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DOUTORADO

RAPHAEL FABRICIO DE SOUZA

EFEITO DE VOLUMES DE TREINAMENTO E SIMULAÇÃO DA CORRIDA DE ULTRA-ENDURANCE NA CAPACIDADE ANTIOXIDANTE CEREBELAR, DANO MUSCULAR E PERFORMANCE FÍSICA DE ROEDORES

Recife
2020

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Tese apresentada ao programa de Pós-graduação em Neuropsiquiatria e Ciências do Comportamento do Centro de Ciências da Saúde da Universidade Federal de Pernambuco como requisito à obtenção do título de doutor em Neurociências.

Área de concentração: Neurociências

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RESUMO

A corrida de ultra-endurance (UE) tem sido associada a alterações metabólicas cerebrais, mas ainda não se sabe quais regiões são vulneráveis. Este estudo investigou se altos volumes de corrida em roedores, mesmo sob baixas intensidades, podem induzir o estado oxidativo e inflamatório cerebelar. Quarenta e cinco ratos adultos foram divididos em 6 grupos, de acordo com um período de treinamento, seguido ou não de um teste de exaustão (TE) que simula a UE: controle (C), controle + TE (C- TE), moderado volume de treinamento (MV) e MV-TE, elevado volume de treinamento (EV) e EV- TE. Uma corrida contínua em esteira foi realizada 5 vezes / semana. O período de treinamento foi de 30 (MV) e 90 (EV) min / dia por 3 meses. Após 24 horas, o TE foi realizado e os níveis séricos de lactato foram avaliados. Homogenatos séricos e cerebelares foram obtidos 24 horas após o TE. Os níveis séricos de creatina quinase (CK), lactato desidrogenase (LDH) e corticosterona foram avaliados. Lipoperoxidação (LP), óxido nítrico (NO), proteínas da interleucina 1β (IL-1β) e proteína ácida fibrilar glial (GFAP), nível de glutationa reduzida (GSH) e oxidada (GSSG), atividades da superóxido dismutase (SOD) e catalase (CAT) foram quantificadas no cerebelo . As concentrações séricas de lactato foram menores em MV-TE (~ 20%) e EV-TE (~ 40%) em comparação ao grupo C-ET. Os níveis de CK e corticosterona foram aumentados mais de 2 vezes pelo treinamento de EV em comparação ao controle. TE aumentou os níveis de CK no grupo MV-TE vs MV ($p = 0,026$). O EV induziu níveis mais altos de LP (~ 40%), mas um efeito aditivo de TE foi observado apenas no grupo MV-TE ($p = 0,02$). A atividade da SOD foi maior em todos os grupos treinados vs C e C-TE ($P < 0,05$). A atividade de CAT, no entanto, foi intensificada apenas no grupo MV ($P < 0,02$). Os níveis de GFAP de 50 kDa foram aprimorados em C-TE e MV-TE versus os respectivos controles, enquanto os níveis das isoformas de 42 kDa (~ 40%) e 39 kDa (~ 26%) foram reduzidos. No grupo EV-TE, a quantidade da isoforma de 50 KDa foi reduzida ~ 40-60% em comparação com os outros grupos e a isoforma de 39 KDa aumentou ~ 7 vezes. Os níveis de LDH, IL-1β, a razão GSH / GSSG e NO não foram modificados. Os dados mostram que a resiliência cerebelar ao dano oxidativo pode ser mantida durante o treinamento com MV, mas é reduzida após UE. O EV por si induziu alterações metabólicas sistêmicas e no estado oxidativo cerebelar. A UE após o treinamento EV foi capaz de prejudicar o perfil reativo dos astrócitos do cerebelo.

Palavras -chave: Estresse oxidativo. Sistema nervoso central. Catalase. Lipoperoxidação. Proteína ácida fibrilar glial.

ABSTRACT

Ultra-endurance race (UE) has been associated with brain metabolic changes, but it is still unknown which regions are vulnerable. This study investigated whether high race volumes in rodents, even under moderate intensity, can induce cerebellar oxidative and inflammatory status. Forty-five adult rats were divided into 6 groups according to a training period, followed or not by an exhaustion test (ET) that simulates UE: control (C), control+ET (C-ET), moderate training volume (MV) and MV-ET, high training volume (HV) and HV-ET. A continuous running on a treadmill was performed 5 times/week. The training period was 30 (MV) and 90 (HV) min/day for 3 months. After 24h, the ET was performed and serum lactate levels were evaluated. Serum and cerebellar homogenates were obtained 24h after ET. Serum creatine kinase (CK), lactate dehydrogenase (LDH) and corticosterone levels were assessed. Lipoperoxidation (LP), nitric oxide (NO), Interleukin 1β (IL-1β) and glial fibrillary acidic protein (GFAP) proteins, reduced (GSH), oxidized (GSSG) glutathione levels, superoxide dismutase (SOD) and catalase (CAT) activities were quantified in the cerebellum. Serum lactate concentrations were lower in MV-ET (~20%) and HV-ET (~40%) compared to C-ET group. CK and corticosterone levels were increased more than ~2 fold by HV training compared to control. ET increased CK levels in MV-ET vs MV group ($p=0.026$). HV induced higher LP levels (~40%) but an additive effect of ET was only seen in the MV-ET group ($p=0.02$). SOD activity was higher in all trained groups vs C and C-ET ($P <0.05$). CAT activity, however, was intensified only in the MV group ($P<0.02$). The 50 kDa GFAP levels were enhanced in C-ET and MV-ET vs respective controls, while 42 kDa (~40%) and 39 kDa (~26%) isoform levels were reduced. In the HV-ET group, the 50 KDa isoform amount was reduced ~40-60% compared to the other groups and the 39 KDa isoform increased ~7fold. LDH, IL-1β levels, GSH/GSSG ratio and NO production were not modified. Data shows that cerebellar resilience to oxidative damage may be maintained under MV training, but it is reduced by UE running. HV per se induced systemic metabolic changes and cerebellar oxidative status. UE after HV training was able to impair the cerebellum astrocyte reactive profile.

Keywords: Oxidative stress. Central nervous system. Catalase. Lipoperoxidation. Glial fibrillary acidic protein.

LISTA DE ILUSTRAÇÕES

LISTA DE ABREVIATURAS E SIGLAS

CAT	Catalase
GPX	Glutadiona peroxidase
SOD	Superóxido dismutase
EROS	Espécies reativas do Oxigênio
$O2^{\bullet -}$	Aníon Superóxido
OH•	Radical hidroxila
H_2O_2	Peróxido de Hidrogênio
GSH	Glutationa
GFAP	Proteína glial fibrilar ácida
M1	Fenótipo reativo e inflamatório da micróglia
M2	Fenótipo inflamatório e bénifico da micróglia
IL-6	Interleucina 6
IL-7	Interleucina 7
IL-1β	Interleucina 1β
BNDF	Fator Neurotrófico Derivado do Cérebro
TBARS	Substâncias reativas ao ácido tiobarbitúrico
NOS	Óxido nítrico sintase
ON	Óxido nítrico
ONOO	Peróxidonitrito
kDa	kilodalton
CK	Creatina Quinase
LDH	Lactato desidrogenase
C	Grupo controle
MV	Grupo moderado volume
EV	Grupo elevado volume
TE	Teste de exaustão
UE	Ultra-endurance
LP	Lipoperoxidação
ATP	Adenosina tri-fosfato

SUMÁRIO

.

1 INTRODUÇÃO

Nas últimas décadas, houve aumento expressivo no número de participantes finalistas de corridas de longa distância e de resistência extrema (AGNEW et al., 2018). Entretanto, oposto ao treinamento de volume regular, implicações deletérias consequentes do grande volume, repetitivo esforço físico, exaustivas e complexas competições vem sendo investigadas (KNECHTLE e NIKOLAIDIS, 2018). Alguns estudos identificaram acometimentos fisiológicos sistêmicos, resultantes dos desgastes relacionado à provas com volumes superiores a 42 km denominado ultra corridas, abrangendo remodelamento estrutural patológico cardíaco e em grandes artérias (BONSIGNORE et al., 2017; O'KEEFE et al., 2012), acentuado dano muscular e renal (JASTRZĘBSKI et al., 2015), disfunção hepática (RAMA et al., 2015), desgaste cartilaginoso (FRANCIOZI et al., 2013), redução do volume (FREUND et al., 2012) e dano oxidativo aos sistemas nervosos periférico (SNP) e central (SNC) (CHALIMONIUK et al., 2015; MUÑOZ et al., 2017).

O SNP é vulnerável ao dano oxidativo porque apresenta elevada atividade mitocondrial dependente de oxigênio, sendo este intensificado pelo exercício aeróbio, aumentando consumo e fluxo sanguíneo cerebral 40-70% para suprir demanda (CHALIMONIUK et al., 2015). Quando submetido ao desequilíbrio enzimático antioxidante o SNC é importante alvo das espécies reativas do oxigênio (EROs) e indiretamente por reações de subprodutos secundários do estresse oxidativo (CAMILETTI-MOIRÓN et al., 2013; DE SOUZA et al., 2018). Ademais o estresse oxidativo cerebral é um mecanismo etiopatológico associado a mutações (UTTARA et al., 2009), apoptose e neurodegeneração (FLYNN, et. al., 2013). Tal condição oxidativa pode ser induzida pelo exercício físico dependendo da intensidade, volume e duração (DE SOUZA et al., 2018).

A influência do exercício físico na defesa antioxidante cerebral também é discutida uma vez que seu efeito varia de acordo com a região cerebral (ACIKGOZ et. al., 2006; (DE SOUZA et al., 2018)). O cerebelo é uma região que desempenha significativo papel no circuito locomotor (HIBI e SHIMIZU, 2011), e vem sendo demonstrado que o exercício aeróbico moderado amplia projeções dendríticas das células de Purkinje (HUANG et al., 2018; Pysh JJ, 1979) e favorece angiogênese cerebelar (BLACK et al., 1990; LEE, 2007). Entendido como centro integrador, o cerebelo promove controle neural dos movimentos, aprendizagem motora (CELNIK, 2016) equilíbrio, coordenação muscular, postural (HIBI e SHIMIZU, 2011). Durante o exercício físico, compara continuamente a intenção dos centros superiores com o desempenho corporal e, atua em medidas corretivas apropriadas. Danos

cerebelares ocasionam perda do tônus muscular e movimentos desordenados e inseguros SCHMAHMANN, 2019). No entanto, dentre as principais pesquisas que avaliaram o Estresse oxidativo cerebral durante o exercício físico de endurance, foram limitadas as avaliações no cerebelo (CHALIMONIUK et. al. 2015; CECHETTI et al., 2008). A maior concentração das pesquisas foi realizada para investigar efeitos no córtex cerebral (CECHETTI et al., 2008; da CUNHA et. al., 2012; VOLLERT et al., 2011; CHALIMONIUK et. al. 2015; COSKUN et. al., 2005; SOMANI et. al., 1995; NEVES et. al. 2015; AKSU et. al. 2009; AGUIAR et al., 2010; SCHIMIDT et al., 2014; FALONE et. al., 2012; CECHETTI et al. 2012; DEVI e KIRAN, 2004), ou homogenados de tecido cerebral por inteiro (CAKIR et al., 2010, ITOH et al., 1998, MAZZOLA et al., 2011, NAVARRO et al., 2004, LIU et al. 2000, RADAK et. al., 2001)**,** o que representa uma restrição, considerando que o comportamento enzimático antioxidante varia de acordo com a região cerebral.

Assim, embora esteja consolidado o conhecimento sobre o desequilíbrio redox cerebral decorrente do exercício aeróbio de alta intensidade (CHALIMONIUK et al., 2015) e efeito protetor da atividade física moderada e regular (CAMILETTI-MOIRÓN et al., 2013), entendemos que os mecanismos de defesa antioxidante induzidos pelo elevado volume de treinamento permanecem ainda inconclusivos. Considerando que: a) pouco se conhece a respeito dos acometimentos de extremos volumes de corrida no SNC, b) há limitação de estudos com protocolos de ultra corridas avaliados em regiões cerebrais específicas, c) há importante função cerebelar no circuito locomotor, d) e há extenso debate científico sobre as potenciais agressões fisiológicas decorrentes de elevados e ultra volumes de exercício físico (KNECHTLE e NIKOLAIDIS, 2018; MILLET et al., 2018; KNEZ, et al., 2006; JASTRZĘBSKI, et. al., 2015; RAMA et. al., 2015), objetivamos no presente estudo investigar quais as repercussões do treinamento físico de moderada intensidade com moderado e elevado volume per se ou associado a uma simulação experimental de ultra corrida sobre o estado redox e inflamatório do cerebelo de ratos. Acreditamos que esta pesquisa possa contribuir para a compreensão de potenciais mecanismos de resiliência cerebelar após elevados volumes de treinamento.

2 FUNDAMENTAÇÃO TEÓRICA

2.1 Cerebelo e atividade motora

O Cerebelo humano é a segunda maior porção do encéfalo, situa-se dorsalmente ao bulbo e a ponte, repousa sobre a fossa cerebelar do osso occipital, é separado do lobo occipital pela tenda do cerebelo e, unido ao tronco encefálico pelos pedúnculos cerebelares (ROOSTAEI, et al., 2014). Do ponto de vista anatômico ele é composto de dois hemisférios corticais unidos pelo vermis e é subdividido em 3 lobos: anterior, posterior e floculo-nodular, os quais são separados por duas fissuras transversais que separam os lobos anterior e posterior bem como os lobos posterior e floculonodular. O cerebelo contém cerca de 50 a 80% dos neurônios do encéfalo estando os mesmos organizados em densas camadas celulares (ROOSTAEI et al, 2014; VAN ESSEN et al., 2018).

O cerebelo foi tradicionalmente conhecido por regular funções motoras, posturais e equilíbrio (ALLIN, 2016; HIBI, 2012) exercendo papel fundamental na coordenação dos membros durante movimentos voluntários (CASARTELLI et al., 2017;); auxiliando na supervisão da informação neuromuscular e padrão correto da sequência contrátil do músculo esquelético (DE ZEEUW, et al., 2015).

Atualmente um número cada vez maior de estudos em humanos evidenciam importantes relações do cerebelo à tarefas não motoras (KLEIN et al., 2016), cognitivas (WAGNER et al., 2017), processamento sensorial (WAGNER et al., 2017), planejamento, atenção e linguagem (MARIËN e BORGATTI, 2018). Além da reciprocidade entre as conexões anatômicas que o ligam não apenas a áreas motoras, mas também às áreas frontal, parietal e límbica do córtex (BECKINGHAUSEN, 2019; ALLIN, 2016).

Evidências obtidas por técnicas de imageamento bem como por sintomas clínicos de lesões específicas vem demonstrando que as funções motoras e cognitivas são processadas em regiões cerebelares distintas (SCHMAHMANN, 2019) . O lobo anterior cerebelar (lóbulos I a V) e partes do lóbulo VI, juntamente com o lóbulo VIII, recebem aferentes da medula espinhal. Eles também são reciprocamente interconectados com os córtices do motor através das aferências corticopontinas e através de feedback para regiões motoras a partir dos núcleos cerebelares através do tálamo. Essas regiões cerebelares anteriores são também interligadas ao o complexo olivar inferior, que recebe aferencias da medula espinhal. A região do vermis desta região está também inteconectada com os núcleos vestibulares e outros do tronco cerebral envolvidos no controle postural e do equilibrio (SCHMAHMANN, 2019).

Por outro lado, os lobos posteriores do cerebelo não possuem conexões com as áreas sensório-motoras corticais cerebrais e nenhuma entrada da medula espinhal do trato espinocerebelar. Estas áreas são interligadas com as áreas de associação do córtex cerebral relacionadas com comportamento cognitivo e límbico planejado, ou seja, com o córtex pré-frontal, parietal posterior, regiões polimodais temporais superiores, giro cingulado e área parahipocampal posterior. (BECKINGHAUSEN, 2019; ALLIN, 2016; SCHMAHMANN, 2019).

De forma similar ao cérebro, o cerebelo também possui um córtex de substância cinzenta (MOORE et al., 2017), substância branca interna e pequenos núcleos profundos (denteado, o emboliforme, o globoso e o fastigial), sendo o denteado o mais conhecido no qual recebe múltiplas aferências celulares (BOND, et al., 2017). Alguns tipos de neurônios são localizados no córtex cerebelar, incluindo as células estrelares, em cesto, granulares e de Purkinje.

Entretanto, as principais células cerebelares relacionadas com a função motora são as células granulares e de Purkinje (POWELL et al., 2015; JIRENHED et al., 2017). As células granulares permitem representações altamente detalhadas do contexto sensório-motor, favorecendo que células de Purkinje recebam alterações contextuais (HIBI, 2012). Além disso, a célula granular recebe informação de uma diversidade de áreas corticais (WAGNER et al., 2017). As células de Purkinje apresentam dendritos extensivamente ramificados, sendo os principais neurônios que fazem sinapses com os núcleos centrais do cerebelo (BECKINGHAUSEN, 2019).

Duas vias são identificadas como as principais aferências excitatórias para o cerebelo (BENAGIANO, et al., 2018). A primeira identificada pelas fibras trepadeiras, que se originam do núcleo olivar inferior, no qual os axônios projetam para os dendritos das células de Purkinje, bem como para os núcleos cerebelares (BECKINGHAUSEN, 2019; GLICKSTEIN et al., 2011). As fibras trepadeiras transportam informações da medula espinhal, sistema vestibular, núcleo rubro, colículo superior, formação reticular e os córtices sensorial e motor (ROOSTAEI et al, 2014). Acredita-se que a ativação destas fibras sirva como um sinal de erro motor enviado ao cerebelo e é um sinal importante para a temporização do movimento. Além do controle e coordenação dos movimentos, este sistema aferente contribui para o processamento sensorial e tarefas cognitivas, provavelmente codificando o tempo de entrada sensorial independentemente da atenção ou consciência (WU et al 2011) .

A segunda aferência excitatória para o cerebelo é fornecida pelas fibras musgosas, que apresentam terminações em células granulares (BECKINGHAUSEN, 2019;

GLICKSTEIN et al., 2011). Estas fibras originam-se de núcleos pontinos dispersos em toda a base da ponte, e da formação reticular lateral. Carreia informações do córtex cerebral, transmitindo pelo pedúnculo cerebelar médio informações ao cerebelo do plano motor elaborado no telencéfalo (BENAGIANO et al., 2018; ROOSTAEI, et al., 2014). Outros contribuidores desta aferência incluem os núcleos vestibulares, a medula espinhal, formação reticular e núcleos cerebelares profundos.

Lesões cerebelares provocam acentuada atonia, ataxia, distúrbios na marcha, acometimentos na fala, memória e na percepção corporal espacial (MARZBAN et al., 2018; SCHMAHMANN, 2019). Além disso, alterações moleculares cerebelares também foram relacionadas à plasticidade no córtex motor primário (MANG et al., 2016; POPA et al., 2013). Estudos sobre modelos animais mostraram que o cerebelo é susceptível à isquemia, hipóxia, excitotoxicidade, exposição a diferentes tipos de produtos químicos (KOEPPEN et al., 2018), mas pode apresentar elevada resiliência oxidativa quando comparado ao córtex cerebral diante de alguns modelos experimentais de má nutrição (AUGUSTO, et al. 2017).

Por outro lado, o cerebelo não dispõe da habilidade para formação de novos neurônios na vida adulta (GRIMALDI e ROSSI, 2006; KUHN et al., 1997). Nele há o predomínio de neurônios GABAérgicos incluindo células de Purkinje e interneurônios (como as células de Golgi, células estreladas e células em cesto (SASSOÈ-POGNETTO et al., 2017; HIBI, 2012; HIBI e SHIMIZU, 2011;), diferente do predomínio excitatório cortical glutamatérgico (SASSOÈ-POGNETTO et al., 2017; HIBI e SHIMIZU, 2011; HIBI, 2012).

As células de Purkinje podem ser seletivamente vulneráveis a insultos citotóxitos glutamatérgicos, principalmente quando há insuficiências metabólicas e aumento excessivo de cálcio intracelular (DHOUIB et al., 2017; KRITIS et al., 2015; O'HEARN e MOLLIVER, 1997) o que aumenta a produção de EROS induzindo estresse oxidativo e excitotoxicidade cerebelar (DHOUIB et al., 2017; KRITIS et al., 2015; NAKASO et al., 2000). Além disso, o cerebelo é um órgão rico em óxido nítrico sintase (NOS) a qual produz óxido nítrico (ON). Consequentemente uma reação do ON com o radical $O2^{\bullet -}$ formam radicais livres altamente reativos como o peroxinitrito ($ONOO^{-}$) e hidroxila ($OH^{\bullet}$) os quais podem estar relacionados a acometimentos à via olivopontocerebelar (GARCIA et al., 2018; YAMASHITA et al., 2000).

Tidos em conjunto, estes dados prévios levam a crer que insultos motores crônicos decorrentes de períodos prolongados de exercício podem elevar o estresse oxidativo cerebelar. No entanto, embora o cerebelo apresente grande importância motora, poucos estudos

experimentais, investigaram o impacto oxidante nesta região cerebral diante de elevados volumes de treinamento e particularmente após ultra corridas.

Os estudos que avaliaram o efeito do treinamento aeróbio de longa distância no cerebelo de ratos, realizaram protocolos de baixo a médio volume, [20 min (CECHETTI et al., 2008) e 60 min (CHALIMONIUK et al., 2015) de treinamento diário de corrida em esteira]. Recentemente foi relatado que exercício moderado por 4 semanas é capaz de atenuar o dano oxidativo induzido pelo álcool ao cerebelo (LAMARÃO-VIEIRA et. al., 2019). No entanto, todos estes estudos não investigaram as repercussões sobre o metabolismo oxidativo cerebelar após treinamentos de ultra corridas.

2.2 Estresse Oxidativo e defesa antioxidante cerebral

2.1.1 Radicais livres e enzimas antioxidantes

Espécies reativas de oxigênio (EROs) é o termo dado a radicais livres e compostos não radicais derivados de oxigênio que tem a capacidade de modificar lipídios, proteínas, carboidratos e ácidos nucleicos (MARGARITELIS et al., 2018; YASUDA et al., 2015). Similarmente, o termo espécies reativas de nitrogênio (ERNs) refere-se a radicais, onde o centro reativo é o nitrogênio (BRIOCHE et al., 2016).

Os radicais livres primários gerados nas células são o ânion superóxido ($O2^{\bullet -}$) e o óxido nítrico (NO) (HE et al., 2017; BLANCO et al., 2017). Tanto o $O2^{\bullet -}$ quanto o NO são reativos e podem reagir prontamente para formar uma série de EROs e ERNs respectivamente (POWERS et al., 2016). Outros exemplos incluem o radical hidroxila ($OH^{\bullet}$), hipocloroso, dióxido de nitrogênio (NO_2-) e peroxinitrito ($ONOO^-$) (BLANCO et al., 2017). O Peróxido de hidrogênio (H_2O_2) apesar de não ser um radical livre, pela ausência de elétrons não pareados na camada de valência, é uma molécula extremamente deletéria, pois participa de reações que produzem o $OH^{\bullet}$ (POWERS et al., 2008). Embora EROs sejam produzidas naturalmente em processos metabólicos celulares aeróbios, níveis aumentados são citotóxicos e podem resultar em danos oxidativos (SHICHIRI et. al., 2014).

Como estratégia protetora à constante exposição a espécies reativas, o organismo apresenta mecanismos endógenos de defesa antioxidante enzimáticos e não enzimáticos. Um agente antioxidante é uma substância que reduz a extensão do dano oxidativo por meio da criação de um radical ou reduzindo o radical livre prejudicial (HE et al., 2017). As enzimas antioxidantes primárias de maior importância são a superóxido dismutase (SOD), a catalase

(CAT), glutationa peroxidase e glutationa redutase. Estas enzimas são essenciais para a estabilidade redox (CHALIMONIUK et al., 2015), pois a SOD converte o $O2^{\bullet-}$ óxido em H_2O_2; a CAT remove o H_2O_2 convertendo-o em agua e oxigênio; e, a glutadiona peroxidase catalisa H_2O_2 e ácidos graxos peroxidados em água e glutationa oxidada (GSSG) (JACKSON et al., 2016; CHALIMONIUK et al., 2015). Antioxidantes não enzimáticos podem ser encontrados também de forma exógena nas vitaminas E e C, no selênio, no ß-caroteno ou produzidos endogenamente com a glutationa reduzida (GSH) (HE et al., 2017).

O exercício físico pode exercer uma atividade deletéria sobre a resposta antioxidante quando realizado com uma intensidade elevada, desequilibrando este sistema protetor, resultando em estresse oxidativo (POWERS et al., 2016, PEDERSEN, 2017; DE SOUZA et al., 2018). A atividade física extenuante aumenta o fluxo de elétrons através da cadeia transportadora, pela necessidade de maior produção de ATP para aprimorar o metabolismo aeróbico e anaeróbico (AOI et al., 2014), e consequentemente ocorre formação de radicais livres.

Sobretudo, as pesquisas sobre as implicações biológicas do estresse oxidativo induzido pelo exercício avançaram extensivamente nos últimos anos (POWERS et al., 2016). Embora altos níveis de espécies reativas possam danificar componentes moleculares; níveis baixos e moderados desempenham papéis regulatórios no controle da expressão gênica, regulando vias de sinalização celular e modulando a produção da força muscular esquelética (Moulin et al., 2017). Em contraste, após elevada intensidade e volume, além do dano oxidativo no tecido muscular (POWERS et al., 2008), hepático (PILLON BARCELOS et al, 2017) e renal (Tucker et. al., 2015), o cérebro pode ser potencialmente atingido (CHALIMONIUK et al., 2015). Além disso, a neurotransmissão glutamatérgica durante o exercício físico, desencadeia uma maior entrada de cálcio no neurônio pós-sináptico, o que pode resultar em estresse oxidativo (DEUTSCHENBAUR et al., 2015; FERNANDEZ-FERNANDEZ, et al., 2012).

A proteção do sistema nervoso central é de especial importância fisiológica, pois há alta vulnerabilidade energética do cérebro relacionado a dano oxidativo, o que pode ser identificado como um fator promotor de distúrbios neurológicos tais como os neurodegenerativos (FLYNN, et. al., 2013). Os neurônios são células que necessitam de grande demanda energética e, consequentemente são também as mais sensíveis ao estresse oxidativo (BOLAÑOS, 2016). Isto pode ser verificado ao submeter neurônios e astrócitos ao mesmo grau de dano oxidativo mitocondrial, os neurônios sofrem morte celular, enquanto os

astrócitos são mais resistentes e reagem geralmente com aumentados níveis de proliferação ou astrogliose (FERNANDEZ-FERNANDEZ, ET AL., 2012).

2.1.2 Metabolismo astrocitário e astrogliose reativa

Os astrócitos são considerados as células gliais mais numerosas do sistema nervoso central. Representam um grupo de células distintas, diferenciadas por subclasses, conforme a morfologia, desenvolvimento, metabolismo, fisiologia e localização (BEN HAIM et al., 2017; SALOUCI et al., 2014). Dentre as subclasses mais significativas os astrócitos fibrosos estão presentes na substância branca, os protoplasmáticos na substância cinzenta e os astrócitos velados no cerebelo (BEN HAIM et al., 2017).

Estas células participam ativamente no desenvolvimento e funcionamento das sinapses, auxiliam na homeostase iônica (STOGSDILl et al., 2017) e no suporte estrutural neuronal (PEREA et al., 2014). Além disso, apresentam função metabólica para os neurotransmissores glutamado e GABA; e na secreção de fatores tróficos considerados cruciais para a sobrevivência e diferenciação dos neurônios (POSADA-DUQUE et al., 2015). No metabolismo antioxidante e energético, proporciona ao neurônio uma função precisa no equilíbrio redox também auxiliando no suprimento glicolítico (BOLAÑOS, 2016).

Notavelmente apresentam grande capacidade de armazenar glicose na forma de glicogênio, assistindo a atividade neuronal, especialmente durante condições de alta atividade sináptica (FALKOWSKA et al., 2015). A fim de evitar efeitos deletérios bioenergéticos, com o aumento da produção de EROs, o astrócito ativa a transcrição de fatores nucleares como o fator nuclear eritroide 2 relacionado ao fator 2 (Nfr2), estimulando uma produção de enzimas antioxidantes relacionadas ao metabolismo da glutationa como por exemplo a glutamato cisteina ligase e glutamiltransferase (FERNANDEZ-FERNANDEZ, et al., 2012). Consequentemente o conteúdo celular de glutationa é transportado aos neurônios, beneficiando a biossíntese antioxidante neuronal, promovendo neuroproteção.

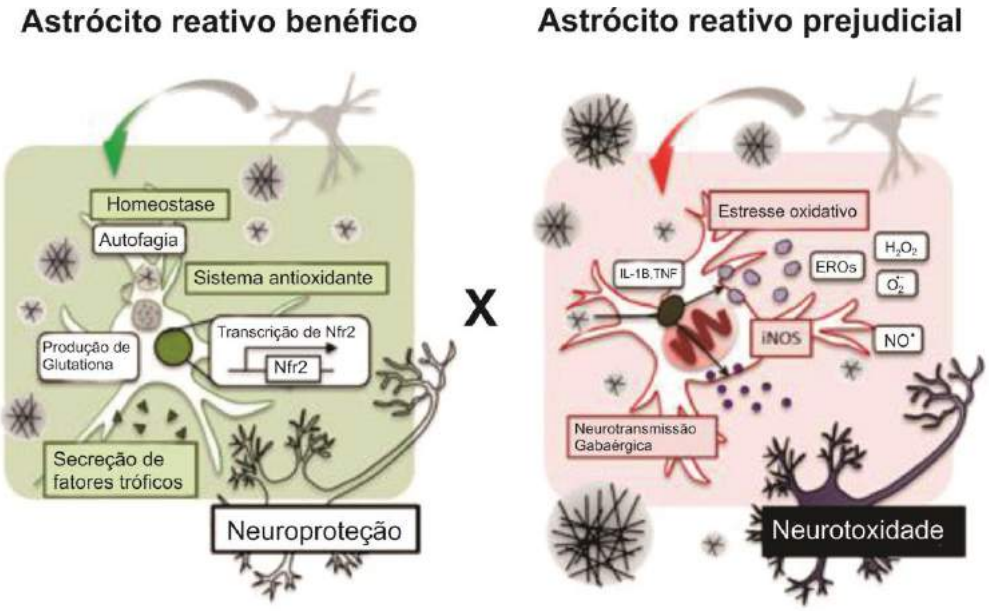
A atividade astrocitária decorrente de lesões traumáticas no sistema nervoso central, isquemias, tumores, doenças infecciosas, neuroinflamatórias e excesso de toxidade é conhecida como astrogliose reativa (HOL et al., 2015), identificado como um mecanismo de defesa. Os astrócitos ainda participam da formação da cicatriz glial necessária para a recuperação da lesão, porém neste caso, pode impedir o crescimento axonal dificultando a total regeneração do Sistema nervoso central (KARIMI-ABDOLREZAEE E BILLAKANTI, 2012).

Além disso, extensas mudanças ocorridas no astrócito reativo alteram suas propriedades morfológicas, transcricionais e funcionais, o que pode ser uma característica prejudicial (LAGOS-CABRÉ et al., 2018). Neste caso, o astrócitos desenvolvem um fenótipo hipertrofiado, complexas ramificações reorganizadas e um número maior de prolongamentos polarizados em direção à injúria ou agregados tóxicos (BARDEHLE et al., 2013). Tais alterações morfológicas relacionadas à plasticidade astrocitária devem-se a modificações em proteínas de seu citoesqueleto. Dentre estas proteínas, o aumento na expressão da proteína ácida fibrilar glial (GFAP) é considerado um marcador padrão de astrócitos reativos (ZENGLI et al., 2019). Além da GFAP, outras proteínas de filamentos intermediários, como a vimentina e a nestina, também são expressas em condições reativas (HOL et al., 2015).

Astrocitos apresentam uma interação recíproca com as citocinas pró-inflamatórias IL-1β e TNF alfa, podendo ser estimulado por estas citocinas inflamatórias liberadas da microglia o que leva a uma produção de EROS e especies reativas de nitrogênio (RAPPOLD e TIEU, 2010). Por outro lado, elevados níveis de espécies reativas e destas citocinas pró-inflamatórias são liberados por astrócitos reativos em um cérebro lesado (Abudara et al., 2015). Experimentos *in vitro* relataram que os astrócitos secretam mais EROs em resposta ao estresse crônico, verificado por exemplo em doenças neurodegenerativas, pela via pentose-fosfato tornando-se tóxicos para os neurônios (Allaman et al., 2010).

Os efeitos bifásicos astrocitários são dependentes do nível de produção de EROs relacionado com o impacto lesivo ou quantidade de agente tóxico (Chun et al., 2018). Ademais, exagerados níveis podem ativar as expressões de óxido nítrico sintetase induzível podendo ocorrer tóxica nitração em neurônios (Bagheri et al., 2017). Portanto o nível de espécies reativas nos astrócitos pode atuar como um determinante chave no destino benéfico ou maléfico desta célula. A figura 1 adaptada de Chun et al., 2018 ilustra algumas das alterações moleculares que podem ocorrer durante a atividade dos astrócitos em condições fisiologicamente normais ou de toxicidade

Figura 1 - Reação astrocitária benéfica e prejudicial



IL-1B: interleucina 1 beta; TNF: fator de necrose tumoral; Nfr2: fator nuclear eritroide 2 relacionado ao fator 2; ROS: espécies reativas de oxigênio; H2O2: perôxido de hidrogênio; O2: ânio superóxido; NO: Oxido Nítrico; iNOS: óxido nítrico sintetase induzível. (Adaptado de Chun et al., 2018).

Pesquisas que buscaram compreender o comportamento astrocitário consequente à pratica esportiva, indicam uma resposta dependente da condição metodológica empregada durante o treinamento físico (LOPRINZI 2019; MATSUI et al., 2017; SANTIN et al., 2011). Variáveis importantes são relacionadas à intensidade, volume e limiares de prescrição.

2.3 Treinamento físico e dano muscular

2.3.1 Treinamento físico em roedores

A resposta adaptativa do treinamento físico é dependente de fatores e princípios desenvolvidos sistematicamente. A intensidade e a duração dos exercícios (DE LADE. et. al., 2018), o tipo de treinamento, a frequência (JAKOVLJEVIC et al., 2019), limitações genéticas (SHIRVANI et. al., 2018) e nível anterior de atividade do indivíduo (ZOLADZ et al., 2017), são variáveis determinantes na periodização do treinamento físico. Além disso, as adaptações

metabólicas e fisiológicas são próprias à natureza da sobrecarga imposta (FORTES, et al., 2015).

Isso quer dizer que o treinamento de força e velocidade, por exemplo, geram alterações diferentes das produzidas pelo treinamento de resistência ou *endurance* onde o principal efeito do treinamento aeróbio sobre o músculo esquelético, relaciona-se à sua capacidade oxidativa (EMAMI, et al., 2016). Além disso, somado ao período de treinamento há necessidade de recuperação regenerativa para proporcionar a adequação neuromuscular (PIN-BARRE, et al., 2017).

Embora, o treinamento físico em humanos seja amplamente estudado, a pesquisa em modelos animais tem sido amplamente desenvolvida a fim de se aprofundar o entendimento das adaptações fisiológicas, estruturais e funcionais que a atividade física produz frente às diversas especificidades da investigação. Com relação aos métodos do treinamento de corrida experimental em roedores, o exercício realizado em rodas ou esteiras vem sendo comumente aplicado (REZAEI et al., 2017).

No entanto, características distintas são verificadas para cada metodologia: a roda permite a avaliação da corrida voluntária (SVENSSON, et al., 2016; REZAEI et al., 2017) já a esteira permite simular um esforço relativamente forçado sob controle de velocidade, inclinação e distância percorrida (TEIXEIRA, et al., 2012). No objetivo de proporcionar um treinamento de corrida em condições funcionais empregando moderados e longos volumes de exercício aeróbio e da simulação de ultra corrida em ratos, a esteira para múltiplos roedores, permite monitorar a intensidade e o volume. Entretanto, quando comparamos os diferentes modelos e metodologias propostas, verificamos que os estudos em roedores tem uma aplicação extensivamente adaptada das pesquisas em humanos. No que diz respeito à prescrição da avaliação do exercício, por exemplo, o VO2 máximo (VO2 max) (REZAEI et al., 2017), limiar de lactato (ABREU, et al., 2016), teste de intensidade máxima (ABREU, et al., 2016; ABREU, et al., 2015) e velocidade crítica (BILLAT et al., 2005) são parâmetros mensuráveis adotados para determinar a intensidade de treino em ratos.

Embora em humanos historicamente a frequência cardíaca (PLEWS, et al., 2014) e recentemente o ritmo (*pace)* (KENNEALLY, et al., 2018) sejam métodos acessíveis e utilizados para monitoração da zona de treinamento,em roedores, as propostas mais aplicadas na determinação da intensidade podem ser verificadas na avaliação do VO2 max classificada em: baixa (≥ 50% do VO2 max), moderada (65 a 70% do VO2 max) e alta (85 a 90% do VO2 max) (de LADE et al., 2018); e pela concentração de lactato: baixa (> 2 mmol\L), moderada (2 a 4 mmol\L) e alta (< 4 mmol\L) intensidade (ABREU, et al., 2016) (Figura 02).

Figura 2 - Transição aeróbico-anaeróbico

Fonte: Abreu et al. 2016.

Curva clássica de transição aeróbico-anaeróbico aplicada para treinamento físico prescrição e avaliação de desempenho. No primeiro ponto, há um aumento da taxa de produção de lactato, devido ao desequilíbrio da capacidade metabólica no músculo em produzir e eliminar lactato sanguíneo. No segundo ponto, com o aumento da intensidade do exercício, a taxa de produção de lactato muscular excede a taxa de eliminação de lactato sistêmico levando a um aumento exponencial da concentração de lactato sanguíneo. (Adaptado de Abreu et al. 2016)

Apesar do elevado grau de confiabilidade científica, o teste do VO2 max por vezes é impraticável devido aos altos custos do equipamento, assim como a avaliação da concentração de lactato sanguíneo, especialmente durante o teste de velocidade incremental, é uma técnica invasiva podendo ser uma variável de estresse e levar o animal a rejeitar o exercício (TEIXEIRA et al., 2012).

Alternativamente, o uso de velocidades arbitrárias é um método potencialmente utilizado, correlacionado parâmetros metabólicos durante os testes de velocidades incrementais (REZAEI et al., 2017; TEIXEIRA et al., 2012). Em recente estudo, após avaliar as velocidades de 10, 13, 14, 15, 16, e 17,5 m/min a fim de determinar o *steady state* lático do rato *wistar*, foi verificado que entre 60 a 70% do pico de velocidade máxima pode ser determinado o limiar anaeróbico do roedor (REZAEI et al., 2017), também corroborando com outros estudos em modelos animais (ABREU et al., 2016; TEIXEIRA et al., 2012; DE SENNA et al., 2015).

Na intenção de aumentar a tolerância à fadiga, a intensidade elevada em curtos períodos de tempos e distâncias, é aplicada metodologicamente durante treinos intervalados (PINTO et al., 2019) ou em *sprints* (MCGINLEY et al., 2016) comuns em finais de provas de longas duração. O emprego do treinamento com intensidade elevada é utilizado principalmente quando se objetiva aumentar a velocidade de corrida (PINTO et al., 2019).

Por outro lado, baixas intensidades e\ou moderadas permitem um treinamento de corrida em limiar aeróbico (ABREU et al., 2016) por médias e longas distâncias, possibilitando benéficas adaptações cardiovasculares e metabólicas (DE LADE, et al., 2018), tradicionalmente conhecidas pelo fortalecimento das fibras vermelhas (BUCKENMEYER, et al., 1990), aumentando a capacidade de correr maiores volumes.

A definição de moderados e longos volumes de corrida em roedores na literatura considera que para moderados volumes são adotados protocolos entre 20 a 30 minutos de atividade (SCHIMIDT et al., 2014; HRNCIC et al., 2013; FALONE et al., 2012; MAZZOLA et al., 2011; DA CUNHA et al., 2012; COSKUN et al., 2005). Protocolos realizados a partir de 60 minutos podem ser considerados representativos de um grande volume de corrida para roedores (CHALIMONIUK et al., 2015; DABIDI et al., 2013; VOLLERT et al., 2011; TEIXEIRA et al., 2009).

Atualmente são poucos os estudos que avaliaram o estado redox cerebral sob protocolos acima de 1 hora de corrida em ratos ou camundongos (RADAK et al., 2001; LIU et al., 2000) e, independente de não estar consolidada a definição de provas de ultra corrida em roedores, eventos concluídos em 4 (MILLET et al., 2011) ou 6 horas de exercício extenuante são considerados ultra corridas, ultra maratona ou ultra *endurance* em humanos TURNER et al., 2014). Além disso, a média de 200 minutos de corrida foi adotada durante protocolos de ultra corrida em roedores (TARINI et al., 2013).

Embora o ultra volume de corrida seja normalmente realizado sob moderada intensidade e, mesmo que o sistema muscular esteja adaptado e treinado, o dano muscular é normalmente verificado como resposta aguda sistêmica em consequência ao elevado desgaste neuromuscular e excessivo esforço físico (RAMOS-CAMPO, et al., 2016).

2.3.2 - Dano muscular provocado por atividade física

O dano muscular provocado pela atividade física pode ser verificado após a realização de exercícios de longa duração, elevada sobrecarga ou intensidade (NIEMAN, et al., 2018; WIEWELHOVE , et al., 2016; KIM, et al., 2013). Este fenômeno ocorre tanto em indivíduos

treinados quando não treinados, porém condições mais acentuadas são constatadas em praticantes menos experientes e não-habituais (NAKAGAWA, et al., 2018).

Outras investigações revelam que ações musculares excêntricas induzem maior lesão muscular (GAVIN, et al., 2016). Isto ocorre porque o músculo é alongado enquanto está ativo, o que aumenta a ruptura estrutural do sarcômero quando comparado às contrações concêntricas e isométricas (DOUGLAS, et. al., 2017; GAVIN, et al., 2016).

O dano no músculo esquelético não apenas caracteriza-se pela ruptura da membrana do sarcolema (HYLDAHL e HUBAL 2014; BAUMERT, et al., 2016), mas também pela expansão da linha Z, sítio de contato das proteínas contráteis e base para transmissão de força (BAUMERT, et al., 2016). Além disso, consequente à perturbação do acoplamento excitação-contração e da sinalização de cálcio muscular, ocorre a ativação da via inflamatória e de degradação proteica (HYLDAHL e HUBAL 2014; PEAKE, et al., 2017). Assim, os mecanismos do dano muscular são discutidos levando em consideração o estresse mecânico e\ou metabólico e pela inflamação (PEAKE, et al., 2017). Este processo tem sido acompanhado por inchaço e uma redução temporária no pico de torque muscular e na amplitude de movimento (BAIRD et al. 2012; PEAKE, et al., 2017).

Limitações metabólicas acontecem especialmente quando o fluxo das vias bioenergéticas, glicolítica e oxidativa, são substancialmente elevados durante a atividade física gerando grande demanda de adenosina tri-fosfato (ATP) (HORN, et. al., 2017). Com o aumento da intensidade ou do exercício físico realizado por grandes volumes, há excesso deste fluxo ocorrendo depleção do glicogênio (GAVIN, et al., 2018). Ademais o estresse gerado também é sucedido pela perda da homeostase de cálcio intramuscular. Pois o comprometimento de seu tamponamento ativa as vias fosfolipolíticas e proteolíticas cálcio-dependentes (calpaínas) degradando as proteínas contráteis e a membrana miofibrilar (HORN, et. al., 2017).

Muitos estudos têm se dedicado à investigação da magnitude do dano muscular e das implicações decorrentes do treinamento físico (RYRSØ, et al., 2018; SON, et al., 2015; PEAKE, et al., 2017; KIM, et al., 2013; BAUMERT, et al., 2016). Como estratégia investigatória, alguns protocolos são utilizados tanto para induzir quanto para quantificar o dano (MINAHAN, et al., 2018) A concentração sangüínea de creatina quinase (CK) e de lactato desidrogenase (LDH) são indicadores indiretos que refletem o grau de dano muscular e da aptidão física constantemente utilizados em provas de longa duração (RUBIO-ARIAS, et al., 2019; BAIRD, et al., 2012).

A CK é a uma enzima que controla o sistema ATP-PC e, a LDH é a principal enzima que dentre outras funções mantém o equilíbrio do catabolismo e anabolismo de carboidratos, catalisando a conversão de piruvato para lactato na etapa final da glicólise. (KIM, et al., 2013; BAIRD, et al., 2012). Outras enzimas como o aspartato aminotransferase e proteínas musculares como a mioglobina, troponina e a miosina também têm sido amplamente avaliadas como marcadores de dano muscular em humanos e animais (SON, et al., 2015; MINAHAN, et al., 2018). Métodos diretos como a biópsia muscular são aplicados com menor frequência em humanos, pelo fato de ser um método invasivo evitando desencadear uma maior extensão ou inflamação da lesão (KOSKINEN, et al., 2017).

Embora a CK seja amplamente utilizada como biomarcador indireto para perturbação muscular, a literatura científica apresenta controvérsia quanto à precisão de refletir o dano muscular consequente ao nível de intensidade de exercício físico (BAIRD et al., 2012). Isto porque, fatores não modificáveis como a etnia, idade e sexo, também podem afetar a resposta enzimática do tecido subsequente aos níveis de CK. Foi constatado que mulheres embora expressem menores níveis enzimáticos basais, apresentam pico superior ao masculino após a atividade física excêntrica (KOMULAINEN, et al., 1999; MILES et al., 1994). Similarmente o efeito do envelhecimento no músculo esquelético em atletas sugere maior dano induzido pelo exercício e um reparo mais lento e resposta de adaptação (FELL et al., 2008). Deste modo, considerado a extensão do efeito do exercício físico na atividade de CK, limites superiores aos normais sugerem ser redefinidos considerando o impacto de fatores determinantes e independentes da intensidade ou da sobrecarga aplicada, bem como diferentemente da LDH plasmática refletir dano muscular a CK é uma enzima que sobretudo representa ajustes metabólicos.

Após o dano muscular, o sistema imunológico em resposta a inflamação instalada atua no processo de remoção e regeneração do tecido lesionado. Ocorre absorção de proteínas plasmáticas e proliferação de células inflamatórias como neutrófilos e macrófagos que por sua vez irão ser responsáveis pela fagocitose no tecido danificado ou necrosado (PEAKE, et al., 2017). Elevados níveis de neutrófilos (LE MOAL, et al., 2017) e cálcio (HORN, et al., 2017) ativam a liberação de enzimas proteolíticas e de EROs degradando o tecido muscular aumentando a permeabilidade da membrana (BAUMERT, et al., 2016; LE MOAL, et al., 2017).

Em um estudo pioneiro, foi verificado aumento nas concentrações de radicais livres no músculo esquelético de ratos após o exercício exaustivo (DAVIES et al. 1982). Desde então, a relação entre dano muscular, EROs e atividade contrátil foi amplamente investigada tanto em

exercícios aeróbicos quanto em anaeróbicos (POWERS, et al., 2016). Atualmente, é reconhecido que baixos níveis fisiológicos de EROs são importantes para a produção de força (MOULIN et al., 2017), no entanto, quando em condições muito elevadas promovem disfunção contrátil, resultando em dano oxidativo proteico e lipídico, desencadeando fraqueza muscular e fadiga (WAN, et al., 2017).

Estudos anteriores mostraram que a realização do exercício de forma exagerada ou em elevada intensidade estão temporariamente associados ao estresse oxidativo muscular (CAMILETTI-MOIRÓN et al., 2015) verificado, desequilíbrio oxidativo em humanos e animais para marcadores sanguíneos e no tecido do músculo esquelético (DE LUCAS, et al., 2014; RYRSØ, et al., 2018; RADAK et al., 2008). Além disso, foi evidenciado que a oxidação do substrato 1 do receptor de insulina é induzido após um exercício exaustivo. Isto pode comprometer a captação de glicose induzida pela insulina no músculo danificado (GAVIN, et al., 2016).

Por outro lado, embora esteja consolidado dano muscular e oxidativo em decorrência da atividade física crônica e principalmente devido a uma ineficiente recuperação (PEAKE, et al., 2017), muitas questões ainda permanecem inconclusivas. Por exemplo, dependendo da característica do exercício, além de sistêmicos, há também efeitos específicos em regiões do Sistema Nervoso Central? E como reagem?. Se a realização de exercício em extremas durações de tempo, mesmo em indivíduos treinados, o cérebro pode apresentar comprometimentos independentemente dos efeitos adaptativos do exercício?

2.4 Volume de treinamento e estado oxidativo cerebral

2.4.1 Exercício aeróbio moderado e regular

O treinamento aeróbio moderado e regular é benéfico para a saúde corpórea e cerebral. No cérebro, auxilia na prevenção de doenças neurodegenerativas (FLYNN, et. al., 2013), potencializa o tratamento de danos traumáticos cerebrais, induz neurogênese, angiogênese, sinaptogênese (PAILLARD, et al., 2015), reduz a neuroinflamação (RYAN e NOLAN, 2015), melhora a adaptação mitocondrial, a vascularização, a plasticidade (CASUSO, et. al., 2014) e melhora o sistema antioxidante (CHALIMONIUK et al., 2015) e

No combate às EROs, o exercício físico moderado eleva a atividade do óxido nítrico expresso constitutivamente no cérebro (KATUSIC et. al., 2014) e estimula a atividade

enzimática da catalase (CAT), glutadiona peroxidase e superóxido dismutase (SOD) (CHALIMONIUK et al., 2015).

É ostensivo o entendimento de que o exercício aeróbio pode aumentar a resistência ao estresse oxidativo como efeito adaptativo ao treinamento (RADAK et al., 2008). Ensaios ou revisões recentes (SOSA et. al., 2015, DOS SANTOS et. al., 2017, NEVES et. al., 2015) explicitam este fato inferindo que durante o treinamento aeróbio de moderado volume há predomínio de baixas concentrações de EROs, o que favorece a hormesis induzida pelo exercício (CABRERA e VIÑA, 2008; RADAK et al., 2008) ou hormesis mitocondrial (RISTOW et al., 2010; RISTOW e SCHMEISSER, 2014).

De acordo com a hormesis, exercício regular de moderada intensidade e duração exerce uma gama de efeitos benéficos, incluindo a modulação da homeostase redox e o aumento na atividade do sistema imune (RADAK et al., 2008). Outras explicações referem o impacto na biogênese, dinâmica (fissão e fusão) e *turnover* mitocondrial (mitofagia) (POWERS et al., 2016).

Os mecanismos de proteção cerebral antioxidante consequente do treinamento aeróbio moderado e regular parecem estar relacionados às ações das células da glia, havendo proliferação e benéficas alterações na morfologia dos astrócitos (SAUR, 2014; LIDDELL, 2017) e uma ativação benéfica da micróglia (JENSEN e YONG, 2014). O astrócito identificado como principal fonte de Glutationa (GSH) e SOD além de funções tróficas, metabólicas e de suporte estrutural para os neurônios (WILSON, 1997; GETHIN & MCBEAN, 2017) atua ativamente na metabolização do glutamato e ácido gama-aminobutírico os quais também contribuem para a síntese de Adenosina trifosfato necessária ao funcionamento neuronal. (MAGI et al., 2013; LEE e YATSU, 1975).

Há evidência de que o exercício aeróbico de intensidade moderada pode induzir astrogliose em algumas regiões do córtex cerebral (LI et al., 2005) bem como na zona subgranular do giro denteado do hipocampo (UDA et al., 2006). No hipocampo, atividade moderada em esteira por curto período (15 dias), aumentou os níveis da Proteína glial fibrilar ácida (GFAP) (FERREIRA et al., 2011).

Quando o exercício moderado foi realizado por 4 semanas (20 min/dia), um aumento na atividade da enzima glutamina sintetase em astrócitos foi observado como mecanismo reativo benéfico, mesmo na ausência de astrogliose (BERNARDI et. al., 2013). Estudos recentes também indicam o relevante papel dos astrócitos sobre regiões do tronco cerebral envolvidas na modulação do ritmo respiratório, que são fundamentais não apenas para as

demandas fisiológicas como também as atividades dos circuitos motores, favorecendo a capacidade para o exercício físico (SHEIKHBAHAEI et. al., 2018).

O treinamento aeróbio moderado e regular também pode modificar o fenótipo reativo e inflamatório da micróglia (M1) para o fenótipo antinflamatório e benéfico M2, mesmo após insultos neurodegenerativos (LIMA et al.,2014; JENSEN e YONG, 2014). A relação da célula glial com o exercício físico está relacionada também à liberação de citocinas, pois mantém uma importante função imunológica no Sistema Nervoso Central (ANG et al., 2007).

A atividade física aumenta os níveis de miocinas (e.g. IL-6, IL-7, BNDF) gerada pela inflamação muscular após o exercício (NYBO et al., 2002), Estas são liberadas pelas fibras musculares para a corrente sanguínea (PEDERSEN et al., 2004) migrando para vários tecidos incluindo o tecido nervoso central (NYBO et al., 2002; VAN WAGONER et al., 1999) sendo capaz de ativar as células da glia (ANG et al., 2007; MORENO-COLLAZOS & ORTIS, 2018).

Por outro lado, o efeito do exercício físico sobre a defesa antioxidante do cérebro pode variar de acordo com protocolo de treinamento empregado e respostas adversas ao treinamento sob moderado volume e intensidade podem ser observadas. Especialmente quando o exercício físico é realizado com grande ação estressante, tanto pela intensidade, altitude como pelo volume (POWERS et al., 2016).

Neste caso, a ação oxidante das EROS pode atingir a membrana celular induzindo alterações em sua estrutura e permeabilidade. Ocorre perda da seletividade na troca iônica e liberação do conteúdo das organelas, formando produtos citotóxicos como o malonaldeído e outras substâncias reativas ao ácido tiobarbitúrico (TBARS) (FALONE et al., 2012) o que atinge alvos intracelulares, incluindo lipídios, proteínas e Ácido Desoxirribonucleico (POWERS e JACKSON et al., 2008; DANIELS et al., 2012). Tais efeitos podem variar de acordo com os órgãos ou regiões cerebrais avaliadas (ACIKGOZ et. al., 2006; POWERS et al., 2016).

2.4.2 Exercício aeróbio crônico e elevado volume

Embora alguns estudos relatem propriedades antioxidantes após programas anaeróbicos (SOMANI et al., 1995; OGONOVSZKY et al., 2005; QIAO et al., 2006), a maioria dos estudos atuais sugere que independentemente da capacidade executada, a alta intensidade aumenta em geral o estresse oxidativo (CAMILETTI-MOIRÓN et al., 2015). Similarmente ao

exercício anaeróbico, acredita-se que o exercício de grande volume aumente o desequilíbrio redox, independente da baixa intensidade empregada, (CAMILETTI-MOIRÓN et al., 2015).

Regimes crônicos de exercício aeróbio de alto volume podem causar alterações metabólicas consequentes à limitação de energia, decorrente da elevada demanda de oxigênio durante a atividade prolongada e, ausência de períodos de descanso necessários para eficiente recuperação, aumentando a vulnerabilidade aos acometimentos citotóxicos e inflamatórios (RADAK et al., 2008).

Estudos que investigam a resposta oxidante cerebral diante do exercício de elevado volume são relevantes haja vista o expressivo aumento no número de participantes finalistas de corridas de longa distância e extrema resistência (AGNEW et al., 2018). Atualmente há uma crescente quantidade de competições organizadas com volumes superiores a 42 km, definidos como ultra corridas, ultra maratonas ou ultra endurance. Estas provas são comumente realizadas de acordo a quilometragem final, tempo (12, 24 e 48 horas) ou por múltiplos dias (4 a 7 dias correndo 200 km e 300 km em média). Muitas destas provas ainda aumentam o grau de dificuldade pelo relevo e condição climática. Recente ensaio com corredores humanos participantes da desafiante competição “Tor des Géants” com 330 km de distância (24.000 m de desnível positivo), variação de altitude (3000 m) e opostas variações climáticas (elevadas e baixas temperaturas, vento frio e gelo) evidenciaram redução no tamanho dos telômeros do DNA possivelmente consequente ao estresse oxidativo (BORGHINI et al., 2015).

Além disso, é conhecido que demandas do esforço extenuante sobrecarregam cronicamente o organismo de maneira geral. Recentes pesquisas consideram alarmantes variados acometimentos fisiológicos resultantes dos desgastes relacionados à ultra corridas, abrangendo: remodelamento estrutural patológico cardíaco e em grandes artérias (BONSIGNORE et al., 2017; O’KEEFE et al., 2012), acentuado dano muscular e renal (JASTRZĘBSKI et al., 2015), disfunção hepática (RAMA et al., 2015), desgaste cartilaginoso (FRANCIOZI et al., 2013) e redução do volume cerebral (FREUND et al., 2012).

Segundo Freund et al. (2012) competidores avaliados antes, durante e depois da TransEurope Foot Race, considerado uma das maiores e mais extremas competições de ultra corridas percorrendo 3.000 milhas, durante 64 dias consecutivos, diminuíram 6% o volume da massa cinzenta cerebral dos corredores, considerando que um encolhimento "normal" associado à velhice é de apenas 0,2% a cada ano.

Em consequência ao grave “esgotamento fisiológico” após o exercício de extrema duração, foi verificado que mesmo em indivíduos altamente treinados, o corpo pode sofrer

“grave exaustão” colocando em risco a saúde (RADAK et al., 2008). Acredita-se que o Sistema nervoso central pode sofrer acometimentos oxidativos concomitantemente aos diversos sistemas fisiológicos (CAMILETTI-MOIRÓN et. al., 2013) após ultra-corridas.

Ademais, substanciais estudos relataram desequilíbrio redox após provas de longas distâncias no músculo e soro humanos (DE LUCAS, et al., 2014; RYRSØ, et al., 2018; ACIKGOZ et. al., 2006), bem como no tecido cardíaco (KNEZ et al., 2006) e renal (LIU et al., 2000) de animais .

Embora alguns ensaios revisaram especificamente a quantidade de volume e intensidade de treinamento no equilíbrio enzimático cerebral de roedores, limitadas interpretações foram obtidas, relacionadas principalmente a elevada heterogeneidade de modalidades esportivas e protocolos abordados, dificultando a elaboração de conclusões objetivas (CAMILETTI-MOIRÓN et. al., 2013).

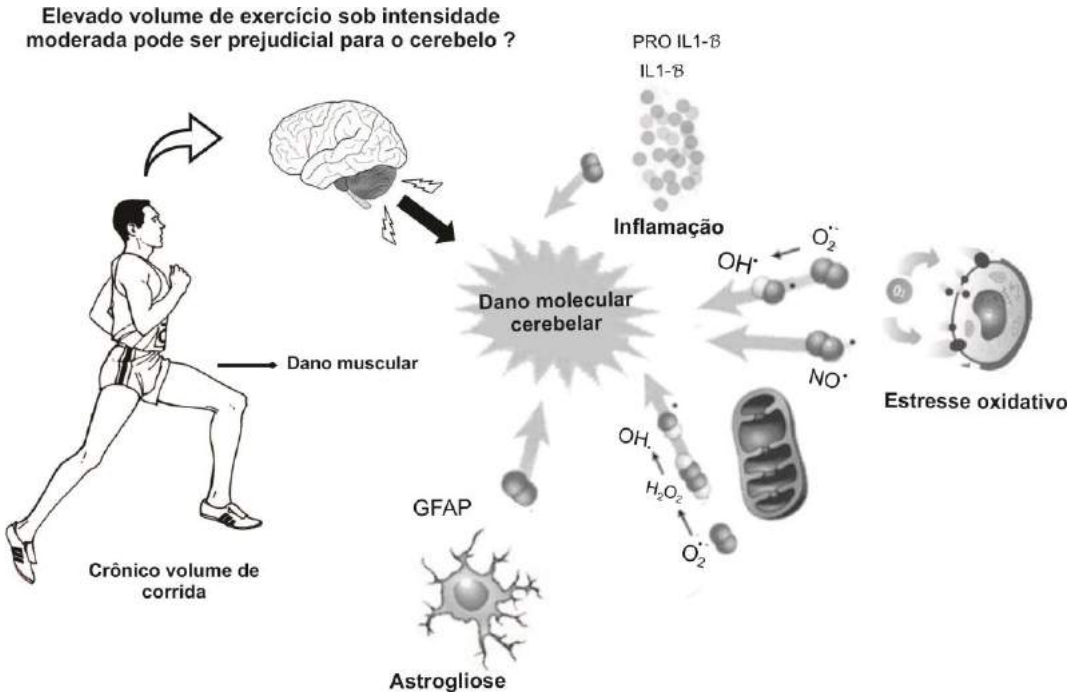
Em uma metanálise recentemente realizada, avaliamos o impacto oxidativo em variadas regiões cerebrais de ratos que realizaram exclusivamente exercício físico moderado de diferentes volumes (DE SOUZA et al., 2018, anexo 01). Neste ultimo estudo, foram excluídos grupos com qualquer utilização de fármacos ou suplementações antioxidantes em roedores durante o exercício. Os dados evidenciaram um aumento na expressão de SOD e CAT cerebral, em períodos de treinamento entre 4 e 8 semanas, no entanto, a grande maioria dos estudos avaliaram principalmente o córtex cerebral (CECHETTI et al., 2008; da CUNHA et. al., 2012; VOLLERT et al., 2011; CHALIMONIUK et. al. 2015; COSKUN et. al., 2005; SOMANI et. al., 1995; NEVES et. al. 2015; AKSU et. al. 2009; AGUIAR et al., 2010; SCHIMIDT et al., 2014; FALONE et. al., 2012; CECHETTI et al. 2012; DEVI e KIRAN, 2004), ou homogenados de tecido cerebral por inteiro (CAKIR et al., 2010, ITOH et al., 1998, MAZZOLA et al., 2011, NAVARRO et al., 2004, LIU et al. 2000, RADAK et. al., 2001)**,** o que representa significante restrição, considerando que o comportamento enzimático antioxidante varia de acordo com a região cerebral, estrutura e função (CAMILETTI-MOIRÓN et. al., 2013; DE SOUZA et. al., 2018).

Tais resultados apontam a falta de conhecimento sobre mecanismos de resiliência tecidual, indicando resultados adversos, com um reduzido número de estudos que de fato avaliem condições similares à ultra corridas em humanos. Regiões relacionadas com a precisão e memória do sistema locomotor como cerebelo, foram muito pouco estudadas.

Assim, considerando que pouco se conhece a respeito dos acometimentos de extremos volumes de corrida no estresse oxidativo cerebelar, alguns questionamentos sobre o comportamento de sua capacidade oxidativa diante de diferentes volumes de treinamento de

corrida e da simulação de ultra corridas; danos fisiológicos sistêmicos, inflamação e reatividade glial foram levantados no presente estudo. A figura 3 ilustra a pergunta norteadora e os principais alvos celulares e moleculares analisados, partindo da hipótese de que os mesmos podem estar envolvidos em efeitos deletérios induzidos pelo exercício de elevado volume sobre a função cerebelar.

Figura 3 – Pergunta norteadora ilustrada



Legenda: IL-1B: Interleucina 1- Beta; GFAP: Proteína glial fibrilar ácida; $O2^{\bullet -}$ - Aníon Superóxido; OH – Radical hidroxila; H_2O_2 – Peróxido de Hidrogênio; ON - Óxido nítrico,

2.2 HIPÓTESE

Hipotetizamos que o cerebelo apresente um desequilíbrio antioxidante endógeno após elevados volumes de treinamento, independente da simulação da corrida de ultra endurance.

2.3 OBJETIVO

Avaliar as repercussões do treinamento físico de moderada intensidade com moderado e elevado volume per se ou associado a uma simulação experimental de ultra-endurance sobre o estado redox cerebelar, performance e dano muscular em ratos *Wistar*

2.3.1 Objetivos específicos

- Avaliar a capacidade antioxidante enzimática e não enzimática cerebelar
- Comparar a concentração de marcadores bioquímicos séricos de dano muscular e níveis de corticosteroides.
- Analisar os níveis proteicos de IL1-β e GFAP como marcadores respectivos de inflamação e reatividade glial cerebelar.
- Avaliar a performance de corrida em animais que realizaram treinamento aeróbico sob moderado e elevado volume, submetidos ou não ao teste de exaustão.

3 MATERIAL E MÉTODOS

3.1 DESENHO EXPERIMENTAL

Quarenta e cinco ratos machos *Wistar* adultos (60 dias; 225,84±24,33g) foram obtidos da unidade de produção animal da Universidade Federal da Paraíba, João Pessoa, PB, Brasil. Os animais foram mantidos no ciclo invertido, ambiente climatizado com temperatura constante (22◦C ± 2◦C) e tiveram livre acesso a alimento e água.

Após o teste de treinabilidade os animais foram randomizados em seis grupos: controle (C: n=8; colocados na esteira desligada e não realizaram treinamento de corrida (TC), controle + teste de exaustão (TE) (C-TE: n=7; colocados na esteira, não realizaram TC, mas realizaram TE), moderado volume de treinamento (MV: n=8, realizaram TC sob moderado volume), moderado volume de treinamento + TE (MV-TE: n=7, realizaram TC sob moderado volume, realizaram TE), elevado volume de treinamento (EV: n=7, realizaram TC sob elevado volume), elevado volume de treinamento + TE (EV-TE: n=8, realizaram TC sob elevado volume, realizaram TE).

3.1.2 Aspectos éticos

Os cuidados com os animais seguiram as diretrizes para o Cuidado e Uso de Animais de Laboratório previamente aprovado pelo Comitê de Ética para Uso de Animais (CEUA) da Universidade Federal de Pernambuco, Brasil (nº 0035-2017).

3.1.3 Local de pesquisa

A aplicação do treinamento de corrida foi realizada no Laboratório de Neuroplasticidade Muscula (sala de treinamento físico) anexo ao Departamento de Anatomia Universidade Federal de Pernambuco (UFPE). As análises bioquímicas foram realizadas no laboratório de Neurofisiologia do Departamento de Fisiologia da UFPE.

3.2 PROTOCOLO DE TREINAMENTO FÍSICO

Para o treinamento de corrida foi utilizado uma esteira motorizada para múltiplos roedores (AVS produtos®) com pistas individuais com choque na porção traseira (2,0 mA). Uma semana anterior ao início do treinamento foi realizado a adaptação dos animais na

velocidade de 5m/min, durante 10min, 5 x por semana. Posteriormente os animais foram submetidos a um teste de velocidade máxima (Vmáx) para determinação do limiar de treinamento. O Vmáx consistiu por uma corrida com limiar graduado e incrementos de velocidade (5 m / min a cada 3 min), iniciando em 5 m / min até a intensidade máxima alcançada por cada animal (SENNA et al., 2011).

O teste de treinabilidade para esteira rolante foi avaliado por uma escala de 1 a 5 seguindo os seguintes critérios: (1) se recusar a correr, (2) corredor abaixo da média (esporadicamente corre, para constantemente de correr), (3) corredor médio (mantem uma corrida constante, porém cai ou para de correr esporadicamente), (4) corredor acima da média (corredor consistente, ocasionalmente cai para trás na esteira) e (5) bom corredor (DISHMAN et al., 1988). Foram randomizados nos grupos para treinamento físico os animais classificados no escore 3 ou superior.

O protocolo de treinamento realizado foi do tipo contínuo, com duração de 12 semanas e sessões diárias de exercício 3 a 5 vezes/semana para os grupos MV e MV-TE e grupos EV e EV-TE (ver tabela 1), com intensidade de 50-70% da intensidade máxima alcançada nos testes de Vmáx). O tempo de treinamento dos grupos de EV e EV-TE foram aumentados gradualmente de 10 a 90 min/dia durante um período de 12 semanas (TARINI et al., 2013) o tempo de treinamento dos grupos MV e MV-TE foram aumentados gradualmente de 10 a 30 min/dia durante um período de 12 semanas (MAROSI et al., 2012). Os grupos C e C-TE não realizarão qualquer treinamento de corrida. Cada sessão de treinamento foi precedida de 5 minutos de aquecimento a 30% do Vmáx e ao término da corrida 5 minutos de recuperação a 30% do Vmáx (SENNA et al., 2011). Estes tempos foram inclusos no período total do protocolo.

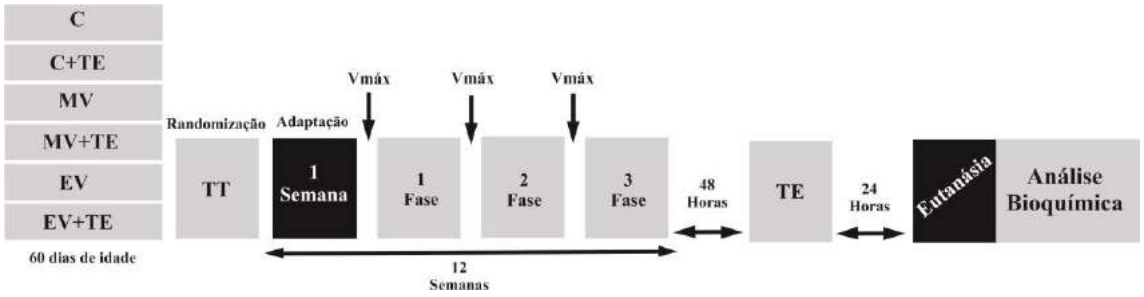
Tabela 1- Protocolo de treinamento físico

Fase do protocolo	**Semana**	**Limiar de treinamento (% - m/min)**	**Tempo (min)**		**Frequência de sessões semanais**	**Recuperação treinamento (horas)**
			MV	**EV**		
Adaptação	1°	5 m/min	10min	10min	3x	24
	2°	50 %	10min	10min	3x	24
1° fase	3°	50 %	15min	20min	3x	24
	4°	50 %	20min	30min	3x	24
	5°	50 %	25min	40min	5x	24
	6°	50 %	30min	50min	5x	24
2° fase	7°	60 %	30min	60min	5x	24
	8°	60 %	30min	70min	5x	24
	9°	60 %	30min	80min	5x	24
	10°	70 %	30min	90min	5x	24
3° fase	11°	70 %	30min	90min	5x	24
	12°	70 %	30min	90min	5x	48
Teste de Exaustão						

MV: Moderado volume de treinamento; EV: Elevado volume de treinamento.

Vinte e quatro horas após o término do período de treinamento, os grupos C-TE, MV-TE e EV-TE foram submetidos ao teste de exaustão. Este teste foi realizado até a máxima distância de corrida realizada em intensidades moderada (50% da velocidade máxima) quando os animais foram considerados esgotados e se recusaram a correr (encostar 10 vezes na caixa de corrida durante 1 minuto) (TARINI et al., 2013) e então seguidos para eutanásia 24 horas ao término do TE. Anteriormente a eutanásia todos os animais foram pesados, anestesiados com isoflurano 100%, seguidos de coleta de sangue diretamente do coração (átrio esquerdo) e decapitados para retirada imediata do cerebelo.

Figura 4 – Designer experimental



C: Grupo controle; C-TE: Grupo controle – teste de exaustão; MV: Moderado Volume; MV-ET: Moderado volume- teste de exaustão; EV: Elevado volume; EV-TE: Elevado volume – teste de exaustão. Vmáx: Teste de velocidade máxima; TE: Teste de exaustão; TT: Teste de treinabilidade.

3.3 ANÁLISE DO ESTRESSE OXIDATIVO

3.3.1 Peróxidação Lipídica

A lipoperoxidação (LP) foi medida pela estimativa dos níveis de malondialdeído (MDA) utilizando um ácido tiobarbitúrico (TBA) para reação (método TBARS) (CARDOSO et al., 2014). A reação foi desenvolvida por adição seqüencial de 80 µL de dodecil sódico a 8,1% sulfato, 600 µL de ácido acético a 20%, pH 3,5 e 600 µL de soluções a 0,8% de TBA fervidas em água durante 60 min pra 100 µL de sobrenadante do cerebelo. Depois da água destilada arrefecimento, 600 µL de n-butanol foram adicionados à amostra, centrifugado a 2500g por 10 min, e a fase orgânica foi lida a 532 nm usando um leitor de placas (Thermo Scientific, varioskan flash espectral digitalização multimodo leitor).

3.3.2 Níveis de óxido nítrico no cerebelo

Níveis de óxido nítrico no cerebelo foram estimados usando o reagente de Griess como indicador da produção de óxido nítrico (NO) (NIJMEH et al., 2018). Volumes iguais (100 μL) de sobrenadante e reagente de Griess foi colocado em placas de 96 poços e reagiu durante 10 min à temperatura ambiente (~ 22 ° C). A absorbância do composto de diazônio foi medido em um comprimento de onda de540 nm. Os resultados foram expressos como nmol por mg de proteína com referência a uma curva padrão construída com concentrações conhecidas de nitrito de sódio.

3.3.3 Atividade da Superóxido dismutase total

Avaliação da atividade da enzima (superóxido-dismutase total) (t-SOD) foi realizada de acordo com (GREEN et al., 1982) a 25° C. Triplicada de sobrenadantes de cerebelo (60 μL) foram previamente incubado em banho-maria a 37 ° C e depois adicionado 920 μL de tampão de carbonato de sódio a 0,05% pH 10,2 EDTA 0,1 mM. A reação foi desenvolvida adicionando 20 μl de epinefrina 150 mM em ácido acético a 0,05%. Alterações de absorvância por 15 s durante um total de 2 min, a 480 nm, foram medidos. Uma unidade de t-SOD foi definida como a enzima quantidade que causa 50% de inibição da oxidação da epinefrina. A atividade enzimática do tecido t-SOD foi expressa em unidades por miligrama de proteína (U / mg de proteína).

3.3.4 Atividade da Catalase

A atividade enzimática da catalase (CAT) foi medida de acordo (MISRA e FRIDOVICH, 1972). Triplicatas de sobrenadantes de cerebelo (60 μL) foram adicionados a 905 μL de tampão fosfato de sódio pH 7,0. A reação foi desenvolvida pela adição de 35 μl de peróxido de hidrogênio 300 mM em tampão fosfato de sódio. A constante de taxa k decomposição de H2O2 sob nossas condições experimentais de temperatura (22 ° C) e pH (7,0) foi determinada como sendo 2.3 para medir as mudanças de absorbância por 10 s, por 1,5 min a 240 nm. A atividade enzimática também foi expressa como unidades por miligrama de proteína (U / mg de proteína).

3.3.5 Níveis de Glutationa reduzida (GSH)

Os níveis de glutationa (GSH) foram analisados de acordo com (AEBI, 1984); 450 µL de tampão fosfato 100 mM com EDTA (5 mM) (pH 8,0) foram adicionados a 50 µL do sobrenadante; 50 µL dessa mistura mais 140 µL de tampão fosfato 100 mM mais 10 µL de soluções de o-phthaldialdehyde (OPT) foram colocadas em placas incubadas por 20 min em temperatura ambiente, protegido da luz. A absorbância foi registrada em um espectrofluorímetro usando um comprimento de onda de 350 nm. Os resultados foram expresso em µmol por mg de proteína com referência a um padrão curva construída com concentrações conhecidas de GSH.

3.3.6 Níveis de Glutationa oxidada (GSSG)

Os níveis de GSH foram analisados de acordo com (AEBI, 1984). 50 µL do sobrenadante foram incubados à temperatura ambiente com 20 µL de N-etilmaleimida (NEM) 0,04M durante 30 minutos a interagir com GSH presente no tecido. Para esta mistura, 430 mL de hidróxido de sódio (NaOH) 0,1 M foi adicionado; 50 µL dessa mistura mais 140 µL de NaOH 0,1 M e 10 mL OPT foram colocados nos poços de uma placa de 96 poços. Esta mistura foi incubada por 15 min em temperatura ambiente e protegido da luz. A leitura foi feita em espectrofluorímetro usando um comprimento de onda de 350 nm para emissão. Os resultados foram expressos em µmol por mg de proteína com referência a um curva padrão construída com glutationa oxidada conhecida (GSSG) concentrações.

3.3.7 Marcadores de dano muscular e sistêmico no soro

Foi quantificado a concentração de creatina quinase (CK) e lactato desidrogenase (LDH) para a análise do dano muscular a partir do soro para cada grupo experimental. Para tanto foi utilizado kit comercial específico (Lab test, Belo Horizonte - Minas Gerais, Brasil)

Foi coletado 4 ml de sangue após a eutanásia mediante punção cardíaca. utilizando-se agulhas para coleta de sangue múltipla alojadas em tubos de coleta (*InjeXvácuo*® com ativador de coágulo); Após a coleta do material biológico os tubos de soro gel foram transportados para o laboratório em caixa térmica com gelo, para centrifugação durante 10 minutos a 800 g a 4 ºC em centrifuga calibrada. Em seguida as amostras do soro foram separadas para análise.

Para coletar o lactato sangüíneo foram utilizados lancetas descartáveis e perfurado a cauda do animal. Foi coletado aproximadamente 25µl de amostra sangue colocada diretamente nas tiras de teste (BM-Lactato) e analisado imediatamente ao término do teste de exaustão por lactímetro Accutrend Lactate Accu-Check (Roche, Brasil).

3.3.8 Níveis de Corticosterona no soro

Para ensaio de corticosterona 50ul de soro sanguíneo foi colocado em eppendorf para descansar sem perturbações durante 30-60 min objetivando coagular a geração. O coágulo foi removido por centrifugação a 2000 rcf durante 15 minutos, em 24 ° C (Centrifugadora de Bancada Frigorífica de Alta Velocidade, 4x100 ml). As análises foram feitas em duplicata por amostra, com uma diluição de 1: 1000. Todo o procedimento foi realizado conforme recomendado pelo kit Corticosterone ELISA (Cayman Chemical, No 501320).

3.3.9 Expressão proteica de GFAP e IL-1β

Níveis de proteína de proteína ácida fibrilar glial (GFAP) e interleucinas 1β de tecido cerebelar foram analisados por Western blotting. O tecido do cerebelo foi homogeneizado em um tampão de lisie contendo ácido etilenodiamino tetracético 10 mM (EDTA), fluoreto de fenilmetilsulfonilo 2 mM (PMSF), fluoreto de sódio 100 mM, pirofosfato de sódio 10 mM, 10 mM ortovanadato ($NaVO_4$), Tris (hidroximetil) aminometano 100 mM, pH 7,4 e 1% de um coquetel de inibidores de protease (AEBSF – [4-(2-Aminoetil)benzonesulfonil fluoreto Hidrocloreto; Aprotinina, Bestatina hidrocloreto, E-64 – [N-(trans-Epoxisucinil)-L-leucina) 4-guanidinobutilamida, hemisulfato de leupeptina pepstatina A); (Sigma-Aldrich, EUA). Os homogenados foram centrifugados a 1000 g a 4 ˚C por 10 min e o sobrenadante foi coletado e armazenado a -80 ˚C até o uso do imunoblot de GFAP. Amostras de homogenatos cerebelares foram diluídos em tampão de amostra e fervidos por 5 min. Quarenta microgramas de proteína por poço foram separados eletroforeticamente em Gel de poliacrilamida-dodecil sulfato de sódio 15% contendo 10% de SDS, 30% solução de acrilamida / bis-acrilamida, 1.5 M TRIS-HCl (pH 8.8), e 10% APS, TEMED a 100V, 0,15A e 300 W. Depois da separação, as proteínas foram transferidas para a membrana de nitrocelulose (Malne Manufacturing, USA, 0.22 micron) durante 1 h e 30min a 250V, 0,35A e 300W. As membranas foram bloqueadas por 1h em solução de Tris-Tween 20 (TBS-T) contendo 5% de BSA. Em seguida, foram incubadas com anticorpo primário policlonal coelho anti- GFAP (Dako Germany, 1: 1000) ou policlonal coelho anti-IL-1β (Sino Biological Inc, Beijing, China, 1:2000) diluído

em TBS-T *overnight* a 4 ° C. Após lavagens (3 vezes, 10min cada) em TBS-T, a membrana foi tratada com anticorpo secundário anti-coelho (Jackson ImmunoResearch, USA; 1: 50.000) diluído em TBS-T durante 1 hora. Posteriormente, as membranas foram lavadas em TBS-T e corado com reagente Luminata (Millipore, USA) via quimioluminescência usando um sistema de imagem ChemiDoc (Bio-Rad, USA). Posteriormente, anticorpos anti-beta-actina monoclonal (Santa Cruz Biotechnology,CA 1: 10.000) e anticorpo secundário anti-mouse (Jackson ImmunoResearch, USA; 1: 50.000) foram utilizados. As figuras digitalizadas das bandas foram analisadas pelo programa Image Lab 6.0.1 (Bio-Rad,USA). Os níveis de GFAP foi normalizada aos valores quantitativos da proteína beta-actina.

3.3.10 Performance de corrida dos animais

Para análise da performance de corrida, foi avaliado o tempo de corrida (min), distância (m) e velocidade (m/min) durante os testes de exaustão.

3.4 ESTATÍSTICA

O teste Shapiro-Wilk foi utilizado para verificar a normalidade dos dados. As comparações entre os grupos foi feita pela análise de variância ANOVA (*two way*), com fatores interagindo entre os grupos (tipo de treinamento vs. teste de exaustão). Quando apropriado, combinações *post-hoc* foram realizadas usando o teste de comparação múltipla de *Tukey* ou LSD. Os resultados não paramétricos foram comparados por meio do teste Friedman. Para encontrar o tamanho do efeito, o teste *d de Cohen* (Nakagawa and Cuthill, 2007) foi aplicado adotando os pontos de 0,02–0,15 para pequeno efeito, 0,16 a 0,35 médio efeito e, maior que 0,35 como grande efeito. O programa SPSS (versão 20.0) foi utilizado, considerado valor de significância $p \leq 0,05$.

4 RESULTADOS

4.1 ARTIGO: ULTRA-ENDURANCE ASSOCIATED WITH MODERATE EXERCISE IN RATS INDUCES CEREBELLAR OXIDATIVE STRESS AND IMPAIRS REACTIVE GFAP ISOFORM PROFILE

Artigo submetido na revista Frontiers in Molecular Neuroscience, fator de impacto 3.72, Qualis Capes: A1 (ANEXO 4).

Título: Ultra-endurance associated with moderate exercise in rats induces cerebellar oxidative stress and impairs reactive gfap isoform profile

5 CONSIDERAÇÕES FINAIS

Em conclusão, os resultados corroboram com a hipótese inicial, indicando que a resiliência cerebelar de ratos a danos oxidativos é mantida durante três meses de volume moderado de treinamento, mas um alto volume de treinamento em intensidade moderada prejudica o sistema de defesa antioxidante enzimática dessa região cerebral. Além disso, demonstramos pela primeira vez que as corridas de UE após alto volume de treinamento são capazes de alterar o perfil da isoforma GFAP relacionado ao status da reatividade dos astrócitos. Os resultados também indicam que o volume moderado de treinamento, sob condição aeróbia por 3 meses, não confere resistência à simulação da UE em ratos, seja para marcadores sistêmicos ou para o status oxidativo e inflamatório do cerebelo. Ao todo, os dados destacam a importância de mais estudos em outras regiões do cérebro, especialmente considerando o aumento do número de participantes em ultramaratonas atualmente.

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APÊNDICE A – ARTIGO DE REVISÃO SOBRE O TEMA DIRETAMENTE RELACIONADO À TESE

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Review article

Endurance training on rodent brain antioxidant capacity: A meta-analysis

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ABSTRACT

The influence of physical exercise on brain antioxidant defense mechanisms has been studied. Nevertheless, the effect of training volume on the brain's redox balance remains unclear. In this meta-analysis, we compared the effect of training volume on antioxidant enzymatic resource and lipid peroxidation on various brain regions. The activities of the enzymes glutathione peroxidase (GPx), superoxide dismutase (SOD), catalase (CAT) and the levels of thiobarbituric acid reactive substances (TBARS) were also evaluated. The effects of training periods (weeks) and exercise duration were compared. Meta-analysis revealed that protocols over 8 weeks were associated with an increase in SOD ($p = 0.0008$) and CAT activities ($p = 0.0001$). Exercise durations for 30 and 60 min were associated with higher CAT activity ($p = 0.04$). Joint analysis revealed that moderate physical exercise over 4 and 8 weeks promoted a healthy enzymatic balance. However, high volumes of exercise over 8 weeks were associated with the increased antioxidant enzymatic activity, indicating higher reactive oxygen species (ROS) levels. The data also indicated that there is still limited research and inaccurate information, on the safety conditions of training periods that simulate tests of ultra resistance in humans.

Contents

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1. Introduction

During exercise, cerebral blood flow increases by 40%–70% to meet the metabolic demand for O_2 (Chalimoniuk et al., 2015). The increase in O_2 consumption results in a higher production of a variety of reactive oxygen species (ROS), such as superoxide radical ($O_2^{\bullet-}$) and hydrogen peroxide (H_2O_2). These ROS react with various intracellular targets, including lipids, proteins, and DNA (Phaniendra et al., 2015). ROS is naturally produced in aerobic cellular metabolic processes. However, increased levels of ROS are cytotoxic and may result in oxidative brain damage (Shichiri, 2014).

The central nervous system (CNS) is highly susceptible to oxidative stress (OS) because it relies mostly on O_2-dependent mitochondrial energy, which is associated with a high concentration of free iron and polyunsaturated lipids, as well lower levels of antioxidant enzymes when compared to other peripheral tissues (Chalimoniuk et al., 2015; Ter-Minassian, 2006). Brain OS is an etiopathological mechanism associated with mutations (Uttara et al.,2009), apoptosis, and neurodegeneration (Flynn and Melovn, 2013).

The influence of physical exercise on brain antioxidant defense mechanisms has also been discussed in the light of various brain regions (Acikgoz et al., 2006) and the training protocol used: the type of exercise, exercise mode, and intensity (Daniels et al., 2012). Although regular exercise is beneficial to the body, exhaustive exercise increases ROS production in the skeletal muscle (Acikgoz et al., 2006) and in the myocardium (Knez et al., 2006). Similarly, high-intensity physical exercise is thought to be a potentiating agent of OS (Camiletti-Moirón et al., 2015), while moderate aerobic training protects the brain from the oxidant action (Chalimoniuk et al., 2015), stimulating neurogenesis and the production of trophic factors (Acikgoz et al., 2006). However, the effect of training volume on the enzymatic equilibrium in the rodent brain remains unclear (Camiletti-Moirón et al., 2013).

The potentially detrimental effects of high and ultra-high volumes of physical exercise are a hot topic in the current scientific debate (Jastrzębski et al., 2015; Knez et al., 2006; Rama et al., 2015). In this systematic review, we conducted a meta-analysis to evaluate the effects of exercise volume on antioxidant enzymes and lipid peroxidation in the brain of experimental animals, considering the importance of this theme for developing healthy strategies in humans (Daskalopoulou et al., 2017).

2. Materials and methods

2.1. Initial query

A search was carried out on articles published between 1995 and January 2018 using PubMed, Web of Science, MEDLINE, Scopus, SciSearch, and DataSearch databases. English-language articles and search results were included in the EndNoteTM online bibliographic management software. Queries were made using the following terms: "oxidative stress," "exercise," "rats," "central nervous system," "strenuous physical activity," and "antioxidants."

2.2. Risk of bias

To assess the risk of bias in the selected studies (Fig. 1), the Cochrane Handbook (Higgins et al., 2011) (Table 1) and the Review Manager (RevMan5.3) program, developed for systematic reviews and available for free download (http://ims.cochrane.org/revman/download) were used.

2.3. Inclusion and exclusion criteria

Studies were included in this review if they satisfied the following criteria: (I) the intervention contained a long-term physical exercise protocol for rodents; (II) OS in at least one cerebral region was evaluated; (III) the animal species studied was clearly indicated; (IV) the endpoints of interest were defined [the type of OS marker: glutathione (GSH), oxidized glutathione (GSSG), glutathione peroxidase (GPx), glutathione reductase (GR), superoxide dismutase (SOD), catalase (CAT) and thiobarbituric acid reactive substances (TBARS); (V) a control group and at least one group that performed physical exercise without drug administration or any other intervention was included.

Articles were included if all of the above-mentioned criteria were met and they were excluded if: (I) they had been published before 1995; (II) the full-text article could not be found; (III) they were not written in English; (IV) they were not performed with rodents; (V) drugs were administered to all experimental groups during physical activity.

2.4. Eligibility criteria

Studies were eligible for inclusion if OS was compared between experimental groups that performed physical exercise and a control group using *in vivo* experiments. Studies using rats and mice of both sexes and treadmill or swimming protocols were eligible. Eligible training protocols had a training duration superior to 20 min independent of exercise intensity: light, moderate or intense loads; sub-maximum or maximum; with or without electrical stimulation; with or without anaerobic threshold identification; and included both interval and continuous training protocols.

The primary endpoints were the evaluation of antioxidant enzyme behavior and lipid peroxidation. The secondary outcomes were the training period (weeks) and training session duration. All the titles, abstracts, and full texts were independently analyzed by two investigators to determine the eligibility of the study for inclusion in the review. After the exclusion of duplicates, the studies were selected by title and abstract, and the type of study, the type of animal and the type of protocol were observed. Then, the full text of the remaining studies was evaluated.

The Review Manager statistical software (version 5.3) was used to analyze the primary and secondary endpoint data. The results were expressed as the standard mean difference (MD) of the 95% confidence intervals (CI) presented by the Forest plot graph. For the analysis, 2 groups were used: 1 control group (sedentary rodents) and 1 experimental group (rodents that performed physical exercise). The studies were combined for trials with parameters (training period and exercise time). For studies with more than one intervention group, we considered only the control and the physical exercise group without drug administration or other interventions. We used Cochran's (Ch^2) and tau-square test (tau^2) to assess heterogeneity. The I^2 statistic was used to assess inconsistency (the percentage of the total variation of heterogeneity) of the effects of exercise (Higgins et al., 2003). We assessed visually and objectively the propagation of the risk of bias the symmetry graphically using

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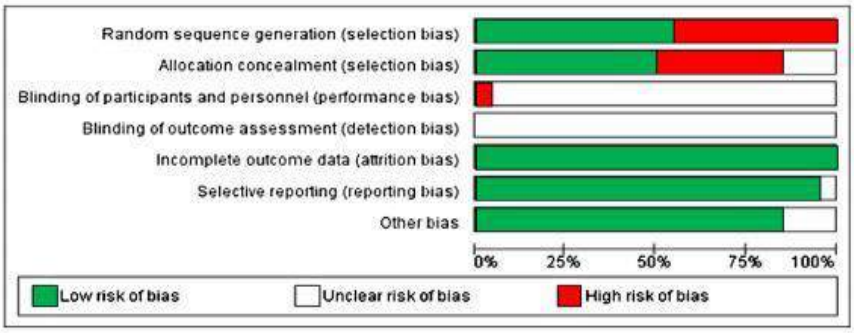

Fig. 1. Bias risk chart.

Table 1
Cochrane Collaboration's tool for assessing risk of bias.

Bias domain	Source of bias	Support judgment
Selection bias	Random sequence generation	The method used to generate the allocation sequence had sufficient detail to allow an evaluation and produce comparable groups
	Allocation concealment	The method used to conceal the allocation sequence, or detailing the intervention allocations could have been previously predicted
Bias performance	Blindness of participants	There was blind trial for participants and researchers
Bias detection	Result evaluation blindness	The measures used for the evaluation of results were blind.
Frequency of friction	Incomplete results data	The conclusion of the results presented exclusions of analyzes or any other friction
Report Bias	Selective reports	How the selective results report was examined and what was found
Another type of bias	Anything else, ideally pre-specified	Important concerns about bias not covered in the other domains in the tool

funnel plots. The threshold for statistical significance was set to $p < 0.05$.

3. Results

3.1. Selection results

In the initial search, 4152 articles were found and 723 duplicate articles were excluded. Of the 3429 articles examined for eligibility, 3159 were excluded based on the title or abstract. The full texts of 270 potentially eligible studies were evaluated. Of these, 27 met the eligibility criteria and were included in the review (Fig. 2).

3.2. Type of training

We found that physical activity on a treadmill (22/27) was more common than swimming (5/27); continuous (24/27) was more common than interval training (2/27), and moderate (24/27) was more common than high-intensity training (5/27). The studies of Somani et al., (1995); Rybak et al., (1995) and Liu et al., (2000) evaluated the effects of both high and moderate intensity training.

3.3. Training period

The most commonly used training period was 8 weeks (Aksu et al., 2009; Cakir et al., 2010; Dabidi et al., 2013; Falone et al., 2012; Liu et al., 2000; Neves et al., 2015; Schimidt et al., 2014). Longer periods were used in studies by Navarro et al., (2004) (50 weeks); Marosi et al., (2012) (15 weeks); Falone et al., 2012 (16 weeks); Camiletti-Moirón et al., 2015; Cechetti et al., 2012; and Devi and Kiran, 2004 (12 weeks) and Teixeira et al., 2009 (11 weeks). Only one study used a shorter period of 2 weeks (Cechetti et al., 2008).

3.4. Weekly frequency and training time

The most commonly used weekly training frequency was 5 training sessions per week (Aksu et al., 2009; Cakir et al., 2010; Chalimoniuk et al., 2015; Coşkun et al., 2005; Dabidi et al., 2013; Devi and Kiran, 2004; Falone et al., 2012; Liu et al., 2000; Marosi et al., 2012; Neves et al., 2015; Radak et al., 2001; Rybak et al., 1995; Schimidt et al., 2014; Sosa et al., 2015; Teixeira et al., 2009; Tuon et al., 2010; Vaghef and Bafandeh Gharamaleki, 2017; Vollert et al., 2011). While the most common training session duration was 60 min (Aksu et al., 2009; Cakir et al., 2010; Chalimoniuk et al., 2015; Rybak et al., 1995; Teixeira et al., 2009; Vaghef and Bafandeh Gharamaleki, 2017; Vollert et al., 2011), the longest training session duration was 120 min (Liu et al., 2000).

3.5. Evaluation of lipid peroxidation and antioxidant activity

Table 2 summarizes the results obtained from the 27 studies evaluating the levels of lipid peroxidation and the redox state of the whole brain or specific brain regions [cerebral cortex (Cx), hippocampus (Hp), caudate and putamen (striatum) (CPu), brainstem (BS); cerebellum (Cb), amygdala (Am), total brain (TB)]. In 62% of these studies (n = 17), moderate physical exercise did not induce an increase in lipid peroxidation in the different brain regions. However, in a subset of studies (15%), increased levels of TBARS were observed. This increase was, in some cases, positively associated with an increase in antioxidant activity (Camiletti-Moirón et al., 2015; Chalimoniuk et al., 2015; Teixeira et al., 2009).

In one study (Liu et al., 2000), a reduction of lipid peroxidation levels and an unaffected redox balance (GSH and GSSG concentration) were observed after a training period of 8 weeks. However, in this study, the enzymatic antioxidant activity was not analyzed. Falone et al., (2012) observed a reduction in TBARS levels and a concomitant reduction in GPx, SOD, and CAT activities after a training period of 8 weeks. However, after 16 weeks of training, the same authors reported increased lipid peroxidation levels and an increased activity of these enzymes. Aguiar et al., (2010) also reported a reduction in GPx activity after 8 weeks of training using an intensive exercise protocol. In 40% (n = 11) of the studies evaluated, no significant difference was observed between pre

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Table 2
Oxidative stress outcomes studied. Sp, species; R/NR, randomized/no randomized; Ex, exercise; GSH, glutathione; GSSG, oxidized glutathione; GPx, glutathione peroxidase; GR, reductase glutathione; SOD, superoxide dismutase; CAT, catalase; TBARS,Thiobarbituric acid reactive substances; LB, local of the brain measured; SD, Sprague–Dawley rat; WS, Wistar; Cx, cerebral cortex; CPu, caudate and putamen (striatum); BS, brainstem; Hp, hippocampus; Cb, cerebellum; Am, amygdala; TB, total brain; ND, no difference between the groups.

References	Sp	R/NR	Ex	Period	Modality	Frequency\ time	GSH	GSSG	GPx	GR	SOD	CAT	TBARS	LB
Mazzolaet al., 2011	WS	NR	Mod	2wk	running	7d.wk 20min	-	-	ND	-	ND	ND	ND	TB
Cechettiet al., 2008	WS	R	Mod	2wk	running	20min diários	-	-	-		-	-	ND	Hp,Cx, Cb,CPu
Itoh et al., 1998	WS	NR	Mod	3 wk	running	1d.wk 30min	ND	Down						TB
Hrncic et al., 2013	WS	R	Mod	4wk	running	1d.wk 30min	-	-	-	-	ND	ND	-	Hp
Da Cunha et al., 2012	WS	NR	Mod	4wk	running	3d.wk 20 min	-	-	ND	-	ND	ND	ND	Cx
Vollert et al., 2011	WS	R	Mod	4 wk	running	5d.wk 60min	-	-	-	-	-	-	ND	Cx,Hp, Am
Chalimoniuk et al., 2015	WS	R	Mod	6wk	running	5d.wk 60min	Up(Cx,Cb,CPu)	-	ND	Up (Cx)	Up (Cb, Cx)	Up (Cb, Cx)	Up (Cb,CPu|)	Cx,Cb, CPu,Hp,BS
Coskun et al., 2005	WS	R	Mod	6.5wk	running	5d.wk 30 min	ND	-	-	-	-	-	ND	Cx
Somani et al., 1995	SD	R	Int and Mod	7.5 wk	running	5d.wk60min	Up (Cx,CPu) Down (Cb)	Up (Cx)	-	-	Up (BS,CPu)	-	-	Cx, Hp, BS, CPu
Dabidi et al., 2013	WS	R	Mod	8 wk	running	5d.wk 64min	-	-	-	-	-	-	ND	Hp
Neves et al., 2015	WS	NR	Mod	8wk	running	5d.wk 50min	ND	-	-	-	-	-	ND	Hp,Cx
dos Santos et al., 2017	WS	R	Mod	8wk	running	3d.wk 20min	-	-	-	-	ND	ND	ND	Hp
Tuon et.al., 2010	WS	NR	Mod	8wk	running	5d.wk	-	-	-	-	ND	-	ND	Hp
Sosaet al., 2015	WS	R	Mod	8wk	swimming	5d.wk 30 min	ND	-	-	-	ND	ND	ND	CPu
Aksu et al., 2009	SD	R	Mod	8wk	running	5d.wk 60 min	-	-	Up (Cx)	-	Down (Hp)	-	ND	Cx,CPu, Hp
Aguiar et al., 2010	SM	R	Int	8wk	running	2x20min 10min intervalado	-	-	Down(Hp)	-	-	-	-	Hp, Cx, CPu
Liu et al., 2000	SD	NR	Mod	8 wk	running	5d.wk 120min	ND	ND	-	-	-	-	Down	TB
Schimidt et al., 2014	WS	R	Mod	8 wk	running	5d.wk 30min	ND	-	ND	-	-	Down (Hp)	ND	Cx,Hp
Cakır et al., 2010	SD	R	Mod	8 wk	swimming	5d.wk60min	Up	-	-	-	-	-	ND	TB
Falone et al., 2012	WS	NR	Mod	8wk	running	5d.wk 20min	-	Down	Down	-	Down	Down	Up	Cx
-	-	-	-	16wk	running	5d.wk 20min	-	Down	Up	-	Up	Up	Down	Cx
Radak et al., 2001	WS	R	Mod	9wk	swimming	5d.wk 90min	-				-	-	ND	TB
Teixeira et al., 2009	WS	R	Int	11wk	swimming	5d.wk 60min	-	-	-	-	-	ND	Up	CPu
Camiletti-Moirónet al., 2015	WS	R	Int	12 wk	running	3d.wk 10x 2min	-	-	ND	-	Up	Up	Up	TB
Cechetti et al., 2012	WS	R	Mod	12 wk	running	3 d.wk 20min	-	-	-	-	ND	-	ND	Hp,Cx, CPu
Devi et al., 2004	WS	NR	Mod	12 wk	swimming	5d.wk 30min	-	-	Up	-	Up	-	ND	Cx,Hp
Morosi et al, 2012	WS	NR	Mod	15wk	running	5d.wk 30min	-	-	Up	-	Up	-	-	Hp
Navarro et al., 2004	Mice	NR	Mod	50 wk	running	7d.wk 5min	-	-	-	-	ND	ND	ND	TB

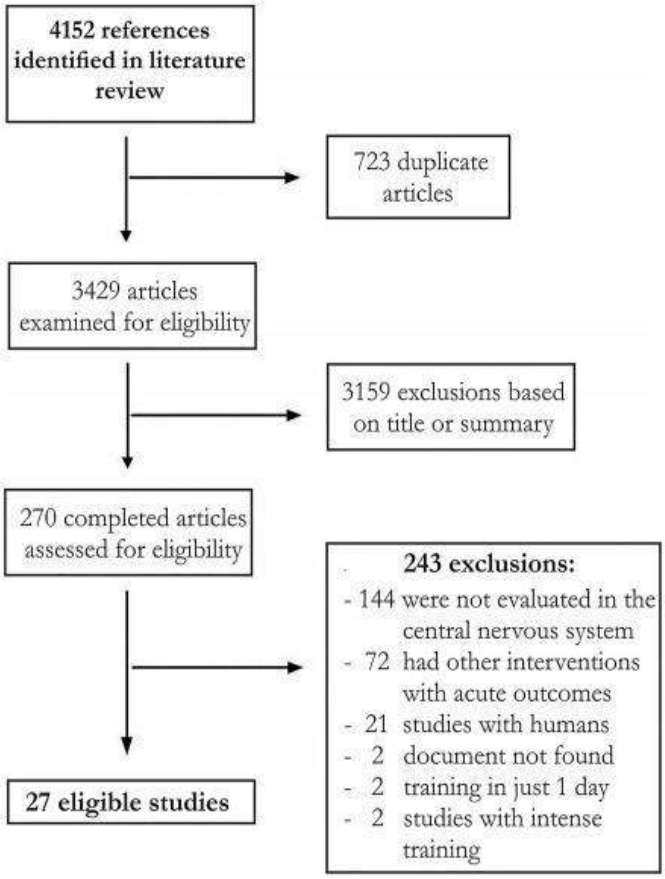

Fig. 2. Flow chart of the search strategy.

and post-exercise conditions for different training periods: 2 weeks (Mazzola et al., 2011), 4 weeks (13) and 8 weeks (Liu et al., 2000; Neves et al., 2015; dos Santos et al., 2017; Sosa et al., 2015; Tuon et al., 2010). Few studies analyzed the effect of longer periods of exercise: 50 weeks was reported in the study of Navarro et al., (2004), followed by 11 and 12 weeks, respectively, described by Teixeira et al., (2009) and Cechetti et al. (2008).

3.6. Meta-analysis

The meta-analysis of the individual studies on effects of GSH (n = 9), GPx (n = 11), SOD (n = 16), CAT (n = 12), and TBARS (n = 23) is summarized in the Tables 3 and 4. When this analysis was conducted, without considering training period or exercise session duration, no significant differences were observed between the exercise and control conditions for GPx (MD 0.17; 95% CI, −2.39, 2.72; p = 0.90), SOD (MD 0.85; 95% CI, −0.60; p = 0.25), CAT (MD 0.29; 95% CI, −0.10, 0.68; p = 0.15), and TBARS levels (MD 0.54; 95% CI −0.31, 0.73; p = 0.06) (Fig. 2).

In the subgroup analysis for different training periods (4 and 8 weeks), it was detected that training periods longer than 8 weeks were associated with increased SOD (MD 2.84; 95% CI, 1.18, 4.49; p = 0.0008) and CAT activities (MD 0.30; 95% CI, 0.14, 0.45; p = 0.0001) (Fig. 3). Training protocols with 30–60 min of exercise

Table 3
Oxidative stress variables used in the included trials.

Variables		Studies (n)
GSH	Glutathione	9
GSSG	Oxidized glutathione	2
GPx	Glutathione peroxidase	11
GR	Oxidized glutathione	1
SOD	Superoxide dismutase	16
CAT	Catalase	12
TBARS	Thiobarbituric acid-reactive substances/malondialdehyde	23

Table 4
Oxidative stress levels of the brain regions analyzed in the included trials.

Local of the brain	Studies (n)
Cerebral cortex	13
Corpus striatum	6
Brainstem	2
Hippocampus	5
Cerebellum	2
Amygdala	1
Total brain	7

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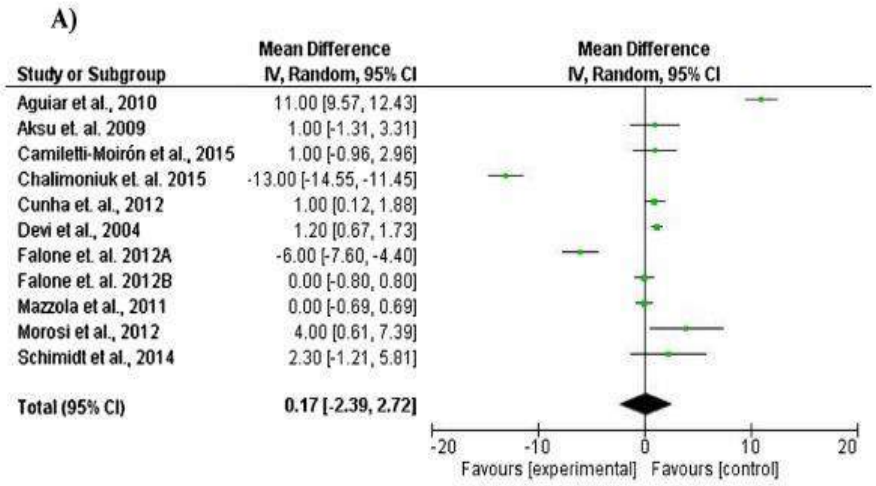

Fig. 3. Meta-analysis of Glutathione Peroxidase (GPx). A) Forest plot. B) Funnel plot.

showed a slight, but significant rise in CAT activity (MD 0.40; 95% CI, 0.01, 0.80; $p = 0.04$). However, no significant difference in the activity of this enzyme was observed when shorter exercise durations (20–30 min) was adopted (MD 0.15; 95% CI, −0.07, 0.38; $p = 0.18$) (Figs. 4 and 5). Evidence of heterogeneity and inconsistency were found for GPx ($Ch^2 = 578.35$, $Tau^2 = 17.74$, $I^2 = 98\%$), SOD ($Ch^2 = 290.09$, $Tau^2 = 7.08$, $I^2 = 94\%$), CAT ($Ch^2 = 2152.55$, $Tau^2 = 0.40$, $I^2 = 100\%$), and TBARS ($Ch^2 = 2408.30$, $Tau^2 = 0.24$, $I^2 = 99\%$).

4. Discussion

The results of the present review lend further support to the notion that repeated moderate exercise triggers the activation of the major defense mechanisms and ROS removal systems in the rodent brain. The results of the meta-analysis indicate an increase in SOD and CAT enzymatic activity after training for a period superior to 8 weeks and duration between 30 and 60 min. Taken together, these results suggest that there is an adaptive response to aerobic exercise that is modulated by training volume and ROS production.

Recent studies (dos Santos et al., 2017; Neves et al., 2015; Sosa et al., 2015) have observed that ROS concentrations are low for moderate intensity exercise. Another previous study suggested a beneficial effect of moderate exercise on antioxidant enzyme expression levels and other mechanisms that define the redox status of the brain (Gomez-Cabrera et al., 2008). Exercise-induced hormesis (Gomez-Cabrera et al., 2008; Radak et al., 2008) and mitochondrial hormesis (Ristow and Schmeisser, 2014; Ristow and Zarse, 2010) have been suggested as an explanation for this phenomenon. According to this hypothesis, regular exercise of moderate intensity and duration exerts a range of beneficial effects, including modulation of redox homeostasis and increased activity of the immune system (Radak et al., 2008).

High volume aerobic exercise regimens have been shown to provoke metabolic changes due to energy limitation, which is induced by the high O_2 demand during prolonged physical activity. Thus, higher activity of SOD and CAT may protect against oxidative damage (Chalimoniuk et al., 2015; Devi and Kiran, 2004).

In the CNS, one of the mechanisms of antioxidant and anti-inflammatory protection derived from moderate aerobic exercise appears to be related to the actions of glial cells, especially astrocytes (Liddell, 2017) and microglia (Jensen and Wee Yong, 2014). Astrocytes provide trophic, metabolic, and structural support for neurons and are the main source of GSH and SOD (Wilson, 1997). In addition, they are involved in the metabolism of glutamate

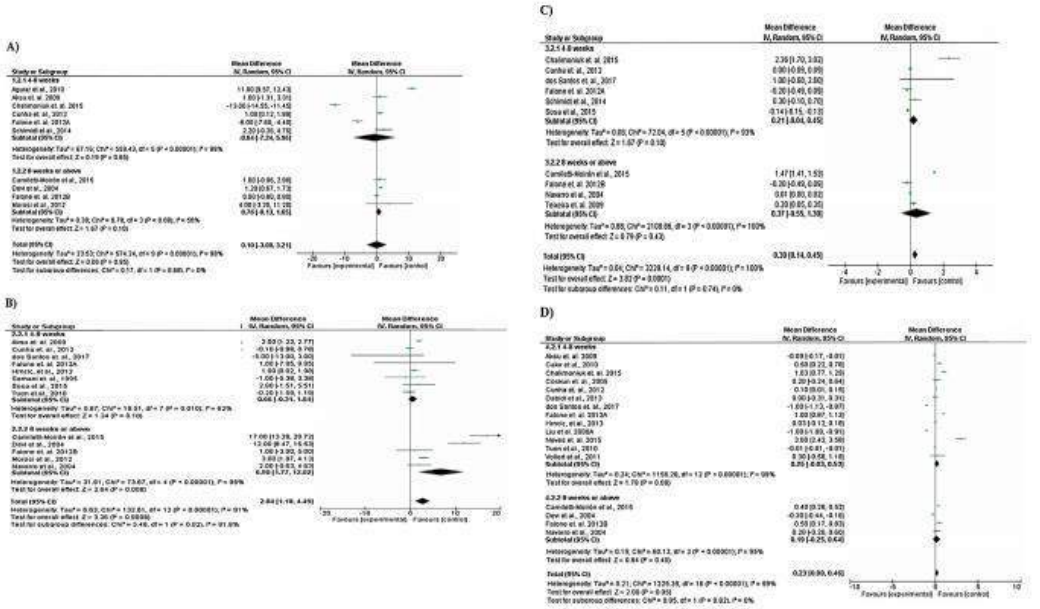

Fig. 4. Comparison and effect among different subgroups relative to the training period for 4–8 weeks and above 8 weeks. A) Glutathione peroxidase (GPx); B) Superoxide dismutase (SOD); C) Catalase (CAT); D) Thiobarbituric acid reactive substances (TBARS).

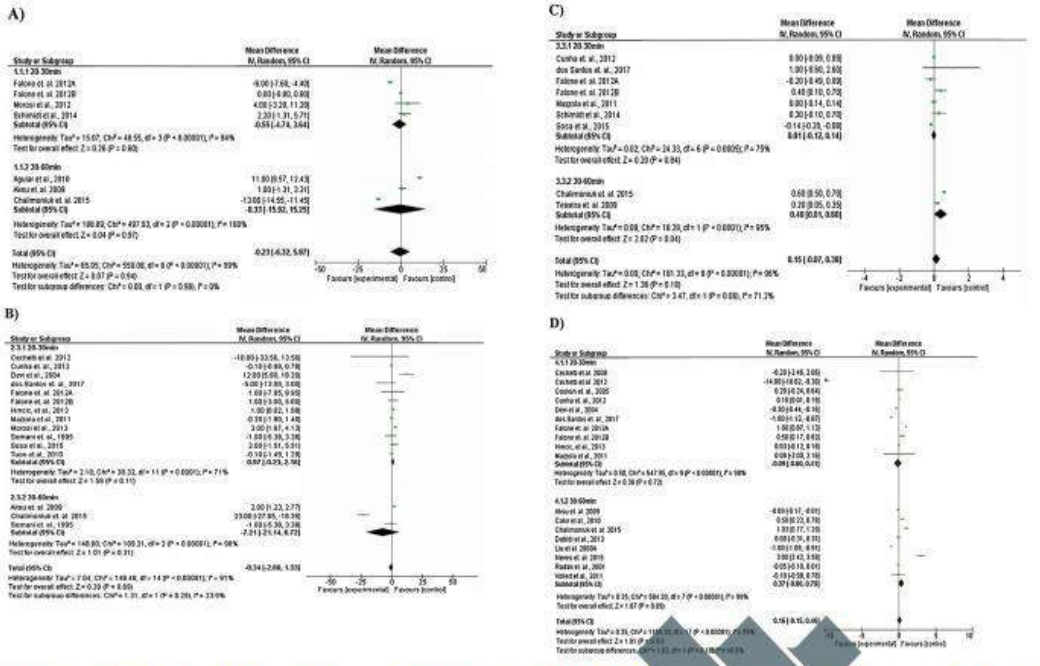

Fig. 5. Comparison and effect between different subgroups relative to the training time for 20–30 minutes and 30–60 minutes. A) Glutathione peroxidase (GPx); B) Superoxide dismutase (SOD); C) Catalase (CAT); D) Thiobarbituric acid reactive substances (TBARS).

and GABA, which contribute to ATP synthesis (Magi et al., 2013; Lee and Yatsu, 1975). Aerobic exercise can induce astrogliosis in some regions of the cerebral cortex (Li et al., 2005) and stimulates the proliferation of astrocytes in the subgranular zone of the dentate gyrus of the hippocampus (Uda et al., 2006). Moderate short-term (15 days) treadmill activity also increased GFAP levels in the hippocampus (Ferreira et al., 2011). Moderate exercise for 4 weeks (20 min/day) increased glutamine synthase enzyme activity in astrocytes even in the absence of astrogliosis. Moderate exercise has also been shown to modify the reactive and inflammatory microglia phenotype (M1) and install an anti-inflammatory and beneficial M2 phenotype, even after neurodegenerative insults (Jensen and Wee Yong, 2014; Lima et al., 2013). The glial cell response to physical exercise is also related to the release of cytokines that maintain immune system function in the CNS (Ang et al., 2007). Physical activity increases myokine levels (e.g., IL-6, IL-7, BNDF) generated by muscle inflammation after exercise (Nybo et al., 2002). Most of these cytokines are released by muscle fibers into the bloodstream (Pedersen et al., 2004), are transported to various tissues, including the CNS (Nybo et al., 2002; Van Wagoner and Benveniste, 1999) and are capable of activating glial cells (Ang et al., 2007).

Recent studies also indicate an important role of astrocytes in brainstem regions that are involved in respiratory rate modulation, fundamental not only for the physiological demands but also for the activity of motor circuits and thus, determining the capacity for physical exercise (Sheikhbahaei et al., 2018).

The brain region most commonly studied in the selected studies was the Cx (35.13%). The reason for this choice is that centers for the voluntary control of motor activity are located there. Homogenates of whole brain tissue were analyzed in 18.91% of the studies (Cakir et al., 2010; Itoh et al., 1998; Liu et al., 2000; Mazzola et al., 2011; Navarro et al., 2004; Radak et al., 2001). This is a limiting factor, considering that antioxidant enzymatic behavior varies across brain regions (Chalimoniuk et al., 2015). The CPu, for example, was analyzed in 16.21% of the studies and it is known to exhibit a high level of resistance to oxidative insults (Teixeira et al., 2009). In contrast, the Hp is considered highly sensitive to oxidative insults. The Hp was analyzed in 13.51%, the Cb in 8.1%, the BS 5.4%, and the Am in 2.7% of the studies.

The heterogeneity of the antioxidant response to physical exercise was also evident in the meta-analysis. The Cb and BS tended to show an increased antioxidant enzymatic activity after training periods between 6 and 7.5 weeks (Chalimoniuk et al., 2015; Rybak et al., 1995). In the Hp, a reduction in the antioxidant enzymatic activity was reported after 8 weeks in two studies (Aksu et al., 2009; Schimidt et al., 2014) with the absence of increased lipoperoxidation levels. In the Cb increased levels of lipid peroxidation together with an increase in antioxidant enzymatic activity were observed after 6 weeks of moderate exercise (Chalimoniuk et al., 2015). In the Cx, after 8 weeks of training, a decrease of antioxidant enzyme activity was detected (Falone et al., 2012). Analysis of total brain homogenates showed that after intense exercise on a treadmill for 12 weeks, the increased levels of lipid peroxidation were accompanied by intensification in SOD and CAT activity (Camiletti-Moirón et al., 2015). In the CPu, after intense swimming exercise for 11 weeks, augmented lipid peroxidation levels were not accompanied by greater CAT activity (Teixeira et al., 2009).

Only four studies analyzed antioxidant enzymatic activity in specific brain regions after exercise protocols > 60 min (Liu et al., 2000; Radak et al., 2001) over 12 weeks (Falone et al., 2012; Navarro et al., 2004). In addition, the results of these previous studies are conflicting. Therefore, an interpretation of the chronic effect of high volume exercise is difficult. This fact demonstrates the need for further research investigating the chronic effects of high volume running and swimming exercise routines in rodents.

Taken together, the present meta-analysis shows that rodent training protocols between 20 and 30 min over a period of 8 weeks do not affect the antioxidant enzymatic equilibrium. In contrast, the aerobic exercise for more than 8 weeks induces increased CAT and SOD activities. Furthermore, the meta-analysis also indicated that there is still limited research and accurate information regarding the safety conditions of training periods between 90 and 200 min that go on for longer than 12 weeks. This occurs due to the lack of homogeneity in the training protocols adopted by the published literature (Tarini et al., 2013) that simulate tests of ultra resistance in humans.

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APÊNDICE B – MANUSCRITO ORIGINAL SUBMETIDO NA FRONTIERS IN MOLECULAR NEUROCIENCE

Título: ULTRA-ENDURANCE ASSOCIATED WITH MODERATE EXERCISE IN RATS INDUCES CEREBELLAR OXIDATIVE STRESS AND IMPAIRS REACTIVE GFAP ISOFORM PROFILE

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ULTRA-ENDURANCE ASSOCIATED WITH MODERATE EXERCISE IN RATS INDUCES CEREBELLAR OXIDATIVE STRESS AND IMPAIRS REACTIVE GFAP ISOFORM PROFILE

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Text pages:
Figures: 07
Tables: 01
Running title: Cerebellar oxidative stress and GFAP reactivity after ultra-endurance

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ABSTRACT

Ultra-endurance race (UE) has been associated with brain metabolic changes, but it is still unknown which regions are vulnerable. This study investigated whether high race volumes in rodents, even under moderate intensity, can induce cerebellar oxidative and inflammatory status. Forty-five adult rats were divided into 6 groups according to a training period, followed or not by an exhaustion test (ET) that simulates UE: control (C), control+ET (C-ET), moderate training volume (MV) and MV-ET, high training volume (HV) and HV-ET. A continuous running on a treadmill was performed 5 times/week. The training period was 30 (MV) and 90 (HV) min/day for 3 months. After 24h, the ET was performed and serum lactate levels were evaluated. Serum and cerebellar homogenates were obtained 24h after ET. Serum creatine kinase (CK), lactate dehydrogenase (LDH) and corticosterone levels were assessed. Lipoperoxidation (LP), nitric oxide (NO), Interleukin 1β (IL-1β) and GFAP proteins, reduced (GSH), oxidized (GSSG) glutathione levels, superoxide dismutase (SOD) and catalase (CAT) activities were quantified in the cerebellum. Serum lactate concentrations were lower in MV-ET (~20%) and HV-ET (~40%) compared to C-ET group. CK and corticosterone levels were increased more than ~2 fold by HV training compared to control. ET increased CK levels in MV-ET *vs* MV group (p=0.026). HV induced higher LP levels (~40%) but an additive effect of ET was only seen in the MV-ET group (p=0.02). SOD activity was higher in all trained groups *vs* C and C-ET (P <0.05). CAT activity, however, was intensified only in the MV group (P<0.02). The 50 kDa GFAP levels were enhanced in C-ET and MV-ET *vs* respective controls, while 42 kDa (~40%) and 39 kDa (~26%) isoform levels were reduced. In the HV-ET group, the 50 KDa isoform amount was reduced ~40-60% compared to the other groups and the 39 KDa isoform increased ~7fold. LDH, IL-1β levels, GSH/GSSG ratio and NO production were not modified. Data shows that cerebellar resilience to oxidative damage may be maintained under MV training, but it is reduced by UE running. HV *per se* induced systemic metabolic changes and cerebellar oxidative status. UE after HV training was able to impair the cerebellum astrocyte reactive profile.

Keywords: Oxidative stress[1], central nervous system[2], high training volume[3], catalase[4], superoxide dismutase[5], lipoperoxidation[6], glial fibrillary acidic protein[7].

INTRODUCTION

In the last decades, a noticeable increase in the number of athletes taking part in long-distance running and ultramarathons has been reported (Agnew et al., 2018). In contrast to regular volume training, a number of deleterious effects have been described as a result of high volume, repetitive physical effort, and exhaustive and complex competitions (Knechtle and Nikolaidis, 2018). Recent studies have identified physiological impairment resulting from ultra-endurance (UE), encompassing cardiac and major artery structural remodeling, indications of pathological condition (Bonsignore et al., 2017; O'Keefe et al., 2012), marked renal and muscle damage (Jastrzębski et al., 2015), hepatic dysfunction (Rama et al., 2015) cartilage wear (Franciozi et al., 2013) and oxidative damage to the peripheral and central nervous systems (CNS) (Chalimoniuk et al., 2015; Muñoz et al., 2017).

The CNS is especially vulnerable to oxidative damage due to its high O_2-dependent mitochondrial activity, intensified by aerobic exercise, increased oxygen uptake and cerebral blood flow (40-70%) to meet energy demands (Chalimoniuk et al., 2015). When subjected to antioxidant enzymatic imbalance, the CNS is an important target for reactive oxygen species (ROS) and oxidative stress (OS) secondary byproducts. Physical exercise can exert neuroprotective or harmful actions in the redox balance, depending on the intensity, volume, duration, and specificity of the brain region, independent of the involvement of this region in the locomotor function (de Souza et al., 2018).

The cerebellum (Cb) plays a relevant role in motor, cognitive and limbic activity (Hibi and Shimizu, 2011; Schmahmann, 2019) and promotes movement control, motor learning (Celnik, 2016) balance, muscle coordination, posture (Hibi and Shimizu, 2011; Jirenhed et al., 2017; Powell et al., 2015) and visually guided locomotion. (Armstrong, 1996; Rabe et al., 2008; Schmahmann, 2019). Moderate aerobic exercise is able to broaden dendritic projections of Purkinje cells (Huang et al., 2018; Pysh, 1979) and favors cerebellar angiogenesis (Black et al., 1990; Lee, 2007). In addition, cerebellar molecular changes induced by exercise promote plasticity in the motor cortex (Mang et al., 2016), attenuate cerebellar deficiencies (Tercero-Pérez et al., 2019), suppress Purkinje cell loss during Parkinson´s disease (Lee et al., 2018) increase mitochondrial biogenesis (Marques-Aleixo et al., 2015) and inhibit oxidative stress,

promoting apoptotic signaling (Marques-Aleixo et al., 2016). Purkinje cells have also been shown to have high levels of monocarboxylate transporters (MCTs), which are key proteins in energy metabolism when stimulated, by treadmill running (Hoshino et al., 2016).

Notwithstanding the importance of the Cb for locomotion, limited experimental studies have investigated the oxidative impact of high volume training and UE tests in this brain region. Studies that evaluated training over 60 minutes per day examined the effects on the entire rodent brain (Hagen et al., 2018; Radak et al., 2001), neglecting metabolic specificities of different brain regions. Another factor not yet investigated has been whether UE could modify astrocyte function in the CB, considering that these cells are active players in brain energy delivery, production, utilization, and storage (Sonnay et al., 2017). Brain glycogen is chiefly located in the astrocytes and it decreases with extensive and prolonged exercise (Matsui et al., 2012, 2017), but it is still unknown whether UE running induces altered glial activity as well as modifies antioxidant defense mechanisms when associated with high volume running training (Jastrzębski et al., 2015; Knechtle and Nikolaidis, 2018; Millet et al., 2018; Rama et al., 2015).

The current discussion on negative effects of high volume and UE on runner health (Knechtle and Nikolaidis, 2018; Millet et al., 2018) and the scarcity of knowledge about these effects in locomotion-related brain regions has motivated the present study. We hypothesized that rodent cerebellar resilience to oxidative injuries may be reduced after high training volumes, regardless of the simulation of UE running.

MATERIALS AND METHODS

Experimental animal groups

Forty-five adult male Wistar rats (60 days; 225.84 ± 24.33g) were obtained from the animal production unit of the Federal University of Paraíba, João Pessoa, Brazil. The animals were kept in the inverted cycle, under a controlled environment with temperature (22 °C ± 2°C) and had free access to food and water.

After the running training test, the animals were randomized into six groups according to the moderate-intensity training period, followed or not by the exhaustion test (ET): control (C: n = 8, placed on the treadmill off), control + ET (C+ET), n = 7),

moderate training volume (MV: *n* = 8) moderate training + TE (MV+TE: *n* = 7), high training volume (TE: *n* = 8) and high training volume + TE (EV+TE: *n* = 7). All the experimental procedures animals followed the guidelines for the Care and Use of Laboratory Animals previously approved by the Animal Use Ethics Committee (CEUA) of the Federal University of Pernambuco, Brazil (# 0035-2017).

Physical training protocol

For running training, a motorized treadmill (AVS products®) with individual tracks and equipped with rear shock (2.0 mA) was used. One week before the beginning of the training, the animals were adapted to a speed of 5m/min for 10 min, 5 times per week. Subsequently, the animals were submitted to a maximum velocity test (Vmax) to determine the training threshold. Vmax consisted of a graded threshold run and speed increments (5 m/min every 3 min), starting at 5 m/min until the maximum intensity achieved by each animal (Senna et al., 2011).

The treadmill trainability test was rated on a scale of 1 to 5 according to the following criteria: (1) refusing to run, (2) below-average runner (sporadically running but stop constantly the running), (3) average runner (maintains a steady run but falls or stops the running sporadically), (4) above-average runner (consistent runner, occasionally falls backward on the treadmill) and (5) good runner (Dishman et al., 1988). Animals classified as score ≥3 were randomized in the groups for physical training.

The training protocol was continuous for 12 weeks. Daily exercise sessions 3 to 5 times/week with low to moderate intensity (50-70% of maximum intensity achieved in Vmax tests) were performed by the groups MV and MV-ET and groups HV and HV-ET (see Table 1). The training times of the MV and MV-T or HV and HV-ET groups were gradually increased from 10 to 30 min or 10 to 90 min per day, respectively, over a 12 week period (Tarini et al., 2013). Each training session was preceded by 5 minutes of warm-up at 30% of Vmax and at the end of the run 5 minutes of recovery at 30% of Vmax (Senna et al., 2011). These times were included in the total protocol period.

Twenty-four hours after the training period, the C-TE, MV-TE and EV-TE groups were submitted to the exhaustion test. This test was performed up to the maximum running distance (low to moderate intensities) when the animals were

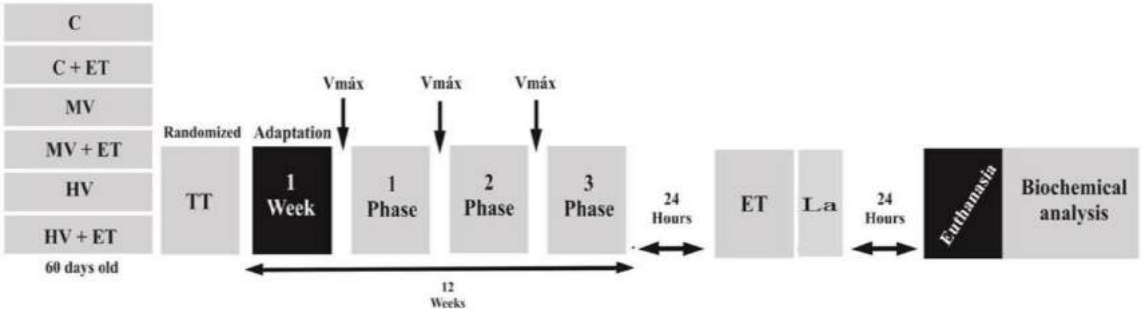
considered depleted and refused to run (touching the running box 10 times for 1 minute) (Tarini et al., 2013) and then tail blood was collected for analysis of serum lactate levels. After 24 h, all animals were weighed, anesthetized with 100% isoflurane, followed by blood collection directly from the heart (left ventricle) before decapitation for immediate removal of the cerebellum tissue.

Table 01- Training protocol

	Week	Training threshold (% - m/min)	Time (min)		Frequency of weekly sessions	Recovery training (hours)
			MV	HV		
Adaptation	1°	5 m/min	10min	10min	3x	24
	2°	50 %	10min	10min	3x	24
1° Phase	3°	50 %	15min	20min	3x	24
	4°	50 %	20min	30min	3x	24
	5°	50 %	25min	40min	5x	24
	6°	50 %	30min	50min	5x	24
2° Phase	7°	60 %	30min	60min	5x	24
	8°	60 %	30min	70min	5x	24
	9°	60 %	30min	80min	5x	24
	10°	70 %	30min	90min	5x	24
3° Phase	11°	70 %	30min	90min	5x	24
	12°	70 %	30min	90min	5x	48

MV: Moderate Volume Training; HV: High volume training

Figure 1 – Experimental protocol of aerobic exercise training under different volumes



Experimental protocol of aerobic exercise training under different volumes. Vmáx: Max speed test, ET: Exhaustion test, TT: Trainability test, La: lactate analysis. C: Control group; C-ET; Control group + exhaustion test; MV: moderate volume group; MV-ET: moderate volume group + exhaustion test; HV: high volume group; HV-ET: high group + exhaustion test.

Running performance

For analysis of running performance, running time (min), distance traveled (meters) and speed (meters/min) were evaluated in the groups submitted to the exhaustion test.

Markers of metabolic changes in the serum

Total Creatine kinase (CK) and lactate dehydrogenase (LDH) concentrations in the serum were quantified as indicators of potential damage to the tissues. The blood samples (~4 mL) were collected after euthanasia by cardiac puncture and put in dry tubes for separating serum (InjeXvácuo®) with a clot activator. They were subsequently centrifuged for 10 min at 800 g and 4ºC and then the serum was separated for analysis using specific commercial kits (Katal Biotechnology, São Paulo, Brazil for LDH and Labtest Diagnóstica S.A.; Minas Gerais, Brazil for CK) according to the manufacturer's instructions.

For quantification of lactate levels, the *tail vein blood sample collection* was used; approximately 25µl of the blood sample was placed directly onto test strips (BM-Lactate) and immediately analyzed with an Accutrend Lactate Accu-Check lactate meter (Roche, Brazil) just after ET.

For the corticosterone assay, 50 µl of serum was used. Analyses were made in duplicate per sample at a 1:1000 dilution. The entire procedure was performed as recommended by the Corticosterone ELISA kit (Cayman Chemical, No 501320).

Biochemical analysis of Cerebellum oxidative status

Cerebellum tissue preparation

The cerebellar tissue was weighed, immersed in an ice-cold saline solution and kept in a deep freezer at -80ºC. Then, this tissue was homogenized at 4 °C in a lysis buffer containing 10mM ethylenediaminetetraacetic acid (EDTA), 2mM phenylmethylsulfonyl fluoride (PMSF), 100mM sodium fluoride, 10mM sodium pyrophosphate (NaVO4), 100mM Tris (hydroxymethyl) aminomethane, pH 7.4 and a 1% cocktail of protease inhibitors (AEBSF- [4- (2-Aminoethyl) benzenesulfonyl fluoride hydrochloride; Aprotinin, Bestatin hydrochloride, E-64-[N-(trans-Epoxysucinyl)-L-leucine) 4-guanidino butylamine, leupeptin hemisulfate pepstatin A; (Sigma-Aldrich, USA) (1:5 w/v). Thereafter the homogenate was centrifuged for 10 min at 1000 g at 4 °C and the supernatants stored at −80 °C for further analyses.

Lipid Peroxidation Analysis

Lipoperoxidation (LP) was measured by estimating malondialdehyde (MDA) levels using thiobarbituric acid (TBA) for reaction (TBARS method) (Ohkawa et al., 1979).

The reaction was developed by sequential addition of 80 µL 8.1% sodium dodecyl sulfate, 600 µL 20% acetic acid, pH 3.5 and 600 µL 0.8% TBA solutions and 200 µL of cerebellum supernatant, boiled in water for 60 min and then cooled in an ice bath. Afterward, 600 µL of n-butanol was added, the mixture was shaken and centrifuged at 2500g for 10 min and the upper phase was collected and analyzed at 532 nm using a plate reader (Thermo Scientific, Varioskan flash spectral scanning multimode reader).

Nitric oxide levels

Nitrite levels were estimated using Griess reagent as an indicator of nitric oxide (NO) production (Green et al., 1982). Equal volumes (100 µL) of supernatant and Griess reagent were placed in a 96-well plate and reacted for 10 min at room temperature (~ 22 °C). The absorbance of the diazonium compound was measured at a wavelength of 540 nm. Results were expressed as nmol per mg protein with reference to a standard curve constructed with known concentrations of sodium nitrite.

Total Superoxide dismutase and Catalase activity

Evaluation of total superoxide dismutase (t-SOD) activity was performed according to Misra and Fridovich, (1972) at 25°C. Triplicates of cerebellum supernatants (60 µL) were previously incubated in a water bath at 37°C. Sodium carbonate (920 µL of 0.05% buffer was added. The reaction was developed by adding 20 µl of 150 mM epinephrine in 0.05% acetic acid. The decay kinetics of absorbance levels at 480 nm was evaluated by measurements every 15 s over a total period of 2 min. One unit of t-SOD was defined as the amount of enzyme that causes 50% inhibition of epinephrine oxidation. The enzymatic activity of t-SOD tissue was expressed in units per milligram of protein (U/mg protein).

Catalase enzymatic activity (CAT) was measured according to Aebi (1984). Triplicates of cerebellum supernatants (60 µL) were added to 905 µL sodium phosphate buffer pH 7.0. The reaction was developed by adding 35 µl of 300 mM hydrogen peroxide in sodium phosphate buffer. The rate constant K decomposition of H2O2 under our experimental conditions of temperature (22 °C) and pH (7.0) was determined to be 2.3 to measure absorbance changes for 10 s for 1.5 min at 240 nm. Enzyme activity was also expressed as units per milligram of protein (U/mg protein).

Reduced Glutathione Levels

Reduced Glutathione (GSH) levels were analyzed according to (Hissin and Hilf, 1976). 450 µL of 100 mM phosphate buffer with EDTA (5 mM) (pH 8.0) was added to 50 µL of the supernatant; 50 µL of this mixture plus 140 µL of 100 mM phosphate buffer plus 10 µL of o-phthaldialdehyde (OPT) solutions were placed in plates and incubated for 20 min at room temperature, protected from light. Absorbance was recorded on a spectrofluorometer using a wavelength of 350 nm. Results were expressed in µmol per mg protein with reference to a curve pattern constructed with known concentrations of GSH.

Oxidized Glutathione levels

Oxidized Glutathione (GSSG) levels were analyzed according to (Hissin and Hilf, 1976). 50 µL of the supernatant was incubated at room temperature with 20 µL of 0.04M N-ethylmaleimide (NEM) for 30 minutes to interact with GSH present in the tissue. To this mixture, 430 mL of 0.1 M sodium hydroxide (NaOH) was added; 50 µL of this mixture plus 140 µL of 0.1 M NaOH and 10 mL OPT were placed in the wells of a 96 well plate. This mixture was incubated for 15 min at room temperature and protected from light. The reading was taken on a spectrofluorometer using a wavelength of 350 nm for emission. Results were expressed in µmol per mg protein with reference to a standard curve constructed with known concentrations of GSSG.

GFAP and IL-1b Protein levels in the cerebellum

GFAP and IL-1b protein levels in the cerebellar tissue were analyzed by Western blotting. Cerebellar homogenate samples were diluted in sample buffer and boiled for 5 min. Forty micrograms of protein per lane were electrophoretically separated in 15% Sodium Polyacrylamide-Dodecyl Sulphate Gel containing 10% SDS, 30% Acrylamide/Bis-Acrylamide Solution, 1.5 M Tris-HCl (pH 8.8), 10% APS and TEMED at 100V, 0.15A and 300W. After separation, the proteins were transferred to a nitrocellulose membrane (Maine Manufacturing, USA, 0.22 micron) for 1 h and 30 min

at 250V, 0.35A and 300W. The membranes were blocked for 1h in a Tris-Tween 20 (TBS-T) solution containing 5% BSA. They were then incubated with rabbit polyclonal anti-GFAP primary antibody (Dako, Agilent Technologies, Germany, 1:1000) or rabbit polyclonal anti-IL-1β (*Sino Biological Inc*, Beijing, China, 1:2000) both diluted in TBS-T overnight at 4 °C. After 3 washes in TBS-T, the membrane was treated with goat anti-rabbit secondary antibody conjugated to HRP (Jackson ImmunoResearch, USA; 1:50,000) diluted in TBS-T for 1 hour. Subsequently, the membranes were washed in TBS-T and stained with Luminata Western HRP substrate (Millipore, USA) via chemiluminescence using a ChemiDoc imaging system (Bio-Rad, USA). Mouse monoclonal anti-beta-actin antibody (Santa Cruz Biotechnology, CA 1:10,000) was used to normalize the quantitative values. The data were analyzed using the Image Lab 6.0.1 software (Bio-Rad, USA).

Statistical analysis

The Shapiro-Wilk test was used to verify the normality of the data. Comparisons between groups were made by two way ANOVA, with interacting factors between groups (the type of training vs. exhaustion test). Post-hoc combinations were performed using Bonferroni's or LSD multiple comparison test. Nonparametric results were compared using the Kruskal Wallis test. To find the size effect, Cohen's d-test (Nakagawa and Cuthill, 2007) was applied by adopting the 0.02–0.15 points for a small effect, 0.16 to 0.35 medium effect, and greater than 0.35 as a large effect. The software SPSS (version 20.0) was used, considering $P \leq 0.05$ as significance value.

RESULTS

Body and cerebellum weights

The body weight of control group was higher than MV (↑ 11.63%; $P = 0.010$; Cohen's d = 1.59), MV-ET (↑ 10.69%; $P = 0.017$; Cohen's d = 2.49) HV (↑ 15.31%; $P < 0.001$; Cohen's d = 2.41) and HV- ET (↑ 14.35%; $P < 0.001$; Cohen's d = 2.51)groups. C- ET was greater than MV (↑ 11.45%; $P = 0.017$; Cohen's d = 1.68), MV-ET (↑ 10.51; $P = 0.028$; Cohen's d = 1.92), HV (↑ 15.14% ; $P < 0.001$; Cohen's d = 2.74) and HV- ET (↑

14.18; *P* = 0.001; Cohen's d = 2.56). Figure 2-a illustrates the body weight gain during the 3 months of training. The graphical representation of the area under the curve of body weight gain can be seen in Figure 2-b. There was no difference in the cerebellar weight (Figure 2-c) or in body/cerebellum weight ratio among the groups (Figure 2-d).

Traveled distance, duration and running speed during exhaustion test

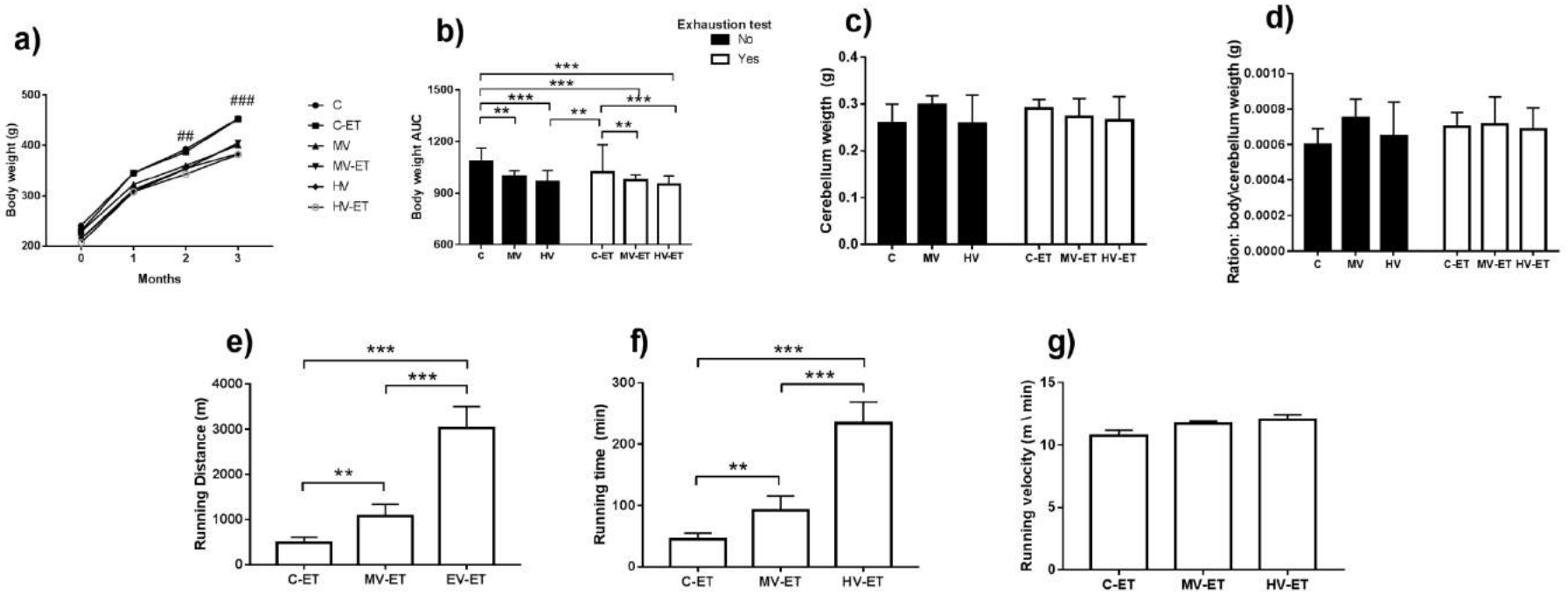
The HV-ET group achieved greater running distance at the end of the exhaustion test when compared to MV-ET (↑ 280.92%; *P* <0.001; Cohen's d = 5.13) and C- ET (↑ 624.75% (*P* <0.001; Cohen's d = 7.43). The running distance traveled by MV-ET was greater than that of the C-TE group (↑ 222.39%; *P* = 0.002; Cohen's d = 2.89) (Figure 2-e). Similarly, the running duration (Figure 2-f) in HV-ET animals was higher than in MV-ET(↑ 253.78%; *P* <0.001 Cohen's d = 4.79) and C-ET groups (↑ 522.00%; *P* < 0.001; Cohen's d = 9.47. Longer running duration was achieved by MV-ET compared to C-ET (↑ 205.68%; *P* = 0.002; Cohen's d = 5.62). No intergroup difference in the running speed was detected (Figure 2-g).

Lactate, Creatine Kinase (CK), lactate dehydrogenase and corticosterone levels in the serum

Figure 3a shows the blood lactate concentration after the ET. The HV-ET (↓ 53.85%; *P* <0.001; Cohen's d = 2.48) and MV-TE (↓ 30.28%; *P* = 0.039; Cohen's d = 4.69) groups had lower lactate levels when compared to C-TE.

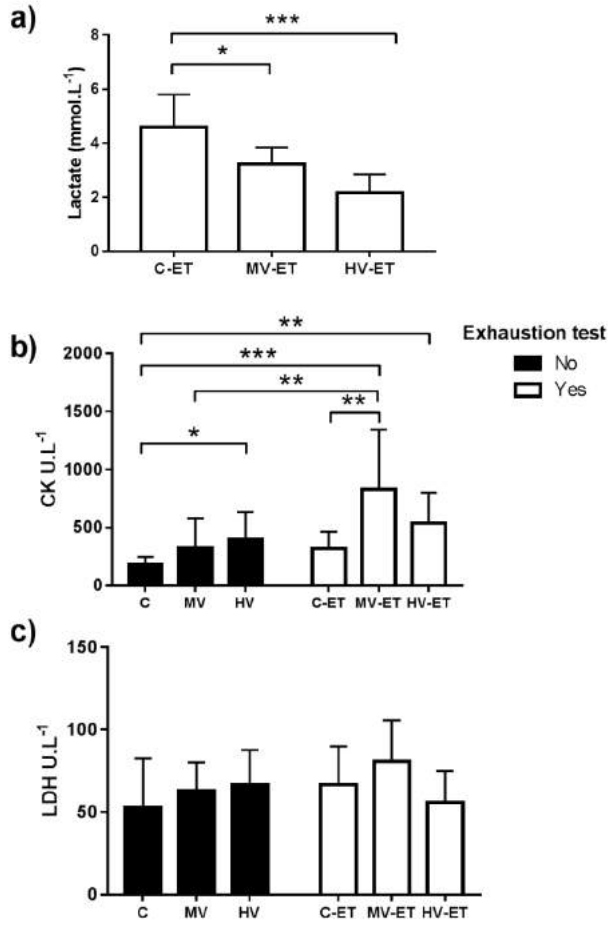
Regarding serum CK concentration, the MV-TE group presented higher CK concentration when compared to C (↑ 460.12%; *P* <0.001; Cohen's d = 1.76), C-ET (↑ 161.68% *P* = 0.026; Cohen's d = 1.34) and MV (↑ 160.72% *P* = 0.033; Cohen's s d = 1.24) groups. In the HV and HV-ET groups, CK values were greater than in the C animals (↑ 223.63%; *P* = 0.03; Cohen's d = 1.31) and (↑ 298.86%; *P* = 0.003; Cohen's d = 1.86) respectively (Figure 3-b). No statistical difference in LDH concentrations was detected among the groups (Figure 3-c).

Figure 2 – Body and cerebellar weights during 3 months of training and running performance after of exhaustion test



Somatic parameters and Running performance a) Body weight during months of training ### C-ET e C vs MV-ET; MV; HV-ET; HV $P<0.001$, ## C-ET e C vs MV-ET; MV; HV-ET; HV $P<0.01$. b) Area under body weight curve C vs MV ($P=0.005$), MV-ET; HV-ET e HV ($P<0.001$); C-ET vs HV-ET ($P<0.001$), MV-ET ($P=0.001$) e HV ($P=0.001$). c) Cerebellar weight; d) Body weight vs cerebellar weight ratio. e) Running distance of exhaustion test: MV-TE vs C-TE; $P = 0.002$. EV-TE vs MV-TE; C-TE; $P < 0.001$; f) Running time of exhaustion test: MV-ET vs C-ET; *P* C-ET; Control group + exhaustion test; MV: moderate volume group; MV-ET: moderate volume group+ exhaustion test; HV: high volume group; HV-ET: high volume group + exhaustion test. * $P \leq 0.05$; ** $P < 0.01$; *** $P < 0.001$. Values represent mean ± S.D.

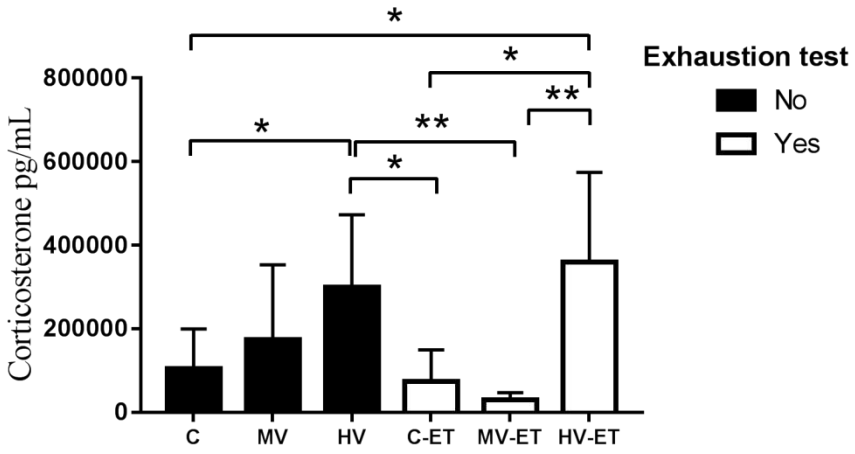
Figura 3 –Serum Lactate, CK and LDH levels



Serum levels of Lactate, Creatine Kinase and Lactate dehydrogenase. a) Lactate: MV-ET vs C-ET (*P* =0.039); HV-ET vs C-ET (*P* < 0.001). b) CK: Creatine Kinase; MV-TE vs C (*P* < 0.001), C-ET (*P* =0.026) e MV (*P* =0.033). EV-TE vs C (*P* = 0.001). EV vs C (*P* = 0.015). c) LDH: Lactate dehydrogenase. C: Control group; C-ET; Control group + exhaustion test; MV: moderate volume group; MV-ET: moderate volume group + exhaustion test; HV: high volume group; HV-ET: high volume group + exhaustion test. * $P \leq 0.05$; ** $P < 0.01$; *** $P < 0.001$. Values represent mean ± S.D. All the experiments were carried out in triplicates.

Figure 4 shows the results of serum corticosterone levels. A significant increase was found in the HV vs C group (↑ 44.44%; *P* = 0.040; Cohen's d = 0.24). Values in HV were also greater than in MV-ET group (↑ 90.06%; *P* = 0.003; Cohen's d = 0.01) and C-TE (↑ 75.17%; *P* = 0.023; Cohen's d =1.33. No intergroup difference was detected between HV and HV-ET groups but corticosterone values in HV-ET group were elevated compared to MV-TE (↑ 91.74%; *P* = 0.002; Cohen's d = 0.32), C-TE (↑ 79.36%; *P* = 0.014; Cohen's d = 0.68) and C (↑ 70.86; *P* = 0.025; Cohen's d = 0.37) groups.

Figure 4 – Corticosterone levels



Corticosterone levels in the serum. HV vs MV-ET (*P* =0.003), C (*P* =0.040) e C-ET (*P* = 0.023). HV-ET vs MV-ET(*P* =0.002), C-ET (*P* =0.014) e C (*P* =0.025). C: Control group; C-ET; Control group +exhaustion test; MV: moderate volume group; MV-ET: moderate volume group+ exhaustion test; HV: high volume group; HV-ET: high volume group +exhaust test. * $P \leq 0.05$; ** $P < 0.01$; *** $P < 0.001$. Values represent mean ± S.D. All the experiments were carried out in triplicates.

Lipid peroxidation in the cerebellum

Figure 5a illustrates increased levels of lipid peroxidation in the cerebellum of MV-TE vs MV (↑ 25.2%; *P* = 0.018; Cohen's d = 1.19) and C (↑ 27.4%; *P* = 0.023; Cohen's d = 1.14). No difference was found between HV and HV-ET groups but between HV and MV (↑ 26.1%; *P* = 0.023, Cohen's d = 1.14) and C (↑ 27.9%; *P* = 0.032, Cohen's d = 1.26). Levels of malondialdehydes were also higher in HV-ET group vs MV (↑ 28.0%; *P* = 0.007; Cohen's d = 1.18), C-ET (↑ 20.9%; *P* = 0.028; Cohen's d = 1.12) and C (↑ 30.3%; *P* = 0.008; Cohen's d = 1.15).

Total SOD and CAT enzymatic activity

Compared to control condition, increased SOD activity was found in the MV group (44.02%; *P* = 0.01; Cohen's d = 0.16) and HV (↑ 41.81%; *P* = 0.02; Cohen's d = 0.11) groups. Similarly, MV-ET and HV-ET groups presented higher SOD activities when compared to C (↑ 55.01%; *P* <0.001; Cohen's d = 0.22) (↑ 58.01%; *P* < 0.001; Cohen's d = 0.26) and C-TE (↑ 31.61%; *P* = 0.01; Cohen's d = 0.15) (↑ 63.75%; *P* = 0.005; Cohen's d = 0.21) respectively (Figure 5d).

CAT activity was higher only in the MV group compared to MV-ET (↑47.82%; *P* = 0.001; Cohen's d = 0.23), HV-ET (↑43.47%, *P* = 0.004, Cohen's d = 0.20), HV (↑34.78%; *P* = 0.034; Cohen's d = 0.14) and C (↑41.44%; *P* = 0.004; Cohen's d = 0.19) groups (Figure 5c).

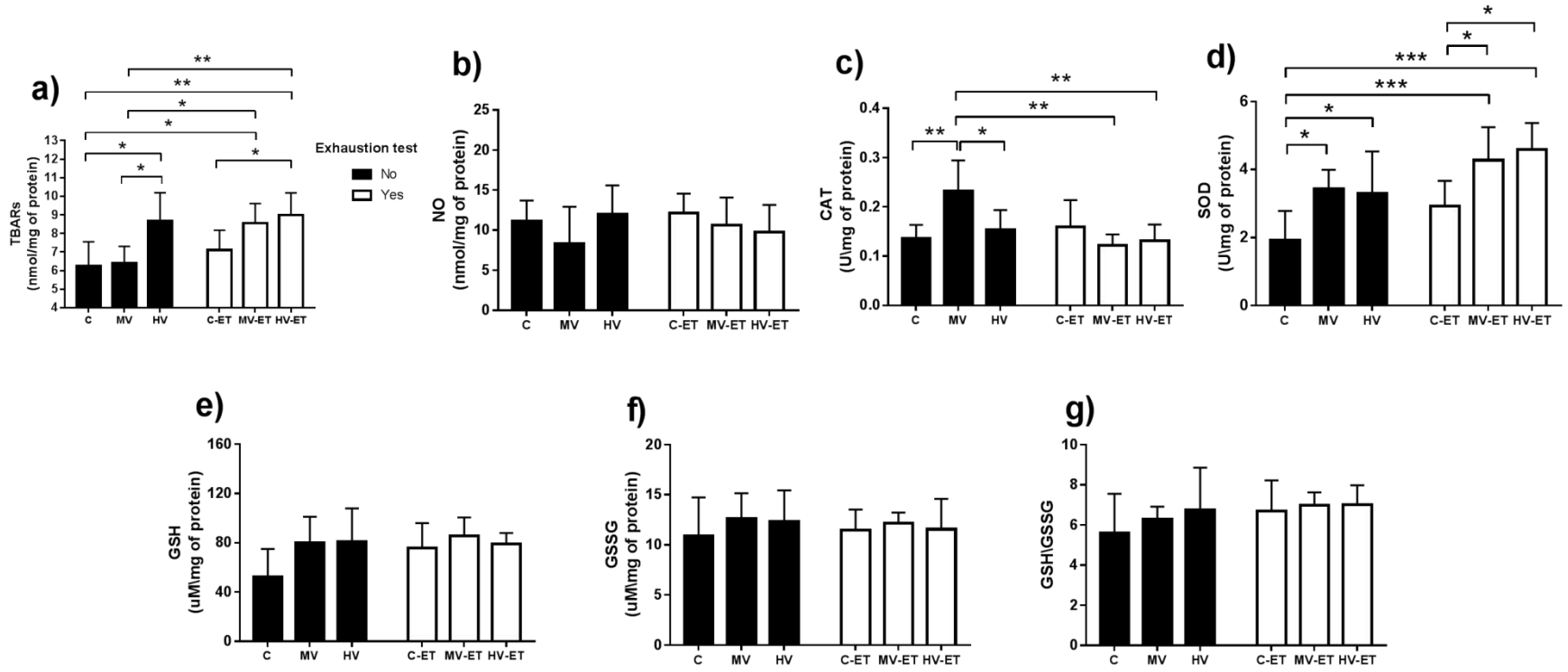
Nitric oxide (NO), Reduced Glutathione (GSH) e oxidized glutathione (GSSG) levels

No intergroup differences were found for NO, (Figure 5b), GSH (Figure 5-e), GSSG (Figure 5f) levels nor for GSH\GSSG ratio (Figure 5g) in the cerebellum.

Interleukin -1b levels

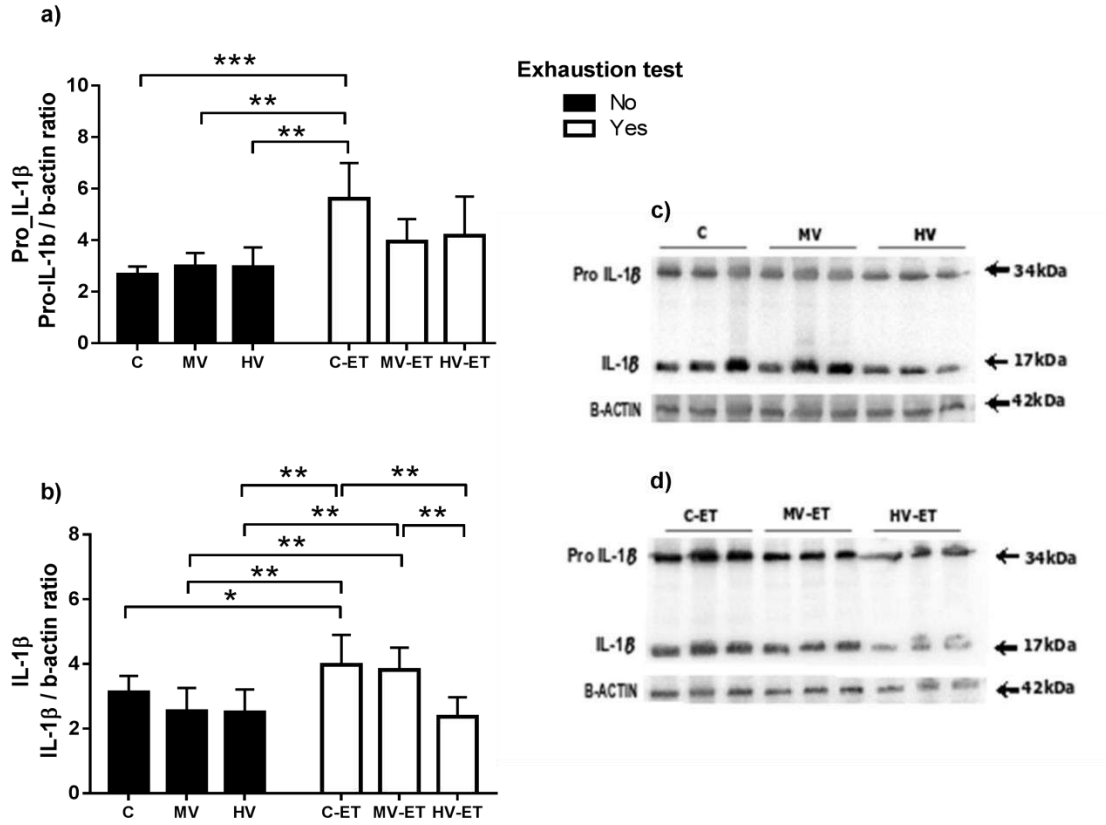
The concentration of pro-IL-1β (33-34 kDa) and the active form of IL-1β (17 kDa) in the cerebellum was not modified by the training volume in both MV and HV groups when compared to the control condition. Nevertheless, 24 h after the ET increased level of pro-IL-1β was detected in the CT-ET compared to the C(↑53.57%; *P* < 0.001; Cohen's d = 2.94), MV(↑46.88%; p = 0.001; Cohen's d = 2.51) and HV(↑41.21%; *P* = 0.001; Cohen's d = 2.37) groups. The amount of the active 17 kDa IL-1β was also intensified by the ET in the C-ET (↑22.92%; *P* = 0.046; Cohen's d = 1.13) and MV-ET (↑33.15%; *P* = 0.006; Cohen's d = 1.79) groups while no change was detected in the cerebellum of HV-ET animals (Figure 6).

Figure 5 – Oxidative Stress markers in the Cerebellum



Oxidative Markers in the cerebellum tissue. a) TBARS: MV-ET vs MV (P =0.018) e C (P =0.023). HV-ET vs C (P =0.008), C-ET (P =0.028) e MV (P =0.007). HV vs C (P =0.032) e MV (P =0.023). b) NO: Nitric Oxide; c) CAT: Catalase; MV vs MV-ET (P =0.001), HV-ET (P =0.004), HV (0.034) e C (P =0.004). d) SOD: Superoxide dismutase; MV vs C (P =0.010); MV-ET vs C (P < 0.001) e C-ET (P =0.019); HV vs C (0.028); HV-ET vs C (P <0.001) e C-ET (P =0.005). HV-ET vs C (P =0.001) e) GSH: Reduced Glutathione; f) GSSG: Oxidized Glutathione; g) GSH\GSSG ratio. C: Control group; C-ET; Control group – exhaustion test; MV: moderate volume group; MV-ET: moderate volume group + exhaustion test; HV: high group; HV-ET: high group + exhaustion test. * $P \leq 0.05$; ** $P < 0.01$; *** $P < 0.001$. Values represent mean ± S.D. All the experiments were carried out in triplicates.

Figure 6 – Interleukin-1Beta levels in the Cerebellum



Pro- IL-1B and active IL-1b protein levels assessed by Western blot in the cerebellar tissue. a) Pro IL-1B: C-ET vs HV (*P* =0.001); MV (*P* =0.001) e C (*P* <0.001). b) IL-1B: C-ET vs C (*P* =0.046); MV (*P* =0.002); HV (*P* =0.001); HV-ET (*P* <0.001). MV-ET vs MV (*P* =0.006); HV (*P* =0.003); HV-ET (*P* =0.001). * $P \leq 0.05$; ** $P < 0.01$; *** $P < 0.001$. c) Representative Western blotting membrane of groups without exhaustion test d) Representative Western blotting membrane of groups with exhaustion test. Values represent mean ± S.D. N=3 independent experiments per group. All the experiments were carried out in triplicates.

GFAP protein expression profile

Four GFAP isoforms were detected in the cerebellum using the Pan-GFAP antibody: the canonical GFAP protein with 50 kDa and the other 3 proteins with 45, 42 and 39 kDa. Figure 7 shows GFAP protein levels illustrated in two different ways. Firstly, we analyzed the total expression of all GFAP isoforms and observed that the HV-ET group had higher values when compared to C (↑ 50.39%; *P* = 0.03; Cohen's d = 4.20), HV (↑ 46 71%; *P* = 0.01; Cohen's d = 3.12) MV-TE (↑ 53.80%; *P* = 0.05; Cohen's s d = 4.31) and MV (↑ 44.88%; *P* = 0.01; Cohen's d = 1.79) groups.

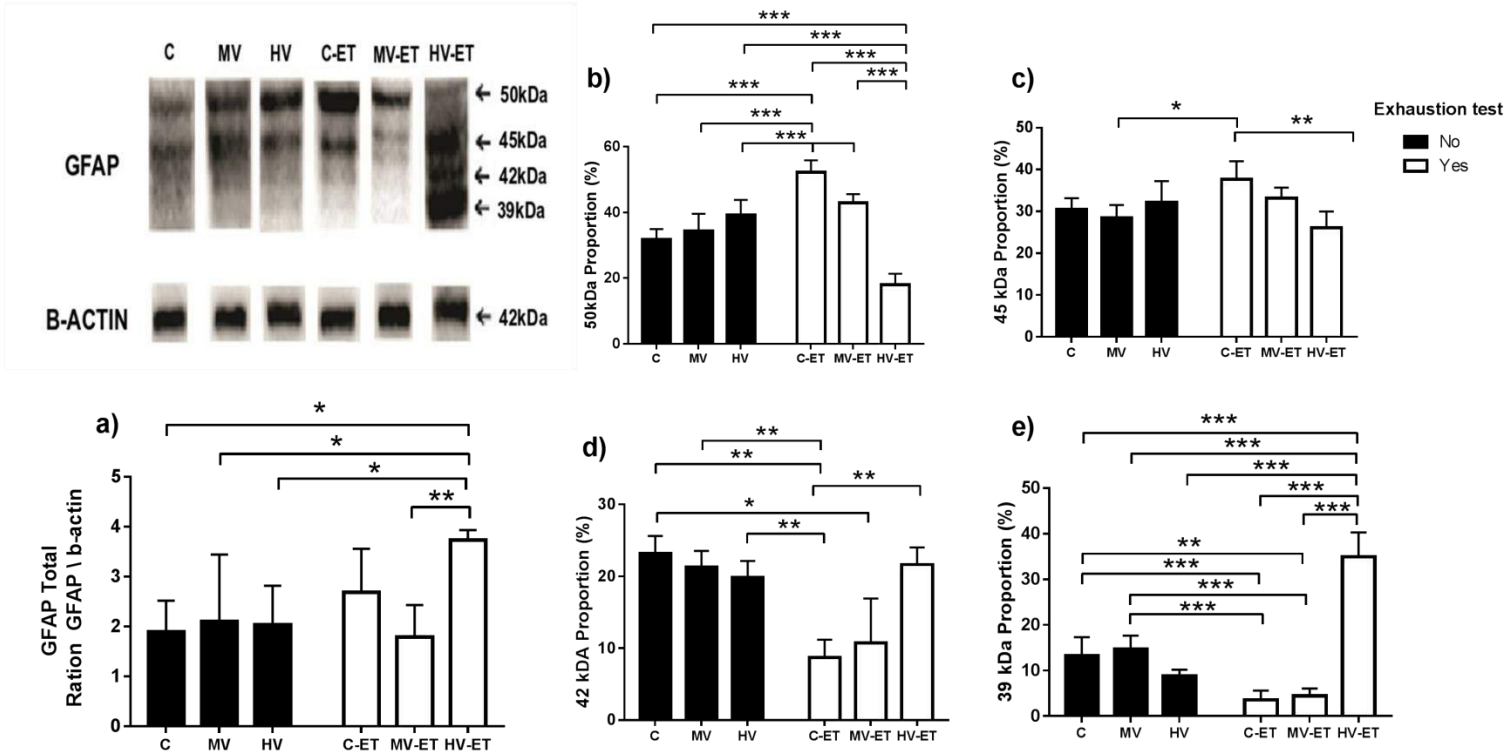
In the separate analysis by isoform, no intergroup difference among C, MV and HV groups was found for any of the 4 isoforms. On the other hand, the 50 kDa isoform increased in the C-ET when compared to C (↑ 36.52%; *P* <0.001; Cohen's d =

4.23), MV (↑ 34.26%; *P* < 0.001; Cohen's d = 3.94), MV-ET (↑ 19.70%; *P* <0.001; Cohen's d = 3.73) and HV (↑ 36.50%; *P* <0.001; Cohen's = 3.11). Higher levels of 50kDa isoform was also found in MV-ET vs C group (↑ 20.94%; p = 0.043; Cohen's d = 2.30). On the other hand, in the HV-ET group, reduced levels of 50kDa isoform were detected when compared to C (↓ 45.74%; *P* <0.001; Cohen's d = 3.46), C-ET (↓ 65.56%; *P* <0.001; Cohen's d = 9.80), MV (↓ 47.61%; *P* <0.001; Cohen's d = 3.70), MV-ET (↓ 42.88%; *P* <0.001; Cohen's d = 9.44) and HV (↓ 54.07%; *P* <0.001; Cohen's d = 5.21).

Regarding 45 kDa GFAP isoform, there was an increase in the C-ET when compared to MV (↑ 24.49%; *P* = 0.048; Cohen's d = 2.45 and HV-ET (↑ 30.76%; *P* = 0.004; Cohen's d = 2) groups. The levels of 42 kDa GFAP in the C-TE (↓ 61.33%; *P* = 0.001; Cohen's d = 6.07) and MV-TE (↓ 62.48%; *P* = 0.05; Cohen's d = 1.19) were lower than those found in the C group. Compared to C-TE group, higher values were found in MV (↑ 59.14%; *P* = 0.001; Cohen's d = 5.30); HV (↑ 56.19%; *P* = 0.004; Cohen's d = 4.66) ; HV-ET (↑ 59.75%; *P* = 0.001; Cohen's d = 5.31) groups.

GFAP isoform with 39kDa was abundant in the cerebellum of HV-ET group compared to C (↑62.17%; *P* <0.001; Cohen's d =4,59); HV (↑74.76%; *P* <0.001; Cohen's d =6.75); MV-ET (↑87.29%; *P* <0.001; Cohen's d =7,80), MV (↑57.87%; *P* <0.001; Cohen's d =0,47) C-ET (↑89.75%; *P* <0.001; Cohen's d =7.82) groups. Reduced values were observed in C-TE group compared to C (↓ 27.08%; *P* <0.001; Cohen's d =2.99); and MV (↓ 24.33%; *P* <0,001; Cohen's d =4,42) groups. Similarly, 39kDa GFAP was less expressed in the MV-TE vs C (↓33.58%; *P* = 0.002; Cohen's d =2.83) and MV (↓30.18%; *P* <0.001; Cohen's d =4.35) groups.

Figure 7 – Glial fibrillary acidic protein isoforms profile



GFAP protein levels assessed by Western blot in the cerebellar tissue . a) Quantification of GFAP total (sum of the 4 isoforms): HV-ET vs C (*P* =0.013); MV-ET (*P* =0.002); MV (*P* =0.024); HV (*P* =0.015). b, c, d and e): The expression profile of each GFAP isoform. b) 50 kDa GFAP: HV-ET vs C; C-ET; MV; MV-ET; HV (*P* <0.001). C-ET vs C; MV; MV; HV (*P* <0.001). MV-ET vs C (*P* =0.043) c) 45 KDa GFAP: C-ET vs HV-ET (*P* =0.004); MV (*P* =0.048). d) 42kDa: C-ET vs C (*P* =0.001); MV (*P* =0.001); HV (*P* =0.004); HV-ET (*P* =0.001). C vs MV-TE (*P* = 0.05) e) 39 kDa GFAP: HV-ET vs C; MV; HV;C-ET;MV-ET (*P* <0.001); C vs C-ET (*P* <0.001); MV-ET (*P* =0.002). MV vs C-ET; MV-ET (*P* <0.001) C: Control group; C-ET; Control group + exhaustion test; MV: moderate volume group; MV-ET: moderate volume group+ exhaustion test; HV: high group; HV-ET: high group + exhaustion test. * $P \leq 0.05$; ** $P < 0.01$; *** $P < 0.001$. Values represent mean ± S.D. N=3 independent experiments per group. All the experiments were carried out in triplicates.

DISCUSSION

The findings of the present study indicate that both the moderate and high training volumes of aerobic exercise in the treadmill were able to reduce the rat body weight similarly and did not modify the running performance during ET simulating UE running. However, a differential response of serum metabolic markers such as CK and corticosterone levels, as well as cerebellum oxidative status, were observed in the animals trained under the two distinct conditions of exercise volume. Three months of high training volume at moderate intensity impaired the cerebellum antioxidant defense system resulting in lipid peroxidation. In addition, when associated with ET, an altered profile of astrocyte reactivity was detected in this brain region.

Systemic effects

To ascertain whether our exercise training volume did indeed enhance muscular function and provoke adaptive responses, we performed exercise-exhaustion tests on treadmills simulating UE. Although there is no consensus on the definition of UE in rodents, events completed at 4 (Millet et al., 2011) or 6 hours of running (Turner et al., 2014) are considered comparable to UE in humans. Our results showed that animals submitted to the ET maintained considerable aerobic resistance for approximately 4 hours. In the animal groups submitted to moderate or high training volume, a lower serum lactate concentration was detected 24 h after ET compared to the control. In this latter condition of absence of training, serum lactate concentration exceeded the anaerobic threshold, which typically can be characterized by a lower mitochondrial oxidative capacity, phosphocreatine depletion, increased H^+, and recurrent substrates for adenine nucleotide catabolism (Marcinek et al., 2010; Lewis et al., 2010; Sahlin et al., 1990). The low lactate concentration observed in the trained groups corroborated the adaptive response for more effective resynthesis, high mitochondrial sensitivity in oxidizing the pyruvate and muscle angiogenesis (Booth et al., 2015; Gaesser and Poole, 1988). Although during long-distance competitions in humans muscle metabolic changes are accompanied by lactate elevation (Lewis et al., 2010), this happens not only because of the exercise volume but especially due to the maximum effort exerted during competition. In our study, there was no intensity variation during ET. In addition, the UE run simulation was performed at a lower intensity (50% of maximum speed) than the previous protocol of training (70%). Therefore, the findings observed in the MV and HV groups are in

agreement with previous evidence that rats submitted to running training under aerobic threshold present lactic stability (Abreu et al., 2016). Moreover, it is well established that aerobic endurance exercise increases fat consumption as an energy source by decreasing glycide oxidation and pyruvate production (Ferreira et al., 2016).

Ultra-endurance exercises also promote a catabolic state by activating protein degradation (Dohm et al., 1987). For this reason, plasma CK is often used as a biomarker for myocardial and skeletal muscle damage or changes in myocyte membrane permeability after exercise (Yamashita K. and Yoshioka T, 1991). An increase of plasma CK has been also observed in rats after running even in the absence of histological damage (Kuipers H., 1994). In the present study, a significant increase in the serum CK levels was detected in the HV group and this increase was maintained without modification 24 h after ET. On the other hand, the insult induced by ET was able to elevate CK levels in the MV group. Two peaks in the serum CK levels have been reported after a marathon run and intense exercise, associated with early transient changes in the membrane permeability and a late inflammatory response during 24-48h of recovery (Clarkson PM and Ebbeling C, 1988). Nevertheless, other studies in humans have shown that plasma CK concentration after ultramarathon runs (~200 Km) declines to basal levels after 24 h of recovery (Son et al., 2015).

The serum CK concentrations here detected in the MV-TE and HV groups were higher than those observed after moderate-intensity protocols in animal models (De Araujo et al., 2012) even when submitted to the eccentric contraction exercise by downhill running (Isanejad et al., 2015) or even when performed at high intensity (Choi et al., 2013; Rezaei et al., 2017). Wistar rats, when subjected to progressive exercise intensities, can reach anaerobic thresholds at velocities exceeding 15m/ min and a maximum CK concentration close to 350 U / L at 17.5m /min (Rezaei et al., 2017). In the MV-TE and HV groups, higher serum CK concentration was detected under the aerobic threshold, at a velocity not exceeding 12m / min and in the absence of increased levels of LDH in the serum. Hepatic and renal dysfunction can also become impaired after UE run (Lipman et al., 2014; Jastrzębski et al., 2015; Knechtle and Nikolaidis, 2018). Acute kidney injury is relatively common in ultramarathon races (Hoffman et al., 2013; Lipman et al., 2014) and post-race serum CK and creatinine concentrations have been significantly correlated in humans (Hoffman and Weiss, 2016; Hodgson et al., 2017). The exhaustive swimming exercise was also able to provoke kidney injury in rats (Wu et al., 2012). Although we did not investigate the renal function and histological markers in the muscle of MV-TE and HV animals, we cannot discard the possibility that their increased serum CK levels are related to different types of muscle or

renal insults inducing modifications in the clearance of CK (Hodgson et al., 2017). Our findings in rodents are partly in agreement with recent evidence in humans demonstrating that an ultraendurance mountain race performed under low intensity and aerobic conditions simultaneously increases CK and LDH levels in the athletes (Ramos-Campo et al., 2016).

Serum corticosterone levels were also elevated by the training volume in the HV group when compared to the control or MV groups. However, similarly to what was detected for CK, no additive effect was induced by the ET. Various aspects must be considered in a discussion of these findings. It is well established that exercise induces increased glucocorticoid secretion by stimulation of the hypothalamus-pituitary-adrenal (HPA) axis (Chennaoui et al., 2002). Karkoulias et al. (2008) demonstrated that marathon running in humans causes plasma cortisol elevation just after running and the recovery to basal levels may take a week. According to these authors, the degree of cortisol elevation is dependent on the duration of the run. The importance of glucocorticoids in determining muscle strength and endurance lies in their catabolic effects, especially hyperglycemic effects, and facilitates the conversion of proteins to glycogen, as well as providing amino acids for gluconeogenesis (Chen et al., 2017). These effects are probably involved in the relative resistance of the HV groups. On the other hand, it has also been shown that under the long-term exercise of moderate intensity, adaptive mechanisms reducing the sensitivity to glucocorticoids in target tissues including HPA axis can occur (Chennaoui et al., 2002; Duclos, Guinot and Le Bouc, 2007). These mechanisms can prevent deleterious effects of corticosterone elevation, decreasing additional muscle insult. Thus, we cannot discard the possibility that this type of adaptive mechanism occurred in the HV-TE group.

Effects on the Cerebellum

The main hypothesis of the present study was that rodent cerebellar resilience to oxidative injuries could be reduced after high training volumes, regardless of the simulation of UE running. Indeed, we found LP in the HV, MV-ET and HV-ET groups but not in the MV group. Considering the unchanged levels of NO production or GSH/GSSG ratio in the trained animals, compared to the control condition, the data indicated that LP herein detected was especially due to unbalanced levels of enzymatic antioxidant resources. Only in the MV group, an intensified CAT activity occurred concomitant to the increased SOD activity which was able to efficiently degrade hydrogen peroxide (H_2O_2) produced by this latter enzyme.

Usually, the exhaustion test in the treadmill can induce systemic oxidative stress depending on the intensity or muscle fatigue (Acikgoz et al., 2006). In the MV-ET group, most of the animals displayed a relative resistance, reaching their point of *exhaustion* after 2 hours of running. The unbalanced levels of SOD/catalase ratio detected in the cerebellum associated to the increased levels of systemic CK detected in this group, indicated that the previous moderate training volume for 3 months was not able to confer resistance to a UE simulation neither by the peripheral organs nor by neural regions involved in locomotion control. Chalimoniuk et al., (2015) reported increased levels of LP in the cerebellum of rats after 6 weeks of moderate exercise performed during 60 min/day. On the other hand, in the cerebral cortex, a decrease of antioxidant enzyme activity was detected after 8 weeks of training (5 days/week for only 20 min), but not after 16 weeks (Falone et al., 2012). Despite the differential vulnerability of these brain regions to oxidative insults, the present data reinforce the hypothesis that high exercise volume *per se* can be deleterious to the brain even under moderate intensity. However, it is not clear at this moment why the exhaustion test did not provoke an additive effect in the HV group.

Long-term exercise at low-to-moderate intensity negatively has been documented as regulating neuroinflammation and glial activation, inducing adaptive responses (Mee-Inta et al., 2019). The association between free radical accumulation and the evolution of inflammatory responses has been described (Haddad and Harb, 2005). In the present study, moderate and high training volumes did not affect the 33-34 -kDa Pro-IL-1β or the active 17-kDa IL-1β levels in the cerebellum. Nevertheless, increased levels of both protein forms were detected 24 h after the ET in the C-ET animals and only 17-kDa IL-1β in the MV-ET group. In the healthy brain, the levels of the active IL-1β are low but can be increased due to a number of conditions, including local damage and peripheral inflammation (Pitossi et al., 1997; Laye et al., 2000). Brain IL-1β is mainly produced by microglia, peri-vascular infiltrating macrophages (Herx et al., 2000), astrocytes (Rappold and Tieu, 2010) but can be released from some neurons (Silverman et al., 2005) under pathological conditions. Neurons are particularly susceptible to IL-1β-mediated toxicity. In the cerebellum, for example, Purkinje neurons undergo apoptosis and excitotoxic death when IL-1β and TNF-α levels are increased (Kaur et al., 2014).

Imbalance in antioxidant and anti-inflammatory neuroprotective mechanisms may be related to astrocyte reactivity (Liddell, 2017). These glial cells are the main source of GSH and SOD in the central nervous system (Fernandez-Fernandez et al., 2012) and also play trophic, metabolic, and neuronal support functions (Matsui et al., 2017). In addition, they are

actively involved in glutamate and GABA metabolism, which contribute to ATP synthesis (Magi et al., 2013). These beneficial actions of astrocytes characterize their neuroprotector phenotype, also called A2 (Cunningham et al., 2019). On the other hand, when overactivated, astrocytes can be neurotoxic acquiring an A1 phenotype and are the main targets of IL-1β released by microglia (Liddell et al., 2017). Under this condition, astrocytes have a reciprocal interaction with IL-1β, leading to the production of reactive oxygen species (Rappold and Tieu, 2010).

Exercise can induce astroglial proliferation depending on the brain's demand for energy, especially due to the involvement of these cells in the blood-brain barrier and their synaptic functions (Li et al., 2005). The GFAP gene can be alternatively spliced giving rise to at least nine proteins that differ from the first described isoform, called GFAPα with 50 kDa: GFAPβ, GFAPɣ, GFAPδ/ε, GFAPk, GFAPΔ135, GFAPΔ164, GFAPΔexon6, GFAPΔexon7 and GFAPζ (review in Middeldorp and Hol 2011). Usually, the astrocyte reactivity is accompanied by increased levels of GFAPα protein isoform which is phosphorylated and mainly expressed in mature and proliferating stages. Augmented levels of this GFAP isoform has been also related to inflammatory status in some pathological conditions (Catts et al., 2014; Sullivan, 2014).

Analyzing the profile of GFAP protein expression, we identified 4 distinct isoforms in the cerebellum with 50, 45, 42 and 39 kDa respectively. We have found that neither moderate nor high training volume modified the cerebellum GFAP expression profile of these isoforms when compared to the control condition. However, the exhaustion test provoked in the CT-ET and MV-ET increased the levels of the 50 kDa GFAP isoform while it concomitantly reduced the 42 and 39 kDa isoforms. An opposite effect was found in the HV-ET group where the 50 kDa isoform was about 40% of that found in the control animals and 50-60% of the values in the cerebellum of C-ET, MV-ET and HV groups. Simultaneously, augmented levels of 42 and 39 kDa GFAP were found in HV-ET.

Changes in the expression level of the GFAP isoforms influence the intermediate filament network which can modify astrocyte mobility and structure (Sullivan, 2014; Sereika et al., 2018). Usually, GFAP phosphorylation contributes to the extensive remodeling of glial networks in mitosis and can also affect its interactions with other intracellular proteins (Moeton et al., 2014). In some neurodegenerative diseases as well as in the astrocytoma of grade IV, GFAP isoforms with lower molecular weight were better expressed when compared to the control condition (Hol et al., 2003; Sereika et al., 2018). Reactive gliosis associated with a drop in GFAP phosphorylation has been reported, for example, in the

neurodegenerative condition in Parkinson's disease (Clairembault et al., 2014). It is not clear at this moment which mechanisms are involved in the changed GFAP isoform profile here detected in the cerebellum of HV-ET group. A recent study using proton magnetic resonance spectroscopy demonstrated that acute exhaustive endurance exercise in rats previously trained on a treadmill increased glutamate signals in the cerebellum. These findings suggested a disequilibrium in the turnover in the glutamate-glutamine cycle and a delay in the return of glutamine from astrocytes to neurons (Świątkiewicz et al., 2017). Astrocyte dysfunction contributes to the central fatigue phenomenon. Therefore, considering the importance of astrocytes for brain metabolism and function, it is possible that the modified GFAP profile found in the cerebellum of HV-ET group is involved in the impaired reactivity of this region to oxidative and inflammatory insult induced by the UE simulation.

CONCLUSION

In conclusion, the results corroborate the initial hypothesis indicating that rat cerebellar resilience to oxidative damage is maintained during three months of moderate training volume, but high training volume at moderate intensity impaired the enzymatic antioxidant defense system of this brain region. In addition, we demonstrated for the first time that UE racing after high training volume is able to alter the GFAP isoform profile related to astrocyte reactivity status. The findings also indicate that moderate training volume, under the aerobic condition for 3 months, does not confer resistance to UE simulation in rats, either for systemic markers or the oxidative and inflammatory status of the cerebellum. Altogether, the data highlights the importance of further studies in other brain regions, especially considering the increased number of participants in ultra-marathons nowadays.

AUTHOR CONTRIBUTIONS

Design the experiment RFS, BLSAC and SRAS; running training FBS, RFS, DDP; biochemical and western blot analysis DDP, RLA, RFS, LVPG, FMAS and GMMM; data curation R.F.S and B.L.S.A.C.; writing original draft RFS and BLSAC. All authors contributed to the final version of the manuscript.

CONFLICT OF INTEREST

The authors declare that they have no conflicts of interest.

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APÊNDICE C - ABSTRACT PUBLICADO

Título: EFFECT OF EXHAUSTIVE ULTRA-ENDURANCE VS MODERATE EXERCISE ON RODENT CEREBELLUM ANTIOXIDANT CAPACITY

Revista: Frontiers

DOI: 10.3389/conf.fncel.2019.01.00018

EFFECT OF EXHAUSTIVE ULTRA-ENDURANCE VS MODERATE EXERCISE ON RODENT CEREBELLUM ANTIOXIDANT CAPACITY

Raphael F. De Souza[1,2*], Ricielle L. Augusto[1], Silvia R. Moraes[1], Lillian Vanessa[1],Danielle D. Pereira[1], Gisele M. Moreno[1], Fabio B. Souza[1], Fernanda M. Souza[3] andBelmira L. Andrade- Da- Costa[1]

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Ultra-endurance (UE) running increases O2 consumption and reactive oxygen species production in peripherical systems but it is still unknown how this type of training can affect brain regions involved in the movement control. The present study test the hypothesis that in addition to muscle damage, the cerebellum redox balance can be impaired by increasing training volumes. Forty five adult male Wistar rats were randomly subdivided in 6 groups according to the moderate training period followed or not by exhaustion test (ET) : control(C), Control + ET, moderate training volume (MV) moderate training volume + ET (MV-ET), high training volume (HV) and high training volume + ET (HV-ET). A continuous running characterized by low to moderate intensity was performed 5 times a week on a treadmill. The training period was gradually increased to 30 (MV) and 90 (HV) min/day over 12-week period. The velocity during ET, and the serum concentration of lactate (Lac), Creatine Kinase (CK) lactate dehydrogenase (LDH) and corticosterone were assessed. The cerebellum redox balance was analyzed by lipoperoxidation (LP) levels, reduced/ oxidized Glutathione GSH/GSSG ratio and the superoxide dismutase (SOD) and catalase (CAT) activities. Serum lactate levels were reduced in the MV-ET and HV-ET compared to CT-ET group ($p<0.05$) while CK levels were increased in the MV-ET, HV-ET and HV groups ($p<0.03$). Corticosterone concentration was higher in the HV and HV-ET contrast to MV-ET and C-ET groups ($p\leq0.05$). Higher LP levels in the cerebellar tissue were induced by HV training despite the presence of ET. CAT activity was higher in the MV group compared to MV-ET, HV-ET and C groups ($p<0.02$). On the other hand, increased SOD activity was seen after ET independent

of the running volume compared to C group (p≤0.05). Running velocity, GSH/GSSG ratio in the cerebellum, serum LDH and NO levels were not modified by the training. The data shows that the resilience of cerebellum to oxidative damage can be kept under moderate training volume but it is reduced by high volume in ultra-endurance running especially due to unbalanced antioxidant enzymatic activity. Keywords: Oxidative stress; central Keywords: nervous system; exercise volume; catalase; superoxide dismutase; lipoperoxidation.

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Keywords: Oxidative stress ., Lipoperoxidation, superoxide dismutase ., Catalase, Central Nervous System
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Received: 26 Feb 2019; **Published Online:** 26 Apr 2019.
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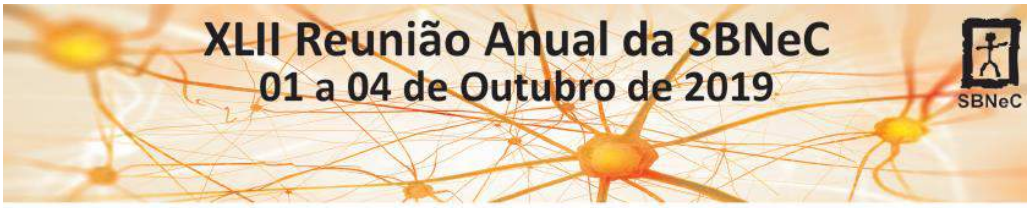
APENDICE D – TRABALHOS SOB CO-PARTICIPAÇÃO RELACIONADO A TESE



Certificado

Certificamos que o trabalho **ULTRARRESISTÊNCIA EXAUSTIVA ASSOCIADA AO EXERCÍCIO MODERADO EM RATOS INDUZ A INFLAMAÇÃO SISTÊMICA, LIPOPERXIDAÇÃO CEREBELAR E PREJUDICA O PERFIL DA ISOFORMA DO GFAP REATIVO.**, de autoria de **LILIAN VANESSA DA PENHA GONÇALVES, RICIELLE LOPES AUGUSTO, RAPHAEL FABRICIO DE SOUZA , SILVIA REGINA ARRUDA DE MORAES, MATHEUS NUNES GAMA, DANIELLE DUTRA PEREIRA, FERNANDA MARIA ARAUJO DE SOUZA, BELMIRA LARA DA SILVEIRA ANDRADE-DA-COST** foi apresentado na forma de POSTER E PRÊMIO JN na **XLII Reunião Anual da Sociedade Brasileira de Neurociências e Comportamento - SBNeC**, realizada no período de 01 a 04 de outubro de 2019, no Campos do Jordão Convention Center, em Campos do Jordão, SP.

Prof. Dr. Anderson Manoel Herculano Oliveira da Silva
Presidente da SBNeC

I CONGRESSO NEURO-FISIOLOGIA
UFPE – 06 A 08 DE NOVEMBRO

Modulação do perfil de expressão de GFAP astrocitário em processos de injúria no sistema nervoso central

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INTRODUÇÃO

Os astrócitos são células de suporte do sistema nervoso que envolvem neurônios e capilares sanguíneos no sistema nervoso central, fazendo a manutenção das células neurais e participando funcionalmente da barreira hematoencefálica (ZHOU, 2018). A proteína ácida fibrilar glial (GFAP) é uma das proteínas de filamento intermediário presente no

com a primeira, como em "*reactive astrocyte AND neuroinflammation*".

CRITÉRIOS DE INCLUSÃO

O critério inicial teve foco em artigos de, no máximo, 10 anos atrás. Destes, foram achados mais de 10 mil artigos que foram escolhidos de acordo com o título e abstract. Os

APENDICE E – CAPÍTULO DE LIVRO PUBLICADO COM A ORIENTADORA NÃO RELACIONADO A TESE

Título: Epigenetic effects of omega-3 fatty acids on neurons and astrocytes during brain development and senescende

Editora: Elsevier

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APENDICE F – ARTIGO NÃO RELACIONADO À TESE PUBLICADO COM A ORIENTADORA

Título: Low omega-6/omega-3 ratio in a maternal protein-deficient diet promotes histone-3 changes in progeny neural cells and favors leukemia inhibitory factor gene transcription

Periódico: Journal of Nutritional Biochemistry

Qualis Capes: A1

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Journal of Nutritional Biochemistry

Low omega-6/omega-3 ratio in a maternal protein-deficient diet promotes histone-3 changes in progeny neural cells and favors leukemia inhibitory factor gene transcription☆

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Abstract

Omega-3 (n-3) fatty acids modulate epigenetic changes critical to genesis and differentiation of neural cells. Conversely, maternal protein-malnutrition can negatively modify these changes. This study investigated whether a low n-6/n-3 ratio in a maternal diet could favor histone-3 (H3) modifications, gene transcription and differentiation in the offspring neural cells even under protein-deficiency. Female rats fed a control (Ct), or 3 types of multideficient diets differing in protein levels or linoleic/alpha-linolenic fatty acid ratios (RBD, RBD-C, RBD-SO) from 30 days prior to mating and during pregnancy. Cerebral cortex tissue and cortical cultures of progeny embryonic neurons and postnatal astrocytes were analyzed. H3K9 acetylation and H3K27 or H3K4 di-methylation levels were assessed by flow cytometry and/or immunocytochemistry. In astrocyte cultures and cortical tissue, the GFAP protein levels were assessed. Glial derived neurotrophic factor (GDNF) and leukemia inhibitory factor (LIF) gene expression were evaluated in the cortical tissue. GFAP levels were similar in astrocytes of Ct, RBD and RBD-C, but 65% lower in RBD-SO group. Higher levels of H3K9Ac were found in the neurons and H3K4Me2 in the astrocytes of the RBD group. No intergroup difference in the cortical GDNF mRNA expression or the H3K27Me2 levels in astrocytes was detected. LIF mRNA levels were higher in the RDB ($P=.002$) or RBD-C ($P=.004$) groups than in the control. The findings indicate the importance of dietary n-3 availability for the brain, even under a protein-deficient condition, inducing Histone modifications and increasing LIF gene transcription, involved in neural cell differentiation and reactivity.

Keywords: Protein malnutrition; Histone post-translational changes; Astrocytes; Neurons; Leukemia inhibitory factor; GDNF

APENDICE G – ARTIGOS PUBLICADOS SOBRE CORRIDA NÃO RELACIONADO À TESE

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Print

The effect of ibuprofen on muscle, hematological and renal function, hydric balance, pain, and performance during intense long-distance running

Raphael Fabricio de Souza;Dihogo Gama de Matos;Alexandre Pires Ferreira;Philip Chilibeck;Natalie de Almeida Barros;Alan Santos de Oliveira;Luana Cercato;Danielle Soares da Silva;Felipe Aidar;

Abstract

The aim of this study was to investigate the effect of prophylactic use of nonsteroidal anti-inflammatory drugs (NSAID, i.e. Ibuprofen) on physical performance, vertical jump, muscle biomarkers, liver, kidney, acute pain and hydration status of participants in the 42 km Trail Running Challenge, a long-distance race integrated over mountain routes. The sample consisted of 20 males randomly divided into two groups: a control group (CG) and an experimental group (EG) with 12 completing the race (41.1±8.8 y; 75.7±12.1 kg) and included in the final analysis. The EG were administered an ibuprofen capsule (400 mg) fifteen minutes before the beginning of the race, and again after 5 hours of racing if the route was not yet complete. There were significant time main effects for creatine kinase (CK) (p=0.001; f2 Cohen=0.25), lactate dehydrogenase (LDH) (p<0.001; f2 Cohen=2.05), aspartate aminotransferase (AST) (p=0.002; f2 Cohen=1.53), creatinine (p=0.002; f2 Cohen=2.24), urea (p=0.001; f2 Cohen=2.25), heart rate (HR) (p<0.001; f2 Cohen=4.88) and pain scores (p<0.001; f2 Cohen=1.93) which all increased during the race. There was a group × time interaction for squat jump (SJ) which significantly decreased in only the CG (p= 0.045; f2 Cohen=2.17). This may have been related to increased frequency of pain reported after the race in the gastrocnemius of the CG compared to the EG (p<0.05). It was concluded that ibuprofen intake did not reduce muscle damage during the competition but maintained leg muscular power performance (i.e. vertical jump), possibly by reducing gastrocnemius muscle pain.

Is sodium a good hyperhydration strategy in 10k runners?

Raphael Fabricio de Souza, Laion Samy de Oliveira, Dihogo Gama de Matos, Osvaldo Costa Moreira, Thays Costa da Silva, Phil Chilibeck, Alexandre Reis Ferreira, Aristela de Freitas Zanona, Felipe Jose Aidar

Abstract

The objective of the present study was to evaluate the effect of pre-exercise hyperhydration with sodium (PEHS), on the state of hydration and performance in runners of a 10K. Ten male runners (age 40.5 ± 9.7 yrs, weight 72.5 ± 8.4 kg, body fat 18.8 ± 4.5%) participated in the study and performed 10 km of street running under two different forms of prehydration: pre-exercise hydration (PEH), consisting of water intake ad libitum, and pre-exercise sodium hyperhydration (PEHS), consisting of sodium ingestion (12 mg of sodium for each 5 mL of water) diluted 1 h before the test. The variables evaluated were heart rate (HR), body temperature (BT), body mass (BM), blood pressure (BP), relative dehydration (RD), absolute dehydration (AD), total ingested water (TH2OING), degree of dehydration (DD), sweating rate (SR), specific gravity of urine (SGU), urine pH, and performance time (PT). There was no difference between intervention groups in the variables HR, BT, BM, BP, SGU, urine pH, and PT. RD (0.76 ± 0.41 kg vs. 1.16 ± 0.43 kg; Cohen's d = 0.95; p = 0.042); AD (0.63 ± 0.36 kg vs. 0.99 ± 0.43 kg; Cohen's d = 0.90; p = 0.038); DD (0.63 ± 0.52% vs. 1.35 ± 0.56%; Cohen's d = 1.33; p = 0.009); SR (2255.03 ± 1297.25 mL vs. 3550.06 ± 1527.35 mL; Cohen's d = 0.91; p = 0.048) were lower in the state of PEHS. PEH presented greater TH2OING (0.16 ± 0.12 mL vs. 0.34 ± 0.41 mL; Cohen's d = 0.59; p = 0.008). It was concluded that PEHS produces better hydration in runners during long distance running.

Keywords

Sodium; Sports performance; Hypertonic saline solution

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Do Compression Stockings Improve the Performance of Street Runners?

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ABSTRACT

Souza RF. Ferreira ARP. Matos DG. Silva EPS. Silva TC. Zanona AF. Junior HA. Schemly PAL. Brito ACNL. Aidar FJ. Do Compression Stockings Improve the Performance of Street Runners? **JEPonline** 2018;21(2):277-285. This study evaluated the use of compression stockings (CS) in runners. Ten subjects (29.1 ± 10.47 yrs) performed 10 km of running with CS and without CS (WCS). The subjects mean velocity, pace and total time, peak torque of the hip flexors (Pt), fatigue index (FI), heart rate (HR), diastolic pressure (DBP), and systolic pressure (SBP) were determined. Findings indicate that heart rate was significantly higher compared to the conditions in time: HR ($P<0.01$, Cohens' d = 0.30). SBP ($P=0.02$, Cohens' d = 1.99), and DBP ($P=0.01$, Cohens' d = 0.86) post-run in both conditions. No significant differences were observed in conditions time vs. groups (between groups) in all variables. There was no evidence on the contribution of the use of compression stockings in the performance of street runners.

Key Words: Compression Socks, Muscle Fatigue, Running

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SINErGIA II

Analysis of Race Performance on Different Floors Using Cushioned Sneakers And Barefoot

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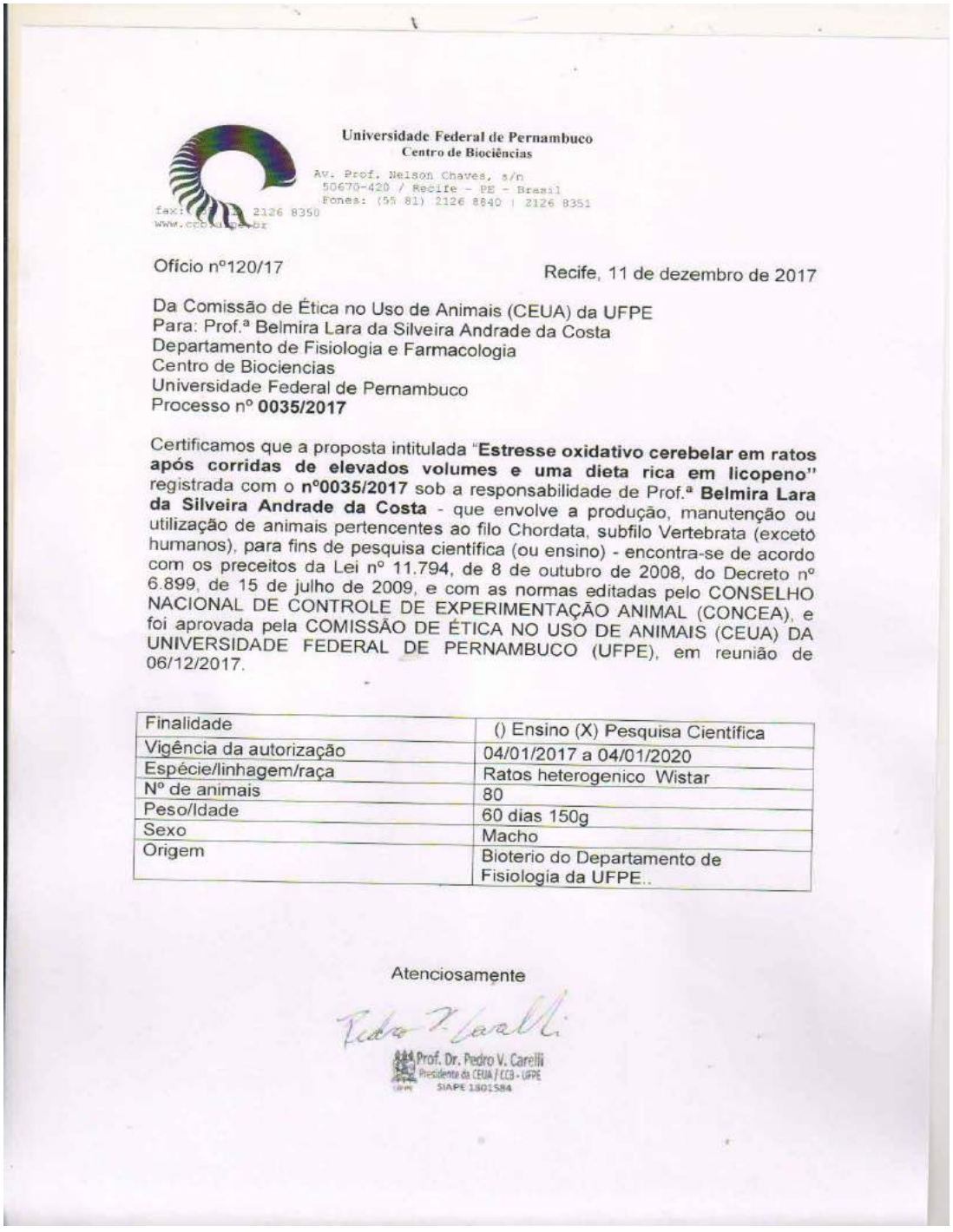
ARTIGO ORIGINAL | *ORIGINAL ARTICLE*

ABSTRACT

The purpose of this study was to analyze the effect of different ground and different types of running equipment on race performance. Fifteen recreational runners were evaluated in two conditions: cushioning sneakers and barefoot; during 3 different times, distances and ground: 15 minutes of running on a synthetic track, 3000m of running in the sand and 6 minutes of running on the treadmill. The rating of perceived exertion (RPE), pain, time and distance of race, pace, average speed, stride frequency, stride amplitude and body angulation were evaluated. The RPE when running with sneakers on synthetic track was higher when compared with running barefoot (6.4 ± 1.42 vs 4.9 ± 1.52 p = 0.036, Cohen's d = 0.10). Running barefoot on synthetic track increased pain (3.30 ± 3.33 vs. 0.50 ± 1.58 p = 0.027, Cohen's d = 1.07). Running without sneakers did not influence the performance, but presented a lower RPE when compared to the use of sneakers with cushioning in the synthetic floor. On the other hand, running without shoes on synthetic ground, increased the acute pain.

Keywords: running, athletic performance, barefoot.

ANEXO A – CARTA DE APROVAÇÃO DA COMISSÃO DE ÉTICA NO USO DE ANIMAIS (CEUA)



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Ofício nº120/17

Recife, 11 de dezembro de 2017

Da Comissão de Ética no Uso de Animais (CEUA) da UFPE
Para: Prof.ª Belmira Lara da Silveira Andrade da Costa
Departamento de Fisiologia e Farmacologia
Centro de Biociencias
Universidade Federal de Pernambuco
Processo nº **0035/2017**

Certificamos que a proposta intitulada "**Estresse oxidativo cerebelar em ratos após corridas de elevados volumes e uma dieta rica em licopeno**" registrada com o **nº0035/2017** sob a responsabilidade de Prof.ª **Belmira Lara da Silveira Andrade da Costa** - que envolve a produção, manutenção ou utilização de animais pertencentes ao filo Chordata, subfilo Vertebrata (excetó humanos), para fins de pesquisa científica (ou ensino) - encontra-se de acordo com os preceitos da Lei nº 11.794, de 8 de outubro de 2008, do Decreto nº 6.899, de 15 de julho de 2009, e com as normas editadas pelo CONSELHO NACIONAL DE CONTROLE DE EXPERIMENTAÇÃO ANIMAL (CONCEA), e foi aprovada pela COMISSÃO DE ÉTICA NO USO DE ANIMAIS (CEUA) DA UNIVERSIDADE FEDERAL DE PERNAMBUCO (UFPE), em reunião de 06/12/2017.

Finalidade	() Ensino (X) Pesquisa Científica
Vigência da autorização	04/01/2017 a 04/01/2020
Espécie/linhagem/raça	Ratos heterogenico Wistar
Nº de animais	80
Peso/Idade	60 dias 150g
Sexo	Macho
Origem	Bioterio do Departamento de Fisiologia da UFPE..

Atenciosamente

Prof. Dr. Pedro V. Carelli
Presidente da CEUA / CCB - UFPE
SIAPE 1801584

ANEXO B – CARTA DE SUBMISSÃO FRONTIERS IN MOLECULAR NEUROSCIENCE

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Seg, 30/12/2019 02:33

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We are pleased to inform you that we have received the manuscript "ULTRA-ENDURANCE ASSOCIATED WITH MODERATE EXERCISE IN RATS INDUCES CEREBELLAR OXIDATIVE STRESS AND IMPAIRS REACTIVE GFAP ISOFORM PROFILE" to be considered for publication in Frontiers in Molecular Neuroscience.

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----------MANUSCRIPT DETAILS---------- Manuscript title: ULTRA-ENDURANCE ASSOCIATED WITH MODERATE EXERCISE IN RATS INDUCES CEREBELLAR OXIDATIVE STRESS AND IMPAIRS REACTIVE GFAP ISOFORM PROFILE Manuscript ID: 523509 Authors: Raphael Fabricio De Souza, Ricielle Lopes Augusto, Silvia Regina Arruda de Moraes, Fabio Borges de Souza, Lillian Vanessa da Penha Gonçalvez, Danielle Dutra Pereira, Gisele Machado Magalhães Moreno, Fernanda Maria Araujo de Souza, Belmira Lara Da Silveira Andrade- Da- Costa Journal: Frontiers in Molecular Neuroscience Article type: Original Research Submitted on: 30 Dec 2019

Research Topic: Meeting of the Portuguese Society for Neurosciences SPN2019

----------ADDITIONAL INFORMATION----------

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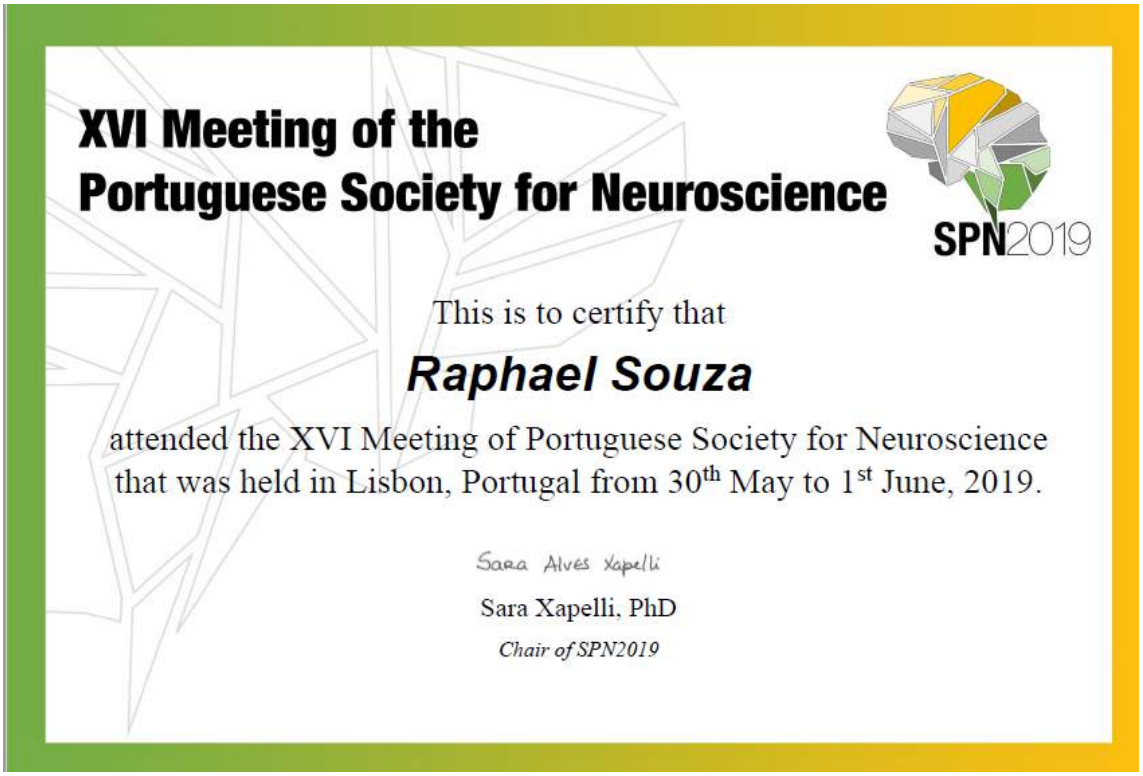
ANEXO C – XVI MEETING OF THE PORTUGUESE SOCIETY FOR NEUROSCIENCE



XVI Meeting of the
Portuguese Society for Neuroscience

SPN2019

This is to certify that

Raphael Souza

attended the XVI Meeting of Portuguese Society for Neuroscience that was held in Lisbon, Portugal from 30th May to 1st June, 2019.

Sara Alves Xapelli

Sara Xapelli, PhD

Chair of SPN2019