAMENTO INTERVALADO DE ALTA INTENSIDADE

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A cafeína tem sido usada como uma substância ergogênica com a finalidade de melhorar a performance atlética ou atenuar os mecanismos geradores de fadiga. No entanto, o efeito ergogênico da cafeína em exercícios de alta intensidade permanece controverso e pouco esclarecido, parecendo ser dependente de alguns fatores como duração do exercício, protocolo utilizado e estado de treinamento dos indivíduos. O objetivo do presente trabalho foi avaliar os feitos da cafeína em parâmetros bioquímicos musculares, neuroquímicos e inflamatórios em ratos submetidos ao treinamento intervalado de alta intensidade (HIIT). Os animais foram divididos em seis grupos: controle, cafeína 4 mg/kg, cafeína 8 mg/kg, HIIT, HIIT/cafeína 4 mg/kg e HITT/ cafeína 8 mg/kg. Os ratos foram treinados utilizando-se um protocolo de natação, três vezes por semana, durante 6 semanas, totalizando uma carga de trabalho de 23% do peso corporal no final do experimento. A cafeína foi administrada oralmente 30 minutos antes da sessão de treinamento. Em músculo foi avaliado níveis de glicogênio, atividade das enzimas acetilcolinesterase (AChE) e Ca^{2+}ATPase e alterações histológicas. Em sistema nervoso foi avaliado o comportamento ansiolítico, a atividade da enzima Na^{+} K^{+} ATPase e parâmetros de estresse oxidativo. O impacto da cafeína e do HIIT na sensibilidade e proliferação dos linfócitos, níveis de IL-6 e IL-10 e atividade das enzimas NTPDase, adenosina desaminase (ADA) e AChE em linfócitos também foi determinado. Nossos resultados demonstraram um aumento nos níveis glicogênio em todos os grupos quando comparado ao grupo controle. HIIT aumentou a espessura do ventrículo esquerdo bem como causou um aumento na atividade de Ca^{2+} ATPase (67,43%- agudo e 34,51% - crônico) e uma diminuição na atividade da AChE (20,69%) em músculo gastrocnêmio. O tratamento com cafeína preveniu as alterações nas atividades enzimáticas, bem como a adaptação à hipertrofia ventricular esquerda induzida pelo HIIT. O treinamento induziu um comportamento ansiolítico e aumentou a atividade da Na^{+} K^{+} ATPase (46,13% em córtex cerebral e 50,13%em hipocampo) e da glutationa peroxidase (38%) os níveis de TBARS (60% e 37,66%) e alterou a atividade da superóxido dismutase e da catalase em córtex cerebral, hipocampo e estriado de ratos. O tratamento com cafeína foi capaz de prevenir as alterações em sistema nervoso causada pelo HIIT. Quando cafeína foi associada com HIIT ocorreu um aumento na proliferação de linfócitos T (147, 61%) e na sensibilidade destas células a glicocorticoides. No protocolo crônico o HIIT induziu a diminuição na hidrólise de ATP (19,88%) e ADP (31,28%) em linfócitos sendo que cafeína foi capaz de reverter apenas alterações na hidrólise de ATP. HIIT causou um aumento nas atividades ADA (30,27%) e AChE (54,76%) nos linfócitos e este efeito foi mais pronunciado em ratos treinados e tratados com cafeína (95,99%). O nível de IL-6 foi aumentado (138,5%) enquanto que o nível de IL-10 foi diminuído (41%) em animais treinados (HIIT) e a cafeína foi capaz de reverter este efeito. Nossos resultados demonstram que a cafeína pode modular vias cruciais para a contração muscular além de prevenir alterações inflamatórias e no estado redox induzidas pelo HIIT.

Palavras chaves: exercício, cafeína, linfócitos, acetilcolinesterase, Na^{+} K^{+} ATPase, estresse oxidativo, Ca^{2+} ATPase, ectonucleotidases, citocinas

ABSTRACT

EFFECTS OF CAFFEINE ON MUSCULAR BIOCHEMICAL, NEUROCHEMICAL AND INFLAMMATORY PARAMETERS OF RATS SUBMITTED TO HIGH-INTENSITY INTERVAL TRAINING

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Caffeine has been used as ergogenic substance for the purpose of improving athletic performance or attenuating fatigue mechanisms. However, the ergogenic effect of caffeine on high-intensity exercise remains controversial and unclear, others factors such as exercise duration, protocol used, and individuals' training status may interfere in response. Objective of this study was to evaluate the effects of caffeine on biochemical muscle, neurochemical and inflammatory parameters in rats submitted to high-intensity interval training (HIIT). The animals were also divided in six groups: Control group, Caffeine 4 mg, Caffeine 8 mg, HIIT, HIIT + caffeine 4 mg and HIIT + caffeine 8 mg. The rats of groups HIIT, HIIT + 4mg and HIIT + 8mg were trained three times a week for 6 weeks for a total workload 23% of the body weight at the end of the experiment. The exercise protocol (Swimming) was performed three times per week on alternate days (48h of recovery between sessions) always at the same time. Caffeine was administered 30 minutes before training, orally. In muscle was evaluated glycogen levels, enzymes acetylcholinesterase/Ca^{2+}-ATPase activity and histological changes. In CNS was evaluated anxiolytic behavior, enzyme Na^{+}, K^{+}-ATPase activity and oxidative stress parameters. In lymphocytes, was evaluated the effects of caffeine and HIIT on proliferation, cellular immune response to phytohemagglutinin (PHA) and cytokines (IL-06 – IL-10). Our results demonstrated an increase in glycogen levels all groups when compared to control group. HIIT increased thickness in the left ventricle as well as caused an increase in Ca^{2+}-ATPase (67,43%- acute and 34,51% - chronic) activity and decrease in AChE (20,69%) activity in gastrocnemius muscle. Caffeine treatment prevented changes in enzymatic activities, as well as adaptation to left ventricular hypertrophy induced by HIIT. HIIT induced anxiolytic behavior and increased the activity of Na^{+},K^{+}-ATPase (46,13% cerebral cortex and 50,13% hippocampus) and glutathione peroxidase (38%), TBARS levels (60% - cerebral cortex and 37,66% - hippocampus) and altered the activity of superoxide dismutase and catalase in cerebral cortex, hippocampus and striatum of rats. Caffeine treatment was able to prevent CNS alterations HIIT-induced. In T lymphocytes, caffeine associated to HIIT promoted an increase in T lymphocyte proliferation (147, 61%) and glucocorticoid sensitivity. HIIT induced a decrease in ATP (19,88%) and ADP (31,28%) hydrolysis lymphocytes already caffeine was able to reverse only alterations in the ATP hydrolysis. HIIT caused an increase in ADA (30,27%) and AChE (54,76%) activities in lymphocytes and this effect was pronounced in rats trained and treated with caffeine. IL-6 level (138,5%) was increased while IL-10 level (41%) was decreased in trained animals (HIIT) and caffeine was able to reverse this effect. Our results demonstrate that caffeine can modulate pathways for muscle contraction in addition to preventing inflammatory, redox state changes induced by HIIT.

Key-words: Exercise, caffeine, lymphocytes, acetylcholinesterase, Na^{+}, K^{+}-ATPAse, oxidative stress, Ca^{2+}-ATPase, ectonucleotidases, cytokines.

LISTA DE ABREVIAÇÕES

•OH	Radical Hidroxila
A_1R	Receptor de adenosina A_1
$A_{2A}R$	Receptor de adenosina A_{2A}
$A_{2B}R$	Receptor de adenosina A_{2B}
ADA	Adenosina Deaminase
ADO	Adenosina
ADP	Adenosina Difosfato
AMP	Adenosina Monofosfato
AMPc	Adenosina Monofosfato cíclico
AMPK	Proteína Quinase ativada pelo AMP
ATP	Adenosina Trifosfato
Ca^{2+}	Íon Cálcio
CAT	Catalase
CYP 1A1	Citocromo P450, família 1, membro 1ª
DNA	Ácido Desoxirribonucleico
eN	Ecto-5'-nucleotidase
ENPPs	Ectonucleotídeo Pirofosfato/fosfodiesterases
ERO	Espécies Reativas de Oxigênio
GABA	Ácido Gama Aminobutírico
GLUT	Transportadores de Glicose
GPx	Glutationa Peroxidase
GSH	Glutationa Reduzida
H_2O_2	Peróxido de Hidrogênio
HIIT	Exercício Intervalado de Alta Intensidade
IFN	Interferon
IL	Interleucina
IP3	Inositol Trifosfato
K^+	Íon Potássio
LDL	Lipoproteína de Baixa Densidade
MDA	Malondialdeído
Mn	Manganês
Na^+	
NAD	Íon Sódio
NADPH	Nicotinamida Adenina Dinucleotídio

NFκβ	Nicotinamida Adenina Dinucleotídio Fosfato Reduzido
NO	Fator Nuclear Kappa β
O_2	Oxido Nítrico
O_2^-	Oxigênio Molecular
$P2X_1R$	Ânion Superóxido
$P2Y_1R$	Receptor $P2X_1$
$P2Y_{12}R$	Receptor $P2Y_1$
RE	Receptor $P2Y_{12}$
RyR	Retículo Endoplasmático
SH	Receptor de Rianodina
SR	Grupos Tióis
SS	Retículo Sarcoplasmático
SNC	Estado estável *"Steady state"*
SOD	Sistema Nervoso Central
TNF-α	Superóxido Dismutase
VO_2max	Fator de Necrose Tumoral Alfa
	Consumo Máximo de Oxigênio

SUMÁRIO

1. INTRODUÇÃO

Exercício físico é qualquer atividade física praticada de forma sistematizada, que mantém ou aumenta a aptidão física em geral podendo ser usado para promoção da saúde ou para melhorar a *performance* física (Guedes, 2006). Estudos epidemiológicos vêm demonstrando a importância da prática de atividade física realizada de forma regular no combate ao sedentarismo, bem estar físico e psicossocial (Cotman e Berchtold, 2002; Antunes, 2006) uma vez que o exercício provoca uma série de benefícios ao sistema nervoso central (SNC) incluindo a formação de novas células nervosas (Shen, Tong *et al.*, 2001; Cotman e Berchtold, 2002; Mattson, Duan *et al.*, 2004); aumento nos níveis de serotonina, endorfinas, noradrenalina (Mustroph, Chen *et al.*, 2012), bem como, elevação nos níveis de atividade cardiovascular e locomotora (Mattson, Duan *et al.*, 2004).

O treinamento intervalado pode ser classificado com base na sua intensidade em: treinamento intervalado de alta intensidade ou HIIT ("*High Intensity Interval Training*"), treinamento intervalado de explosão ou SIT ("*Sprint Interval Training*") e treinamento contínuo (Macinnis e Gibala, 2016). O HIIT é definindo como esforço submáximo ou muito próximo do máximo, geralmente, em intensidades superiores a 80% do VO_2máx com baixo volume e breves períodos de recuperação (Zwetsloot, John *et al.*, 2014; Macinnis e Gibala, 2016). Cabe ressaltar que na busca de um desempenho esportivo de alto nível tem-se utilizado inúmeros recursos ergogênicos com o objetivo de potencializar a performance atlética ou atenuar os mecanismos geradores de fadiga (Gomes et al., 2002). Neste sentido, a cafeína tem sido utilizada como substância ergogênica (tem por objetivo aumentar *performance* física, prevenindo o início da fadiga ou aumentando a potência/força muscular) previamente a realização de exercícios de resistência e alta intensidade (Graham, 2001; Ganio, Klau *et al.*, 2009).

É bem estabelecido na literatura que a cafeína possui propriedade estimulante (Armstrong, Pumerantz *et al.*, 2005), antioxidante (Arnaud, 2006), neuroprotetora (Rosso, Mossey *et al.*, 2008) e anti-inflamatória (Horrigan, Kelly *et al.*, 2006). Acredita-se que a cafeína como recurso ergogênico possua mecanismos de ação central e periférica que podem desencadear importantes alterações metabólicas e fisiológicas melhorando assim a *performance* física via retardo da instalação do processo de fadiga (Graham, Hibbert *et al.*, 1998a). Por possuir propriedades lipofílicas, a cafeína ultrapassa a barreira hematoencefálica inibindo as fosfodiesterases e mobilizando cálcio (Ca^{2+}) (Davis, Zhao *et al.*, 2003; Ribeiro e

Sebastiao, 2010). A cafeína também atua bloqueando os receptores de adenosina no SNC, consequentemente, isso pode estar relacionado a um retardo na sensação de cansaço, aumentando, por sua vez, o desempenho em exercícios de resistência (Davis, Zhao *et al.*, 2003; Tarnopolsky, 2008; Davis e Green, 2009). Além disso, ao impedir a interação de adenosina com seu receptor, este composto provoca uma série de respostas incluindo liberação de catecolaminas (Graham, Hibbert *et al.*, 1998a; Arnaud, 2006) aumento da pressão sanguínea, liberação de adrenalina/insulina, ritmo cardíaco, lipólise e ativação do SNC (Davis, Zhao *et al.*, 2003; Tarnopolsky, 2008; Davis e Green, 2009). Contudo, os efeitos e o mecanismo de ação da cafeína associado ao exercício, especialmente ao HIIT, ainda são controversos.

A acetilcolinesterase (AChE) é uma importante enzima regulatória responsável pela hidrólise do neurotransmissor acetilcolina nas sinapses do SNC bem como na junção neuromuscular (Soreq e Seidman, 2001). Tem sido demonstrado também que uma redução na atividade desta enzima leva alterações na função muscular incluindo as propriedades contráteis e a falta de resistência à fadiga (Mouisel, Blondet *et al.*, 2006; Vignaud, Fougerousse *et al.*, 2008). Por outro lado, acetilcolina também pode alterar muitas funções dos linfócitos, desta forma, tem disso demonstrado que a AChE possui um papel crucial na regulação das respostas imunes e inflamatórias (Kawashima e Fujii, 2003; 2004). Neste contexto, trabalhos prévios tem demonstrado que o exercício físico pode modular a atividade da AChE em linfócitos (Cardoso, Abdalla *et al.*, 2014).

A Ca^{2+} ATPase e a Na^{+}, K^{+} ATPase são enzimas chaves na manutenção de gradientes de eletróliticos em células excitáveis como as musculares e os neurônios (Verbist, Gadella *et al.*, 1991; Panayiotidis, Bortner *et al.*, 2006; Huang, Nagaraja *et al.*, 2010; Jimenez, Sanchez *et al.*, 2010). As alterações transitórias nos níveis intracelulares de Ca^{2+} regulam uma grande variedade de vias de sinalização e vários processos fisiológicos e patológicos (Callewaert, Parys *et al.*, 2003; Ruknudin e Lakatta, 2007; Huang, Nagaraja *et al.*, 2010). A Na^{+}, K^{+} ATPase é responsável pelo transporte ativo de Na^{+} e K^{+}, sendo crucial para manter o gradiente iônico através da membrana e, assim, regular a excitabilidade neuronal (Kaplan, 2002; Jorgensen, Hakansson *et al.*, 2003; Jimenez, Sanchez *et al.*, 2010). Neste contexto, uma diminuição da atividade e expressão da Na^{+} K^{+}-ATPase afeta diretamente a sinalização de neurotransmissores, prejudicando a aprendizagem e a memória, bem como a atividade locomotora e comportamento de ansiedade em ratos (Lingrel, Williams *et al.*, 2007; Moseley, Williams *et al.*, 2007). Embora o mecanismo molecular que regula o efeito mediado pelo

exercício físico sobre os genes que codificam a Na^+, K^+-ATPase ainda não foram completamente elucidados (Thompson e Mcdonough, 1996).

Dados da literatura também apontam que diferentes protocolos de HIIT estão relacionados ao aumento de espécies reativas de oxigênio e, consequente, resposta adaptativa do sistema de defesa antioxidante (Bogdanis, Stavrinou *et al.*, 2013; Gillen e Gibala, 2014; Pimenta, Bringhenti *et al.*, 2015). Além disso, HIIT tem sido associado a alterações imunes, incluindo a redução da função linfocitária e níveis elevados de citocinas pró – inflamatórias (Mars, Govender *et al.*, 1998; Gleeson, 2007)

A ativação de linfócitos assim como a libertação de citocinas está relacionada com sinalização mediada pelo ATP e adenosina (Junger, 2011). No entanto, a eficácia destas moléculas é dependente de algumas enzimas específicas, que são expressas na membrana das células imunes (Junger, 2011). O ATP liberado para o espaço extracelular pode ser rapidamente hidrolisado para ADP e AMP pela NTPDase. O AMP produzido pode ser convertido em adenosina pela ação da 5'- nucleotidase, e a adenosina pode ser degradada em inosina pela adenosina desaminase (ADA). Alguns estudos tem demonstrado que protocolos de exercício podem alterar a atividade destas enzimas em soro (Siqueira, Elsner *et al.*, 2010; Moritz, Teixeira *et al.*, 2016), bem como diminuir a expressão da NTPDase em linfócitos de ratos (Cardoso, Abdalla *et al.*, 2015).

Neste contexto, este estudo teve como objetivo avaliar as alterações em enzimas chaves na manutenção dos gradientes de concentração iônicos e nas respostas imunes tais como Na^+,K^+-ATPase, Ca^{2+}-ATPase, AChE, NTPDase e ADA em ratos submetidos a um protocolo de HIIT e tratados com cafeína nas doses de 4 e 8 mg/kg. Em adição a estas análises, o presente trabalho também avaliou parâmetros de ansiedade e estresse oxidativo em SNC, níveis de glicogênio muscular e possíveis alterações histológicas em músculo de ratos submetidos ao exercício de alta intensidade e/ou tratados com cafeína.

2. REVISÃO BIBLIOGRÁFICA

Exercício físico pode ser definido como qualquer atividade física realizada de forma estruturada, planejada, levando-se em conta as variáveis fisiológicas (Gomes, Silva *et al.*, 2012). Existem diferentes tipos de exercícios: aeróbico ou anaeróbico, de *endurance* ou de força (Hawley, Hargreaves *et al.*, 2014). De acordo com a especificidade do treinamento, exercícios aeróbicos caracterizam-se por utilizarem prioritariamente o metabolismo aeróbico melhorando a resistência à fadiga, enquanto que exercícios anaeróbicos estão mais ligados ao treinamento resistido promovendo aumento na massa muscular e produção de força (Egan e Zierath, 2013; Hawley, Hargreaves *et al.*, 2014).

Os exercícios aeróbicos são caracterizados pela utilização de oxigênio (O_2) e estão associados a melhora na capacidade oxidativa (Hawley, Hargreaves *et al.*, 2014). São exercícios de longa duração e baixa intensidade onde o exercício é sustentando de forma contínua utilizando prioritariamente a via oxidativa de fornecimento de energia, ou seja, a maior parte do ATP (adenosina trifosfato) é gerado pela fosforilação oxidativa. Este tipo de exercício aprimora, principalmente, a adaptação das fibras tipo I (vermelhas, de contração lenta) com grande capacidade oxidativa, densidade capilar e mitocondrial, e grandes estoques intracelulares de triglicerídeos o que lhes confere uma alta resistência (Maughan, 2005). Este tipo de exercício possui um papel importante na aptidão física, pois fornecem suporte fisiológico para que novas cargas de trabalho sejam implementadas (Tang, Sibley *et al*., 2009; Armstrong, Tomkinson *et al*., 2011). Sendo assim, ao se iniciar um exercício aeróbico, o consumo de O_2 aumenta muito nos primeiros minutos até atingir um estado estável (Steady State) definido como o ponto de equilíbrio entre a energia necessária pelos músculos (consumo) e a produção pelo metabolismo aeróbico (Raper, Love *et al.,* 2014). Este é o ponto em que todo o lactato gerado não se acumula sendo, posteriormente, reconvertido à glicose pelo ciclo de Cori (Madrid, Oliveira Pires *et al*., 2016). O consumo máximo de oxigênio (VO_2max) é o ponto em que ocorre um platô na utilização de oxigênio celular, ou seja, aumenta-se o ritmo do exercício mas o consumo permanece inalterado (Sloth, Sloth *et al.*, 2013).

Por outro lado, os exercícios anaeróbicos apresentam características diferentes, pois as fibras rápidas e glicolíticas ou do tipo II, produzem em curto período de tempo (< 30s) alta intensidade utilizando-se de substratos energéticos como ATP, creatina fosfato (Cr) e glicose. Estas fibras possuem alta capacidade metabólica anaeróbica, as quais possuem altos níveis

intramusculares de fosfocreatina e glicogênio muscular como substratos energéticos predominantes e, por isso, a limitação no tempo de esforço (Staron, Hagerman *et al.*, 2000). Este fornecimento de energia se dá pela degradação anaeróbica da glicose pela glicólise. Sob condições de hipóxia a produção de nicotinamida adenina dinucleotídeo reduzido (NADH) gerado pela glicólise anaeróbica é muito superior à capacidade da célula de regenerar o NADH em nicotinamida adenina dinucleotídeo oxidado(NAD^+) (Li, Dash *et al.,* 2009). Este excesso na produção de NADH resulta na redução da molécula de piruvato à lactato pela ação da enzima lactato desidrogenase (LDH) (Li, Dash *et al.,* 2009). Quando a intensidade do exercício é aumentada, o lactato sanguíneo começa a acumular-se exponencialmente no sangue (limiar de lactato) (Lantis, Farrell *et al.,* 2016). A partir deste ponto para que o exercício continue é necessário o fornecimento de energia por processos metabólicos que não envolvem o O_2 (Edwards, Challis *et al*., 1999; Lantis, Farrell *et al.*, 2016), caracterizando assim o exercício anaeróbico. Desta forma, ocorre um platô onde a demanda do exercício excede a capacidade de fornecimento de energia pela via oxidativa o que é definido como limiar de lactato. Ou seja, nada mais é do que o ponto onde a produção de lactato excede a capacidade de reconversão à piruvato.

É bem estabelecido que o treinamento intervalado pode provocar adaptações especificas tanto ao treinamento de *endurance* quanto ao treinamento de força (Macinnis e Gibala, 2016). Por exemplo, um protocolo de treinamento intervalado semelhante ao teste de Wingate é um potente estimulador do conteúdo mitocondrial e da capacidade aeróbica (Tabata, Irisawa *et al.*, 1997; Burgomaster, Howarth *et al.*, 2008), enquanto que o treinamento intervalado utilizando o peso corporal como resistência aumenta VO_2 máx e força muscular (Tabata, Nishimura *et al.*, 1996; Mcrae, Payne *et al.*, 2012; Ramos-Campo, Rubio-Arias *et al.*, 2016).

Inúmeras são as variáveis utilizadas para a prescrição do exercício (Buchheit e Laursen, 2013; Tschakert, Kroepfl *et al.*, 2015). Dentre elas, a intensidade pode ser manipulada de diversas formas como: quantidade de carga, tempo de recuperação breve, tipo de contrações utilizadas (concêntrica, excêntrica ou isométrica) e velocidade de execução (Silva, Oliveira *et al.*, 2014). Desta forma, o treinamento intervalado pode ser classificado com base na sua intensidade em: treinamento intervalado de alta intensidade (HIIT – *High Intensity Interval Training*), treinamento intervalado de explosão (SIT - *Sprint interval training*) e o treinamento contínuo (TC) em intensidade moderada (< 75% do VO_2max) (Macinnis e Gibala, 2016). O HIIT é definido como esforço submáximo ou muito próximo do

máximo, geralmente, em intensidades superiores a 80% do VO_2max com baixo volume e breves períodos de recuperação (Zwetsloot, John *et al.*, 2014; Macinnis e Gibala, 2016). O SIT é caracterizado por esforços máximos ou supramáximos, porém intervalos de recuperação maiores (Sloth, Sloth *et al.*, 2013; Martin, Buchan *et al.*, 2015). Já o TC é usado para fins comparativos onde o exercício é realizado de forma contínua e com intensidades moderadas (Macinnis e Gibala, 2016).

Vários estudos tem demonstrado que o HIIT provoca alterações especificas no sistema de fornecimento de energia, representados por aumentos significativos no VO_2 max (Tabata, Nishimura *et al.*, 1996; Burgomaster, Hughes *et al.*, 2005; Terada, Kawanaka *et al.*, 2005) e alterações mitocondriais (Steiner, Murphy *et al.*, 2011; Zhang, Wu *et al.*, 2012; Macinnis e Gibala, 2016). Entretanto, nas últimas décadas, também tem se discutido as alterações que ocorrem na resposta imunológica no organismo em relação a esse tipo de exercício. Sabe-se que o estresse fisiológico provocado por um protocolo de exercício ativa diversos sistemas tais como neuroendócrino e imune (Pedersen e Toft, 2000; Taub, 2008). Entretanto, a magnitude do estresse provocado depende de fatores como: estado nutricional, efeitos a sessões agudas ou crônicas, intensidade (velocidade, carga, intervalos de recuperação) e volume (frequência, tempo, séries, repetições) (Fragala, Kraemer *et al.*, 2011).

A resposta imune celular tem se mostrado efetiva ao exercício, principalmente pelo aumento de linfócitos circulantes durante uma sessão de exercício aeróbico (Gleeson, 2007). Porém, em atletas bem treinados há uma redução das células T, inibição na produção de citocinas (IL-4, IL-10) e queda na síntese de imunoglobulinas, o que predispõe o organismo a infecções especialmente do trato respiratório (Gleeson, Walsh *et al.*, 2012). Estudos tem documentado também uma redução no número de linfócitos durante a recuperação (2-6 horas pós-exercício) em exercícios de *endurance* (>1h, em intensidade que varia de 55 a 75% do VO_2max). Todavia, exercícios extenuantes com alto volume, ou em intensidades supramáximas parecem ter respostas distintas. Certamente, a magnitude desta resposta depende da intensidade e volume do protocolo de exercício adotado (Fisher, Schwartz *et al.*, 2011; Kruger e Mooren, 2014; Ahmadizad, Avansar *et al.*, 2015; Cullen, Thomas *et al.*, 2016).

As citocinas são mediadores importantes da resposta aguda ao exercício físico. Frequentemente associadas à propagação da resposta imunológica, as citocinas se ligam a receptores específicos nas células-alvos, desencadeando vias de transdução de sinal (Tonet, Karnikowski *et al.*, 2008). As citocinas são produzidas por subpopulações de células como

linfócitos T e macrofágos, sendo capazes de influenciar a atividade, a diferenciação e a proliferação de várias células imunes (De Oliveira, Sakata *et al*., 2011). As citocinas podem ser pró-inflamatórias como, por exemplo, a interleucina-1β (IL-1β), interleucina-2 (IL-2), interleucina-6 (IL-6), interleucina-8 (IL-8), fator de necrose tumoral - alfa (TNF-α - *Tumor necrosis fator-alpha*) e o interferon-gama (IFN-γ) (De Miguel, Kraychete *et al.*, 2014) ou anti-inflamatórias como a interleucina-4 (IL-4) e a interleucina-10 (IL-10) (De Miguel, Kraychete *et al.*, 2014).

Modelos experimentais e estudos em humanos têm demonstrado que o exercício moderado exerce papel anti-inflamatório (Gleeson, Bishop *et al.*, 2011), enquanto que o exercício de alta intensidade pode aumentar o risco de adquirir infecções (Pedersen e Toft, 2000; Moreira, Delgado *et al.*, 2009; Gleeson, Bishop *et al.*, 2013). Os níveis de citocinas pro tendem a aumentar após o exercício de alta intensidade, porém, a magnitude do exercício é crucial para que não haja uma superexpressão de citocinas pro-inflamatórias (De Almeida, Gomes Da Silva *et al.*, 2013). Zwetsloot e colaboradores (2014) demonstraram um aumento nos níveis de IL-6, IL-8, IL-10 e TNF-α após um protocolo de duas semanas de HIIT (Zwetsloot, John *et al.*, 2014). Ahmadizad e colaboradores (2015) verificaram que tanto o HIIT como o TC tem efeitos similares sobre citocinas pro-inflamatórias como a IL-6 e o TNF-α (Ahmadizad, Avansar *et al.*, 2015). Recentemente foi verificado que uma única série de 45 minutos em ciclo ergômetro (intensidade moderada) tem efeitos adversos sobre citocinas pro-inflamatórias (IL-1β, IL-6, IL-10) do que 6 x 30s/ 240s (séries x tempo de exercício em segundos / recuperação em segundos) em intensidade supramáxima de um protocolo HIIT (Cullen, Thomas *et al.*, 2016).

Neste contexto é importante salientar que após uma sessão de exercício ocorre microlesões musculares e, consequente, liberação de IL-6. Isto é, inicia-se o influxo de células inflamatórias para o local lesado, então, ocorre um aumento nos níveis de citocinas pro-inflamatórias (Heavens, Szivak *et al*., 2014). Desta forma, linfócitos T circulantes apresentam diminuição após sessão de exercício físico (3 – 24h) favorecendo o surgimento de imunossupressão transitória (Gleeson, 2007). Sendo assim, o impacto de uma série aguda pode levar a exacerbação de citocinas pro-inflamatórias as quais se ligam a receptores no hipotálamo promovendo a um aumento nas prostaglandinas o que pode levar a febre, perda de apetite, sede, queda da libido, depressão e alterações no humor (Smith, 2004). Entretanto, estes efeitos só ocorrem quando a demanda do exercício é supramáxima e não há descanso adequado após o treinamento (Navalta, Tibana *et al.*, 2014). Sendo assim, o processo

inflamatório gerado após o exercício pode ser benéfico, pois promove uma resposta ao estimulo (intensidade) o que pode gerar um reparo das estruturas lesadas (Zaldivar, Wang-Rodriguez *et al*., 2006). Entretanto, tal benefício só será conseguido se o estado nutricional e descanso forem respeitados, uma vez que estudos tem demonstrado que após sessão de HIIT o consumo de carboidratos e proteínas é essencial para a obtenção de melhores respostas imunológicas (Gleeson, 2013; Gleeson e Williams, 2013; Hashimoto, Ishijima *et al.*, 2014).

O exercício físico também pode induzir uma série de adaptações no sistema nervoso central (SNC) (Tabata, Nishimura *et al.*, 1996; Laursen e Jenkins, 2002; Rakobowchuk, Tanguay *et al.*, 2008; Koshinaka, Kawasaki *et al.*, 2009). Trabalhos prévios descritos na literatura demonstraram que o TC de baixa à moderada intensidade aumenta uma série de proteínas neurotróficas em diferentes regiões cerebrais tais como o fator neurotrófico derivado do cérebro (BDNF - *Brain-derived neurotrophic fator*) e o fator neurotrófico derivado de células gliais (GDNF - *Glial cell-derived neurotrophic fator)* (Afzalpour, Chadorneshin *et al*., 2015), bem como, melhora a memória (Snigdha, De Rivera *et al.*, 2014), além de prevenir doenças como Alzheimer (Ohman, Savikko *et al.*, 2016) e depressão (He, Tang *et al.*, 2012).

Ainda, dados da literatura têm demonstrado que o HIIT estimula a neurogênese (Swain, Harris *et al.*, 2003; Swain e Franklin, 2006; Van Der Borght, Kobor-Nyakas *et al.*, 2009) e aumenta a expressão de fatores neurotróficos, os quais estão diretamente relacionados com aumentos nos níveis de peróxido de hidrogênio (H_2O_2) no SNC (Afzalpour, Chadorneshin *et al.*, 2015). Além disso, um aumento nos níveis de glicogênio cerebral (Matsui, Ishikawa *et al.*, 2012) e modulação da neuroplasticidade através do aumento na expressão de receptores de dopamina no estriado (Vuckovic, Li *et al.*, 2010) também tem sido relatado. HIIT também é capaz de aumentar a expressão de BDNF, a proliferação celular na região hipocampal e os níveis de citocinas anti-inflamatórias como a IL-10 (Afzalpour, Chadorneshin *et al.*, 2015; Marusiak, Zeligowska *et al.*, 2015; Hwang, Brothers *et al.*, 2016). Além disso, Stavrinos e Coxon (2016) demonstraram que o HIIT possui efeitos positivos na consolidação de memória motora através de inibição via ácido gama-aminobutírico (GABA) do córtex motor primário (Stavrinos e Coxon, 2016).

Embora a literatura tenha documentado que o HIIT induz respostas benéficas no SNC, os efeitos deste tipo de exercício na enzima Na^+ K^+ ATPase cerebral tem sido pouco investigado. A enzima Na^+ K^+ ATPase é uma proteína integral de membrana sendo responsável pelo co-transporte de três íons de Na+ para o meio extracelular e dois íons K+ para o meio intracelular. Essa enzima desempenha um papel crucial no SNC por atuar na

manutenção dos gradientes iônicos e na propagação do impulso nervoso (Takada, Matsuo et al., 2009; Kurita, Xu *et al.*, 2015). Estudos prévios tem demonstrado que esta enzima está intimamente ligada à memória e ao comportamento ansiogênico (Moseley, Williams *et al.*, 2007; Baldissera, Souza *et al.*, 2016). Além disso, alterações nas subunidades alfa, na expressão, ou atividade desta enzima vêm sendo associadas com várias patologias (Crema, Schlabitz *et al.*, 2010; Carvalho, Gutierres *et al.*, 2016). Embora o mecanismo molecular que regula o efeito mediado pelo exercício físico sobre os genes que codificam a Na^+, K^+- ATPase ainda não foram completamente elucidados (Thompson e Mcdonough, 1996; Mota, Pereira *et al.*, 2012).

Considerando a vulnerabilidade da Na^+ K^+- ATPase ao estresse oxidativo causado pelo efeito de uma série aguda de HIIT, é plausível sugerir que o exercício intervalado de alta intensidade pode afetar a atividade desta enzima através da produção de EROS (Deminice, Trindade *et al.*, 2010). Sabe-se que a alta produção destas espécies é responsável por várias ações deletérias, tais como aumento nos níveis da peroxidação lipídica das membranas, aumento na carbonilação de proteínas e danos ao DNA. No entanto, a susceptibilidade das células ao dano oxidativo depende do estado da sua defesa antioxidante (Gomes, Silva *et al.*, 2012).

O sistema de defesa antioxidante é composto por enzimas como a superóxido dismutase (SOD), catalase (CAT) e glutationa peroxidase (GPx) e por compostos não enzimáticos como as vitaminas C e E, carotenoides, glutationa e ácido úrico (Gomes, Silva *et al.*, 2012). O radical ânion superóxido ($O_2^{-\bullet}$) é formado constantemente em organismos aeróbicos durante a respiração, sendo detoxificado pela enzima (Pimenta, Bringhenti *et al.*, 2015). A SOD age rapidamente na dismutação do $O_2^{-\bullet}$ produzindo oxigênio (O_2) e H_2O_2 (Lamprecht e Williams, 2012). Posteriormente, a catalase (CAT) converte do H_2O_2 convertendo em O_2 e água (Revan, Balci et al., 2010). Além da CAT, o H_2O_2 também pode ser removido pela ação da GPx. Uma vez que a molécula de glutationa opera em ciclo, na qual pode existir sob a forma reduzida (GSH). Onde o grupo sulfidril (-SH) age como doador de elétrons fazendo uma varredura nas células. Por outro lado, também participa sob a forma oxidada (GSSG). Neste processo, a forma oxidada (GSSG) usa elétrons do NADPH para regenerar a molécula de glutationa em GSH. (Ehrlich, Viirlaid *et al.*, 2007). Sendo assim, a GPx, é responsável pela redução de superóxidos, convertendo a GSH à GSSG removendo o H_2O_2 e formando água. Protegendo assim lipídeos, proteínas e ácidos nucleicos da oxidação (Gomes, Silva *et al.*, 2012).

Os dados descritos na literatura relacionando o exercício de alta intensidade e o estresse oxidativo ainda são bastante controversos. Pesquisadores já relataram que o HIIT (após sessão aguda) aumenta a produção de EROS, bem como aumenta a síntese de enzimas do sistema de defesa antioxidante (Gomes, Silva *et al.*, 2012; Bogdanis, Stavrinou *et al.*, 2013) desta forma, o desequilíbrio causado no estado redox pelo HIIT pode ser uma alternativa eficiente para aumentar a quantidade endógena de defesas antioxidantes (Revan, Balci *et al.*, 2010; Lamprecht e Williams, 2012; Bogdanis, Stavrinou *et al.*, 2013). Em relação ao SNC, tem sido discutido que o exercício regular aeróbico moderado é responsável por aumentar as defesas antioxidantes enquanto que exercícios anaeróbicos ou de alta intensidade podem diminuir essas defesas (Camiletti-Moiron, Aparicio *et al.*, 2013). Rosa e colaboradores (2007) demonstraram que um protocolo de exercício intenso e exaustivo induz déficits de memória e aumenta os níveis de peroxidação lipídica e proteína carbonil em SNC (Rosa, Takahashi *et al.*, 2007). Neste contexto, é importante considerar que alterações em parâmetros de estresse oxidativo em SNC parecem ser dependentes de alguns fatores como duração do exercício e protocolo utilizado.

Durante a atividade muscular intensa, a produção de $O_2^{-\bullet}$ aumenta muito, uma vez que a demanda energética pode superar em até 100 vezes o fluxo de O_2 em todo o corpo (Gomes, Silva *et al.*, 2012). Assim, durante a sua realização ocorre um grande aumento no consumo máximo de O_2 e, consequente, aumento na produção de EROs. Entretanto, têm sido relatado que exercícios de alta intensidade são ferramentas eficientes para melhorar a capacidade física dos indivíduos (Gleeson, Bishop *et al.*, 2011). O HIIT pode melhorar a adaptação do músculo, aumentar o volume do reticulo sarcoplasmático (RS), a expressão de receptores de rianodina (RyR), melhorar a liberação e recaptação de Ca^{2+} em células musculares e, além disso, aprimorar a produção de ATP pela via glicolítica (Hostrup e Bangsbo, 2016).

Visto que as concentrações citosólicas de Ca^{2+} cumprem importante função para produção de força das células musculares (Hostrup e Bangsbo, 2016), as taxas de liberação e captação de Ca^{2+} são reguladas pela atividade da enzima Ca^{2+}ATPase, a qual está localizada nas mitocôndrias, na membrana plasmática, ou em membranas intracelulares no RS, formando a Ca^{2+}ATPase do RS (SERCA). Esta enzima é responsável pelo sequestro de Ca^{2+} presente no citosol de células musculares reconduzindo-os até o RS, onde os receptores de RyRs são responsáveis pela liberação de Ca^{2+} (Tupling, 2004). A atividade desta enzima é primordial para a contração muscular, uma vez que, no músculo em contração, o Ca^{2+} é liberado a partir do RS dentro de células musculares através da membrana via ativação dos

RyRs (Toyoshima, 2009). A seguir, durante o relaxamento a Ca^{2+}-ATPase passa por fosforilação e desfoforilação o que leva a uma série de alterações conformacionais, bombeando dois íons Ca^{2+} contra o gradiente para dentro do RS (Toyoshima, 2009). Sabe-se que a cafeína atua como um agonista dos receptores de RyRs elevando a liberação de Ca^{2+} dentro das células musculares contribuindo, desta forma, para o esgotamento dos níveis intracelulares deste íon (Posterino e Dunn, 2008; Endo, 2009).

Durante uma sessão de HIIT estruturas intracelulares reduzem a excitabilidade sarcolemal, a liberação de Ca^{2+} e, consequentemente, reduzem a produção de força pelo músculo esquelético levando a instalação do processo de fadiga (Hostrup e Bangsbo, 2016). Por sua vez, alterações nas concentrações de Na^{+}, K^{+} e Ca^{2+} limitam a produção energia pelo músculo esquelético (Hostrup e Bangsbo, 2016). Matsunaga e colaboradores (2008) avaliaram a oxidação e captação de Ca^{2+} pelo RS logo após única série de exercício intenso e observaram que as EROS geradas durante o exercício provocam modificação conformacional na enzima interferindo na recaptação de Ca^{2+} (Matsunaga, Mishima *et al.*, 2008). Já Ortenblad e colaboradores (2013) afirmam que suprimentos limitados de energia como glicogênio muscular, após exercício exaustivo, podem afetar diretamente a atividade da enzima Ca^{2+}ATPase (Ortenblad, Westerblad *et al.*, 2013). Curiosamente, ainda são escassos os achados sobre a influência do HIIT na atividade da enzima Ca^{2+}ATPase em músculo.

Sendo o processo de fadiga multifatorial, este também pode ser agravado devido a alterações nos níveis de acetilcolina (ACh) na junção neuromuscular (Blotnick e Anglister, 2016). Estudos tem demonstrado que a enzima acetilcolinesterase (AChE) está envolvida em processos de fadiga muscular, uma vez que ela é a enzima responsável pelo hidrólise da ACh (Abbracchio e Cattabeni, 1999; Wen, Hui *et al.*, 2009; Blotnick e Anglister, 2016). A AChE é expressa em diversos tecidos dentre eles o encéfalo, junção neuromuscular, eritrócitos e linfócitos (Soreq e Seidman, 2001). Desta forma, alterações na atividade e expressão desta enzima em músculo associada a diferentes protocolos de exercício já têm sido documentado na literatura (Vignaud, Fougerousse *et al.*, 2008; Wen, Hui e*t al.*, 2009; Marrero, Rossi *et al.*, 2011; Blotnick e Anglister, 2016).

Inúmeros recursos ergogênicos tem sido utilizados na busca do desempenho esportivo de alto nível com o objetivo de potencializar a *performance* atlética ou atenuar os mecanismos geradores de fadiga. Neste sentido, a cafeína tem sido utilizada como substância ergogênica previamente a realização de exercícios de *endurance* e anaeróbicos (Thein, Thein *et al.*, 1995).

A cafeína é um alcaloide purínico derivado do grupo das metil-xantinas, conhecida quimicamente como 1,3,7- trimetilxantina (C8H10N4O2) (Zulak, Liscombe *et al.*, 2007). É uma substância de fácil acesso a população sendo consumida em sua maior parte através de cafés, chocolates, chás, suplementos (pré- treino/termogênicos) e bebidas energéticas (Arnaud, 2006; Mccusker, Goldberger *et al.*, 2006). A cafeína é rapidamente absorvida no trato gastrointestinal e, após sua ingestão pode atingir o pico na concentração plasmática entre 15 e 120 minutos (Sinclair e Geiger, 2000). Desta forma, a maioria dos estudos relata a administração oral entre 30 e 60 minutos antes do exercício. A metabolização da cafeína ocorre no fígado, principalmente, no citocromo P450 (enzima) CYP 1A2 (isoforma) onde ocorre a demetilação na posição 3 formando o principal metabólito paraxantina (Ursing, Wikner *et al.*, 2003). Devido a este processo, o catabolismo da cafeína leva aproximadamente de 4 a 6 horas, dependendo da dose (Van Soeren e Graham, 1998a). Além disso, alguns fatores como sexo, idade, uso de cigarros e álcool, dieta, gravidez, uso de agentes contraceptivos e exercícios podem alterar o seu metabolismo (Jusko, Gardner *et al.*, 1979; Benowitz, Hall *et al.*, 1989; Collomp, Anselme *et al.*, 1991).

Sendo utilizada há séculos como um psicoestimulante (Walsh, Muehlbach *et al.*, 1990) a cafeína por possuir propriedades lipofílicas, ultrapassa a barreira hematoencefálica e assim estimula o SNC (Chen e Pedata, 2008), inibindo as fosfodiesterases, bloqueando receptores GABA e mobilizando Ca^{2+} intracelular (Ribeiro e Sebastiao, 2010). Além da sua propriedade estimulante (Fisone, Borgkvist *et al.*, 2004) outros estudos também tem descrito propriedades antioxidantes (Abreu, Silva-Oliveira *et al.*, 2011) e anti-inflamátorias desta substância (Rosso, Mossey *et al.*, 2008).

Estudos prévios tem relatado que doses entre 1,5 e 30 mg/kg de cafeína são capazes de estimular o SNC a liberar neurotransmissores como catecolaminas (Van Soeren e Graham, 1998a; Fredholm, Yang *et al.*, 2016), ACh (Carter, O'connor *et al.*, 1995), glutamato (Wang, 2007) serotonina e ainda aumentar a expressão de receptores A_1 de adenosina (Shi, Nikodijevic *et al.*, 1993; Fredholm, Yang *et al.*, 2016). Estes efeitos podem melhorar o estado de atenção, aprendizagem, memória e outras funções cognitivas (Arnaud, 2006; Fredholm, Yang *et al.*, 2016). No entanto, em altas doses (≥25mg/kg), esta metilxantina atua no sistema neuroendócrino através da mediação de corticosteroides em soro, beta-endorfinas (Plaskett e Cafarelli, 2001; Gerull, Manser *et al.*, 2013) e promove diminuição nos níveis de tireotrofina e homônimos do crescimento (GH), contudo, estes efeitos são temporários (Spindel, 1984; Wu e Lin, 2010). Além disso, doses acima de 15 mg/kg parecem induzir dores de cabeça,

ansiedade, irritabilidade, tremores musculares, palpitação, taquicardia e enjoos (Bracco, Ferrarra *et al.,* 1995; O'neill, Newsom *et al.,* 2016; Zuchinali, Souza *et al.,* 2016). Desta forma, é importante ressaltar que a cafeína possui efeito bifásico, ou seja, doses muito elevadas (acima de 500mg/dia) podem inibir os efeitos mediados por doses mais baixas (Fredholm, Battig *et al.,* 1999; Fredholm, Yang *et al.,* 2016).

Embora o consumo de cafeína visando efeitos estimulantes datam de muitos séculos, a sua utilização por atletas com a intenção de melhorar a performance tem se tornado popular nas últimas décadas, devido aos estudos sobre seus efeitos ergogênicos (Graham e Spriet, 1991b; Graham, Hibbert *et al.,* 1998a; Tarnopolsky, 2008). Diversos trabalhos têm proposto que a ingestão de 3 à 9 mg/kg de cafeína pode retardar, em torno de 20 a 50%, a instalação do processo de fadiga durante o exercício intenso prolongando (Spriet, Maclean *et al.*, 1992; Graham Te, 1998; Greer, Mclean *et al.*, 1998a; Davis, Zhao *et al.*, 2003; Woolf, Bidwell *et al.*, 2009).

Um dos principais mecanismos que tem sido usado para explicar o potencial ergogênico da cafeína durante o exercício se refere a sua ação poupadora de glicogênio devido à mobilização ácidos graxos livres do tecido adiposo, via liberação de epinefrina (Costill, Dalsky *et al.,* 1978; Davis e Green, 2009), entretanto esses mecanismos são controversos. Para Graham e colaboradores (2008), embora a cafeína, em doses moderadas (5,5 mg/kg), mobilize ácidos graxos livres do tecido adiposo através do bloqueio seletivo dos receptores $A_{1,}$ não há base científica de que a cafeína exerça ação sobre o metabolismo dos ácidos graxos e carboidratos, uma vez que, as concentrações de glicogênio muscular, glicose-6-fosfato, acetil - CoA e citrato não apresentam diferenças significativas entre grupos exercitados com e sem o uso de cafeína (Graham, Battram *et al.,* 2008). Entretanto, esta metilxantina é capaz de aumentar a concentração de AMPc durante o exercício (60-80% do VO_2max) (Graham, Battram *et al.,* 2008). Outros estudos ainda argumentam a favor do uso da cafeína devido ao fato desta melhorar a oxidação de ácidos graxos, porém as variáveis estudadas giram em torno da frequência cardíaca, pressão arterial e variáveis respiratórias (Jo, Lewis *et al.,* 2016).

A hipótese atual mais aceita é a de que a cafeína atue bloqueando aos receptores de adenosina presentes no SNC, sendo um dos seus efeitos o retardo na sensação de fadiga durante exercício físico (analgesia) (Graham, Battram *et al.,* 2008; Davis e Green, 2009). Existem quatro tipos de receptores de adenosina A_1, A_{2A}, A_{2B} e A_3, os quais estão acoplados a proteínas G (Fredholm, Arslan *et al.*, 2000). No SNC, ao se ligar a esses receptores a

adenosina desempenha muitas funções fisiológicas importantes, dentre elas destacam-se a ação neuromoduladora e neuroprotetora (Latini e Pedata, 2001; Chen e Pedata, 2008; Gomes, Kaster *et al.*, 2011).

A cafeína atua bloqueando receptores A_1 de adenosina seletivamente e, A_{2A} não seletivamente (Gomes, Kaster *et al.*, 2011). Os receptores A_1 são responsáveis pela inibição da liberação de neurotransmissores, insulina/glucagon, sono, redução do ritmo cardíaco e analgesia (Fredholm, 2010; Chen, Eltzschig *et al.*, 2013). Sendo assim, o bloqueio dos receptores A_1 pela cafeína ativa a transmissão excitatória, eleva a liberação de insulina e aumenta o ritmo cardíaco (Gomes, Kaster *et al.*, 2011). Já os receptores A_{2A} atuam em processos fisiológicos como atividade locomotora, processos neurodegenerativos, imunossupressão, vasodilatação, angiogênese e inibição da agregação plaquetária (Fredholm, 2010; Gomes, Kaster *et al.*, 2011).

Uma vez que, a cafeína antagoniza estes efeitos, seu potencial terapêutico também tem sido amplamente estudado. Ao impedir que a adenosina se ligue a um receptor A_{2A} a cafeína tem sido alvo de muitas pesquisas que envolvem doenças como Alzheimer, Parkinson, Epilepsia e Depressão (Gomes, Kaster *et al.*, 2011). É interessante notar que a expressão de receptores A_1 de adenosina é aumentada após o uso crônico da cafeína, o mesmo não ocorre com os receptores A_{2A} (Jacobson, Von Lubitz *et al.*, 1996; Yang, Chen *et al.*, 2009). Este desequilíbrio na razão dos receptores $A1/A_{2A}$ de adenosina que serve de base para indicar a tolerância aos efeitos da cafeína na atividade motora após tratamento crônico (Ciruela, Casado *et al.*, 2006).

Por ser uma molécula envolvida em vários processos fisiológicos como neurotransmissão e inflamação, a adenosina, possui um nível basal (Antonioli, Colucci *et al.*, 2012). Sendo assim, em situações de estresse, por exemplo, exercício intenso ou trauma tecidual, os níveis de adenosina extracelular podem se elevar (Andine, Rudolphi *et al.*, 1990; Fredholm, 2010) através de dois mecanismos principais: (1) formação intracelular e exportação via transportadores; (2) formação de adenosina no espaço extracelular através da degradação dos nucleotídeos de adenina (Figura 1) (Pedata *et al.*, 2007).

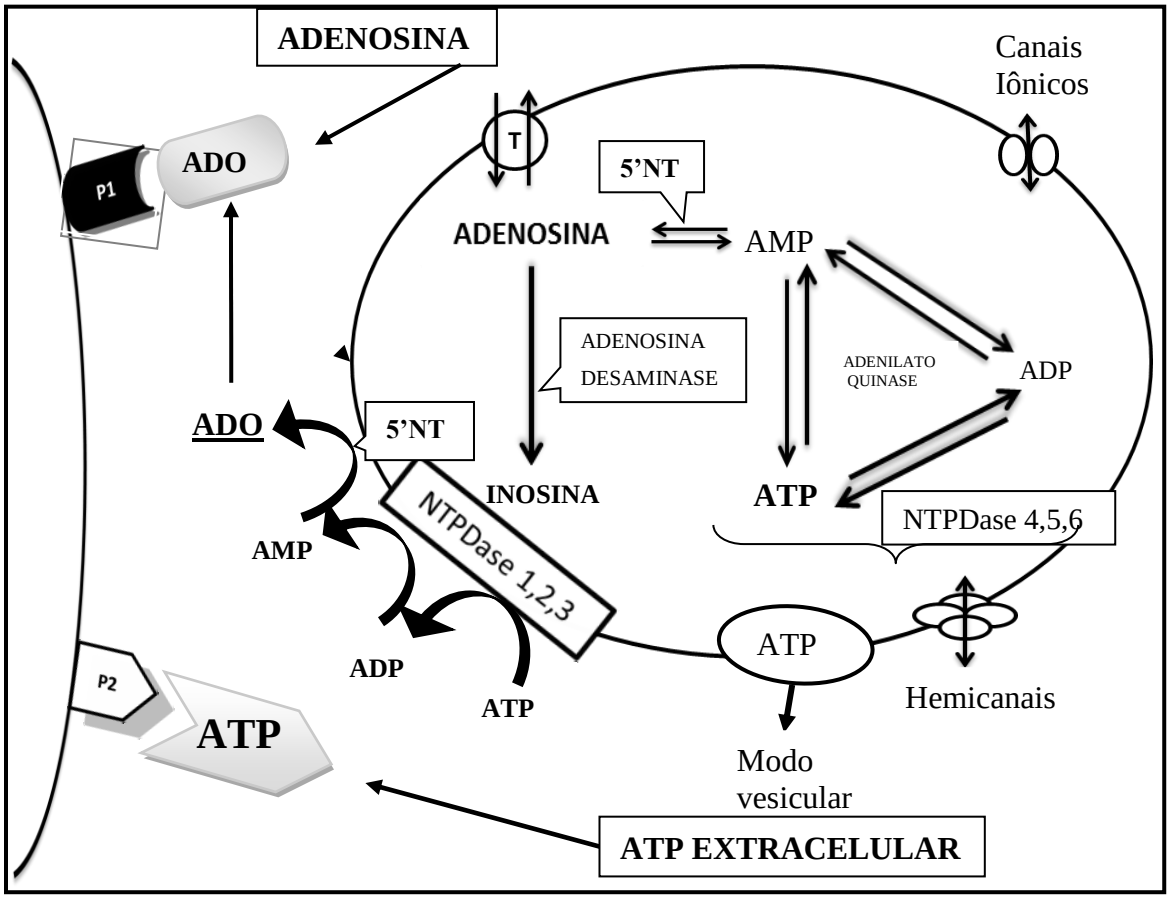

Figura 1: Formação extracelular da adenosina. Adaptado de Pedata et al. (2007).

A adenosina, então, é formada intracelularmente sempre que houver diferenças entre síntese e degradação de ATP, ou seja, quando há um aumento na demanda energética e o suprimento de glicose ou oxigênio for limitado (Lloyd, Lindstrom *et al.*, 1993; Fredholm, 2010). Desta forma, altos níveis extracelulares de adenosina transmitem sinais parácrinos destinados a coordenar a atividade metabólica em grupos de células que formam tecidos (Shryock e Belardinelli, 1997; Bours, Swennen *et al.*, 2006; Gomes, Kaster *et al.*, 2011). Assim, a utilização de antagonistas como as metilxantinas podem aumentar os níveis extracelulares de adenosina podendo ser utilizados com alto potencial terapêutico (Fredholm, 2010). Além da adenosina, os nucleotídeos de adenina ATP, ADP também são considerados moléculas sinalizadoras (Yegutkin, 2008). Tem sido demonstrado que o ATP e o ADP juntamente com a adenosina regulam processos relacionados à tromboregulação, sinalizam vias que são cruciais para o SNC e modulam respostas imunes e inflamatórias (Burnstock, 2002).

Tanto o ATP quanto a adenosina estão envolvidas na ativação de receptores purinérgicos de células imunes, uma vez que, o ATP exerce funções pró-inflamatórias a adenosina possui ações anti-inflamatórias (Cekic e Linden, 2016; Cheng, Azarbal *et al.*, 2016). Desta forma, quando por algum motivo (reposta a antígeno, apoptose ou lesão celular) ocorre, a resposta pode ser dividida em três fases: (1) Fase aguda – ocorre imediatamente após a lesão ou patógeno (minutos a horas) onde as concentrações extracelulares de ATP se acumulam ativando células imunes como os linfócitos; (2) Fase subaguda – ocorre nas próximas horas e dias onde a razão extracelular dos níveis ATP/adenosina apresenta redução. Consequentemente, ocorre diminuição do ATP extracelular e aumento na ativação dos receptores A_{2A} e A_{2B} de adenosina que servem para limitar a extensão da inflamação; (3) Fase crônica – ocorre nos próximos dias ou semanas está associado a baixos níveis de ATP e ADO iniciando, portanto o processo de remodelação do tecido (Cekic e Linden, 2016). Ou seja, após lesão tecidual, as funções ATP aumentam a ativação das células T efetoras. Ocorre inflamação aguda por elevação de Ca^{2+}, enquanto que a adenosina suprime a ativação subaguda de células T ativando os receptores A_{2A} (Herrera, Casado *et al.*, 2001; Cekic e Linden, 2016; Cheng, Azarbal *et al.*, 2016).

A sinalização induzida pelo ATP e adenosina no microambiente inflamatório pode ser controlado pela ação de enzimas como a NTPDase e a adenosina desaminase (ADA). A NTPDase hidrolisa tanto ATP como ADP formando AMP na presença de íons Ca^{2+} e Mg^{2+} (Zimmermann, 1996; Yegutkin, Henttinen *et al.*, 2001; Robson, Sevigny *et al.*, 2006) sendo expressa em várias células imunes tais como neutrófilos, macrófagos, células dendriticas e linfócitos B e T (Burnstock e Boeynaems, 2014). A ADA é responsável pela desaminação da adenosina à inosina sendo a principal enzima responsável por regular as concentrações extracelulares desse nucleosídeo (Yegutkin, 2008). Esta enzima desempenha um papel chave na maturação, proliferação e ativação de linfócitos (Bours, Swennen *et al.*, 2006)

Alguns estudos têm demonstrado que o exercício físico pode modular a atividade das ectonucleotidases. Um protocolo de exercício moderado (corrida em esteira) por duas semanas durante 20 minutos/dia foi capaz de reduzir a hidrólise de ATP e ADP em sinaptossomas de hipocampo de ratos (Siqueira, Elsner *et al.*, 2010). Uma diminuição na hidrólise de ADP e AMP em sinaptossomas de córtex cerebral de ratos após um mês de exercício também tem sido relatado (Ben, Soares *et al.*, 2009). Outros estudos demonstraram que protocolos de exercício podem alterar a atividade destas enzimas em soro (Siqueira *et al.*, 2010; Moritz *et al.*, 2016) bem como diminuir a expressão da NTPDase em linfócitos de ratos

(Cardoso *et al.*, 2015). Além disso, cabe destacar também que a cafeína por via oral durante 14 dias nas concentrações de 0,3 g/L ou 1 g/L não afetou a hidrólise dos nucleotídeos (Da Silva, Bruno *et al.*, 2003). Entretanto a administração (aguda) intraperitoneal (ip) de 30 mg/kg de cafeína foi capaz de aumentar a hidrólise de ATP e ADP em hipocampo e estriado de ratos (Da Silva, Bruno *et al.*, 2003). Esses efeitos na atividade das ectonucleotidases pela cafeína têm sido atribuídos como um mecanismo compensatório devido ao efeito antagonista dessa molécula nos receptores de adenosina.

Considerando que o exercício de alta intensidade promove uma série de adaptações fisiológicas no organismo e que os efeitos ergogênicos da cafeína neste tipo de exercício são controversos e pouco esclarecidos, o objetivo do presente trabalho foi avaliar os efeitos da cafeína em diferentes doses (4 e 8 mg/kg) associada a um protocolo de HIIT em enzimas chaves na manutenção dos gradientes de concentração iônicos e nas respostas imunes tais como Na^+,K^+-ATPase, Ca^{2+}ATPase, AChE, NTPDase e ADA. Além destas análises, o presente trabalho também avaliou parâmetros de ansiedade e estado redox em SNC, níveis de glicogênio em músculo (gastrocnêmio) e possíveis alterações histológicas em músculo gastrocnêmio e cardíaco, respostas de ativação e proliferação de linfócitos bem como níveis de citocinas inflamatórias.

3. OBJETIVOS

3.1 Objetivo geral

Avaliar o treinamento intervalado de alta intensidade (HIIT) sobre parâmetros bioquímicos de ratos tratados com cafeína.

3.2 Objetivos específicos

1) Determinar os níveis de glicogênio muscular, atividade da enzima acetilcolinesterase e Ca2+ ATPase em músculo gastrocnêmio de ratos submetidos ao exercício de alta intensidade e/ou tratados com cafeína (agudo e crônico).
2) Verificar possíveis alterações histológicas em músculo gastrocnêmio e cardíaco de ratos submetidos ao treinamento (crônico) de alta intensidade e/ou tratados com cafeína.
3) Analisar parâmetros de estresse oxidativo como níveis de substâncias reativas ao ácido tiobarbitúrico (TBARS), atividade enzimática da Superóxido dimutase (SOD), Catalase (CAT), Glutationa peroxidase (GPx) e níveis do conteúdo de glutationa reduzida (GSH) em homogeneizado de diferentes estruturas cerebrais (córtex cerebral, hipocampo e estriado) de ratos submetidos ao exercício intervalado de alta intensidade e/ou tratados com cafeína.
4) Analisar parâmetros de ansiedade através da tarefa labirinto em cruz elevado na atividade da enzima Na^+, K^+ ATPase em córtex cerebral, hipocampo e estriado de ratos submetidos ao protocolo crônico de exercício intervalado de alta intensidade e/ou tratados com cafeína.
5) Verificar a atividade das enzimas NTPDase e adenosina desaminase (ADA) em linfócitos de ratos controles ou submetidos ao protocolo crônico de exercício intervalado de alta intensidade tratado e/ou com cafeína.
6) Avaliar níveis de citocinas (IL-6; IL-10) em ratos submetidos ao protocolo crônico de exercício intervalado de alta intensidade e/ou tratados com cafeína.
7) Avaliar *ex vivo* resposta celular imune a antígeno ("*phytohemagglutinin"* - PHA) em ratos submetidos ao protocolo crônico de exercício intervalado de alta intensidade e/ou tratados com cafeína.

4. RESULTADOS

Os resultados desta tese estão apresentados na forma de três manuscritos. Os itens materiais e métodos, resultados, discussão e referências bibliográficas encontram-se nos próprios manuscritos e representam a íntegra deste estudo. Os manuscritos estão estruturados de acordo com as normas das revistas científicas para os quais foram submetidos.

4.1 ARTIGO I

Caffeine prevents changes in muscle caused by high-intensity interval training

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Abstract

The use of ergogenic substances such as caffeine has become a strategy to enhance sports performance. In the present study we evaluated the effects of high-intensity interval training (HIIT) associated with caffeine intake on acetylcholinesterase (AChE) and Ca^{2+}ATPase activity and glycogen levels in the muscles of rats. The animals were divided in groups: control, caffeine 4 or 8 mg/kg, HIIT, HIIT *plus* caffeine 4 or caffeine 8 mg/kg. Our results showed a decrease in glycogen levels in muscle in all trained groups after acute session exercise, while that an increase in glycogen levels was observed in all groups in relation to control in chronic exercise protocol. HIIT increases the thickness of the left ventricle and the Ca^{2+}-ATPase activity and decrease the AChE activity in gastrocnemius muscle. Caffeine treatment prevents changes in enzymes activities as well as left ventricular hypertrophy adaptation induced by HIIT. Our findings suggest that caffeine modulates crucial pathways for muscle contraction in HIIT.

Key Words: physical exercise, caffeine; heart and gastrocnemius muscles, sarcoplasmic reticulum calcium ATPase, acetylcholinesterase.

Introduction

High-intensity interval training (HIIT) is exercise characterized by explosive and vigorous activity in muscle tissue with short periods of recovery [1]. It is well described that strenuous exercise produces a number of benefits in the body such as increases in plasma and systolic volume, myoglobin content, peripheral vascular resistance reduction and angiogenesis [2-4]. In muscle, normally this type of exercise also induces important adaptations such as increases in expression of mitochondrial enzymes, increase in glycogen levels and insulin responsiveness [5-7].

The use of ergogenic substances has become a common strategy to enhance sports performance beyond the effects of training. Caffeine is an alkaloid compound with psychostimulant effects and it has been consumed mainly as tea or coffee [8]. In recent decades, athletes have being using caffeine to improve athletic performance [9]. Studies have suggested that the intake of 3 to 9 mg/kg of caffeine can slow the process of fatigue during long-term exercise, prolonging the activity by 20-50% by acts with non-selective antagonist of adenosine receptors (ARs) [10,11].

During physical exercise, the action potential reaches presynaptic neurons and voltage-dependent Ca^{2+} channels. Most neurons release ATP as a fast co-transmitter together with classical fast transmitters, such as noradrenaline and acetylcholine [12]. After being released, acetylcholine is cleaved into choline and acetate by acetylcholinesterase (AChE). AChE is one of the most efficient enzymes known for rapidly hydrolyzing the neurotransmitter acetylcholine at cholinergic synapses as well as the neuromuscular junction [13,14]. Studies have demonstrated alterations in the activity and expression of AChE in muscle since this enzyme has a key-role in the contraction and adaptation to exercise [15-17].

Another important enzyme in muscular contraction is Ca^{2+}-ATPase, which regulates intracellular Ca^{2+} levels in skeletal muscle and modulates muscle contraction and relaxation [18]. Some studies have indicated that HIIT can reduce Ca^{2+}-ATPase activity and Ca^{2+} uptake by the sarcoplasmic reticulum (SR) and that the activity of this enzyme is sensitive to metabolites such as pH, ATP, Ca^{2+} and glycogen [19-22].

There are few studies reporting the effects of association between caffeine consumption and HIIT performance on AChE and Ca^{2+}-ATPase activity in the muscles. Moreover, the growing consumption of energy drinks containing caffeine has been the subject of numerous controversies. The main aim of this study was to determine the effects acute or

chronic HIIT protocol associated with caffeine consumption on glycogen levels, AChE and Ca^{2+}- ATPase activity and on histological parameters in skeletal and heart muscles of rats.

Materials and Methods

2.1 Chemicals

Caffeine (1,3,7 trimethylxanthine), Coomassie Brilliant Blue G, acetylthiocholine iodide (ASCh), and 5,5′-dithio-bis-2-nitrobenzoic acid (DTNB) were obtained from Sigma Chemical Co. (St. Louis, MO, USA). Bovine serum albumin, K_2HPO_4, was obtained from Reagen. All the other chemicals used in this experiment were of the highest purity.

2.2 Animals

Male Wistar rats (90–100 days; 250–280 g) from the Central Animal House of Federal University of Santa Maria were used in this experiment. Animals were maintained at a constant temperature (23±1 °C) on a 12 h light/dark cycle with free access to food and water. All animal procedures were approved by the Animal Ethics Committee from the Federal University of Santa Maria (protocol under number: 077/2011).

2.3 *Adaptation protocol to exercise*

Swimming was used as the HIIT exercise. Initially, all rats were adapted to water before beginning training. The adaptation was to keep the animals in shallow water (5 cm in depth) at 32 °C, 20 min per day for 5 days. The purpose of the adjustment was to reduce stress, without, however, promoting adaptation to the training. After this time, the animals were adapted to exercise where they were put to swim in a circular tank with 115 x 90 cm (diameter and deep, respectively) with water temperature of around 32 °C for 10 days. The adjustment period (10 days) was as follows: day 1: 20 minutes of swimming, without load; day 2: 20 minutes of swimming with only "backpacks" (no load); day 3: 15 minutes of swimming, corresponding to 3 % body weight load; day 4: 20 minute swim with 3 % of body weight; day 5: 15 minutes of swimming with the equivalent of 5.5 % of body weight load; day 6: 20 minute swim with 5.5 % of body weight; day 7: 15-minute swim with 8 % of body weight (3 intervals of 1min for recovery at 4, 8 and 12 minutes); day 8: swim for 20 minutes with 8% of body weight (4 intervals of 1min for recovery at 4, 8, 12 and 16 minutes); day 9 swim for 15 minutes with 10.5% of body weight (4 intervals of 1 min for recovery at 3, 6, 9

and 12); day 10: 20 minute swim with a load equivalent to 10.5% of body weight (4 intervals of 1min for recovery at 4, 8, 12 and 16 minutes). More information on the adaptation period and HIIT protocol can be found in Table 1.

2.4 Lactate levels

Forty-eight hours after the adaptation period and before the start of training, rats were submitted to a test to assess maximal lactate obtained by the adapted protocol, as previously described [23]. For lactate evaluation, the test of progressive loads equivalent to 4.5, 6.5, 8.5 and 10.5% of the body-weight of each animal with 30 seconds of exercise was conducted. Lactate was collected during a pause of 60 seconds while their load was adjusted [23,24,25]. Blood samples from the distal end of the tail of rats at rest were collected for lactate measurements. This test was used to prove that animals submitted to HIIT protocol produced lactate levels above of the 5.5 mmol/l. Forty- eight hours after lactate evaluation the animals were submitted to the acute or chronic exercise protocol and more information can be found in Figure 2B.

2.5 Acute protocol for HIIT and caffeine treatment

Forty-eight rats were divided in six groups (n= 8 each): Control group, Caffeine 4 mg/kg, Caffeine 8 mg/kg, HIIT, HIIT *plus* caffeine 4 mg/kg, and HIIT *plus* caffeine 8 mg/kg. Acute exercise was conducted 48 hours after the adaption period (as described above). The animals of groups caffeine 4mg/kg, caffeine 8mg/kg, HIIT + 4mg/kg and HIIT + 8 mg/kg received orally 4 or 8 mg/kg of caffeine diluted in saline 0.9% (1 ml/kg) 30 minutes before the start of the training session. The acute protocol exercise consisted of 12 sets of 25 seconds with loads equivalent to 13% of body weight with the recovery period of 35 seconds between the sets. After the 12th set the animals were submitted to euthanasia and the gastrocnemius muscle was collected for enzymatic assays. Acute protocol after 48h adaptation protocol represented 12 sets it swimming just only one day of HIIT with 13%/ body weight (kg). Showed time ~25” in exercise *per* ~35” in rest. Chronic protocol included adaptation protocol and lactate test represented 6 weeks of HIIT. Showed progressive increases loads (2.5%) during 6 weeks. This exercise protocol consisted of three workouts *per* week with progressive increases (2.5 % of body weight) every week. The rats of were trained three times a week for 6 weeks for a total workload 23% of the body weight at the end of experiment. The exercise protocol was performed three times per week on alternate days. The caffeine treatment started

after the period of adaptation to exercise. Caffeine was diluted with saline 0.9% (1 ml/kg) and was administered 30 minutes before training, 5 days a week orally. More information can be found in Figure 1A.

2.6 Chronic protocol for HIIT and caffeine treatment

Forty-eight rats were also divided in six groups (n = 8 each): Control group, Caffeine 4 mg/kg, Caffeine 8 mg/kg, HIIT, HIIT *plus* caffeine 4 mg/kg and HIIT *plus* caffeine 8 mg/kg. The rats of groups HIIT, HIIT + 4mg/kg and HIIT + 8mg/kg were trained three times a week for 6 weeks for a total workload 23% of the body weight at the end of the experiment. The animals were submitted to 12 sets of swimming exercise of 25 seconds alternated with about 35 seconds of recovery as described in the literature [24,25,26]. This protocol is characterized by high-intensity and short duration of activity with successive intermittent increases in workloads and is mainly based on the principle of overload and adaptation. The animals in groups Control, Caffeine 4mg/kg and Caffeine 8mg/kg were placed in shallow water at 32 ± 1ºC, 12 min three times a week, so as to be subjected to the same stress, without, however, suffering the effects of physical training. The exercise protocol was performed three times per week on alternate days (48h of recovery between sessions) always at the same time, for 6 weeks. The caffeine treatment started after the period of adaptation to exercise. Caffeine was diluted with saline 0.9% (1 ml/kg) and was administered 30 minutes before training, 5 days a week orally at a dose of 4 mg/kg in groups caffeine 4 mg/kg and HIIT plus caffeine 4 mg/kg; and the dose of 8 mg/kg in groups caffeine 8 mg/kg and HIIT plus caffeine 8 mg/kg, whereas groups Control and HIIT received only 0.9% saline. The animals were treated with caffeine during 6 weeks. The purpose of caffeine use 5 times a week was saturating adenosine receptors. At the end of caffeine treatment and, therefore, 48 hours after the last training session, the animals were submitted to euthanasia and the gastrocnemius and heart muscles were collected for histological and enzymatic assays. More information can be found in Figure 1B.

2.7 Determination of Muscular Glycogen

Muscle glycogen (µmol glucosyl–glucose) was estimated as proposed in the literature [27]. The tissues were weighed (80 and 100 mg), KOH (30%) and ethanol (95%) were added (1 and 3 ml, respectively), for hydrolysis and precipitation of glycogen. Heat water 100 ºC for 10 minutes. After cool down, add 100 µl 20% sodium sulfate (Na2So4) to digest and shake

(vortex). Add 1 ml of ethanol 95% wait precipitate. After centrifuged for 15mintes at 3.000 rpm. Wash pellet with 2 ml 70% ethanol (vortex), then centrifuge at 3000 rpm for 1 min. Discard sup, and resuspend pellet in 4 N H2SO4 (250ul for muscle), heat for 100º C for 10 minutes. Neutralize with equal volume KOH. The samples measured by absorbance at 340 nm.

2.8 Determination of AChE activity in the gastrocnemius muscle

The gastrocnemius muscle was homogenized in Tris HCl 10 mM (pH 7.2). AChE was determined by a modification of the method as previously describe (Ellman, Courtney *et al.*, 1961). The reaction mixture (2 ml final volume) contained 100 mM K+-phosphate buffer, pH 7.2 and 10 mM 5,5'- dithiobis (nitrobenzoic acid) (DTNB). The method is based on the formation of the DTNB yellow anion, measured by absorbance at 412 nm during 3-min incubation at 25 ◦C. The enzyme (40–50 µg of protein) was pre-incubated for 2 min. The reaction was initiated by adding 0.8 mM acetylthiocholine iodide (AcSCh). The enzyme activity was expressed in µmol AcSCh/h/mg of protein.

2.9 Determination of gastrocnemius muscle Ca^{2+}ATPase activity

Protein extraction of membranes from rat gastrocnemius muscle tissue fractionation was carried out as previously described [29] with some modifications. Gastrocnemius muscle was homogenized with Micro Retífica Dremel Série 4000 in buffer containing (in mM) 20 Tris-HCl, 250 sucrose, 0.1 EGTA, pH 7.4. To isolate a purified fraction, the resulting homogenate was centrifuged for 10 min at 800 g, 4ºC. The supernatant containing partial membrane fractions was centrifuged again for 30 min at 20.000 g and the resulting pellet was suspended. This purified membrane fraction containing Ca^{2+}-ATPase used for the enzymatic assay. Ca^{2+}-ATPase activity was measured as previously described [30] with minor modifications [31].

Briefly, the assay medium consisted of (mM) 30 Tris-HCl buffer (pH 7.4), 0.1 EGTA, 3 $MgCl_2$ and 100 µg/ml of protein in the presence or absence of 0.4 $CaCl_2$, in a final volume of 200 µL. The reaction was started by the addition of ATP to a final concentration of 3 mM. After 60 min at 37ºC, the reaction was stopped by the addition of 50 µL of 50% (w/v) trichloroacetic acid. Saturating substrate concentrations were used and the reaction was linear with protein and time. Appropriate controls were included in the assays for non-enzymatic

hydrolysis of ATP. The amount of inorganic phosphate (Pi) released was quantified colorimetrically, as previously described [32], using KH_2PO_4 as reference standard. The Ca^{2+}-ATPase activity was determined by subtracting the activity measured in the presence of Ca^{2+} from that determined in the absence of Ca^{2+} (no added Ca^{2+} *plus* 0.1 mM EGTA) and expressed in nmol of Pi/min/mg of protein.

2.10 Histological Analysis

Samples of gastrocnemius and cardiac muscle were collected in 10% buffered formalin. Samples were embedded in paraffin for routine processing and 5 μm sections were produced and stained with hematoxylin and eosin (HE). The images were captured using a digital camera, AxioCam HRc, connected to a microscope, Carl Zeiss Axio Scope A1, for histological analysis..

2.11 Statistical Analysis

Data are presented as mean ± SEM and were analyzed statistically by two-way ANOVA, followed by Bonferroni post-hoc tests. Differences between groups were considered to be significant at $P<0.05$. In histological sample data are presented as mean ± SEM and were analyzed statistically by one-way ANOVA (Graphpad – Prism 5.0).

Results

Figure 2 shows a decrease in lactate levels in the training groups when compared to the sedentary group (Figure 2B, $P<0.05$). These results demonstrate that the animals were adapted to exercise, present values above 5.5 mmol/l characterizing the anaerobic exercise, validating the model of HIIT. Figure 2 (C and D) shows the acute effects of HIIT on gastrocnemius muscle glycogen levels. The results showed a decrease in glycogen levels in trained animals and caffeine treatments 4 or 8 mg/kg did not prevented this effect ($P<0.05$). On the other hand, in the chronic exercise protocol, there was an increase in glycogen levels in rats only trained rats and in rats trained and treated with caffeine (4 mg/kg and 8mg/kg) when compared to the control group (Figure 2D, $P<0.05$). Caffeine *per se* also was able to increase muscle glycogen levels at doses of 4 mg/kg and 8 mg/kg in relation to the control group (Figure 2D, $P<0.05$).

Figure 3 shows the effects of acute and chronic HIIT protocols alone or in association with caffeine consumption on AChE activity. As can be observed, the acute exercise protocol did not alter gastrocnemius muscle AChE activity in trained rats or treated with caffeine 4 and 8mg/kg (Figure 3A, $P>0.05$). In the chronic protocol, AChE activity in the gastrocnemius muscle was decreased only in the group submitted to HIIT and caffeine treatment was able to prevent this effect (Figure 3B, $P<0.05$). Caffeine *per se* did not alter gastrocnemius muscle AChE activity in the chronic protocol.

Figure 4 shows that Ca^{2+}- ATPase activity was increased in trained rats with both the acute and chronic HIIT protocols (Figures 4A and B, $P<0.001$). Treatment with caffeine at doses of 4 and 8 mg/kg was able to prevent this effect. Caffeine *per se* did not alter Ca^{2+}-ATPase activity in rat gastrocnemius muscle (Figures 4A and B).

Histological analysis and weights of heart and gastrocnemius muscle are presented in Figure 5. Increased thickness in the left ventricle was observed in rats submitted to HIIT (Figures 5A and 5E) ($P<0.05$). However, caffeine at both doses (4mg/kg or 8mg/kg) reduced this increase in rats submitted to physical exercise (Figure 5E). Additionally, histological analyses performed on gastrocnemius rat muscle revealed that there was no change in the density of muscle fiber in all groups tested (Figure 5B) since no difference was observed in the weight and thickness of the gastrocnemius muscle (Figures 5C and 5D).

Discussion

In this study we evaluated the acute and chronic effects of HIIT associated with caffeine consumption on enzymes that regulate muscle contraction and on histological parameters in rat muscle. The ATP production occurs with high efficiency by anaerobic reactions, however, during the HIIT, there is an accumulation of lactate that exceeds its removal, resulting in muscle fatigue [33,34]. Therefore, blood lactate is an excellent indicator of the performance and is an important physiological marker for transition from aerobic to anaerobic metabolism [35]. Our results demonstrated a decrease in lactate levels in animals adapted to exercise. In addition, values above 5.5 mmol/l indicate a predominance of anaerobic metabolism during the HIIT [36,37] that animals are adapted to HIIT validating the protocol used in this study.

In the acute protocol, muscle glycogen (gastrocnemius) stores were reduced in the trained group, while glycogen stores were increased in trained animals submitted to the chronic protocol. This effect was not reverted by caffeine 4 or 8 mg/kg. Muscle glycogen is an

immediate reserve source of glucose and an important substrate for skeletal muscle contraction during exercise. Glycogen depletion has been implicated in the mechanisms involved in muscle fatigue [38]. The decrease of glycogen stores in muscle of animals submitted to the acute HIIT protocol can be explained by the muscle glycogen depletion due to rapid ATP consumption [39]. On the other hand, the increase in the glycogen content observed in chronic HIIT appears to be associated to increase in the in free fatty acid oxidation [40], oxidative capacity of mitochondrial enzymes, enhance in GLUT 4 receptors and hexokinase activity in muscle [5]. It is plausible that overexpression of GLUT 4 leads to increased glucose uptake and thus the accumulation of glycogen levels in muscle associated with more time of HIIT [41,42].

Studies have reported that caffeine delays the fatigue process and enhances performance during endurance exercise [43-46]. One possible mechanism involved in these ergogenic effects is related to the increase in the lipid oxidation that has been presumed to preserve muscle glycogen stores. Ryu and colleagues demonstrated that caffeine (6 mg/kg) administered 1h prior to exercise in rats and caffeine (5 mg/kg) administered also 1h prior to exercise in human subjects increased blood free fatty acid levels resulting from spare stored glycogen [47]. On the other hand, 5 mg/kg caffeine administered to rats 30 min before swimming exercise did not alter the liver or muscle glycogen at rest or during exercise.

Caffeine is rapidly absorbed from the gastrointestinal tract after its ingestion and can reach peak plasma concentrations after 15–120 minutes [48]. Caffeine acts on the central nervous system (CNS) by blocking GABA receptors, inhibiting phosphodiesterase and blocking ARs [11] and increasing intracellular Ca^{2+} for the sarcoplasmic reticulum [49]. Thus, a number of biochemical processes may occur as a result including activation of AMPK, which is responsible for stimulating glucose uptake, oxidation of fatty acids, increase in the AMP/ATP ratio and reduction in processes such as protein and lipid synthesis [41]. Of particular importance, the inhibitory effects of caffeine on enzyme glycogen phosphorylase have been reported [50]. Taken together; these findings could explain the effects of caffeine on the glycogen stores observed in this study. However, caffeine was able to preserve the glycogen stores only when administered over a longer period. In addition, our studies that this effect of caffeine is not potentiated when associated to exercise

It is well established in the literature that the cholinergic system and Ca^{2+} pump play important roles in the process of muscle contraction. AChE is highly concentrated at the neuromuscular junction where it plays an important role in cholinergic transmission by

terminating the action of acetylcholine (ACh) [51,52]. Sveistrup and colleagues [17] reported an increase in AChE activity and expression in rat muscle subjected to chronic enhancement of neuromuscular activation. An increase in the G4 tetrameric form of AChE also was observed in fast-twitch fibers in hind limb skeletal muscles after treadmill exercises [53]. Of particular importance, Pedzikiewicz demonstrated that after one exercise session, AChE activity was increased in skeletal rat muscle, however, after long term training, the activity of this enzyme was decreased in muscle [54].

In the present study, AChE activity was not altered in gastrocnemius rat muscle submitted to an acute exercise protocol. However, chronic HIIT caused a decrease in AChE activity. In support of our results, Wen and colleagues, in a protocol of exercise-induced fatigue, also showed that the expression and activity of AChE decreased transiently in gastrocnemius neuromuscular junctions, suggesting that theses alterations would lead to a overstimulation of the cholinergic receptors and inefficiency to degrade the ACh in neuromuscular junction [15].

Considering the repetitive nerve stimulation that occurs during exercise, ACh accumulation could induce a desensitization of ACh receptors resulting in the failure to generate muscle action potentials [15]. However, in a model of endurance training an increase in the number of ACh receptors at the neuromuscular junction has been observed in rat skeletal muscle [17]. Our results suggest that the decrease observed in muscle AChE activity after HIIT in the chronic protocol may lead to an increase in ACh at the neuromuscular junction, promoting an increased response to exercise and contraction mechanisms. This finding may indicate a specific adaptive reaction of the muscle to enhance motor response, suggesting that AChE plays a crucial importance in neuromuscular transmission during HIIT. This hypothesis could be sustained by the fact that no histological changes were observed in the gastrocnemius muscle.

Previous studies have demonstrated that caffeine can interact directly with AChE, causing its inhibition [55]. In addition, it is important to consider that in the CNS, the caffeine can increase the release of several excitatory neurotransmitters, such as glutamate and ACh [56]. Increased release of ACh at the neuromuscular junction can explain in part the effects of caffeine to restore AChE activity in the gastrocnemius muscle and, which helps to explain the delayed fatigue properties of caffeine. However, more studies are necessary to evaluate the importance of this effect of caffeine on AChE activity in endurance exercise.

Caffeine is a non-selective antagonist of adenosine receptors (ARs) that is bound by A_1 and A_{2A} receptors. Studies have shown that activation of ARs are involved in the increase in intracellular Ca^{2+} concentration [57,58,59]. The non-selective A_{2A} agonist, the CGS, and adenosine promote increased Ca^{2+} influx and activation of atrial fibrillation through of RyD2 receptors (ryanodine receptor) [58,59], which are mainly responsible for regulating sarcoplasmic reticulum calcium ATPase (SERCA) and releasing intracellular Ca^{2+}. In line with these findings, both HIIT protocols (acute and chronic) promoted an increase in Ca^{2+}-ATPase activity in rat gastrocnemius muscle and caffeine treatment was able to prevent this effect.

In support of our results, other studies have also reported that physical exercise promotes an increase in both the activity and expression of Ca^{2+}-ATPase SERCA-2a [60]. The properties of caffeine responsible for preventing this effect are possibly related to phosphorylation of ryanodine by adenosine receptors. Since caffeine acts as a non-selective antagonist for $A_{2A}R$, it could reduce the intracellular Ca^{2+}concentration and modulate the activity of Ca^{2+}-ATPase by adenosine receptor-dependent signaling pathways.

Muscular fatigue can be induced by repetitive exercise, so a reduction in Ca^{2+}- ATPase activity may be a result of ATP depletion in muscle [61]. Our acute protocol exercise induced an increase in SERCA activity, which may be due to the action of intracellular signaling pathways activated by adenosine receptors (cAMP/PKA) that can phosphorylate SERCA, thus regulating its activity. In contrast, a blockade of $A_{2A}R$ by caffeine could reverse this modulatory effect. However, we cannot rule out the possibility that the exercise protocol developed could result in an adaptive response, thus promoting an increase in the expression of Ca^{2+}- ATPase.

Histological analysis of the heart rat muscle submitted to the chronic exercise protocol showed an increase in thickness of the left ventricle. Cardiac hypertrophy is an important physiological process considered to be compensatory or adaptive and necessary to maintain optimal cardiac performance under conditions of overload hypertrophy [62]. Prolonged and intensive physical training induces cardiovascular adaptations that allow the heart to improve and sustain high physical performance [63,64]. However, it is important to note that the use of caffeine as a supplement to enhance physical performance reduced hypertrophy of the heart muscle. In our study, HIIT promoted an increase in thickness of the left ventricle as an adaptive response; caffeine prevented this effect.

The use of caffeine as an ergogenic substance is a strategy to enhance sports performance. Moreover, the growing consumption of energy drinks containing caffeine in order to increase performance has been the subject of debate. We investigated the association of caffeine consumption during acute and chronic HIIT. The activity of two important enzymes that regulate muscle contraction, AChE and Ca^{2+}-ATPase, was observed. Caffeine prevented adaptive responses to the activity of these enzymes induced by HIIT. In parallel, morphological studies of cardiac muscle showed that caffeine prevented left ventricular hypertrophy during chronic HIIT. These findings show that more studies are needed to verify the potential beneficial effect of caffeine associated with exercise of high intensity, because it prevented several biochemical and morphological adaptations induced by HIIT. Our results point to an acute use for moderate doses of caffeine. Notably, chronic administrations seems to reverse the effect of HIIT. Thus, our results can contribute to a change in the pattern of consumption of substances containing caffeine and energy drinks, teas, coffee and thermogenic supplements.

Conflict of Interest statement

There are no conflicts of interest.

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Legends

Table 1 – Adaptation protocol for the high- intensity interval training (HIIT). As described in materials and methods.

Figure 1 – Schematic of acute and chronic HIIT protocols.

Figure 2 – Chemical structure of caffeine (A). Lactate threshold in trained animals (B), effects of caffeine (CAF, 4 mg/kg and 8 mg/kg) on glycogen storage in gastrocnemius muscle in acute (C) and chronic protocol (C). Data are presented as mean ± SEM, one or two-way ANOVA. Groups are statistically different (*) for $P<0.001$, n= 8 per group.

Figure 3 – Effect of caffeine on AChE enzyme activity in rats submitted to the HIIT protocol. AChE activity in gastrocnemius muscle of animals treated with caffeine (CAF, 4 mg/kg and 8 mg/kg) in acute (A) and chronic (B) protocol of high-intensity interval training (HIIT). Data are presented as mean ± SEM, two-way ANOVA using Bonferroni post-test. Groups are statistically different (*) for $P<0.05$, n= 8 per group.

Figure 4 – Caffeine effect on enzyme activity Ca2+-ATPase in rats submitted to HIIT protocol. Ca2+-ATPase activity in gastrocnemius muscle of animals treated with caffeine (CAF, 4 mg/kg and 8 mg/kg) in acute (A) and chronic (B) protocol of high-intensity interval training (HIIT). Data are presented as mean ± SEM, two-way ANOVA using Bonferroni post-test. Groups are statistically different (*) for $P<0.01$, n=8 per group.

Figure 5 – Histological analysis of the gastrocnemius and heart muscles. Representative Hematoxylin & Eosin (H&E) staining sections from the heart (A), gastrocnemius muscle (B), weight of gastrocnemius muscle and heart (C), gastrocnemius thickness (D) and heart thickness (E) of animals treated with caffeine (CAF, 4 mg/kg and 8 mg/kg) and submitted to chronic protocol of high-intensity interval training (HIIT). Images from the first line of rectangular boxes at a magnification of 40 x and the second line of rectangular boxes at a magnification of 10x. Data show mean ± SEM. One or Two-way ANOVA. Significant differences (*) for $P <0.05$, n = 6 per group.

Table 1. Adaptation protocol for the high- intensity interval training (HIIT). Workload (%) based on body weight. Time of exercise in min. Recovery time through 1 minute in the times described in table (recovery time).

Day	Workload (%)	HIIT time (min)	Interval (nº)	Recovery Time
1	0	20	0	no recovery
2	backpag	20	0	no recovery
3	backpag *plus* 3%	15	0	no recovery
4	backpag *plus* 3%	20	0	no recovery
5	backpag *plus* 5.5%0	15	0	no recovery
6	backpag *plus* 5.5%	20	0	no recovery
7	backpag *plus* 8%	15	2	5 and 10 min
8	backpag *plus* 8%	20	3	5, 10 and 15 min
9	backpag *plus* 10.5%	15	4	3, 6, 9 and 12 min
10	backpag *plus* 10.5%	20	4	4, 8, 12 and 16 min

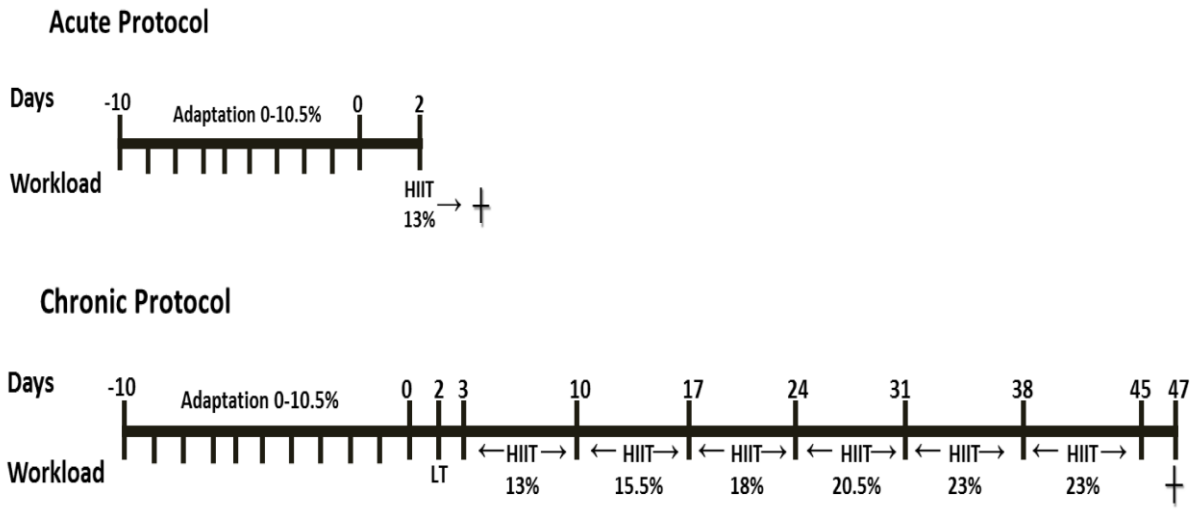

Figure 1

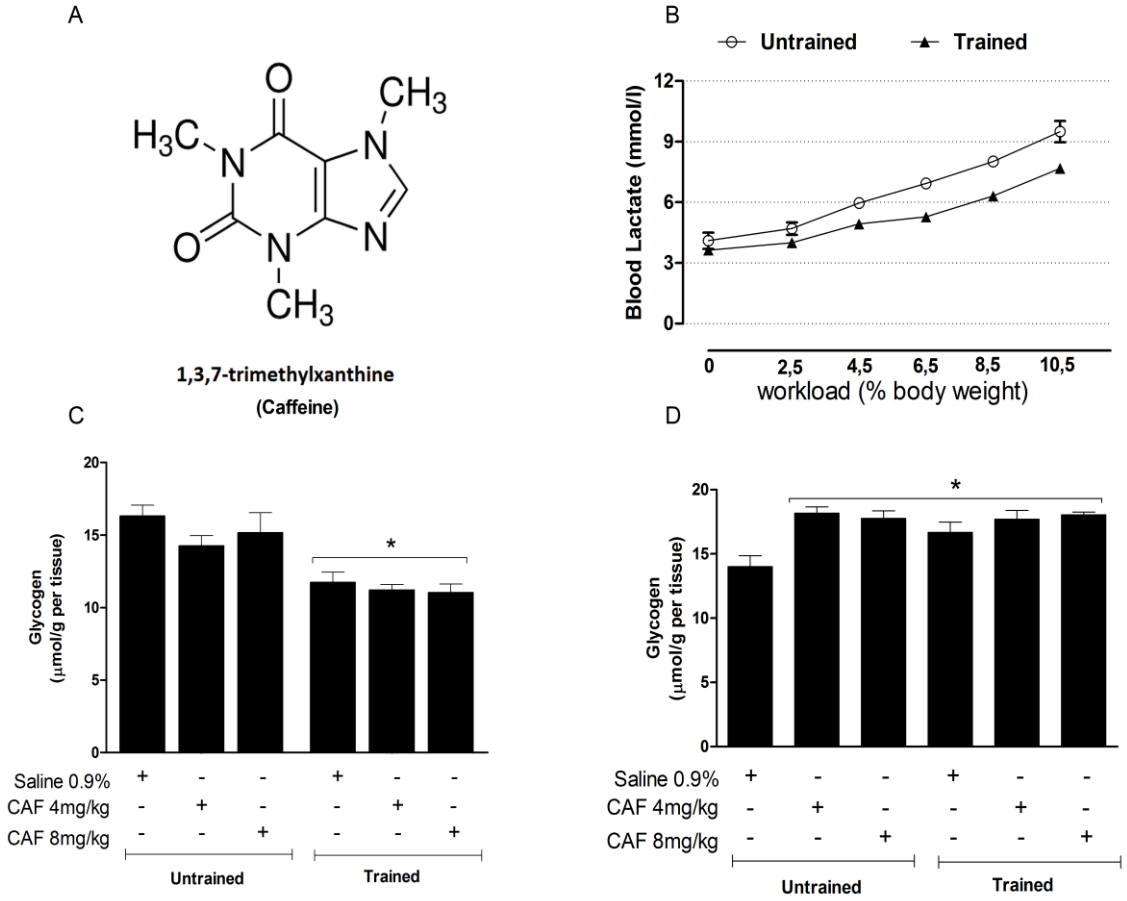

Figure 2

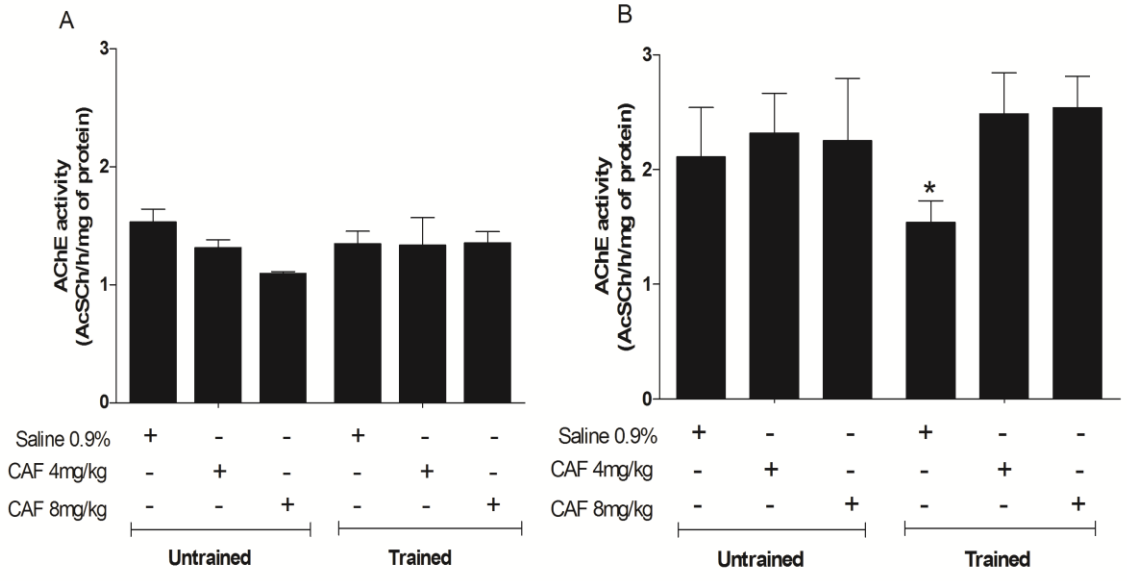

Figure 3

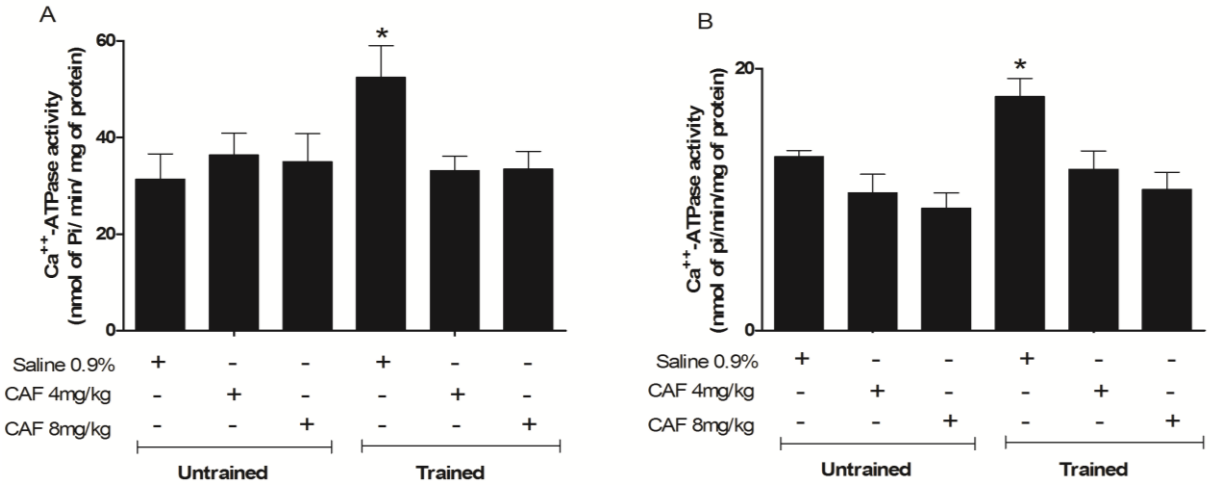

Figure 4

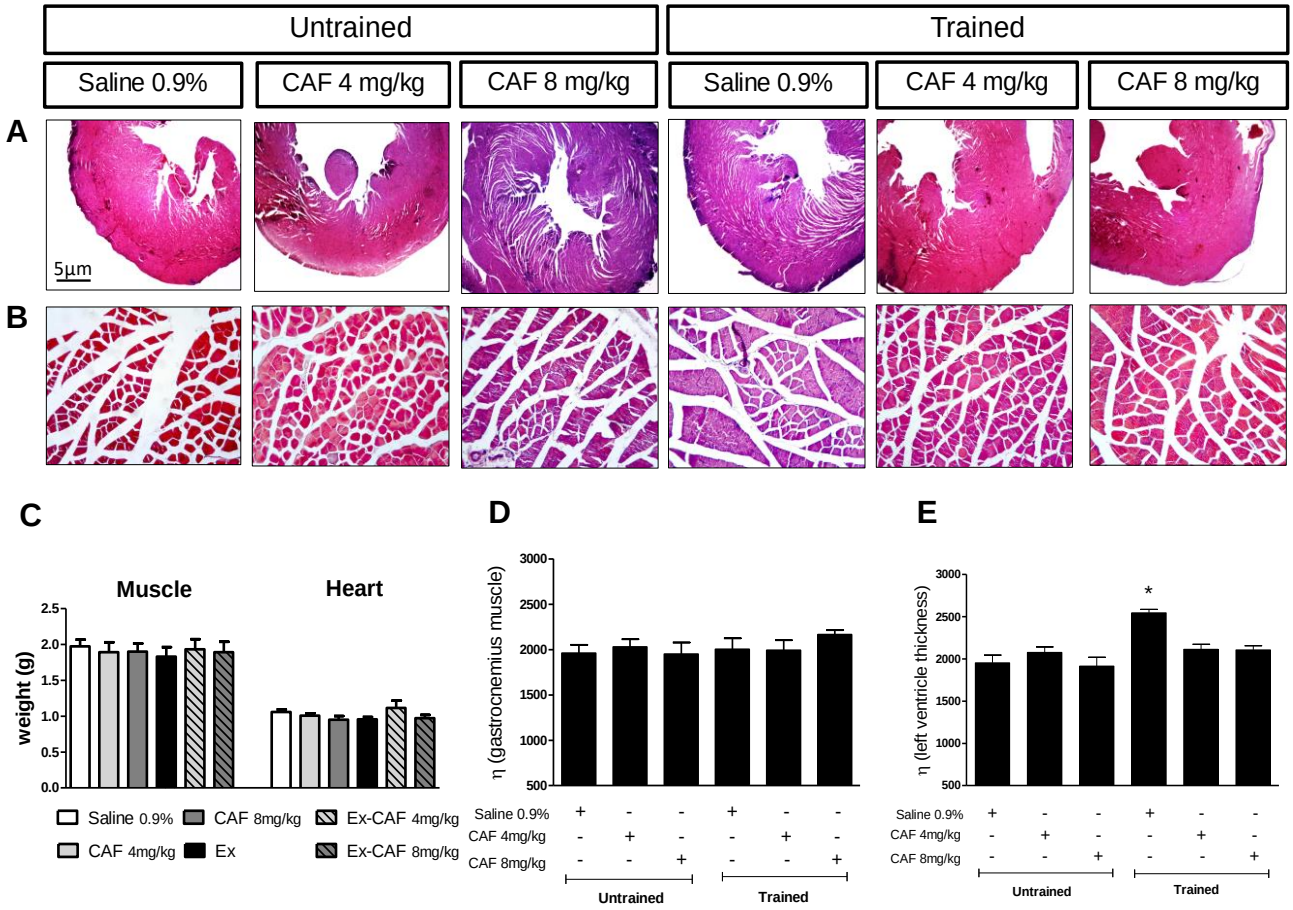

Figure 5

4.2 MANUSCRITO II

Caffeine prevents high intensity exercise-induced increase in the activities of the enzymatic antioxidant system and Na^+-K^+-ATPase, and reduction of anxiolytic like-behavior in rats

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Caffeine prevents high intensity exercise-induced increase in the activities of the enzymatic antioxidant system and Na^+-K^+-ATPase, and reduction of anxiolytic like-behavior in rats

Running Title

Effects of caffeine consumption in combination with chronic HIIT on the antioxidant system and anxiolytic like-behavior in rats

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Abstract

Objective: We investigated the impact of chronic high-intensity interval training (HIIT) and caffeine consumption on the activities of Na^+-K^+-ATPase and enzymes of the antioxidant system and anxiolytic-like behavior in the rat brain.

Methods: Animals were divided into several groups: control, caffeine 4mg/kg, caffeine 8mg/kg, HIIT, HIIT *plus* caffeine 4 mg/kg and HIIT *plus* caffeine 8 mg/kg. Rats were trained three times a week for 6 weeks, and vehicle or caffeine were administered 30 minutes before the training. We assessed anxiolytic-like behavior with the elevated plus-maze task. We also measured the activity of Na^+-K^+-ATPase, superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GPx), and levels of reduced glutathione (GSH) and malondialdehyde (MDA) in the brain.

Results and Discussion: HIIT induced anxiolytic-like behavior, increased Na^+-K^+-ATPase and GPx activities and MDA levels, changed the activity of SOD and CAT in different brain regions, and decreased GSH levels. Caffeine, however, elicited anxiogenic like-behavior and blocked HIIT effects. The combination of caffeine and HIIT prevented the increase in SOD activity in the cortex and GPx activity in the three brain regions. Our results show that caffeine promoted anxiolytic-like behavior and prevented HIIT-induced changes in the antioxidant system and Na^+-K^+-ATPase activities.

Keywords: caffeine; redox status; Na^+ K^+ pump; anxiety; brain; physical exercise..

Introduction

Healthier lifestyles are frequently associated with the regular practice of physical exercise, which disrupts cellular homeostasis by stimulating muscular activity [1]. For this reason, researchers have sought to elucidate the benefits of high-intensity interval training (HIIT), a training characterized by brief periods (around 30s) of extreme stress (≥ 90% - 100% of VO_2 max) intercalated with short periods of recovery [2]. HIIT exercise affects all body systems, increasing muscle glycogen levels [3], aerobic capacity and muscle hypertrophy [4], and also improves certain brain functions [5]. Recent studies show that HIIT increases the levels of brain-derived neurotrophic factor (BDNF) and insulin receptors, improving glucose uptake and metabolism [6, 7]. Moreover, HIIT has been shown to improve blood flow in the brain [8] and activate signaling pathways that promote adult hippocampal neurogenesis [9].

Na^+-K^+-ATPase (EC 3.6.3.9), a ubiquitous plasma membrane protein, plays a key role in the maintenance of intracellular electrolyte homeostasis in virtually all tissues [10]. Reduction of Na^+-K^+-ATPase levels and activity directly impairs neurotransmitter signaling with deleterious consequences on learning and memory, and increases locomotor activity and anxiety [11, 12]. Impairment of Na^+-K^+-ATPase activity and/or mutations in its alpha subunits lead to neuronal dysfunction and may trigger depression, anxiety and bipolar disease [13 - 15]

Intake of ergogenic substances has become a common strategy to enhance sports performance beyond the effects of training. Caffeine is an alkaloid compound with psychostimulant effects that is mainly consumed through tea and coffee [16], and that acts as a non-selective adenosine receptor antagonist that modulates the A_{2A} receptors [17] with antioxidant [18] and neuroprotective effects [19 - 22]. In recent decades, athletes have used caffeine to improve their performance [23 - 25] and several studies have suggested that intake of 3 to 9 mg/kg of caffeine can slow the process of fatigue during long-term exercise, prolonging activity by 20-50% [25-28]. Caffeine doses greater than 15 mg/kg, however, trigger several undesired effects, including headaches, anxiety, irritability, tachycardia and nausea [29, 30]

Given that HIIT exercise has beneficial effects on the central nervous system (CNS), we investigated the effect of caffeine in combination with this type of training in anxiolytic-like behavior, and in the activity of Na^+-K^+-ATPase and enzymes of the antioxidant system in the rat brain.

Materials and Methods

Animals

Male Wistar rats (100 days; 250–280g) from the Central Animal House of the Federal University of Santa Maria (UFSM) were maintained at a constant temperature (23±1 °C) on a 12 h light/dark cycle with free access to food and water. All animal procedures were approved by the Animal Ethics Committee from the UFSM (protocol number 077/2011).

Protocol of adaptation to HIIT

The HIIT exercise used the swimming. Initially all rats were adapted to water before beginning training. The adaptation was to keep the animals in shallow water (5 cm in depth) at 32 °C, 20 min per day for 5 days. The purpose of the adjustment was to reduce stress, without, however, promoting adaptation to the training. After this time, the animals were adapted to exercise where they were put to swim in a circular tank with 115 x 90 cm (diameter and deep, respectively) with water temperature of around 32 °C for 10 days. More information to the adaptation period and HIIT protocol can be found in the table 1 and in the figure 1.

Chronic protocol for HIIT and caffeine intake

Forty eight rats were divided into six groups with eight animals per group: vehicle, caffeine 4 mg/kg, caffeine 8 mg/kg, HIIT, HIIT plus caffeine 4 mg/kg, and HIIT plus caffeine 8 mg/kg. The exercise groups were trained three times a week (with 48h of recovery between sessions) for 6 weeks for a total workload of 23% body weight at the end of the experiment. The animals were submitted to 12 sets of swimming exercise of 25 seconds alternated with 35 seconds of recovery as previously described [31-33]. This protocol is characterized by high-intensity and short duration with successive and intermittent increases in workloads. It is mainly based on the principle of overload and adaptation. No-exercise groups were placed in shallow water at 32 ± 1ºC, 12 min three times a week, so as to be subjected to the same stress, without, however, suffering the effects of physical training.

The caffeine treatment started after the period of adaptation to HIIT. Caffeine (HPLC standert, >99 of purity) was purchased from Sigma Chemical Co. (St. Louis, MO, USA). Caffeine was diluted with saline 0.9% (1 ml/kg) and was administered 30 minutes before training, 5 days a week orally at a dose of 4 or 8 mg/kg. Vehicle and HIIT groups received

only 0.9% saline. The animals were treated with caffeine during 6 weeks. Caffeine was administered until 48 hours after last training. After behavioral tasks, the animals were submitted to euthanasia (see the protocol in the fig.1).

Elevated plus maze task

Anxiolytic-like behavior was evaluated using the elevated plus maze task as previously described [34]. The apparatus consists of a structure raised to 50 cm from the floor and is composed of 4 arms of the same size, with two closed-arms (walls 40 cm) and two open-arms. Initially, the animals were placed on the central platform of the maze in front an open arm. The animal had 5 minutes to explore the apparatus. Was recorded the time spent and the number of entries in center, open and closed arms, fecal pellets and head in deep. The apparatus was thoroughly cleaned with 30% ethanol between each session.

Sample preparation for biochemical parameters

Cerebral cortex, hippocampus and striatum were separated and homogenized in a solution of 10 mM Tris–HCl, 0.1 mM EDTA, pH 7.4, on ice and processed as previously described [35]. An aliquot of the homogenate was separated. After centrifugation of 1.500 g at 4ºC for 15 min, aliquots of the supernatant were stored at −80 °C until the biochemical analyses.

Na^+,K^+-ATPase activity

Na^+,K^+-ATPase activity was measured in the supernatant as previously described [36] with minor modifications[37]. The assay medium consisted of (in mM) 40 Tris-HCl buffer (pH 7.4), 0.1 EDTA, 50 NaCl, 5 KCl, 6 $MgCl_2$, 150 μg of protein in the presence or absence of ouabain (4 mM). The reaction was started by the addition of ATP (3 mM). After 30 min at 37ºC, the reaction was stopped by the addition of 30 μL of 50% (w/v) TCA. Appropriate controls were included in the assays for the non-enzymatic hydrolysis of ATP. The amount of inorganic phosphate (Pi) released was quantified colorimetrically, as previously described [38] using KH_2PO_4 as the reference standard. The absorbance was measured at 630 ηm. The specific Na^+,K^+-ATPase activity was calculated by subtracting the ouabain-insensitive activity from the overall activity (in the absence of ouabain) and was expressed in ηmol of Pi/mg of protein/min.

Superoxide dismutase (SOD) activity

The SOD activity was performed in supernatant and this method is based on reaction autoxidation adrenaline to adenochrome. The intermediate in this reaction is superoxide, which is scavenged by SOD. Results were expressed as U SOD/mg of protein. One SOD unit was defined as the enzyme amount to cause 50% inhibition of adrenaline autoxidation [39].

Catalase (CAT) activity

CAT activity was carried out as previously described [40] with minor modifications [41]. The activity was determined by following the decomposition of 30 mM H2O2 in 50 mM potassium phosphate buffer (pH 7.0) at 240 nm for 120 s in a thermostated (37°C) Hitachi U-2001 spectrophotometer. Results were expressed as μmol H_2O_2/mg of protein and appropriate controls for nonenzymatic decomposition of H_2O_2 were included in the assays.

Glutathione peroxidase (GPx) activity

GPx activity was measured using a commercial kit (RANSEL®; Randox Lab, Antrim, United Kingdom). GPx catalyzes glutathione (GSH) oxidation by cumene hidroperoxide. GPx activity was determined in supernatant using glutathione reductase and NADPH. The method is based on the oxidation of NADPH, wich is indicated by a decrease in absorbance at 340 nm. The enzymatic activity was expressed as μmol NADPH /min/ mg of protein.

Glutatione reduced (GSH) levels

Reduced glutathione (GSH) was determined in supernatant as previously describe [42]. Aliquots of samples adjusted to 1mg/ml of protein content were added to a phosphate buffer 0.3 mol/l , pH 7.4 and the product of reaction was read at 412 nm after the addiction of 10 mM DTNB (0.05 ml). The results were expressed as μmol of GSH/mg of protein.

Brain malondialdehyde (MDA) measurement

MDA levels were obtained from homogenate by the method previously described [43] with a few modifications [44]. In short, the reaction mixture contained 200 μl of samples or standard (MDA, malondialdehyde 0.03 mM), 200 μl of 8.1% sodium dodecylsulfate (SDS), 750 μl of acetic acid solution (2.5 M HCl, pH 3.5) and 750 μl of 0.8% TBA. The mixtures

were heated at 95ºC for 90 min. The absorbance was measured at 532 nm and results were expressed as µmol MDA/ mg of protein.

Statistical analysis

Results were analysed statistically by one or two-way ANOVA, followed by Tukey post hoc test (Graph Pad Prism 5.0). Differences between groups were considered to be significant when $P<0.05$. Dates were expressed as mean ± standard error medium (SEM).

Results and Discussion

HIIT induces anxiolytic-like behaviour in rats: Caffeine blocks this effect

Anxiety disorders are the most common mental illness in the general population with prevalence about 25% [45]. The clinical symptoms are often accompanied by cognitive impairment, suggesting that interactions between affective state and cognition may underlie the debilitating nature of pathological anxiety [46]. In this context, physical activity has been proposed as an alternative for improving mental health since the sport practice has been associated with improvement in symptoms of anxiety, depression and cognitive[46,47]. Based on the evidence, our results (see figure 2 and 3) show that chronic HIIT was able to induce an anxiolytic-like behavior in rats. HIIT decreased the number of entries to closed-arms [$F_{5,54}$=5.960, $p<0.001$, graph 2A] and increased the number of entries to open-arms [$F_{5,54}$= 6.618, $p<0.001$, graph 2B]. In addition, caffeine 4 and 8 mg/kg did not change the entries to open and closed-arms, but prevented the anxiolytic-like behavior induced by HIIT. Furthermore, caffeine 4 and 8 mg increased the time spent in the closed-arm [$F_{5,54}$= 8.072, $p<0.001$, graph 2C] and also reduced it in the open-arms [$F_{5,54}$= 5.504, $p<0.001$, graph 2D]. HIIT was able to prevent the anxiogenic-like behavior induced by caffeine in the time spent in open-arms.

Caffeine also showed an anxiogenic effect reducing the time spent in center [F5,54= 12.020, $p<0.001$, graph 3A]. There were no significant differences between groups to head in deep [F5,54= 0.366, $p>0.05$, graph 3B]. Caffeine 4 mg/kg increased the number of fecal pellets and HIIT prevented this effect [F5,54= 2.930, $p<0.05$, graph 3C]. No significant differences were observed between groups in the number of crossings in the arms [F5,54=0.821, $p>0.05$, graph 3D]. As seen above, caffeine intake showed an opposite effect to HIIT inducing an anxiogenic-like behavior, and blocking the beneficial effects of HIIT on anxiety parameters. The fact, our findings corroborate evidence in the literature that point a

psychostimulant effect of caffeine since it is able to increase the anxiety in rats and humans [48-50].

Caffeine prevents the increase in the Na^+,K^+-ATPase activity induced by chronic HIIT

Inhibition of Na^+, K^+-ATPase activity is found in various neuropathological conditions, including neurodegenerative diseases [51] and some psychiatric disorders [13, 52]. An impairs in the ion homeostasis, by reduction in the Na^+,K^+-ATPAse activity, is found in patients with depression [53]. Furthermore the ouabain, an inhibitor of this enzyme, mimics some symptoms of bipolar disorder in rats [54]. In our study, HIIT increased the Na^+,K^+-ATPase activity only in the cerebral cortex [$F_{5,54}$= 8.350, $p<0.001$, graph 4A] and hippocampus [$F_{5,54}$= 6.151, $p<0.01$, graph 4B] and caffeine prevented this effect. A reduction in the activity of this enzyme has been associated with an increase in anxiety [12, 52]. We verified an increase in the activity of this enzyme as well as a reduction of anxiety in animals subjected to HIIT.

Chronic HIIT alters SOD and CAT activity in brain of rats

Since the brain is a highly sensitive to reactive oxygen species, and Na^+,K^+-ATPase activity is known to be affected by the redox state of the cell [37], we verified the activity of key enzymes that compose the antioxidant system. HIIT increased the SOD activity in cerebral cortex [$F_{5,54}$= 5.068, $p<0.01$, graph 5A]. On the other hand, this exercise reduced the SOD activity in hippocampus [$F_{5,54}$= 7.175, $p<0.001$, graph 5B] and striatum [$F_{5,54}$= 11.450, $p<0.001$, graph 5C]. Caffeine prevented the effects of HIIT in the cerebral cortex.

A similar effect was observed to CAT activity. HIIT increased the CAT activity in cerebral cortex [$F_{5,54}$= 5.764, $p<0.001$, graph 5D] and reduced in the hippocampus [$F_{5,54}$=3.898 6.618, $p<0.01$, graph 5E], however caffeine were not able to prevent the effects induced by HIIT. No significant differences were observed among groups in the striatum [$F_{5,54}$= 1.081, $p>0.05$, graph 5F].

Experimental studies have indicated that caffeine intake provides neuroprotective effects against several neurological disorders such as Alzheimer's and Parkinson's diseases [56-58]. A possible mechanism occurs due to a modulation of the A_{2A} receptor since there are studies showing blocking this receptor reduces the production of reactive species and cellular death [59, 60].

Caffeine did not prevent alterations in the GPx activity and reduction in GSH levels induced by HIIT in the brain of rats

Following this line, the HIIT exercise increased the GPx activity in the cerebral cortex [F5,54= 10.370, p<0.0001, graph 6A], hippocampus [F5,54= 5.768, p<0.001, graph 6B] and striatum [F5,54= 13.750, p<0.0001, graph 6C] and caffeine prevented this effect. Furthermore, HIIT reduced the GSH content only in hippocampus, and caffeine did not show any significant effect in restore de GHS levels [$_{F5,54}$= 10.450, p<0.0001, graph 6E]. There were no significant differences between groups in the cerebral cortex [$_{F5,54}$= 1.009, p>0.05, graph 6D] and striatum [$F_{5,54}$= 0.528, p>0.05, graph 6F].

Caffeine prevents the increase in the MDA levels induced by HIIT in the brain of rats.

HIIT increased the MDA levels in cerebral cortex [F5,54= 7.229, p<0.001, graph 7A], hippocampus [F5,54= 3.970, p<0.01, graph 7B] and striatum [F5,54= 11.560, p<0.01, graph 7C]. Caffeine 4 and 8 mg/kg prevented this effect only in the cerebral cortex and hippocampus.

Neurochemical and behavioral changes found in rats submitted to chronic HIIT associated with caffeine intake

Although the effects of physical exercise are beneficial to the health, as to reduce anxiety or promote an increase in the activity of enzymes of the antioxidant system, we verified an increase in MDA content, suggesting an increase in lipoperoxidation. In the brain, the high content of polyunsaturated fatty acids and the high utilization of oxygen account for the susceptibility to free radical damage. The chronic HIIT induces an adaptive response in the brain redox system since it increases the activity of antioxidant enzymes SOD, CAT and GPx. Possibly, the increase in cerebral blood flow induced by HIIT leads to an increase in tissue oxygen supply and the glycolytic metabolism culminating in a higher production of reactive oxygen species from the mitochondria. Once the physical exercise induces an increase in the number of cellular mitochondria, this event can promote the reactive species production. In this context, an improve in the enzymatic antioxidant system can be an adaptive response induced by HIIT. In addition, it has been describe that HIIT increases BDNF and GDNF concentrations in the brain of rats, and these alterations is related to the higher concentrations of H_2O_2 and TNF-α produced during the exercise [61]. In the brain after HIIT, an increase in the hippocampal TNFα and BDNF concentrations also was reported by,

and this condition is associated to stress oxidative, specially to H_2O_2 levels, and hypoxic condition during HIIT [61, 62]. These reports can explain the neurochemical alterations in the enzymatic activity of the antioxidant system, possibly in response to an increase in the H_2O_2 production. Furthermore, we found that HIIT caused an increased in TBARS content, signaling the establishment of oxidative stress in the CNS. On the other hand, we find that the caffeine blocked this effect. Since caffeine is one neuroprotective and antioxidant molecule, the chronic caffeine intake acted preventing the production of reactive species and an adaptive response of the redox system front to chronic HIIT. In conclusion the present study we demonstrated that HIIT induces adaptations in the enzymatic antioxidant system, increases the Na^+,K^+-ATPase activity and anxiolytic-like behavior. Furthermore, it is not clear whether caffeine has a beneficial or harmful role when associated with HIIT since it prevented the effects induced by the physical exercise. Differences in metabolism, diet, frequency of caffeine intake, type of exercise are some of the factors that can determine how an individual will react to association between caffeine and HIIT. In addition, more studies are necessary to better elucidate these alterations caused by caffeine associated with HIIT (see figure 8).

Acknowledgments

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Conflicts of Interest statement

We have not received any financial support or other benefits from commercial sources for the work reported here. None of the authors has financial interests that could create a potential conflict of interest or the appearance of a conflict of interest with regard to this work.

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Legends

Table 1. High- intensity interval training (HIIT) protocol. Workload (%) based on body weight. Time of exercise in min. Recovery time through 1 minute in the times described in table (recovery time).

Figure 1. Experimental protocol to chronic high-intensity interval training (HIIT) associated to caffeine intake (4 and 8 mg /kg). The adaptation occurred between -10 to 0 day (performing a gradual increase in workload to 10.5% body weight). On day 2 was measured the blood lactate content (LT). On day 3 started the HIIT, three times a week, with interval of 48 hours between each session. The workload (%) can be found in the timeline. The last HIIT session was performed at 45 day. After 48 hours, the elevated plus maze task was carried out and next, the animals were submitted to euthanasia.

Figure 2. Effects of chronic high-intensity interval training (HIIT) associated to caffeine (4 and 8 mg/kg) on behaviour in the elevated plus maze task. (A) Number of closed-arm entries, (B) number of open-arms entries, (C) time spent in the closed-arm and (D) time spent in the open-arms. Data are expressed as mean ± SEM. $P<0.05$ was considered to represent a significant difference. *Denotes a significant difference from the vehicle group. # Denotes a significant difference compared with the HIIT group (ANOVA one-way followed by post-hoc Tukey, n = 8-10).

Figure 3. Effects of chronic high-intensity interval training (HIIT) associated to caffeine (4 and 8 mg/kg) on behaviour in the elevated plus maze task. (A) Time spent in center, (B) head in deep, (C) number of faecal pellets and (D) number of entries to arms. Data are expressed as mean ± SEM. $P<0.05$ was considered to represent a significant difference. *Denotes a significant difference from the vehicle group. # Denotes a significant difference compared with the HIIT group (ANOVA one-way followed by post-hoc Tukey, n = 8-10).

Figure 4. Effects of chronic high-intensity interval training (HIIT) associated to caffeine (4 and 8 mg/kg) on Na^+,K^+-ATPase activity in the cerebral cortex (A), hippocampus (B) and striatum (C). Data are expressed as mean ± SEM. $P<0.05$ was considered to represent a significant difference. *Denotes a significant difference from the vehicle group. # Denotes a

significant difference compared with the HIIT group (ANOVA one-way followed by post-hoc Tukey, n = 8-10).

Figure 5. Effects of chronic high-intensity interval training (HIIT) associated to caffeine (4 and 8 mg/kg) on superoxide dismutase (SOD) and calatase (CAT) activities. SOD activity in the cerebral cortex (A), hippocampus (B) and striatum (C). CAT activity in the cerebral cortex (D), hippocampus (E) and striatum (F). Data are expressed as mean ± SEM. $P<0.05$ was considered to represent a significant difference. *Denotes a significant difference from the vehicle group. # Denotes a significant difference compared with the HIIT group (ANOVA one-way followed by post-hoc Tukey, n = 8-10).

Figure 6. Effects of chronic high-intensity interval training (HIIT) associated to caffeine (4 and 8 mg/kg) on glutathione peroxidase (GPx) activity and on reduced glutathione levels (GSH). GPx activity in the cerebral cortex (A), hippocampus (B) and striatum (C). GSH levels in the cerebral cortex (D), hippocampus (E) and striatum (F). Data are expressed as mean ± SEM. $P<0.05$ was considered to represent a significant difference. *Denotes a significant difference from the vehicle group. # Denotes a significant difference compared with the HIIT group (ANOVA one-way followed by post-hoc Tukey, n = 8-10).

Figure 7. Effects of chronic high-intensity interval training (HIIT) associated to caffeine (4 and 8 mg/kg) on malondialdehyde (MDA) levels in the cerebral cortex (A), hippocampus (B) and striatum (C). Data are expressed as mean ± SEM. $P<0.05$ was considered to represent a significant difference. *Denotes a significant difference from the vehicle group. # Denotes a significant difference compared with the HIIT group (ANOVA one-way followed by post-hoc Tukey, n = 8-10).

Figure 8. Neurochemical and behavioral changes found in rats submitted to chronic HIIT associated with caffeine intake.

Table 1. High- intensity interval training (HIIT) protocol. Workload (%) based on body weight. Time of exercise in min. Recovery time through 1 minute in the times described in table (recovery time).

Day	Workload (%)	HIIT time (min)	Interval (nº)	Recovery Time
1	0	20	0	no recovery
2	backpag	20	0	no recovery
3	backpag *plus* 3%	15	0	no recovery
4	backpag *plus* 3%	20	0	no recovery
5	backpag *plus* 5.5%0	15	0	no recovery
6	backpag *plus* 5.5%	20	0	no recovery
7	backpag *plus* 8%	15	2	5 and 10 min
8	backpag *plus* 8%	20	3	5, 10 and 15 min
9	backpag *plus* 10.5%	15	4	3, 6, 9 and 12 min
10	backpag *plus* 10.5%	20	4	4, 8, 12 and 16 min

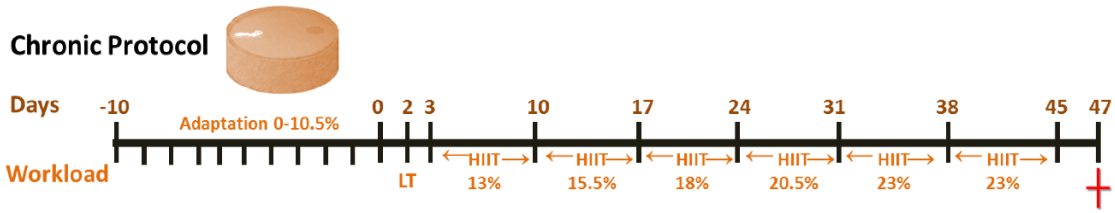

Figure 1

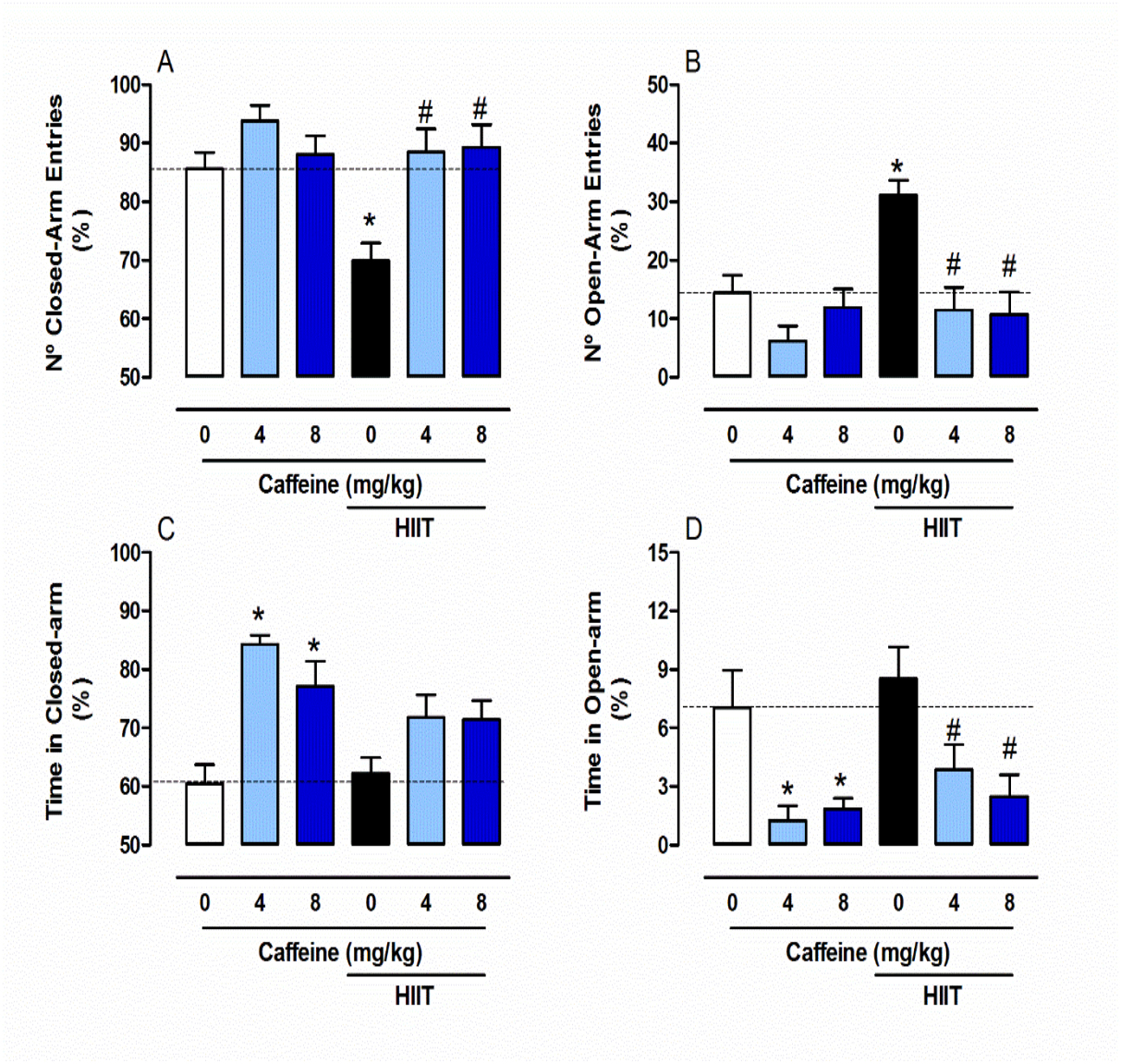
A
Nº Closed-Arm Entries (%)
100
90
80
70
60
50
*
#
#
0
4
8
0
4
8
Caffeine (mg/kg)
HIIT
B
Nº Open-Arm Entries (%)
50
40
30
20
10
0
*
#
#
0
4
8
0
4
8
Caffeine (mg/kg)
HIIT
C
Time in Closed-arm (%)
100
90
80
70
60
50
*
*
0
4
8
0
4
8
Caffeine (mg/kg)
HIIT
D
Time in Open-arm (%)
15
12
9
6
3
0
*
*
#
#
0
4
8
0
4
8
Caffeine (mg/kg)
HIIT

Figure 2

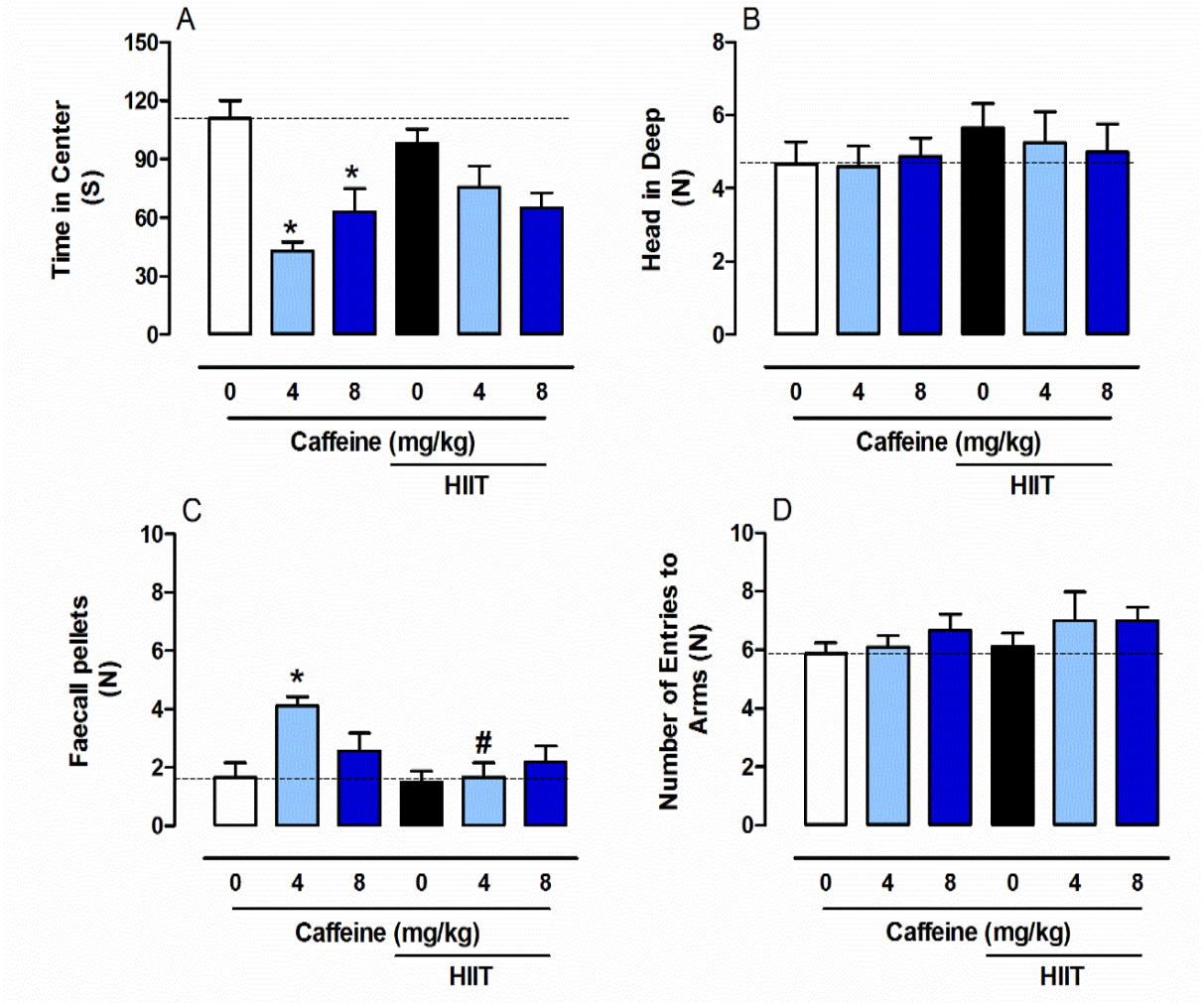
A
Time in Center (S)
150
120
90
60
30
0
*
*
0 4 8 0 4 8
Caffeine (mg/kg)
HIIT
B
Head in Deep (N)
8
6
4
2
0
0 4 8 0 4 8
Caffeine (mg/kg)
HIIT
C
Faecall pellets (N)
10
8
6
4
2
0
*
#
0 4 8 0 4 8
Caffeine (mg/kg)
HIIT
D
Number of Entries to Arms (N)
10
8
6
4
2
0
0 4 8 0 4 8
Caffeine (mg/kg)
HIIT

Figure 3

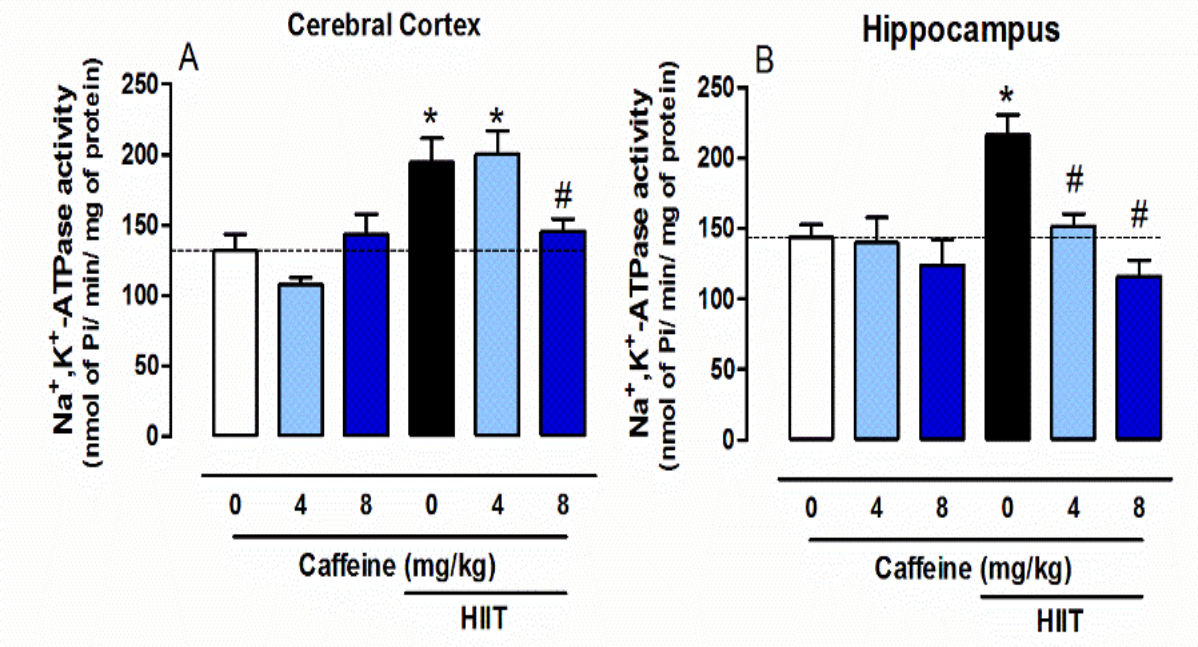
Cerebral Cortex
A
Hippocampus
B
Striatum
C
Na+,K+-ATPase activity
(nmol of Pi/ min/ mg of protein)
250
200
150
100
50
0
*
*
#
#
#
0
4
8
0
4
8
Caffeine (mg/kg)
HIIT

Figure 4

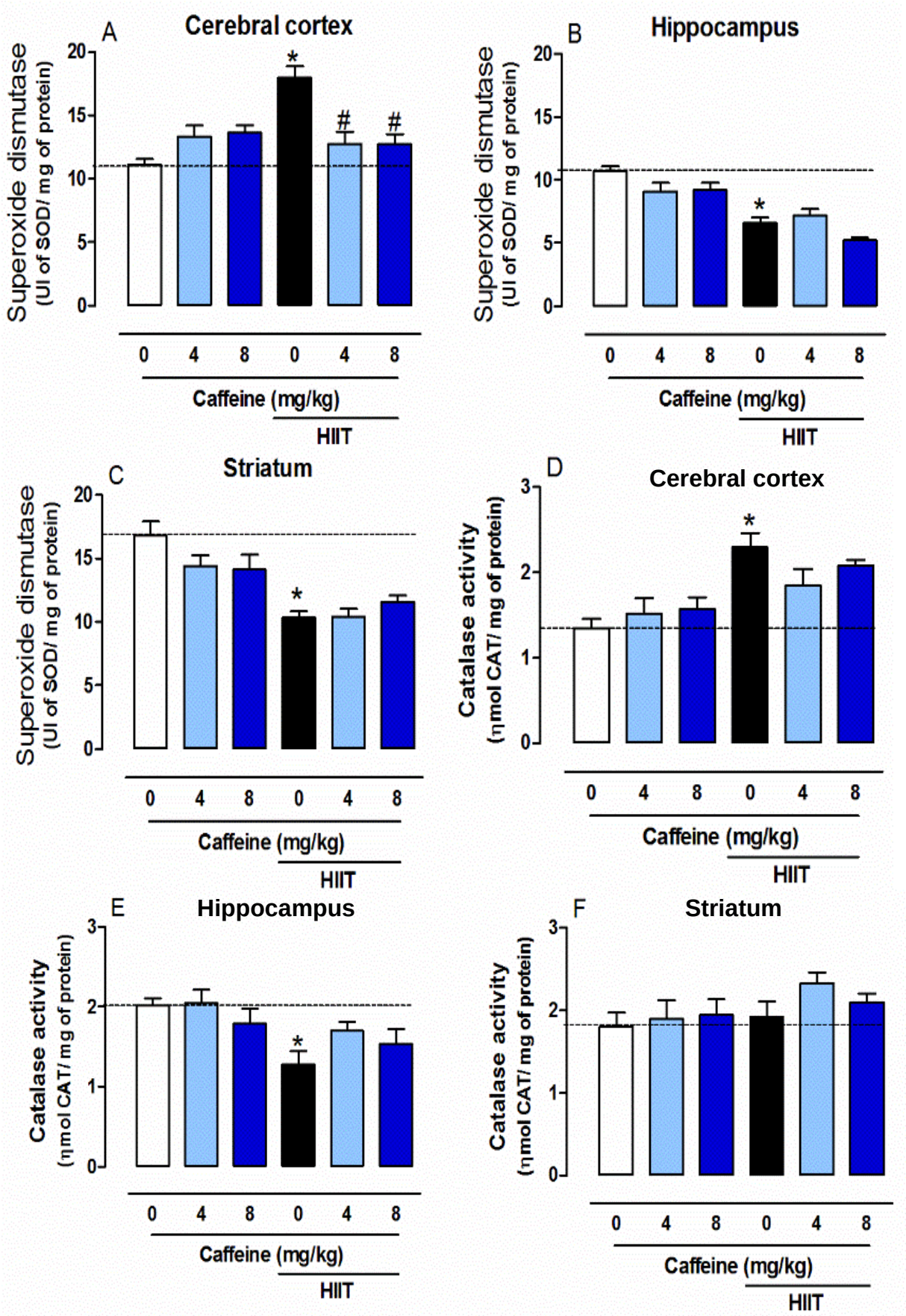
A
Cerebral cortex
Superoxide dismutase
(UI of SOD/ mg of protein)
20
15
10
5
0
*
#
#
0 4 8 0 4 8
Caffeine (mg/kg)
HIIT
B
Hippocampus
Superoxide dismutase
(UI of SOD/ mg of protein)
20
15
10
5
0
*
0 4 8 0 4 8
Caffeine (mg/kg)
HIIT
C
Striatum
Superoxide dismutase
(UI of SOD/ mg of protein)
20
15
10
5
0
*
0 4 8 0 4 8
Caffeine (mg/kg)
HIIT
D
Cerebral cortex
Catalase activity
(ηmol CAT/ mg of protein)
3
2
1
0
*
0 4 8 0 4 8
Caffeine (mg/kg)
HIIT
E
Hippocampus
Catalase activity
(ηmol CAT/ mg of protein)
3
2
1
0
*
0 4 8 0 4 8
Caffeine (mg/kg)
HIIT
F
Striatum
Catalase activity
(ηmol CAT/ mg of protein)
3
2
1
0
0 4 8 0 4 8
Caffeine (mg/kg)
HIIT

Figure 5

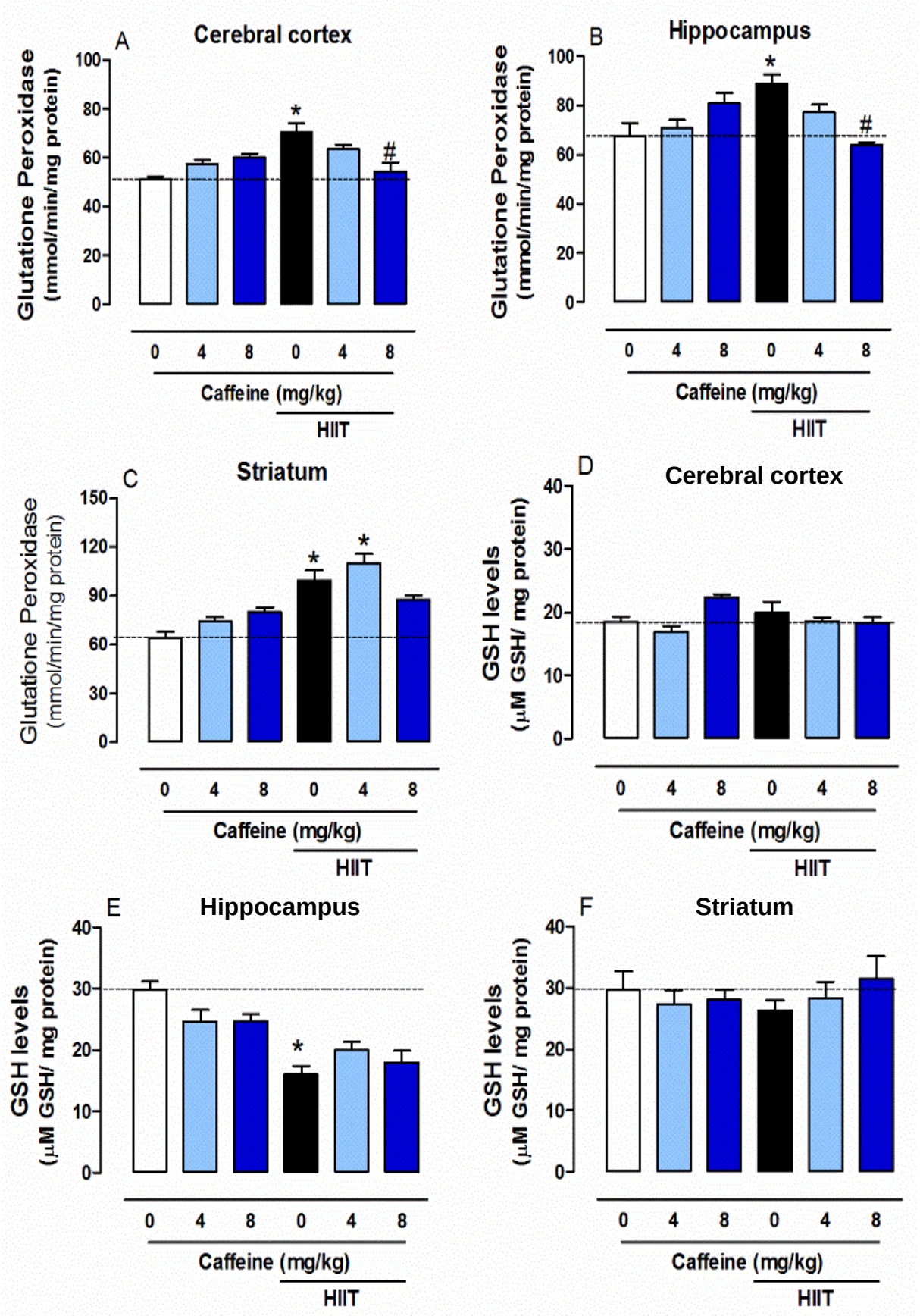
A
Cerebral cortex
Glutatione Peroxidase
(mmol/min/mg protein)
100
80
60
40
20
0
*
#
0 4 8 0 4 8
Caffeine (mg/kg)
HIIT
B
Hippocampus
Glutatione Peroxidase
(mmol/min/mg protein)
100
80
60
40
20
0
*
#
0 4 8 0 4 8
Caffeine (mg/kg)
HIIT
C
Striatum
Glutatione Peroxidase
(mmol/min/mg protein)
150
120
90
60
30
0
*
*
0 4 8 0 4 8
Caffeine (mg/kg)
HIIT
D
Cerebral cortex
GSH levels
(µM GSH/ mg protein)
40
30
20
10
0
0 4 8 0 4 8
Caffeine (mg/kg)
HIIT
E
Hippocampus
GSH levels
(µM GSH/ mg protein)
40
30
20
10
0
*
0 4 8 0 4 8
Caffeine (mg/kg)
HIIT
F
Striatum
GSH levels
(µM GSH/ mg protein)
40
30
20
10
0
0 4 8 0 4 8
Caffeine (mg/kg)
HIIT

Figure 6

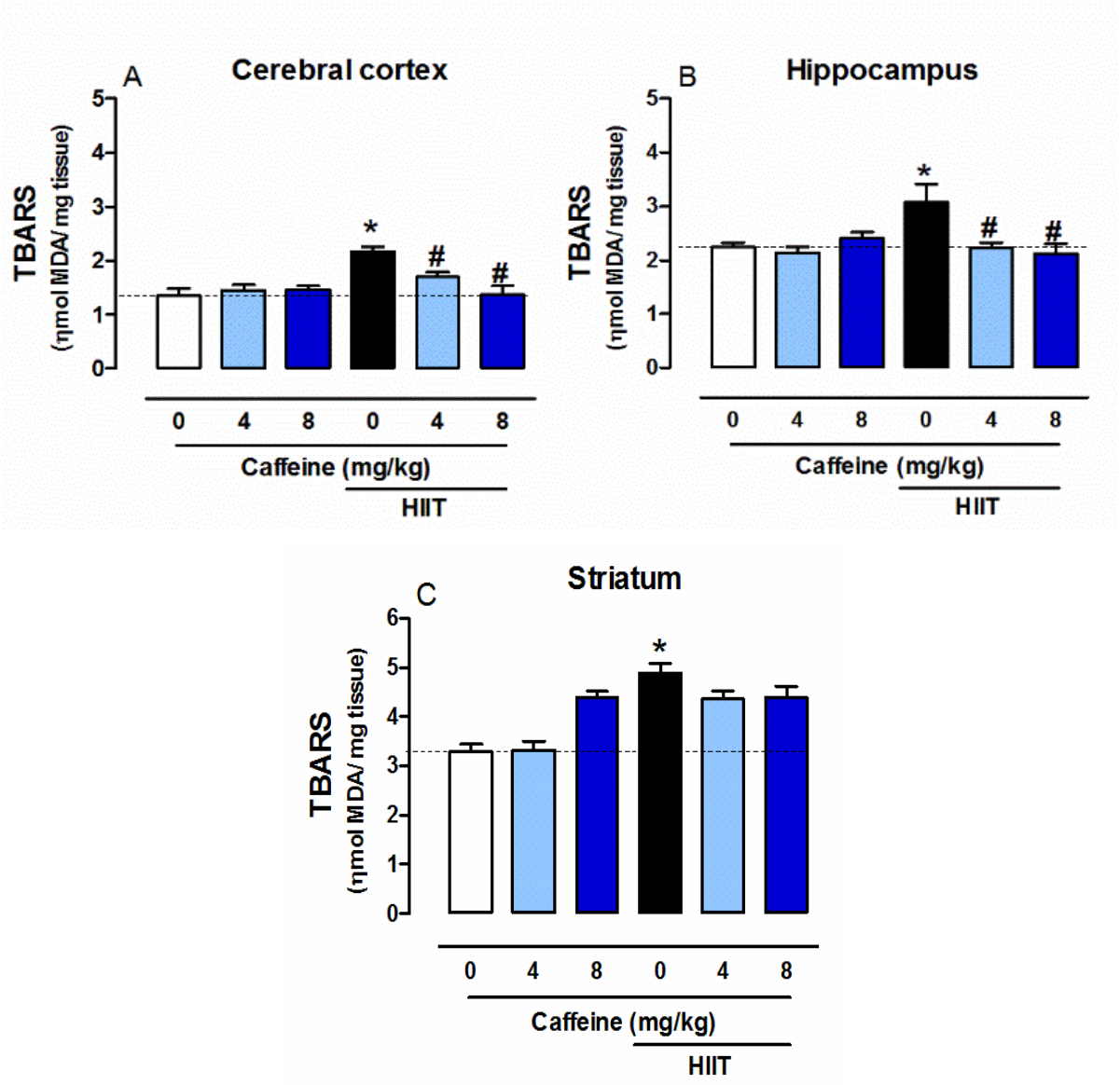
A
Cerebral cortex
TBARS
(ηmol MDA/ mg tissue)
0
1
2
3
4
5
*
#
#
0
4
8
0
4
8
Caffeine (mg/kg)
HIIT
B
Hippocampus
TBARS
(ηmol MDA/ mg tissue)
0
1
2
3
4
5
*
#
#
0
4
8
0
4
8
Caffeine (mg/kg)
HIIT
C
Striatum
TBARS
(ηmol MDA/ mg tissue)
0
1
2
3
4
5
6
*
0
4
8
0
4
8
Caffeine (mg/kg)
HIIT

Figure 7

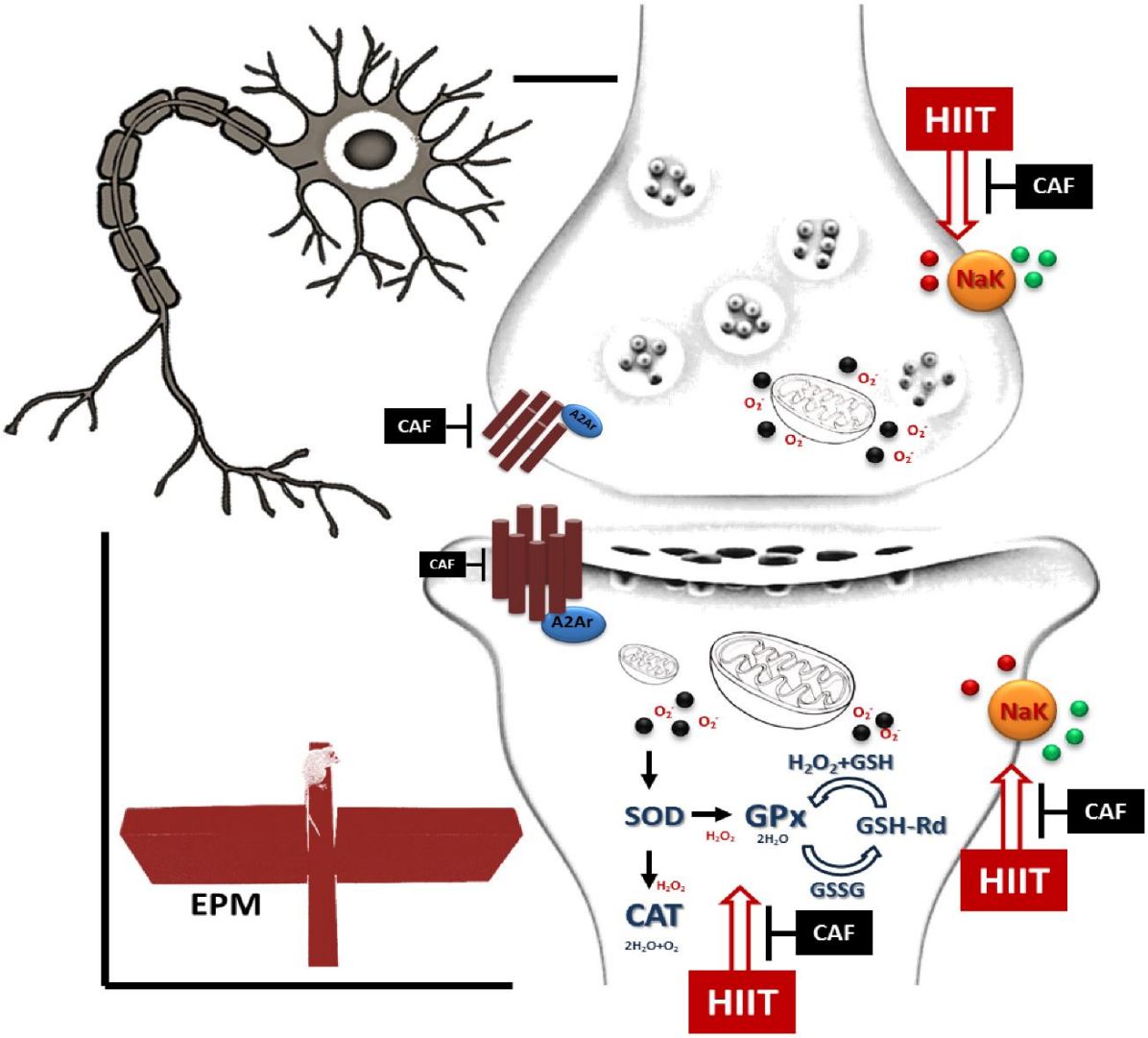
HIIT
CAF
NaK
CAF
A2Ar
CAF
A2Ar
EPM
NaK
CAF
HIIT
H_2O_2+GSH
SOD
GPx
GSH-Rd
GSSG
CAT
CAF
HIIT

Figure 8

4.3 MANUSCRITO III:

Caffeine and high intense exercise: impact in purinergic and cholinergic signaling in lymphocytes and cytokines levels

Juliano Marchi Vieira, Jessié Martins Gutierres, Fabiano Barbosa Carvalho, Andréia Cardoso Machado, Naiara Stefanello, Liziele Oliveira, Vera Maria Morsch, Michele Mainardi Pillat, Marta Duarte, Maria Rosa Chitolina Schetinger, Roselia Spanevello

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Caffeine and high intense exercise: impact in purinergic and cholinergic signaling in lymphocytes and cytokines levels

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Abstract

Caffeine has been used by athletes as ergogenic substance to improve performance. This study evaluated the effects de caffeine in combination with high-intensity interval training (HIIT) in the sensibility and proliferation of the lymphocytes, IL-6 and IL-10 levels and NTPDase, adenosine deaminase (ADA) and acetylcholinesterase (AChE) activities in lymphocytes the rats. The animals were divided in groups: control, caffeine 4 mg/kg, caffeine 8 mg/kg, HIIT, HIIT *plus* caffeine 4 mg/kg and HIIT plus caffeine 8 mg/kg. The rats were trained three times a week for 6 weeks for a total workload 23% of body weight at the end of the experiment. Caffeine was administrated orally 30 minutes before the training session. When lymphocytes were stimulated with phytohemagglutinin no changes were observed in proliferative responses between trained and sedentary animals, however when caffeine was associated with HIIT occurred an increase in T lymphocytes proliferation and in the sensibility of the lymphocytes to glucocorticoides. ATP and ADP hydrolysis was decreased in lymphocytes of the animals only trained and caffeine treatment prevented alterations in ATP hydrolysis. HIIT caused an increase in the ADA and AChE activities in lymphocytes and this effect was more pronounced in rats trained and supplemented with caffeine. The level of IL-6 was increased while that the level of IL-10 was decreased in trained animals (HIIT) and caffeine was capable to prevent this exercise effect. Our findings suggest that caffeine ingestion appear to attenuate, as least in part, the alterations immune and inflammatory following prolonged HIIT protocol.

Key Words: Caffeine; Acetylcholinesterase; Cytokines; high-intensity exercise; lymphocytes; NTPDase; Adenosine deaminase

1.Introduction

High intensity exercise training is characterized by alternating periods of maximum intensity exercise with short rest periods for a specific number of sets (Gibala et al., 2006). This type of training has been used by athletes not only to improve performance, but it also improves ability of the muscles e induces beneficial metabolic adaptations (Rakobowchuk et al., 2008; Koshinaka et al., 2009; Osawa et al., 2014). However, exhaustive physical exercise has been associated with immune changes such as reduced lymphocyte function and increase in pro - inflammatory cytokines levels (Mars et al., 1998; Pedersen et al., 2000; Gleeson, 2007).

It is well established in the literature that purinergic and cholinergic signaling can modulate the inflammatory and immune status. The activation of lymphocytes as well as the release of pro and/or anti-inflamatory citokines is related to changes in extracellular circulating nucleotides and nucleosides such as ATP and adenosine and by acetylcholine (Ach) molecule (Das, 2007; Junger, 2011). ATP released by cells may acts as a danger signal eliciting immune responses, while adenosine and ACh have anti-inflammatory properties (Junger, 2011; DI Virgilio, 2005; Ravichandran, 2010, Das, 2007). However, the effectiveness of these molecules is dependent of some specific enzymes, which are expressed on immune cells membrane (Junger, 2011; Das, 2007). ATP released into the extracellular space can be rapidly hydrolyzed to ADP and AMP by ecto-NTPDases enzymes family. AMP produced can be converted into adenosine by the action of ecto-5'nucleotidase, and adenosine can be degraded to inosine by adenosine deaminase (ADA). The availability of ACh is dependent of the acetylcholinesterase (AChE) enzyme activity (Das, 2007). Thus, the complex network of purinergic and cholinergic enzymes has a central role in the immune responses controlling the pro and anti-inflammatory effects the molecules such as ATP, adenosine and Ach. Previous studies from our research group have showed that moderate aerobic exercise is capable to modulate the cholinergic and purinergic system enzymes (Cardoso et al. 2012; 2014; 2015).

Caffeine is a secondary plant metabolite found in coffee and tea that has been used by athletes as ergogenic substance to improve performance endurance (Graham e Spriet, 1991a; Graham, Hibbert *et al.*, 1998b). Studies have suggested that intake 3 to 9 mg/kg of caffeine slows down the process of fatigue during intense exercise (Spriet, 1992; Greer, Mclean *et al.*, 1998b; Graham, 2001; Davis, Zhao *et al.*, 2003). The mechanism involved in the benefits of

caffeine in exercise performance not is clear but several hypotheses has been proposed such as antagonism of adenosine receptors (Ribeiro e Sebastiao, 2010).

Considering the points raised above, the aim of this study were evaluated the effects de caffeine in combination with high intensive exercise (HIIT) in the sensibility and proliferation of the lymphocytes and in the NTPDase, ADA and AChE activities in this cells. The levels of citokines IL-6 and IL-10 in serum also were evaluated.

2.Material and Methods

2.1 Chemicals

Nucleotides, trizma base, ficcoll hypaque, adenosine, acetylthiocholine iodide (ASCh), 5, 5′-dithio-bis-2-nitrobenzoic acid (DTNB) and caffeine were purchased from Sigma Chemical Co. (St. Louis, MO, USA). All other reagents used in the experiments were of analytical grade and of the highest purity.

2.2 Animals

Male Wistar rats (60 days; 250–280g) from the Central Animal House of the Federal University of Santa Maria were used in this experiment. Animals were maintained at a constant temperature (23±1 °C) on a 12 h light/dark cycle with free access to food and water. All animal procedures were approved by the Animal Ethics Committee from the Federal University of Santa Maria (protocol under number: 077/2011).

2.3 Experimental exercise protocol

The intermittent exercise used in this study was swimming. Firstly, all rats were adapted to water before the beginning of the training. The adaptation was to keep the animals in shallow water (5 cm in depth) at 31 ± 1 °C, 20 min by day for 5 days. The purpose of the adjustment was the reduction of stress, without, however, promoting adaptations to the training. After this time the animals were adapted to exercise where they were subjected for 10 days to swim in a tank of 115 X 90 X 90 with the water temperature around 32 °C. The adjustment period is: day 1: 20 minutes of swimming, without load; day 2: 20 minutes of swimming only "backpacks" (no charge); day 3: 15 minutes of swimming, corresponding to 3 % body weight load; day 4: 20 minute swim with 3 % of body weight; day 5: 15 minutes of swimming with the equivalent of 5.5 % of body weight load; day 6: 20 minute swim with 5.5

% of body weight; day 7: 15 minute swim with 8 % of body weight (3 intervals of 1min each , in 4, 8 and 12 minutes for recovery); day 8: swim for 20 minutes to 8% of body weight (4 intervals of 1min each in times 4', 8', 12' and 16' minutes to recovery); day 9 swim for 15 minutes and 10.5% of body weight (4 intervals of 1 min each in three minutes, 6', 9 'and 12' for retrieval); day 10: 20 minute swim with a load equivalent to 10.5% of body weight (4 intervals of 1min each in 4 minutes for recovery). Forty- eight hours after the adjustment period and therefore, before the start of training will apply a stress test to assess maximal lactate obtained by the adapted protocol. Blood samples from the distal end of the tail of the rats at rest for lactate measurements are collected. Immediately after, a test of progressive loads equivalent to 4.5, 6.5, 8.5 and 10.5% of the body-weight of each animal lasted 30 seconds exercise will be conducted. A pause of 60 seconds about to collect lactate and their trade cargo will be held. After this procedure the animals were submitted chronic protocol exercise.

2.4 Caffeine treatment

Forty - eight male wistar were divided into six groups (n= 8 each): I (Control), II (Caffeine 4 mg/kg), III (Caffeine 8 mg/kg), IV (Trained), V (Trained *plus* caffeine 4 mg/kg), and VI (Trained *plus* caffeine 8 mg/kg). The exercise protocol consists of three work outs per week with progressive increases (2.5 % of body weight) every week. The animals were weighed every week for the calculation of workloads. The rats of groups IV, V and VI were trained three times a week for 6 weeks for a total workload 23% of the body weight at the end of the experiment. The animals were submitted to 12 sets of exercise with alternating births of about 25 seconds of activity for 35 seconds as described in the literature. This protocol is characterized by high-intensity and short duration with successive increases in workloads, being carried out intermittently, is mainly based on the principle of overload and adaptation. The animals in groups I, II and III were placed in shallow water at 32 ± 1ºC, 12 min for three times a week, as the goal of being subjected to the same stress, without, however, suffering the effects of physical training. The exercise protocol was performed three times per week on alternate days always at the same time, for 6 weeks.

The caffeine treatment starts after the period of adaptation to exercise. Caffeine was diluted with saline 0,9% water (1 ml/kg) and was administered 30 minutes before training, 5 days a week orally at a dose of 4 mg/kg in the animals of the groups II and V and dose of 8 mg/kg in animals of the groups III and VI, while animals of the groups I and IV will receive

only saline 0,9%. The animals were treated with caffeine during 6 weeks. After this time, the animals were submitted to euthanasia and blood was collected for culture experiments and biochemical determinations.

2.5 Lymphocytes culture experiments

Five milliliters of peripheral blood were collected in EDTA tubes. All samples were analyzed within 4h after collection. Peripheral blood mononuclear cells (PBMCs) were isolated by centrifugation over a Ficoll-hypaque (Sigma) gradient (900 g, 30 min). The viability of cells was found to always exceed 95%, as judged from the cells' ability to exclude trypan blue (Sigma). PBMCs were resuspended in culture medium RPMI-1640 with fetal calf serum (FCS) 10% with concentration adjusted to 3×10^6 cells/mL.

2.6 Lymphocytes proliferation viability assay

PBMCs were cultured in flat-bottomed 96-well microplates at 1.5×10^5 cells/well in complete culture medium for 96 h at 37 °C in an atmosphere with 5% CO^2. Stimulation was performed with selective T-cell mitogen 1% phytohemagglutinin (PHA, from Gibco, USA) or 50 ng/mL of synthetic diacylglycerol phorbol 12-myristate 13-acetate (PMA, from ACROS Organics, Belgium) plus 250 ng/mL of calcium ionophore ionomycin (all from Invitrogen, USA). The proliferative response was determined by a modified colorimetric (MTT) assay as previously described (Luz et al., 2006). The optical density (OD) was determined using a Biorad ELISA plate reader at a wavelength of 570 and 620 nm. Proliferation-viability was expressed as ΔOD (OD of stimulated−OD of unstimulated cultures).

2.7 Lymphocytes sensitivity to glucocorticoids

Cellular sensitivity to GCs was evaluated by the ability of dexamethasone (DEX, a selective GC receptor agonist; Sigma, USA) to suppress T-cell proliferation in vitro. DEX (10^{-9} to 10^{-5}M) was added in duplicates (50 µL/well) to PBMC cultures stimulated with PHA or unstimulated. To address the role of ERK and p38 over cellular GC sensitivity, specific MAPK inhibitors (U0126 and SB203580) were also used in some cultures when indicated DMSO was used as negative control. Data are shown as percentage of basal proliferation (100%=proliferation with different stimuli without DEX).

2.8 NTPDase, ADA e AChE determination in lymphocytes

Lymphocytes were isolated from blood collected with EDTA as anticoagulant and separated on Ficoll–Histopaque density gradients as described by Böyum (1968). Then, lymphocytes were suspended in saline solution and the final protein concentration was adjusted to 0.1–0.2 mg/mL. In lymphocytes, the NTPDase activity was determined as described by Leal et al. (2005). The reaction medium contained 0.5 mM $CaCl_2$, 120mM NaCl, 5 mM KCl, 6 mM glicose and 50 mM Tris HCl buffer pH 8.0, at a final volume of 200 µL. In both cases, 20 µL of the enzyme preparation (8–12 µg of protein) was added to the reaction mixture and pre-incubated at 37°C. The reaction was initiated by the addition of ATP, ADP or AMP. Reactions were stopped by the addition of 10% trichloroacetic acid (TCA). Released inorganic phosphate (Pi) was assayed using malachite green as the colorimetric reagent and KH_2PO_4 as standard. Controls were carried out to correct for non-enzymatic hydrolyses of nucleotides by adding platelets after TCA addition. All samples were run in triplicate. Enzyme specific activities were reported as ηmol Pi released/min/mg of protein.

The adenosine deaminase activity was measured in lymphocytes using the method of Giusti (1974). The reaction was started by the addition of the adenosine to a final concentration of 21 mmol/L and incubations were carried out for 1 h at 37 °C. The pH of enzymatic assay of ADA was 6.5. The reaction was stopped by adding 106 mmol/L/0.16 mmol/Lphenol–nitroprusside/ml solution. The reaction mixtures were immediately mixed to 125mmol/L/11mmol/L alkaline hypochlorite (sodium hypochlorite). Ammonium sulfate of 75 lmol/l was used as ammonium standard. The ammonia concentration is directly proportional to the absorption of indophenol at 620 ηm. The specific activity is reported as U/L. One unit (1 U) of ADA is defined as the amount of enzyme required to release 1mmol of ammonia per minute from adenosine at standard assay conditions.

AChE activity in lymphocytes was determined as described by the colorimetric method of Ellman et al. (1961) modified by Fritzgerald and Costa (1993). The reaction mixture was composed of 1.0 mM acetylthiocholine, 0.1 mM 5,5′-dithio-bis-2-nitrobenzoic acid (DTNB), 0.1 M phosphate buffer (pH 8.0) and 100 µL of the intact mononuclear cells suspended in saline solution was added to the reaction. The proteins of all samples were adjusted to 0.1–0.2 mg/mL. The absorbance was read on a spectrophotometer at 412 nm before and after incubation for 30 min at 27°C. All samples were run in triplicate and the activity of lymphocyte AChE was expressed as µmol/h/mg of protein.

2.9 Protein Determination

Protein was measured by the Coomassie blue method according to Bradford (1976) using serum albumin as standard.

2.10 Citokines quantification

Cytokine quantification was assessed by ELISA using commercial kits for IL-6 and IL-10 (R&D Systems) the according to the manufacturer's instructions. The presence and concentration of the cytokines were determined by the intensity of the color measured by spectrometry in a micro ELISA reader.

2.11 Statistical analysis

The proportion differences between groups were analyzed by chisquare tests. One way ANOVA was performed to analyse cell proliferation (non-stimulated versus stimulated) data. Multiple comparisons among levels were analyzed with Tukey post hoc test. Proliferation/sensitivity data were analyzed by repeated measures ANOVA that included one between-subjects variable (untraiend vs trained) and one within subjects variable (mitogen or DEX concentrations). Enzymes activities and cytokines levels were analyzed statistically by two-way ANOVA, followed by Bonferroni post-tests (Graphpad – Prism 5.0). Differences between groups were considered to be significant when $P < 0.05$.

3. Results

3.1 Effects of exercise and caffeine treatment in lymphocytes proliferation and sensibility of glucocorticoide

The results in relation to lymphocytes proliferation is showed in the figure 1. Lymphocytes obtained of the animals trained or treated with caffeine were stimulated with phytohemagglutinin (PHA) (an activator of lymphocytes T).Our results showed that did not changes in proliferative responses were observed between trained and sedentary animals. However when trained animals were treated with caffeine, both doses (4 and 8 mg/kg), caused an increase in T lymphocytes proliferation ($P<0.05$). Caffeine *per se* in the dosage of the 8 mg/kg also caused an increase in T lymphocytes proliferation ($P<0.05$).

In the next set the experiments, we evaluated the effects of the exercise and caffeine treatment in the immunosuppressant effect caused by dexamethasone *in vitro*. Our results

showed no statistical differences between lymphocytes of trained and sedentary rats (Figure 1 A). When animals trained were treated with caffeine an increase in the sensibility of glucocorticoide was observed in relation the control groups ($P<0.05$) (Figure 2 C). Caffeine *per se* in the dosage of the 8 mg/kg also caused an increase in the sensibility of glucocorticoide in sedentary animals ($P<0.05$) (Figure 2 B).

3.2 Effects of exercise and caffeine treatment in lymphocytes enzymes activities

Our results showed that NTPDase activity was altered in lymphocytes the rats trained. As can be observed the ATP hydrolysis was decreased in lymphocytes of the animals only trained ($P<0.05$). When rats trained were treated with caffeine no alterations in ATP hydrolysis were observed in relation the control group (Figure 3A). However, in ADP hydrolysis we observed a decreased in NTPDase activity in lymphocytes the rats only trained and also in rats trained and supplemented with caffeine 4 or 8 mg/kg (Figure 3 A). Is important to note that caffeine *per se* did not alter the NTPDase activity for both ATP and ADP substrates (Figure 3 A and B).

In this study was observed also an increase in the ADA activity in lymphocytes the rats only trained. This increase was more pronounced when rats trained was supplemented with caffeine in the doses of 4 and 8 mg/kg ($P<0.05$). Caffeine *per se* (8mg/kg) also was capable the increase the ADA activity in lymphocytes of sedentary animals ($P<0.05$) (Figure 4).

Figure 5 shows the results obtained for AChE activity. Statistical analysis revealed that the activity of this enzyme is increased in trained group when compared to untrained rats ($P<0.05$). Treatment with caffeine 4 and 8 mg/kg caused a more pronounced increase in the AChE activity when compared to group only trained ($P<0.05$ Figure 5). No significant differences in the AChE activity in lymphocytes were observed in the animals treated only with caffeine 4 and 8 mg/kg in comparison with the untrained/saline group.

3.3 Effects of exercise and caffeine treatment in cytokines levels.

Statistical analysis showed an increase in the IL-6 levels in serum of the all groups the trained rats when compared to control groups (untrained animals) ($P<0.0001$). When rats submitted to exercise were supplemented with caffeine, our results demonstrated a decrease in the levels of this cytokine in both doses caffeine used (4 and 8 mg/kg) when compared to the group only trained ($P<0.05$).(Figure 6).In animals only trained, our results also showed a

decrease in the IL-10 level in serum when compared to untrained animals ($P<0.0001$) and caffeine supplementation was capable to revert this exercise effect ($P<0.05$).(Figure 6).

4. Discussion

Our findings demonstrated that HIIT during six weeks altered the activity of the crucial enzymes for lymphocyte function and the levels of IL- 6 and IL-10 citokines. Caffeine ingestion when associated with HIIT is capable to modulate inflammatory parameters and lymphocyte responses.

Dates from literature have demonstrated that HIIT is associated with a biphasic change of circulating lymphocytes and release of citokines. In the immediate post-exercise period, a pro-inflammatory microenvironment is frequently found because active skeletal muscle synthesizes and releases IL-6. This pro-inflammatory profile plays an important role in the organism's adaptation to exercise, leading to the development of an anti-inflammatory microenvironment (Gleeson, 2007, Gleeson et al., 2011, Fernández-Verdejo et al., 2014). In this line, we showed that HIIT increased the levels of IL-6 and decreased the levels of IL-10. Our findings were consistent with other studies that showed an increase in IL-6 circulating following six weeks of HIIT training protocol on a treadmill (at a speed of 40–54 m/min) in rats (Sarir et al., 2015).

The hypothesis that HIIT induced increase in circulating IL-6 levels is associated with muscle skeletal damage (Bruunnsgaard et al., 1997) and increase level of IL-6 mRNA in stimulated muscle (Jonsdottir et al., 2000). In addition, high levels of reactive oxygen species (ROS) are produced in intensive interval training. ROS can stimulate IL-6 production from skeletal myotubes in a manner that involves transcriptional activation of the IL-6 gene through an NF-κ B–dependent pathway (Kosmidou et al., 2002). In addition, evidences support the hypothesis that IL-6 released from muscle induces several metabolic effects, such as alterations in the glucose metabolism, lipolysis and fat oxidation during exercise (Pedersen and Pedersen, 2005).

Caffeine supplementation decreased the levels of IL-6 and increased the levels of IL-10 in trained animals. Besides, caffeine also increased the proliferation and sensibility of lymphocytes to glucocorticoides suggesting that this substance has immunomodulatory and anti-inflammatory properties when used as ergogenic recourse. Corroborating with our results, Chechella et al. (2014) shows that caffeine supplementation (30 mg/kg/ 4 weeks)

decrease the levels of IL-1 β, IL-6 and IFN - γ in serum from middle aged rats submitted to moderate intensity swmming. On the other hand, an increase of IL-10 level also was observed in subjects supplement with caffeine (6 mg/kg) after run competition (15 km) (Tauler et al., 2016). Although the source for circulatory levels of IL-10 have not yet been elucidated, is plausible suggests that the muscle could be the main contributor for increase the circulatory IL-10 levels after exercise. Based on this, on possible mechanism involved in the alterations of IL-10 levels by caffeine is an increase in the cAMP levels in muscle (Tauler et al., 2016).

Intensive exercise is associated with neutrophilia, lymphocytosis, lymphocytopenia and suppression of lymphocyte proliferative response (MacNeil et al., 1991; Nieman et al., 1994; Siedlik et al., 2016). Following high-intensity exercise, elevations on hormones concentrations may contribute to a transitory decrease the proliferative responses of T lymphocytes (Nieman et al., 1994). Here, we showed that caffeine increase the proliferation and sensibility the lymphocytes to glucocorticoides, especially when associated to HIIT. These effects of caffeine can be associated with elevated concentrations of epinephrine, perturbations in numbers of circulating lymphocytes (CD4+ and CD8+ cells), and increase of glucocorticoid receptor activity (Pettenuzzo, Noschang *et al.*, 2008; Ping, Lei *et al.*, 2012).

In regard the enzymatic activities in lymphocytes, after six weeks the HIIT a decrease in the ATP and ADP hydrolysis was observed in trained rats. Although limited data are available, studies have showed the influence of the exercise protocols in NTPDase activity. Moritz et al., (2016) demonstrated that moderate aerobic exercise on a treadmill increase the ATP and ADP hydrolysis in serum of sedentary individuals. Corroborating with our findings, two weeks of 20 min/day treadmill running also decreased ATP and ADP hydrolysis in serum from rats (Siqueira et al., 2010).

ATP and adenosine are the modulatory messengers implicated in the control of immune and inflammatory process, since that the ATP can promote pro-inflammatory responses, whereas the adenosine may signal anti-inflammatory actions (Bours et al., 2006). Our findings suggest that HIIT could increase ATP and ADP levels, since their hydrolysis was diminished. This modification in adenine nucleotides hydrolysis also can be associated with a decrease in the expression of NTPDase in lymphocytes, as showed by Cardoso et al., (2015) after six weeks of moderate intensity exercise in rats. On the other hand, HIIT also alter the ADA activity contributing to the reduced levels of adenosine. Based on these findings, alterations in the purinergic signaling can contribute to pro-inflammatory condition found after HIIT.

Caffeine can modulate many aspects of the immune and inflammatory responses, such as cytokine production, lymphocytes proliferation, antibody synthesis and immune cells apoptosis (Horrigan et al., 2006). The increases in ATP hydrolysis in rats trained and treated with caffeine demonstrate that this compound can modulate proinflammatory responses mediated by ATP signaling during HITT. These mechanisms can be associated, at least in part, with the alterations in the citokines levels observed in this study. Besides, when associated with the HIIT, caffeine causes a more pronounced increase in the ADA activity in lymphocytes. It well established that caffeine is an antagonist of adenosine receptors (Ribeiro and Sebastiao, 2010). Chronic caffeine ingestion may increase the expression of A_{2A} adenosine receptors as well exacerbate extracellular adenosine levels (Ribeiro and Sebastiao, 2010). Thus, the increase in the ADA activity suggests a compensatory effect to decrease adenosine levels and counteract the antagonist action of caffeine.

Finally, we also showed that HIIT altered the cholinergic signaling in lymphocytes from rats. The increase in the AChE activity may decrease the acetylcholine levels contributing to sustained inflammation and immune dysfunctions (Das, 2007). T lymphocytes have the ability to synthesize ACh and express muscarinic and nicotinic receptors. The activation of these receptors by acetylcholine induces effects such increase in the intracellular Ca^{2+}, regulation the cytokines production and cell proliferation (Mashimo et al., 2016). On the other hand, caffeine, at least at the dosage used in this study, no revert the alterations in the AChE activity induced by HIIT. However in other study, caffeine alone or combined with exercise decreased the plasma AChE activity (Bracelos et al., 2014).

In conclusion, HIIT induces alterations in the NTPDase, ADA and AChE activities in lymphocytes and in serum cytokines levels suggesting that these parameters are associated to a pro - inflammatory response to exercise. Caffeine administration could play an essential role in the inflammatory response to exercise by limiting the production of proinflammatory factors and the tissue damage induced during this inflammatory response. Thus, caffeine supplementation may help to regulate the proinflammatory response and the subsequent muscle regeneration when associated with HIIT. However, caffeine seems to interfere on HIIT-induced adaptations and more studies are necessary to evaluate the impact the caffeine in inflammatory parameters in athletes submitted a strenuous exercise routine or in different exercise-related health treatments.

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Conflict of Interest statement

There are no conflicts of interest.

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Legends of figures

Figure 1 - Lymphocytes proliferation after stimulation of phytohemagglutinin (PHA) (an activator of lymphocytes T). Proliferation responses to PHA X Caffeine curve (NT– blacks / HIIT – reds). ***** Different of the others groups for P<0.05 (n= 10).

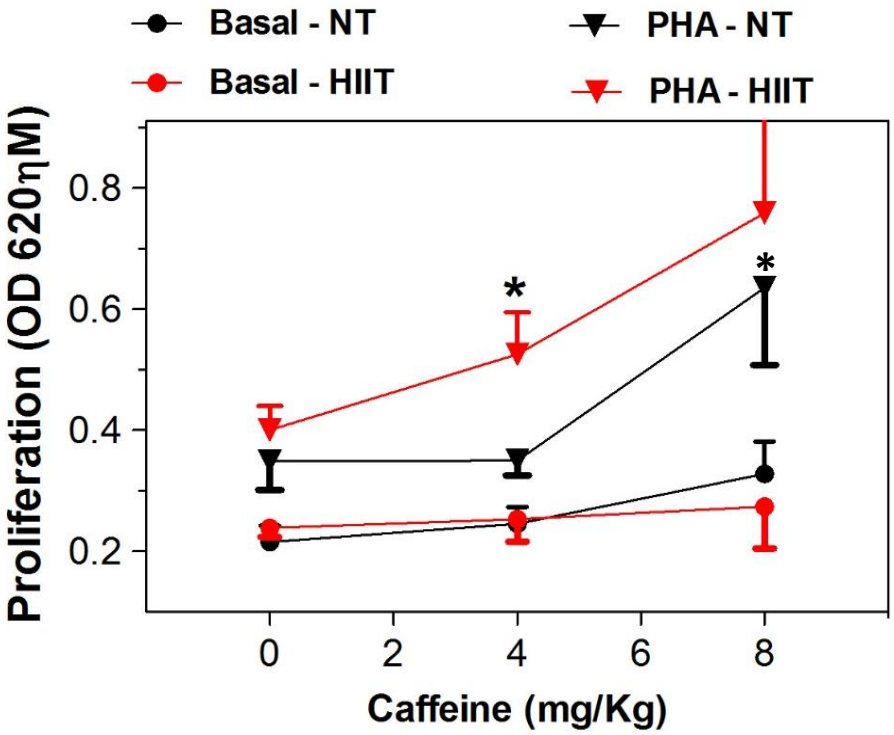
Figure 2 - Effects of the exercise and caffeine treatment in the immunosuppressant effect caused by dexamethasone *in vitro*. * Different of the others groups for P<0.05 (n=10).

Figure 3 - Activity of NTPDase enzyme using ATP and ADP as substrate in lymphocytes of rats trained and supplemented with caffeine (4 mg/Kg and 8 mg/Kg). Data are presented as mean ± SEM. (Two-way ANOVA using Bonferroni post test n=10). *, **, *** Indicate statistical difference in relation of untrained groups for $P < 0.05$, $P < 0.01$ and $P < 0.001$ respectively.

Figure 4 - Adenosine deaminase (ADA) activity in lymphocytes of rats trained and supplemented with caffeine (4 mg/Kg and 8 mg/Kg). Data are presented as mean ± SEM. (Two-way ANOVA using Bonferroni post test n=10). *,*** Indicate statistical difference in relation the other groups for $P < 0.05$ and $P < 0.0001$ respectively. $^{\#, \#\#}$ indicate statistical difference in relation group only trained for $P < 0.05$ and $P < 0.001$ respectively.

Figure 5 – Acetylcolinesterase (AChE) activity in lymphocytes of rats trained and supplemented with caffeine (4 mg/Kg and 8 mg/Kg). Data are presented as mean ± SEM. (Two-way ANOVA using Bonferroni post test, n=8). *,*** Indicate statistical difference in relation of untrained groups for $P < 0.05$ and $P < 0.0001$ respectively.

Figure 6 – Cytokines levels (IL-6 and IL-10) in serum of rats trained and supplemented with caffeine (4 mg/Kg and 8 mg/Kg). Data are presented as mean ± SEM. (Two-way ANOVA using Bonferroni post test n=10). *,*** Indicate statistical difference in relation of untrained groups for $P < 0.05$ and $P < 0.0001$ respectively. $^{\#, \#\#\#}$ indicate statistical difference in relation group only trained for $P < 0.05$ and $P < 0.0001$ respectively.


Basal - NT
Basal - HIIT
PHA - NT
PHA - HIIT
Proliferation (OD 620ηM)
0.8
0.6
0.4
0.2
0
2
4
6
8
Caffeine (mg/Kg)
*
*

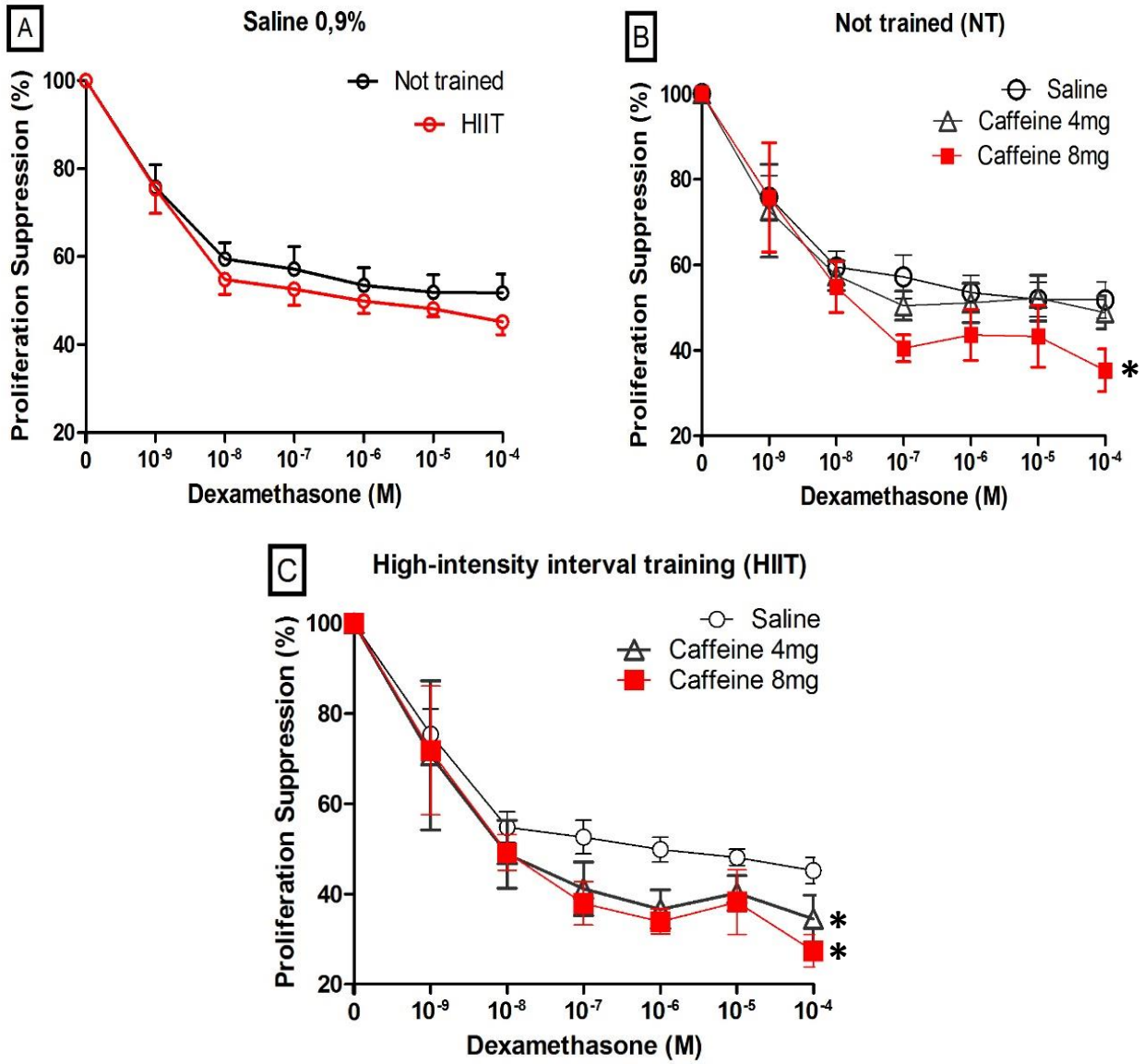

A
Saline 0,9%
Not trained
HIIT
Proliferation Suppression (%)
Dexamethasone (M)
B
Not trained (NT)
Saline
Caffeine 4mg
Caffeine 8mg
*
C
High-intensity interval training (HIIT)
Saline
Caffeine 4mg
Caffeine 8mg
*
*

Figure 2

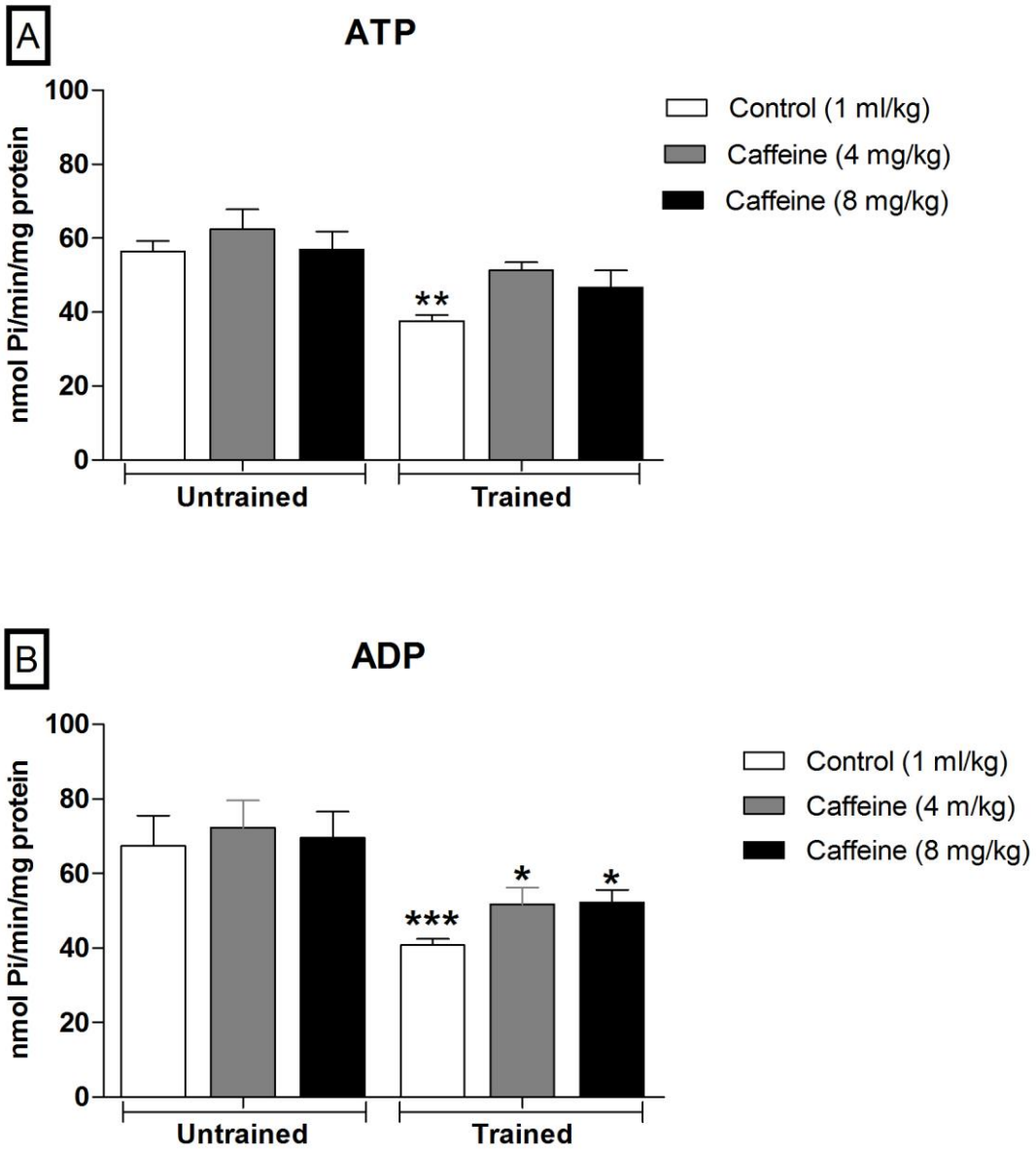

A
ATP
nmol Pi/min/mg protein
100
80
60
40
20
0
**
Untrained
Trained
Control (1 ml/kg)
Caffeine (4 mg/kg)
Caffeine (8 mg/kg)
B
ADP
nmol Pi/min/mg protein
100
80
60
40
20
0
***
*
*
Untrained
Trained
Control (1 ml/kg)
Caffeine (4 m/kg)
Caffeine (8 mg/kg)

Figure 3

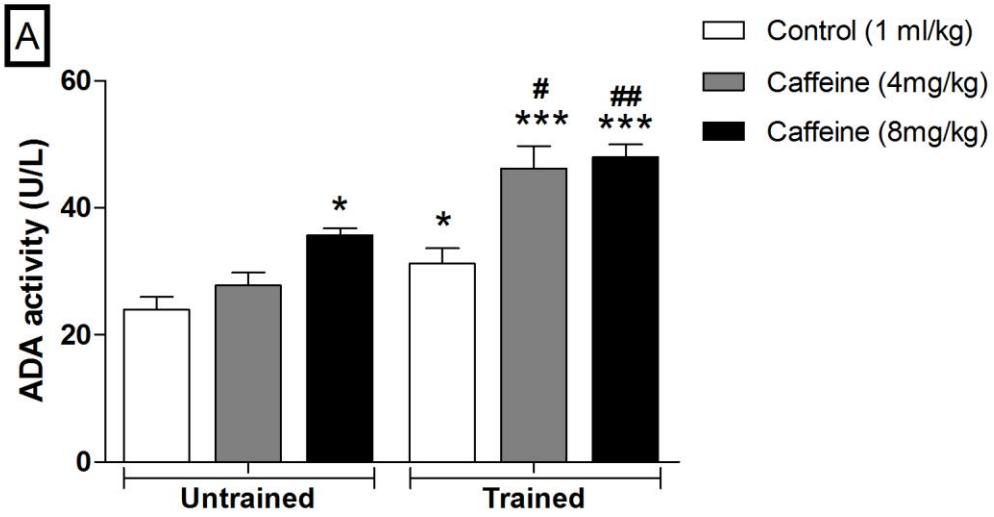


Figure 4

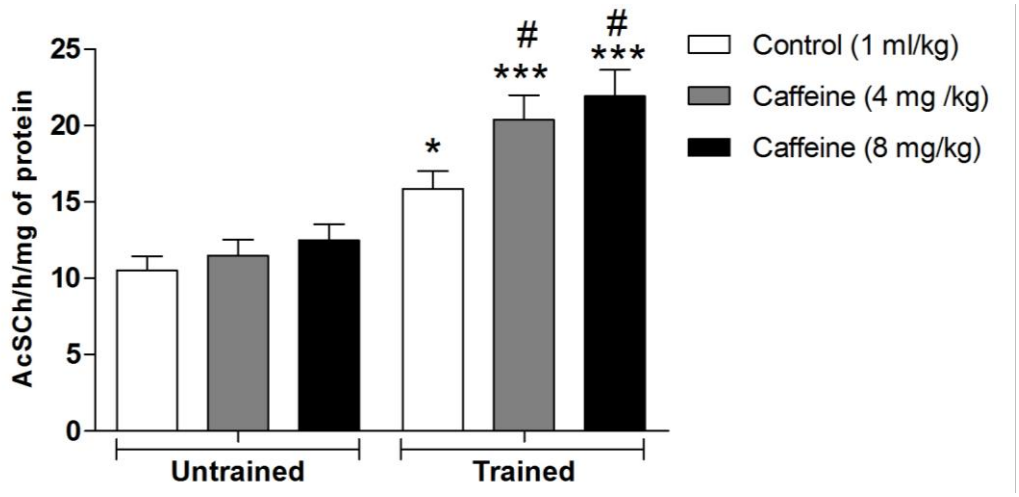


Figure 5

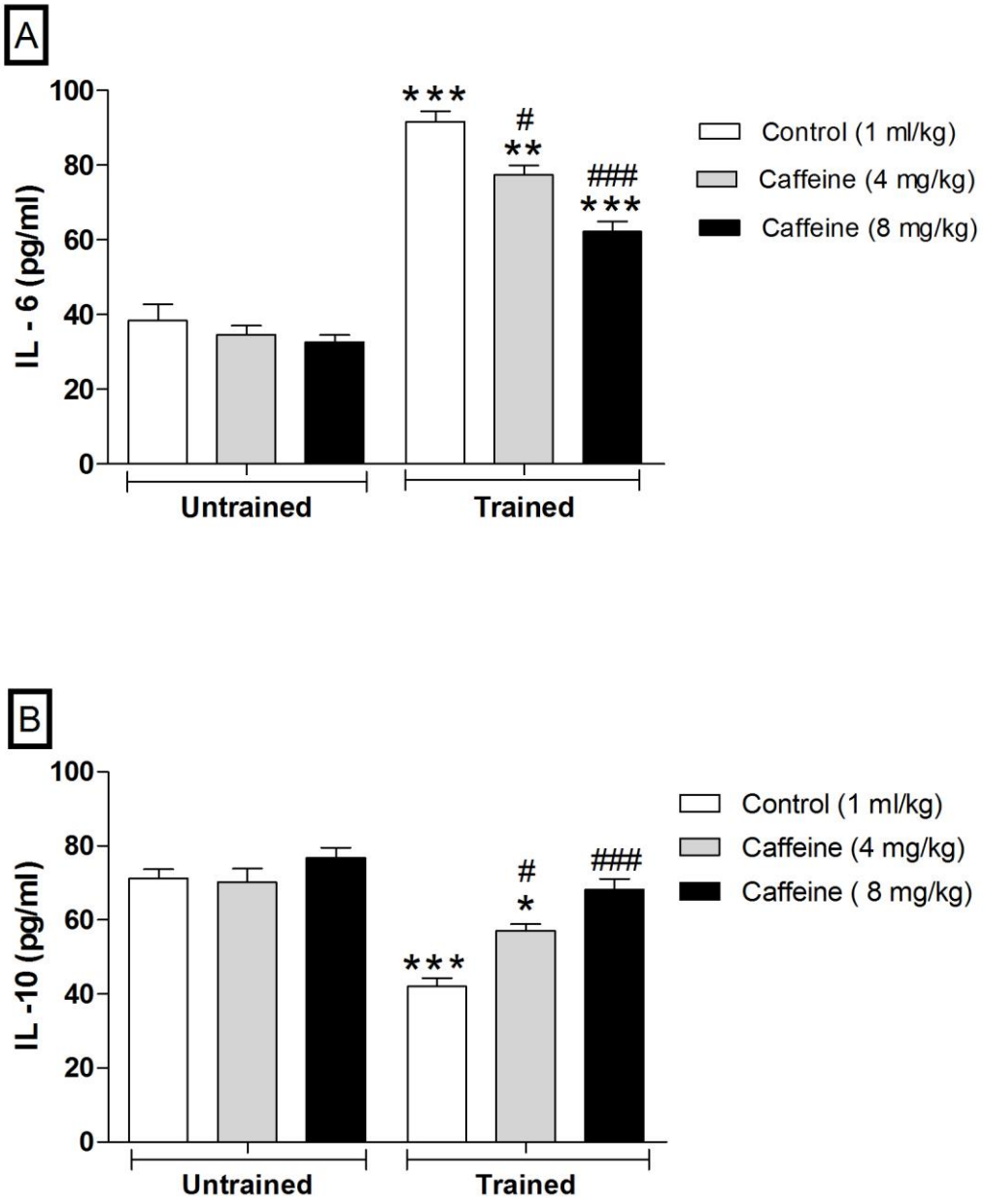

A
100
80
60
40
20
0
IL - 6 (pg/ml)
***
#
**
###
***
Control (1 ml/kg)
Caffeine (4 mg/kg)
Caffeine (8 mg/kg)
Untrained
Trained
B
100
80
60
40
20
0
IL -10 (pg/ml)
***
#
*
###
Control (1 ml/kg)
Caffeine (4 mg/kg)
Caffeine (8 mg/kg)
Untrained
Trained

Figure 6

5. DISCUSSÃO

A associação de dois temas relevantes como a cafeína, uma substância consumida mundialmente, e exercício físico despertam grande curiosidade tanto do meio científico quando da sociedade atual. Na última década, muita atenção tem sido dada principalmente, no que diz respeito a prática de atividade física não apenas como meio de culto ao corpo, mas também na promoção à saúde. Por outro lado, a cafeína ganhou destaque no meio científico tanto para os estudiosos do exercício físico como para pesquisadores que voltam seus olhares em pesquisas com doenças neurodegenerativas como Parkinson e Alzheimer. Recentes pesquisas têm demonstrado os efeitos neuroprotetores e antioxidantes da cafeína, porém estes estudos em sua maioria são condicionados a doenças neurodegenerativas ou metabólicas. Por outro lado, muitas pesquisas ainda buscam elucidar o efeito ergogênico da cafeína associada a protocolos de exercício, epecialmente no retardo da instalação do processo de fadiga (Tarnopolsky, 2008).

Primeiramente, neste trabalho foi avaliado os efeitos da administração da cafeína nos níveis de glicogênio e atividade das enzimas Ca^{2+}-ATPase e AChE em músculo esquelético de ratos submetidos a um protocolo agudo e crônico de HIIT. No protocolo agudo, devido à alta demanda requerida pelo músculo esquelético para gerar ATP ocorreu redução do conteúdo de glicogênio muscular. Este dado é facilmente explicável, pois o HIIT utiliza prioritariamente a via glicolítica lática para produção de ATP no miócito (Pederson, Cope *et al.*, 2005) Desta forma, o conteúdo de glicogênio do músculo gastrocnêmio de animais submetidos à uma única sessão de HIIT encontra-se reduzido. Além disto, a cafeína não foi capaz de reverter esta alteração nos níveis de glicogênio em apenas uma sessão de HIIT. Por outro lado, após 6 semanas de HIIT verificou-se uma supercompensação no conteúdo de glicogênio muscular tanto em animais tratados com cafeína (4 e 8 mg/kg) quanto em animais submetidos ao HIIT. Devido ao somatório dos efeitos agudos (sessões) do HIIT inúmeras modificações ocorrem após um período de treinamento (Hawley, Hargreaves *et al.*, 2014). Dentre estes efeitos destacam-se o aumento da biogênese mitocondrial (Zhang, Wu *et al.*, 2012), aumento da expressão de enzimas oxidativas como citrato sintase, piruvato desidrogenase e citocromo c oxidase (Burgomaster, Heigenhauser *et al.*, 2006), maior eficiência dos receptores de GLUT-4 (Terada, Yokozeki *et al.*, 2001) e atividade da hexoquinase em músculo (Greenberg, Jurczak *et al.*, 2006). Provavelmente, esta resposta ocorre devido a uma superexpressão dos receptores GLUT – 4 os quais sinalizam para captação da glicose de forma mais eficiente.

Em adição, pode-se observar que em muitos estudos a cafeína melhora a *performance* física em exercícios de longa duração, devido ao bloqueio seletivo do A_1R permitindo, assim, uma maior liberação de catecolaminas que por sua vez aumentam a liberação de ácidos graxos livres (Van Soeren e Graham, 1998b; Ribeiro e Sebastiao, 2010). Entretanto, a cafeína também estimula inúmeros processos bioquímicos dentre eles está o aumento de AMPK (proteína cinase mitógeno ativada), a qual é responsável por melhorar a captação de glicose, aumentar a liberação de ácidos graxos e reduz processos de síntese (Jensen, Rose *et al.*, 2007; Jensen e Richter, 2012). Em outro estudo, Rush & Spriet (2001) demonstram que a cafeína exerce efeito inibitório na enzima glicogênio fosforilase (Rush e Spriet, 2001). Desta forma, sugere-se que o tratamento com cafeína (4 e 8mg/kg) estimula a supercompensação de glicogênio via diversos processos (aumento da degradação de ácidos graxos e aminoácidos), bem como inibição de enzimas chave da degradação do glicogênio muscular. Neste caso, tanto o tratamento crônico com cafeína como o protocolo de HIIT melhoram o conteúdo de glicogênio muscular. Entretanto, a ingestão crônica de cafeína não foi capaz de potencializar o efeito causado pelo HIIT nos níveis de glicogênio.

Já é bem estabelecido na literatura que tanto a AChE quanto a Ca^{2+}-ATPase desempenham papel chave nos processos de contração muscular (Rotundo, 2003; Llach, Molina *et al.*, 2011; Finkel, Etlin *et al.*, 2014). Pesquisadores, demonstram em seus estudos que o exercício melhora a ativação neuromuscular em fibras rápidas (Sveistrup, Chan *et al.*, 1995), entretanto o desempenho da AChE é reduzido quando ativado por longos períodos (Pedzikiewicz, Piaskowska *et al.*, 1984). Nossos resultados demonstraram que nenhuma alteração ocorreu na atividade da AChE em músculo gastrocnêmio quando os animais foram submetidos a um protocolo agudo de HIIT. Todavia, no protocolo crônico observou-se uma redução significativa na atividade desta enzima. Supõe-se que a repetida estimulação neuromuscular imposta pelo HIIT leve a um acumulo de ACh o que pode dessensibilizar seus receptores levando a incapacidade de gerar novos potenciais de ação o que é um dos mecanismos envolvido no processo de fadiga (Wen, Hui *et al.*, 2009). Os resultados apontam para uma resposta adaptativa ao exercício, uma vez que com a atividade da AChE reduzida, hipotetiza-se, que ocorra mais ACh na fenda sináptica o que se relaciona com os mecanismos de fadiga. Por outro lado, a cafeína reverte estes efeitos, uma vez que, restabelece os níveis da atividade da AChE em animais submetidos ao HIIT a níveis de animais controles. Sendo assim, retarda a instalação do processo de fadiga. Estudos já demonstraram a capacidade desta

metilxantina em inibir a atividade da AChE (Pohanka e Dobes, 2013). Todavia, mais estudos são necessários para confirmar esta hipótese.

Outra importante enzima que também está diretamente envolvida na contração muscular é a Ca^{2+}-ATPase na qual controla a captação e liberação de íons Ca^{2+} no RS para sarcoplasma (Tupling, 2004). Tanto no protocolo agudo como crônico o HIIT aumentou a atividade desta enzima em músculo gastrocnêmio. Assim, outros estudos também verificaram que o exercício aumenta tanto a atividade quanto a expressão da SERCA (Pshennikova, Khaspekov *et al.*, 1999; Nogueira, Shiah *et al.*, 2013). Neste caso, a cafeína preveniu os efeitos do HIIT, por atuar como antagonista não-seletivo dos receptores A_{2A} de adenosina e consequente, fosforilação dos RyR, reduzindo desta forma, as concentrações de íons Ca^{2+} e modulando assim a atividade da enzima. Sabe-se que a instalação do processo de fadiga é multifatorial, o acumulo de íons de H^+, redução dos níveis de ATP levam a queda da produção de força (Hostruo & Bangsbo, 2016). Porém, todos estes fatores servem de base para que o organismo crie mecanismos adaptativos que ao longo de séries agudas melhoram a *performance* física. Portanto, a cafeína ao previnir estas alterações estaria bloqueando adaptações induzidas pelo exercício que são úteis ao organismo.

O exercício intenso provoca inúmeras alterações no organismo dentre elas o aumento na espessura do ventrículo esquerdo, sendo este considerado um fenômeno adaptativo que visa suportar a sobrecarga imposta pelo exercício. A análise histológica revelou que o HIIT provoca hipertrofia do ventrículo esquerdo, entretanto, quando os animais submetidos ao HIIT foram tratados com cafeína observou-se uma prevenção deste efeito. Desta forma, pode-se concluir que embora muitos estudos discutam o potencial ergogênico da cafeína, os resultados deste trabalho demonstram que ela é capaz de reverter importantes efeitos induzidos pelo HIIT em musculo esquelético. Cabe salientar que muitos suplementos pré-treino e/ou termogênicos contêm cafeína em sua composição e a combinação de um protocolo intenso de exercício aliado ao consumo de cafeína ($\geq$4mg/kg) deve ser visto com cautela.

Pela sua estrutura química semelhante à molécula de adenosina, a cafeína atua no bloqueio dos receptores de adenosina inibindo seus efeitos (Ribeiro e Sebastiao, 2010). Muitos artigos foram publicados avaliando os efeitos terapêuticos da cafeína em doenças neurodegenerativas como Parkinson e Alzheimer (Gomes, Kaster *et al.*, 2011), sendo estes associados ao antagonismo não seletivo sobre os receptores A_{2A} (Ribeiro & Sebastião, 2010). Entretanto, os benefícios ergogênicos da cafeína associados a protocolos de exercício de alta intensidade em parâmetros neuroquímicos não tem sido ainda bem esclarecidos. Assim,

procurou-se neste trabalho investigar o efeito desta metilxantina sobre a ansiedade, enzima Na^+-K^+-ATPase e parâmetros de estresse oxidativo em SNC de animais submetidos ao HIIT.

A cafeína, em ambas as doses, foi capaz de prevenir o efeito ansiolítico do exercício, tanto em animais submetidos ao protocolo de HIIT quanto animais controle, pois aumentou o tempo nos braços fechados e reduziu o tempo nos braços abertos. Além disto, a cafeína também induziu tanto em animais controles quanto treinados a um aumento no tempo gasto no centro do aparato, bem como aumentou o número de *pellets* fecais o que demonstra comportamento ansiogênico. Jin e colaboradores (2016) utilizaram diferentes parâmetros comportamentais para avaliar comportamento ansiogênico e depressivo em adolescentes que consumiam habitualmente cafeína e obtiveram correlação positiva para o desenvolvimento depressão e insônia severa (Jin, Yoon *et al.,* 2016). Em adição, Hughes & Hancock (2016) avaliaram comportamento relacionado a ansiedade e aprendizado habitual em 3 diferentes linhagens de ratos (PVG/c; Long-Evans e Wistar) mediante uso agudo de 50 mg/kg de cafeína. Foram utilizados 3 testes (campo aberto, labirinto em cruz elevado e caixa claro-escuro). Os autores concluíram que a cafeína exerce efeito ansiogênico nas 3 diferentes cepas (Hughes e Hancock, 2016). Todavia, convém salientar que a dose de 50 mg/kg é extremamente alta. Embora, muitos estudos ressaltem o papel da cafeína nos processos de aprendizagem/memória, já é bem estabelecido que a cafeína induz a ansiedade e previne o efeito do exercício (Hughes e Hancock, 2016). Tem sido demonstrado que o exercício é um excelente adjuvante no tratamento a doenças neurodegenerativas (Garcia-Mesa, Colie *et al.*, 2016). Corroborando com esta hipótese ao verificar-se um aumento na atividade da enzima Na^+-K^+-ATPase no grupo treinado associado a redução nos parâmetros comportamentais como redução na entrada de braços fechados e aumento do tempo em braços abertos denotando o efeito ansiolítico do exercício. Já é bem descrito na literatura que protocolos de exercícios em intensidades moderadas produzem bons efeitos sobre proteínas como BDNF, bem como melhoram o desempenho cognitivo (Berchtold, Castello *et al.,* 2010). Outros pesquisados também verificam que o HIIT melhora a expressão de proteínas neurotróficas e expressão de outras neurotrofinas correlacionados com aumento de ERO induzido pelo exercício intenso (Siamilis, Jakus et al., 2009; De Almeida, Gomes Da Silva et al., 2013; Afzalpour, Chadorneshin et al., 2015; Cabral-Santos, Castrillon et al., 2016; Hwang, Brothers et al., 2016)

Uma vez que, reduções na atividade da enzima Na^+-K^+-ATPase estão associadas a surtos de depressão e desordens bipolares (Kirshenbaum, Burgess *et al.*, 2014),). Verificou-se,

em nosso estudo, que o HIIT aumentou a atividade desta enzima em córtex cerebral e hipocampo. Por outro lado, a cafeína em ambas as doses usadas (4 e 8 mg/kg) preveniu este efeito. Como o exercício físico altera diferentes vias metabólicas com aumento pronunciado de íons como Ca^{2+}, Na^{+}, K^{+}, lactato o que leva a mecanismo adaptativo induzido pela redução nos níveis de ATP, aumentos significativos de lactato, aumento no VO_2max que elevam por consequência a produção de ERO (Hostrup e Bangsbo, 2016). Indiretamente o HIIT afetaria a atividade da enzima Na^{+}-K^{+}-ATPase, uma vez que, num primeiro momento leva a aumento na produção de H_2O_2 (Bogdanis, Stavrinou et al., 2013). Entretanto, são estas alterações que sinalizam para que os mecanismos adaptativos sejam aprimorados. Desta forma, sugere-se que o HIIT influencie vias metabólicas, pois aprimora a tolerância a íons de H^{+}, lactato, aumento expressão de AMPK facilitando o uso de glicose (Koshinaka, Sano *et al.*, 2008; Sano, Koshinaka *et al.*, 2012) por aumentar a sensibilidade aos receptores de GLUT-4 (Terada, Yokozeki *et al.*, 2001), melhora o fluxo sanguíneo facilitando para que outras enzimas e proteínas desempenhem seu papel de forma mais eficiente. Aprimorando o estado redox e protegendo enzimas como Na^{+}-K^{+}-ATPase do ataque de ERO. Por outro lado, a cafeína aumenta as vias de degradação dificultando o efeito gerado pelo HIIT. Assim, verificou-se que o HIIT altera enzimas que participam do sistema de defesa antioxidante (SOD, CAT, GPx), níveis de GSH e também altera níveis de MDA.

O HIIT promoveu o aumento da SOD em córtex cerebral, mas não em hipocampo ou estriado. Como se sabe a SOD é uma enzima capaz de dismutar um $O_2^{\bullet -}$ em H_2O_2. Já é bem estabelecido que o HIIT aumenta processos de biogênese mitocondrial (E, Burns et al., 2014), sendo este processo diretamente correlacionado com ativação da SOD2 (Leick, Lyngby *et al.*, 2010). Além disso, outros estudos estabelecem que o exercício regular melhora o sistema de defesa antioxidante em diferentes regiões do cérebro (Radak, Taylor *et al.*, 2001; Marosi, Bori *et al.*, 2012; Tuon, Valvassori *et al.*, 2012). Kamsler e colaboradores (2007) relacionou a superexpressão da SOD com melhoras na performance no "*Morris Maze test*" (Kamsler, Avital et al., 2007). Siamilis e colaboradores (2009) administraram pequenas doses de H_2O_2 em ratos controles e treinados em cultura de células (hipocampo) e verificaram uma redução dose-dependente de proteínas neurotrófica como CREB e BDNF (Siamilis, Jakus *et al.*, 2009). Azfalpour e colaboradores (2015), descreveram que elevados níveis de H_2O_2 eram necessários para que a sinalização de proteínas neurotróficas fosse "inicializada" (Afzalpour, Chadorneshin *et al.*, 2015). Desta forma, concluímos que o HIIT prejudica o SNC uma vez que aumenta níveis de ROS. Contudo, aumentos nos níveis de H_2O_2 podem ter importante

papel nos fenômenos adaptativos, pois atuam na sinalização de enzimas do sistema de defesa antioxidante (Radak, Zhao *et al.*, 2013)..

Efeitos similares foram encontrados sobre a atividade da enzima CAT. Neste caso, o HIIT o provocou o aumento na atividade da enzima apenas em córtex cerebral, mas o efeito oposto foi observado tanto em hipocampo e estriado. Além disso, a cafeína não foi capaz de prevenir estes efeitos. Por sua vez, a CAT também cumpre importante papel no sistema de defesa antioxidante convertendo o H_2O_2 em O_2 e água.

Outro achado interessante deste trabalho é o aumento no conteúdo de GSH na atividade da GPx induzido pelo HIIT. Neste caso, a cafeína preveniu estes aumentos. Desta forma, a cafeína atua bloqueando as alterações provocadas pelo HIIT. Diferentes tipos de exercício (intensidades e tempo de duração) promovem diferentes repostas do organismo frente a ativação na produção ERO. Gomez – Cabrera 2008, afirmam que a suplementação com antioxidantes previne biogênese mitocondrial induzida pelo HIIT por suprimir a superexpressão de enzimas SOD e GPx, ou seja, o uso de antioxidantes pode atenuar o efeito do exercício físico reduzindo a atividade de enzimas envolvidas no estado redox (Gomez-Cabrera, Domenech *et al.*, 2008; Gomez-Cabrera, Salvador-Pascual *et al.*, 2015; Gomez-Cabrera, Vina *et al.*, 2016). Wadlwey 2016 verificou que um protocolo de baixo volume e alta intensidade (10 x 1 min 90% VO_2max) melhora o nível do sistema de defesa antioxidante total devido aumentos no conteúdo de peróxido de hidrogênio (Wadley, Chen *et al.*, 2016). Desta forma, o efeito antioxidante da cafeína pode estar associado a prevenção da biogênese mitocondrial induzida pelo HIIT. Uma vez que o HIIT promove aumento de enzimas do sistema de defesa antioxidante, podem estimular os mecanismos antioxidantes celulares e aumentar a resistência a lesões induzidas pelo exercício (Finkel e Holbrook, 2000; Brentano e Martins Kruel, 2011). Cabe salientar que a maior parte dos efeitos induzidos pelo exercício físico (aumento da massa muscular, melhora do sistema cardiovascular, redução da incidência de doenças e infecções) é devida, principalmente, às adaptações induzidas sobre os diversos sistemas corporais, incluindo o sistema antioxidante endógeno (Powers, Radak *et al.*, 2016).

Já é bem descrito na literatura que as adaptações impostas pelo treinamento físico (reparação do dano muscular) ativam o sistema imune (Gomez-Cabrera, Vina *et al.*, 2016). Neste contexto, nossos resultados demonstraram que HIIT foi capaz de alterar a atividade de enzimas cruciais na função de linfócitos e níveis de citocinas inflamatórias como IL-6 e IL-10. A cafeína foi capaz de modular parâmetros inflamatórios e respostas de proliferação e ativação de linfócitos.

Dados da literatura têm demonstrado que o HIIT está associado a maior liberação na circulação de linfócitos e citocinas. Imediatamente após cessar o exercício ocorre um período pró-inflamatório. Devido à alta atividade contrátil do músculo esquelético que acaba por sinalizar a liberação de altos níveis de IL-6 (Peake, Della Gatta *et al.,* 2015). Este perfil pro-inflamatório da IL-6 desempenha um importante papel na sinalização de um microambiente anti-inflamatório (Gleeson, 2007; Gleeson, Bishop et al., 2011; Fernandez-Verdejo, Casas *et al.,* 2014). Assim, nossos resultados demonstraram que o HIIT induziu a um aumento de IL-6 e a redução dos níveis de IL-10. Outros estudos também já demonstraram aumentos nos níveis de IL-6 após 6 semanas de HIIT em esteira rolante (velocidade de 40 -54 m/mim) em ratos (Sarir, Emdadifard *et al.,* 2015). Em contra partida, Monteiro e colaboradores (2016) verificaram que 8 semanas de HIIT (1:1 à 100% do VO_2max) associado ao treinamento de força (80% de 1 Repetição Máxima) não causou diferenças nos níveis de IL-6 e IL-10 (Monteiro, Campos *et al.,* 2016).

É bem estabelecido que a produção de citocinas ocorre por uma variedade de células. Sendo assim, o músculo esquelético tem sido identificado como potencial liberador de IL-6 (Peake, Della Gatta *et al.,* 2015). Níveis elevados de IL-6 estão associados ao dano muscular que acorre durante o exercício (Peake, Suzuki et al., 2005; Della Gatta, Garnham *et al.,* 2014; Peake, Della Gatta *et al.,* 2015). Camundongos *knockout* para esta citocina demonstram baixa regeneração após uma lesão, sugerindo que a sinalização induzida pela IL-6 é necessária para promover a recuperação (Cafferty, Gardiner *et al.,* 2004). Em adição, altos níveis de ERO são produzidos a partir do exercício de alta intensidade, os quais também podem estimular a produção de IL-6 a partir do músculo esquelético envolvido na ativação e transcrição de IL-6 (Kosmidou, Vassilakopoulos *et al.,* 2002). Em adição, evidências suportam a hipótese de que a liberação de IL-6 a partir do músculo esquelético induz a vários efeitos metabólicos como alterações no metabolismo da glicose e oxidação de ácidos graxos durante o exercício (Pedersen e Febbraio, 2008; Pedersen, Lessard *et al.,* 2008; Karstoft e Pedersen, 2016).

A ingestão de cafeína durante 6 semanas reduziu o nível de IL-6 e aumentou nível de IL-10 em animais treinados. Além disto, cafeína também aumentou a proliferação e sensibilidade de linfócitos a glicocorticoides (GCs) sugerindo efeito imunomodulatório e anti-inflamatório quando utilizada como recurso ergogênico. Corroborando com nossos achados, Cechella e colaboradores (2014) demonstraram que altas doses de cafeína (30mg/kg/4 semanas) diminui os níveis de IL-1β, IL-6 e IFN – γ no soro de ratos de meia idade submetidos a um protocolo de nado moderado (Cechella, Leite et al., 2014). Por outro lado,

um aumento da IL-10 foi observado em sujeitos treinados os quais foram suplementados com cafeína (6 mg/kg) (Tauler, Martinez *et al.,* 2013). Além disso, Ritter e colaboradores (2005) postularam que a cafeína inibe a expressão citocinas em linfócitos, sugerindo que este efeito ocorra pela via de sinalização do cálcio (Ritter, Hohenberger *et al.,* 2005). Embora a fonte para níveis circulatórios de IL-10 não tenha sido elucidada, é plausível sugerir que o músculo esquelético contribua para o aumento da circulação de IL-10 após o exercício. Com base nisto, pode-se propor que o possível mecanismo envolvido nas alterações dos níveis de IL-10 pela cafeína seja um aumento dos níveis de AMPc no músculo (Tauler, Martinez *et al.,* 2016).

O exercício intenso está associado com neutrofilia, linfocitose, linfocitopenia, e supressão da resposta proliferativa de linfócitos (Nieman, 1994; Gleeson, Nieman *et al.*, 2004; Nieman, Konrad *et al.,* 2012; Siedlik, Deckert *et al.,* 2016). Após o exercício de alta intensidade, elevações nas concentrações de hormônios podem contribuir para transitória diminuição da resposta proliferativa a linfócitos T (Nieman, 1994; Gleeson, 2006; Gleeson e Williams, 2013). Os resultados estudados demonstraram que a cafeína aumentou a proliferação e sensibilidade de linfócitos a GCs, especialmente quando associados ao HIIT. Este efeito da cafeína pode estar relacionado a elevadas concentrações de epinefrina, perturbações no número de linfócitos circulantes (células CD4+ e CD8+), aumento na atividade de receptores GCs (Pettenuzzo, Noschang et al., 2008; Ping, Lei *et al.,* 2012) bem como alterações na sinalização de cálcio em linfócitos (Ritter, Hohenberger *et al.,* 2005).

Com relação ao sistema purinégico nos linfócitos, após 6 semanas de HIIT observou-se uma redução na hidrólise de ATP e ADP em ratos treinados. Embora sejam escassos os estudos com protocolos de alta intensidade sobre a atividade das NTPDases, existem outros tipos de exercício os quais têm demonstrado sua influência sobre a atividade destas enzimas. Moritz et al., (2016) demonstraram que corrida em esteira reduz a hidrólise ATP e ADP em soro de indivíduos exercitados (Moritz, Teixeira *et al.,* 2016). Corroborando com nossos achados, 2 semanas de corrida em esteira (20min/dia) também causou redução na hidrólise de ATP e ADP em soro de ratos treinados (Siqueira, Elsner *et al.,* 2010).

ATP e ADO são mensageiros modulatórios no controle imune e processos inflamatórios, uma vez que o ATP pode promover respostas pró-inflamatórias, enquanto que ADO exerce ações anti-inflamatórias (Bours, Swennen et al., 2006). Nossos achados sugerem que o HIIT é capaz de aumentar os níveis de ATP e ADP, uma vez que a hidrólise foi reduzida. Esta modificação na hidrólise de nucleotídeos de adenina também pode estar associada com a redução na expressão da NTPDase em linfócitos como proposto por Cardoso

e colaboradores (2015) após 6 semanas de nado moderado em modelo experimental (Cardoso, Abdalla *et al.*, 2015). Por outro lado, o HIIT também pode alterar a atividade da ADA contribuindo para uma redução dos níveis de ADO. Baseados nestes achados, alterações na sinalização purinérgica podem contribuir para uma condição pró-inflamatória após HIIT.

A cafeína pode modular muitos aspectos da resposta imune e inflamatória, bem como produção de citocinas, proliferação de linfócitos, síntese de anticorpos e apoptose de células imunes (Horrigan, Kelly *et al.*, 2006). Aumentos na hidrólise de ATP em ratos treinados e tratados com cafeína demonstram que este composto pode modular a resposta inflamatória mediada pelo ATP durante sinalização induzida pelo HIIT. Este mecanismo pode estar associado em parte com alterações nos níveis de citocinas. Além disso, quando associado ao HIIT, a cafeína promoveu aumento da atividade da ADA em linfócitos. Por sua vez, a cafeína exerce antagonismo aos receptores de adenosina (Ribeiro e Sebastiao, 2010). Uma vez que, o consumo de cafeína por períodos longos faz aumentar a expressão de receptores A_{2A} devido ao aumento da formação extracelular de adenosina (Ribeiro e Sebastiao, 2010). Desta forma, o aumento da atividade da ADA sugere um efeito compensatório para diminuição dos níveis de adenosina e neutralizar a ação antagonista da cafeína.

O presente trabalho também demonstrou que o HIIT altera a sinalização colinérgica em linfócitos de ratos. O aumento na atividade da AChE pode diminuir os níveis de ACh contribuindo para a inflamação e disfunção imune (Das, Sarkar *et al.*, 2007). Os linfócitos T são células que expressam receptores nicotínicos e muscarínicos e têm a capacidade de sintetizar ACh. A ativação destes receptores pela ACh induz efeitos como aumento de intracelular, regulação na produção de citocinas e proliferação celular (Mashimo, Iwasaki *et al.*, 2016). Por outro lado, a cafeína não foi capaz de reverter as alterações provocadas pelo HIIT na atividade da AChE. Todavia, outros estudos demonstram que em doses mais elevadas a cafeína é capaz de diminuir a atividade desta enzima em plasma (Barcelos, Souza *et al.*, 2014).

Desta forma, o HIIT promove alterações na atividade das enzimas NTPDase, ADA e AChE em linfócitos e nos níveis de citocinas sugerindo uma ação pro-inflamatória do treinamento intervalado de alta intensidade. Por outro lado, a ingestão de cafeína reverteu este efeito limitando a extensão da produção de fatores inflamatórios. Contudo a cafeína parece interferir nas adaptações impostas pelo HIIT sendo necessário maiores investigações relacionados ao uso de cafeína e parâmetros inflamatórios durante protocolos de exercícios mais intensos.

6. CONCLUSÕES

Verificou-se que o uso crônico de cafeína 4 mg e 8 mg/kg foi capaz de elevar os níveis de glicogênio muscular tanto animais não-treinados quando em animais treinados. Além disso, a cafeína mostrou-se eficaz ao manter em níveis de AChE similares aos animais controles, sendo este talvez um dos mecanismos no qual ela esteja envolvida com o retardo na instalação do processo de fadiga. Porém, quando avaliado a atividade da enzima Ca^{2+}-ATPase em dois protocolos distintos (agudo e crônico) a cafeína foi capaz de prevenir os efeitos associados ao HIIT.

No protocolo crônico, constatou-se em animais treinados um aumento na espessura do ventrículo esquerdo o que é uma adaptação crônica ao exercício. No entanto, doses de 4 mg/kg e 8 mg/kg foram capaz de prevenir esta adaptação. Sendo esta substância contra indicada para pessoas que praticam exercícios em alta intensidade como HIIT.

Observou-se em parâmetros de estresse oxidativo como níveis de substâncias reativas ao ácido tiobarbitúrico (TBARS), atividade das enzimas (SOD, CAT, GPX e conteúdo de GSH em diferentes estruturas cerebrais (córtex cerebral, hipocampo e estriado). Neste contexto, o HIIT exerceu um efeito pro-oxidante elevando a atividade de enzimas como a SOD, CAT e GPx em córtex cerebral, contudo, a cafeína atenuou esta resposta. A cafeína apresentou efeito parcialmente antioxidante prevenindo alterações do HIIT no estado redox. Uma vez que, o efeito pro-oxidante do HIIT serve de "gatilho" para que o sistema de defesa antioxidante atue de forma mais eficiente.

A partir da tarefa do labirinto em cruz elevado (avalia-se ansiedade) e da atividade da enzima Na^+, K^+ - ATPase (córtex cerebral, hipocampo, estriado). Observou-se que o HIIT foi capaz de induzir efeito ansiolítico, hipótese esta ratificada pelo aumento na atividade da Na^+, K^+ - ATPase em córtex cerebral e hipocampo de animais treinados. Visto que a cafeína (8 mg/kg) previne esta alteração induzindo ansiedade tanto em animais não treinados como animais treinados. Verificou-se, desta forma, que a cafeína exerce um efeito "tampão" na modificações induzidas pelo HIIT.

Em relação ao sistema purinérgico, observou-se alterações na atividade das enzimas NTPDase e ADA em linfócitos, onde o HIIT contribuiu para condição pro inflamatória e a cafeína preveniu parcialmente este efeito. Além disso, a cafeína promoveu significativo aumento da atividade de enzimas como ADA e AChE, o qual está envolvido com o bloqueio aos de AR liberando assim mais adenosina no meio.

O HIIT aumentou níveis de citocinas pro inflamatórias como IL-6 e reduziu níveis de citocinas anti-inflamatória como IL-10. Desta forma, observamos os fatos sob dois aspectos diferentes, uma vez que a cafeína reverte estes efeitos em ambas as doses. Por um lado, o uso da cafeína se torna contraproducente, uma vez que impede a maior parte dos efeitos do HIIT e, consequentemente, impede as adaptações induzidas por este. Por outro lado, observando sobre o ponto de vista da *performance*, o uso cafeína se torna um importante aliado. Uma vez que, esta substância pode auxiliar o sistema imune através de seu efeito anti-inflamatório, sendo importante adjuvante contra infecções que podem surgir após treinamentos intensos/extenuantes e sem o devido descanso. Circunstância comum em atletas de *endurance*, onde o descanso entre as sessões de treinamentos são curtos.

7. PERSPECTIVAS

O uso da cafeína como recurso ergogênico em atividades que visam *performance* física pode se tornar muito importante principalmente em atletas de *endurance*, porém seus efeitos para atletas que visam hipertrofia muscular ou executam treinamentos como HIIT não parecem ser uma boa opção. Por outro lado, investigações mais detalhadas sobre como esta substância altera a atividade de enzimas que compõem o sistema colinérgico em musculo gastrocnêmio durante o HIIT. Podendo auxiliar no retardo a instalação do processo de fadiga em atletas bem treinados.

8. APÊNDICE

UNIVERSIDADE FEDERAL DE SANTA MARIA
PRÓ-REITORIA DE PÓS-GRADUAÇÃO E PESQUISA
COMISSÃO DE ÉTICA NO USO DE ANIMAIS-UFSM

CARTA DE APROVAÇÃO

A Comissão de Ética no Uso de Animais-UFSM, analisou o protocolo de pesquisa:

Título do Projeto: "Efeito de um protocolo de exercício intermitente de alta intensidade na atividade das enzimas do sistema purinérgico e colinérgico em ratos tratados com cafeína".

Numero do Parecer: 077/2011

Pesquisador Responsável: Maria Rosa Chitolina Schetinger

Este projeto foi **APROVADO** em seus aspectos éticos e metodológicos. Toda e qualquer alteração do Projeto, assim como os eventos adversos graves, deverão ser comunicados imediatamente a este Comitê.

Os membros da CEUA-UFSM não participaram do processo de avaliação dos projetos onde constam como pesquisadores.

DATA DA REUNIÃO DE APROVAÇÃO:

Santa Maria, 10 de outubro de 2011.

Marta Lizandra do Rêgo Leal
Coordenadora da Comissão de Ética no Uso de Animais-UFSM

Comissão de Ética no Uso de Animais - UFSM - Av. Roraima, 1000 – Prédio da Reitoria - 2º andar - Campus Universitário 97105-900 – Santa Maria – RS - - Tel: 0 xx 55 3220 9362

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