RAFAEL MARINS REZENDE

EFEITOS DO EXERCÍCIO AERÓBICO ASSOCIADO À SUPLEMENTAÇÃO COM TRIPTOFANO NO CONTROLE DA DOR EM RATAS WISTAR COM FIBROMIALGIA EXPERIMENTAL

Tese apresentada à Universidade Federal de Viçosa, como parte das exigências do Programa de Pós-Graduação em Ciência da Nutrição, para obtenção do título de Doctor Scientiae.

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APROVADA: 27 de junho de 2019.

Rafael Marins Rezende
Autor

Antônio José Natali
Orientador

“Sim, grandes coisas fez o Senhor por nós,

e por isso estamos alegres”. (Sl 126:3).

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LISTA DE ABREVIATURAS E SIGLAS

5-HIAA: ácido 5-hidroxiindolacético

5-HT: 5-hidroxitriptamina

ANG: Aminoácidos Neutros Grandes

CAT: Catalase

CEA – Coeficiente de Eficácia Alimentar

CEUA – Comissão de Ética no Uso de Animais

CON: Grupo controle

F: Grupo com fibromialgia induzida

FE: Grupo com fibromialgia + exercício

FES: Grupo com fibromialgia + exercício + suplementação com triptofano

FiO_2: Fração Inspirada de Oxigênio

FM: Fibromialgia

FS: Grupo com fibromialgia + suplementação com triptofano

HPA – Hipotalâmico-Pituitário-Adrenal

IL-1: Interleucina-1

IL-6: Interleucina-6

IL-8: Interleucina-8

IMC – Índice de Massa Corporal

HPLC: Cromatografia Líquida de Alta Pressão

LP: Peróxido Lipídico

MDA: Malondealdeído

NA: Noradrenalina

PBS – Solução Salina Tamponada com Fosfato

QUINU: Quinurenina

SOD: Superóxido Dismutase

SP: Substância P

SC: Sensibilização Central

SCP: Substância Cinzenta Periaquedutal

SNA: Sistema Nervoso Autônomo

SNC: Sistema Nervoso Central

TBARS – Ácido Tiobarbitúrico

TNF: Fator de Necrose Tumoral

TRP: Triptofano

TRP-l: Triptofano livre

TTF: Tempo Total de Exercício até a Fadiga

VMC: Velocidade Máxima de Corrida

RESUMO

REZENDE, Rafael Marins, D.Sc., Universidade Federal de Viçosa, junho de 2019. **Efeitos do exercício aeróbico associado à suplementação com triptofano no controle da dor em ratas wistar com fibromialgia experimental.** Orientador: Antônio José Natali.

O objetivo deste estudo foi avaliar os efeitos do exercício aeróbico de intensidade baixa associado à suplementação com triptofano no controle da dor em ratas Wistar com fibromialgia experimental. Ratas Wistar (peso corporal inicial: ~ 350 g; idade: 12 meses) foram divididas aleatoriamente em 5 grupos: CON (Controle); F (Fibromialgia); FE (Fibromialgia mais exercício); FES (Fibromialgia mais exercício e suplementação com triptofano) e FS (Fibromialgia mais suplementação com triptofano). A fibromialgia foi induzida com duas injeções (20 µL) de solução salina ácida (pH 4,0) no músculo gastrocnêmio direito, com intervalo de 3 dias. Os animais controles receberam as mesmas doses de solução salina neutra (pH 7,4). Os animais exercitados foram submetidos a exercício aeróbio progressivo em esteira rolante elétrica (10-12 m / min, 30-45 min / dia, 5 dias / semana) por três semanas. Durante esse período, os animais suplementados receberam dieta suplementada com triptofano (210 g / semana), enquanto os demais receberam dieta controle. A hiperalgesia mecânica foi avaliada pré-injeção, pós-injeção e ao final da terceira semana. Após três semanas de intervenções foram avaliados: 1) as concentrações séricas de cortisol e musculares de IL-6 e TNF; 2) a atividade antioxidante da superóxido dismutase (SOD) e da catalase (CAT), bem como as concentrações hepáticas do malondealdeído (MDA) e séricas de ureia e creatinina; 3) as concentrações cerebrais de triptofano, serotonina (5-HT) e quinureninas (QUINU), além das concentrações séricas de substância P. A fibromialgia experimental causou hiperalgesia bilateral da pata traseira, aumentou as concentrações séricas de cortisol e musculares de IL-6, reduziu a atividade de SOD (37,88 %) e CAT (30,50 %) no fígado, mas não influenciou o metabolismo do triptofano cerebral. Após três semanas de intervenção, o exercício aplicado reduziu a hiperalgesia mecânica bilateral (~151%) e as concentrações séricas de cortisol (72%), mas não alterou a atividade de SOD e CAT e as concentrações de MDA hepáticas, bem como as concentrações cerebrais de triptofano e 5-HT entre os animais com fibromialgia experimental. A suplementação com triptofano reduziu a hiperalgesia mecânica bilateral (~67%), reduziu as concentrações séricas de cortisol (67%), influenciou a redução das concentrações de MDA e aumentou a concentração cerebral

de triptofano entre os animais com fibromialgia. A combinação do exercício aeróbico com a suplementação com triptofano reduziu a hiperalgesia bilateral (162%), reduziu as concentrações musculares de IL-6 (68%) e as concentrações séricas de cortisol (54%), mas não afetou a atividade de SOD e CAT e as concentrações de MDA hepáticas, bem como a concentração cerebral do triptofano. A suplementação com triptofano aumentou as concentrações cerebrais de QUINU e a atividade inferida da indolamina 2,3 dioxigenase (IDO) e reduziu a razão 5-HT/QUINU. As intervenções não alteraram as concentrações séricas de substância P ao final das três semanas de intervenções. Em conjunto, os achados deste estudo indicam que a associação da suplementação com triptofano ao exercício aeróbico de intensidade baixa não potencializa a redução da hiperalgesia mecânica bilateral promovida pelo exercício neste modelo de fibromialgia experimental, entretanto uma importante redução em IL-6 é evidente.

ABSTRACT

REZENDE, Rafael Marins, D.Sc., Universidade Federal de Viçosa, June, 2019. **Effects of aerobic exercise associated with tryptophan supplementation on pain control in wistar rats with experimental fibromyalgia.** Adviser: Antônio José Natali.

The objective of this study was to evaluate the effects of low-intensity aerobic exercise associated with tryptophan supplementation on pain control in experimental wistar rats with experimental fibromyalgia. Female Wistar rats (initial body weight: ~ 350 g; age: 12 months) were randomly divided into 5 groups: CON (Control); F (Fibromyalgia induced); FE (Fibromyalgia induced plus exercise); FES (Fibromyalgia induced plus exercise and tryptophan supplementation) and FS (Fibromyalgia induced plus tryptophan supplementation). Fibromyalgia was induced with two injections (20 µL) of acidic saline (pH 4.0) into the right gastrocnemius muscle with a 3-day interval. Control animals received the same doses of neutral saline (pH 7.4). The exercised animals underwent progressive low-intensity aerobic exercise on a treadmill (10-12 m/min, 30-45 min/day, 5 days/week) for three weeks. During this period, the supplemented animals received a tryptophan supplemented diet (210 g/week), while the others received a control diet. The mechanical hyperalgesia was evaluated pre-injection, post-injection and at the end of the third week. After three weeks of interventions, we evaluated: 1) serum cortisol and muscle concentrations of IL-6 and TNF; 2) the antioxidant activity of superoxide dismutase (SOD) and catalase (CAT), as well as hepatic concentrations of malondealdehyde (MDA) and serum levels of urea and creatinine; 3) cerebral concentrations of tryptophan, serotonin (5-HT) and kynurenines (KYN), in addition to serum concentrations of substance P. The experimental fibromyalgia caused bilateral hind paw hyperalgesia, increased serum cortisol and IL-6 concentrations, reduced SOD (37.88%) and CAT (30.50%) activity in the liver, but did not influence metabolism of the cerebral tryptophan. After three weeks of intervention, LIAE reduced bilateral mechanical hyperalgesia (~ 151%) and serum cortisol concentrations (72%), but did not alter SOD and CAT activity and concentrations of hepatic MDA as well as cerebral concentrations of tryptophan and 5-HT among animals with experimental fibromyalgia. Exclusive supplementation of tryptophan reduced bilateral mechanical hyperalgesia (~ 67%), reduced serum cortisol concentrations (67%), influenced the reduction of MDA concentrations, and increased cerebral concentration of tryptophan among animals with fibromyalgia. The combination of

aerobic exercise with tryptophan supplementation reduced bilateral hyperalgesia (162%), reduced muscle concentration of IL-6 (68%) and serum cortisol concentrations (54%) but did not affect SOD and CAT activity and concentrations of hepatic MDA as well as brain concentration of tryptophan. Supplementation with tryptophan increased the brain concentrations of KYN, the inferred indoleamine 2,3-dioxygenase (IDO) activity and reduced the 5-HT / KYN ratio. Interventions also did not change the serum substance P concentrations at the end of the three weeks of interventions. Taken together, the findings of this study indicate that the association of tryptophan supplementation with the aerobic exercise does not potentiate the reduction of bilateral mechanical hyperalgesia promoted by the low-intensity aerobic exercise in this experimental fibromyalgia model, however an important decrease in IL-6 is evident.

1. INTRODUÇÃO

A percepção dolorosa é tipicamente associada à resposta biológica dos sistemas somatossensorial, imunológico, neuronal, autonômico e vascular / circulatório a danos, patógenos ou irritantes [1]. As vias de modulação da dor integram um sistema sensorial complexo de proteção em resposta a estímulos nocivos. Estes estímulos são transmitidos a partir de receptores nociceptivos por fibras neuronais aferentes Aδ e C que se projetam do corno dorsal da medula espinhal e ascendem pelo trato espinotalâmico lateral ao núcleo ventral póstero-lateral do tálamo [2,3]. A informação nociceptiva é, em seguida, transmitida ao córtex somatossensorial, à substância cinzenta periaquedutal [3,4] e a outras áreas do cérebro envolvidas com a memória e aspectos afetivos relacionados à dor, como a amígdala, o hipotálamo e o núcleo accumbens [5]. Neurotransmissores como o glutamato, a calcitonina e a substância P estão diretamente envolvidos na transdução do sinal doloroso [2]. As vias neuronais envolvidas na modulação descendente da dor originam-se, principalmente, no hipotálamo, amígdala e córtex cingulado anterior com projeções para a substância cinzenta periaquedutal e para os núcleos do tronco cerebral, como o locus coeruleus e a medula ventromedial rostral. As vias descendentes que se projetam para a medula espinhal incluem, entre outras, fibras noradrenérgicas, serotoninérgicas e dopaminérgicas [6].

Alterações adaptativas na plasticidade neuronal do sistema nervoso central (SNC) têm sido associadas à fisiopatologia das síndromes dolorosas crônicas [7,8]. As más adaptações plásticas das vias e circuitos de codificação da dor podem causar sensibilização central do sistema nociceptivo, resultando em hipersensibilidade dolorosa e persistente [9]. A sensibilização central pode ser definida como o aumento da responsividade dos neurônios nociceptivos do SNC a estímulos aferentes normais

[10], tendo como possíveis fatores causais a desregulação do sistema imune, do eixo hipotalâmico-pituitário-adrenal (HPA) e do sistema nervoso autônomo [11]. Diversas síndromes dolorosas crônicas tais como, síndrome da fadiga crônica, síndrome do intestino irritável e fibromialgia têm sido associadas à sensibilização central [12].

A fibromialgia é uma síndrome caracterizada por dor crônica difusa, fadiga, ansiedade, distúrbios do sono, cognição e humor [13]. Está presente em 2 a 5% da população geral, particularmente em mulheres entre 50 e 80 anos de idade [14,15]. Pacientes com formas moderadas e graves de fibromialgia frequentemente relatam níveis de incapacidade e má qualidade de vida, além da necessidade de tratamentos médicos contínuos [16]. O diagnóstico da fibromialgia é puramente clínico e é confirmado pela presença de dor à palpação digital de 11 dos 18 pontos específicos do tecido mole, chamados “tenderpoints” [17], e pelos critérios preliminares de diagnósticos definidos pelo Colégio Americano de Reumatologia, os quais incluem a avaliação do estado de fadiga, a qualidade do sono, o estado cognitivo e a presença de outros sintomas associados [18,19].

Sua patofisiologia, ainda longe de ser completamente elucidada, tem sido relacionada a anormalidades neuroendócrinas envolvendo o principal sistema modulador de estresse no corpo, o eixo hipotalâmico-pituitário-adrenal (HPA), e a déficits nos sistemas endógenos de modulação da dor [20-22]. Evidências recentes também têm associado o aumento do estresse oxidativo à etiologia da dor musculoesquelética, um dos principais sintomas ligados à fibromialgia [23-25].

Os sintomas da fibromialgia têm sido diretamente relacionados à incapacidade do eixo HPA em modular o estresse agudo e crônico [26]. O estresse estimula a liberação de hormônio liberador de corticotrofina pelo hipotálamo que, por sua vez, estimula a glândula pituitária a liberar o hormônio adrenocorticotrófico [27]. O aumento

das concentrações plasmáticas do hormônio adrenocorticotrófico estimula a liberação de cortisol pela glândula adrenal. Além disso, o estresse estimula a liberação da isoleucina 1β (IL-1β) no cérebro que inicia a liberação do fator de necrose tumoral (TNF) e ativa diretamente o eixo HPA, culminando na liberação de mais cortisol o qual estimula a produção de isoleucina-6 (IL-6) [28]. As elevadas concentrações séricas de cortisol parecem exacerbar a dor musculoesquelética em pacientes com fibromialgia e potencializar os efeitos pronociceptivos de citocinas inflamatórias como IL-6 e TNF [26,29].

Neste contexto, as citocinas inflamatórias parecem ter um importante papel na patogênese da fibromialgia, em particular as citocinas pró-inflamatórias IL-6 e TNF [30,31]. Para Dina et al. [30-32], a administração intramuscular de IL-6 e TNF induz hiperalgesia musculoesquelética a partir de alterações no limiar nociceptivo de animais com dor crônica difusa experimental. Algumas citocinas, tais como TNF, IL-1, IL-6, e IL-8 também podem estar envolvidas na regulação do eixo HPA e do sistema nervoso simpático, e associadas a sintomas da fibromialgia, tais como fadiga, dor, sono e respostas ao estresse [33]. Estudos prévios [34,35] demonstraram que pacientes com fibromialgia possuem altos níveis circulantes de IL-8, cortisol e noradrenalina que, juntos, aumentam a liberação de citocinas pró-inflamatórias pelos monócitos.

No que se refere às alterações dos sistemas endógenos de modulação da dor, baixas concentrações cerebrais de serotonina e elevadas concentrações de substância P, neurotransmissores inibitórios e excitatórios do SNC, respectivamente, podem estar diretamente relacionados à sensibilização central e ao desenvolvimento da hiperalgesia observada em pacientes com fibromialgia [36-38]. A serotonina (5-hidroxitriptamina: 5-HT) é um neurotransmissor do sistema nervoso central e, tradicionalmente, é conhecida por influenciar uma variedade de comportamentos,

funções fisiológicas e cognitivas [39]. A serotonina possui importante função na modulação da dor atuando como um neurotransmissor antinociceptivo nos tratos descendentes localizados no funículo dorsolateral da medula espinhal [40]. O efeito antinociceptivo da serotonina parece ocorrer pela supressão da produção de substância P, um neurotransmissor nociceptivo que atua nos receptores de neurocinina-1 localizados no corno dorsal [41]. Neste sentido, a redução das concentrações cerebrais de serotonina parece estar diretamente ligada à perda da capacidade de modulação serotoninérgica das vias descendentes resultando na sintomatologia dolorosa frequentemente observada na fibromialgia [40].

Em relação ao papel do aumento do estresse oxidativo na patofisiologia da fibromialgia, estudos têm demonstrado que pacientes com fibromialgia apresentam aumento das concentrações plasmáticas dos biomarcadores de dano oxidativo, principalmente de peroxidação lipídica e proteínas carboniladas, além da diminuição de enzimas antioxidantes tais como a superóxido dismutase (SOD) e a catalase (CAT) [42,43]. Estes marcadores de estresse oxidativo têm sido associados à etiologia da dor musculoesquelética, um dos principais sintomas ligados à fibromialgia [44,45]. Em condições normais, há um equilíbrio entre espécies reativas de oxigênio (ROS) e antioxidantes dentro da célula, nas membranas e no espaço extracelular. Por outro lado, em situações de desequilíbrio oxidativo / antioxidante, os ataques das ROS a ácidos graxos poliinsaturados em lipídios de membrana levam à peroxidação lipídica, perda da fluidez da membrana, alterações nos potenciais de membrana e, eventualmente, ruptura que resulta em liberação de conteúdo celular e organelas [46]. Tais danos oxidativos podem levar a disfunções celulares que contribuem para a fisiopatologia de várias doenças, incluindo a fibromialgia [47].

Neste contexto, o foco do tratamento do paciente com fibromialgia é, atualmente, a dor e as comorbidades como a depressão e a ansiedade. De acordo com Heymann et al. [48], o tratamento farmacológico da fibromialgia passa pela administração de relaxantes musculares, analgésicos, anti-inflamatórios e antidepressivos. Entretanto, terapias não farmacológicas também têm sido recomendadas como coadjuvantes no tratamento da fibromialgia.

Exercícios aeróbicos de intensidade baixa à moderada, por exemplo, são recomendados por reduzirem a concentração sistêmica de biomarcadores de estresse (ex., cortisol e noradrenalina) e inflamação (citocinas) [35,49], além de contribuírem com a redução do estresse oxidativo em pacientes com fibromialgia [50,51]. Adicionalmente, a suplementação com triptofano também tem sido recomendada, uma vez que, baixas concentrações séricas de triptofano foram associadas a condições como síndrome da fadiga crônica e fibromialgia [52,53]. O triptofano tem sido utilizado na tentativa de aumentar sua disponibilidade cerebral, o que potencializaria as ações da serotonina [54-56]. Existem duas vias principais envolvidas no metabolismo do triptofano e que resultam na formação de dois metabólitos diferentes, a serotonina (5-HT) e quinurenina (QUINU). A degradação do 5-HT forma o ácido 5-hidroxiindolacético (5-HIAA), enquanto a QUINU é posteriormente convertida, pela ação da enzima indolamina 2,3 dioxigenase (IDO), em metabólitos neuroativos, como o ácido quinolínico [57]. Evidências suportam a existência de uma relação inversa entre os níveis de triptofano, 5-HT ou 5-HIAA e as medidas clínicas de dor, bem como uma correlação positiva entre os níveis de QUINU e a percepção da dor [58-60].

Como a serotonina é responsável por estimular o eixo HPA em resposta ao estresse [61], o triptofano melhoraria a função da serotonina cerebral e reduziria a liberação de cortisol em situações de estresse. De fato, tem sido demonstrado que a

suplementação de triptofano pode reduzir a liberação de cortisol em pacientes propensos ao estresse [62,63] e beneficiar o tratamento da dor em síndromes de dor aguda e crônica [64,65]. Além disso, a suplementação com tem sido associada à redução de biomarcadores de dano oxidativo, como o MDA, e ao aumento da atividade antioxidante (ex., SOD e CAT), atenuando, assim, os efeitos do estresse oxidativo [66,67].

Por fim, considerando que os efeitos da associação do exercício aeróbico de intensidade baixa à suplementação com triptofano no tratamento da fibromialgia ainda são desconhecidos, este estudo foi desenhado para verificar os efeitos do exercício aeróbico de intensidade baixa associado à suplementação com triptofano no controle da dor em ratas com fibromialgia experimental.

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3. OBJETIVOS

3.1 Objetivo Geral

Verificar a influência do exercício aeróbico de intensidade baixa associado à suplementação com triptofano no controle da dor em ratas com fibromialgia experimental.

3.2 Objetivos Específicos

Analisar os efeitos do exercício aeróbico de intensidade baixa associado à suplementação com triptofano nos seguintes parâmetros de ratas com fibromialgia experimental:

- ✓ Hiperalgesia mecânica bilateral;
- ✓ Concentração sérica de cortisol e muscular de IL-6 e TNF;
- ✓ Atividade antioxidante de SOD e CAT bem como a concentração de MDA no fígado;
- ✓ Concentrações séricas de ureia e creatinina;
- ✓ Concentração cerebral de triptofano, 5-HT e QUINU;
- ✓ Concentração sérica de substância P;

4. RESULTADOS

Em consonância com as normas vigentes no regimento interno do Programa de Pós-Graduação em Ciência da Nutrição, em seu artigo 36 §2º, a apresentação dos resultados obtidos, bem como a discussão dos mesmos, dar-se-ão em forma de artigos científicos.

ARTIGO 1 (Original): Does aerobic exercise associated with tryptophan supplementation attenuates hyperalgesia and inflammation in female rats with experimental fibromyalgia?

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ARTIGO 2 (Original): Effects of aerobic exercise associated with tryptophan supplementation on hyperalgesia and oxidative stress in female rats with fibromyalgia.

ARTIGO 3 (Original): Serotoninergic modulation of hyperalgesia in the acidic saline model of fibromyalgia: effects of aerobic exercise associated with tryptophan supplementation.

ARTIGO 1

Does Aerobic Exercise Associated with Tryptophan Supplementation Attenuates Hyperalgesia and Inflammation in Female Rats with Experimental Fibromyalgia?

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Abstract

The objective of this study was to verify the effects of aerobic exercise associated with tryptophan (TRP) supplementation on hyperalgesia, as well as on cortisol, IL-6 and TNF concentrations in female rats with experimental fibromyalgia (FM). Female Wistar rats (initial body weight: ~ 350 g; age: 12 months) were randomly divided into 5 groups: CON (Control); F (Fibromyalgia induced); FE (Fibromyalgia induced plus exercise); FES (Fibromyalgia induced plus exercise and TRP supplementation) and FS (Fibromyalgia induced plus TRP supplementation). Fibromyalgia was induced with two injections (20 µL) of acidic saline (pH 4.0) into the right gastrocnemius muscle with a 3-day interval. Control animals received the same doses of neutral saline (pH 7.4). The exercised animals underwent progressive low-intensity aerobic exercise (LIAE) on a treadmill (10-12 m/min, 30-45 min/day, 5 days/week) for three weeks. During this period, the supplemented animals received a TRP supplemented diet (210 g/week), while the others received a control diet. Mechanical hyperalgesia was evaluated weekly and serum cortisol and muscle IL-6 and TNF concentrations were assessed after three weeks of interventions. Experimental FM caused bilateral hind paw hyperalgesia and augmented serum cortisol and muscle IL-6 concentrations. After 3 weeks of interventions, LIAE alone reduced hyperalgesia (151 %) and reduced serum cortisol concentrations (72 %). Tryptophan supplementation itself diminished hyperalgesia (57 %) and reduced serum cortisol concentrations (67 %). Adding TRP supplementation to LIAE did not further reduce hyperalgesia significantly (11 %), which was followed by an important decrease in muscle IL-6 concentrations (68 %), though reduction in serum cortisol pulled back to 54 %. Muscle TNF concentrations were not affected. In conclusion, the association of TRP supplementation to LIAE does not potentiate significantly the reduction of bilateral

mechanical hyperalgesia promoted by LIAE in female rats with experimental FM, however an important decrease in IL-6 is evident.

Introduction

Fibromyalgia (FM) is a syndrome characterized by chronic widespread pain, fatigue, anxiety and sleep, cognitive and mood disorders [1]. It is present in 2 to 5% of the general population, particularly in women between 50 and 80 years of age [2,3]. Fibromyalgia has been linked to neuroendocrine abnormalities, involving the main stress modulator system, the hypothalamic-pituitary-adrenal (HPA) axis, and "deficits" in endogenous pain modulation systems [4-6].

Women with FM exhibited high circulating levels of cortisol and noradrenaline as well as increased release of proinflammatory cytokines (i.e. IL-1β, TNFα, IL-6, IL-18) by monocytes [7, 8]. Thus, cytokines such as IL-6 and TNFα appear to be involved in the regulation of the HPA axis and the sympathetic nervous system related to the painful symptomatology in FM [9]. For example, intramuscular administration of IL-6 and TNFα induces musculoskeletal hyperalgesia in animals with chronic widespread experimental pain [10,11], which indicates that cytokines may play an important role in the pathogenesis of FM.

Non-pharmacological therapies have been recommenced for the management of FM. For example, exercise is strongly recommended as it promotes improvements in pain [12-15]. In his sense, low to moderate-intensity aerobic exercises are recommended [16] and moderate-intensity aerobic exercise is reported to reduce the systemic concentration of biomarkers indicative of stress (e.g., cortisol and noradrenaline) and inflammation (e.g. cytokines) in patients with FM [17]. Tryptophan (TRP) supplementation has also been used in the management of FM and other painful

syndromes, inasmuch as low serum concentrations of TRP have been associated with conditions such as chronic fatigue syndrome and FM [18,19]. Tryptophan supplementation has been used in an attempt to increase its cerebral availability, which would potentiate serotonin actions [20, 21, 22]. Since serotonin is responsible for stimulating the HPA axis in response to stress [23], TRP would improve the function of cerebral serotonin and reduce the release of cortisol in stressful situations. Indeed, TRP supplementation has been shown to reduce cortisol release in stress-prone patients [24,25] and to benefit the treatment of pain in acute and chronic pain syndromes [26, 27].

Nevertheless, the benefits of the association of aerobic exercise with TRP supplementation in the management of FM are not known. Therefore, this study was designed to verify the effects of aerobic exercise associated with TRP supplementation on hyperalgesia, as well as on cortisol, IL-6 and TNF concentrations in female rats with experimental FM.

Materials and methods

Animals and experimental procedures

The experimental procedures were approved by the Ethics Committee for Animal Use of the Federal University of Viçosa (process number 21/2015) and were conducted according to the Guide for the Care and Use of Laboratory Animals (2011), National Academy of Sciences (US).

Twelve-month old female adult Wistar rats (initial body weight: ~ 350 g) from the Central Biotherm of the Biological Center for Health Sciences at the Federal University of Viçosa (UFV) were randomly assigned to groups of 8 rats each: CON (Control); F (Fibromyalgia induced); FE (Fibromyalgia induced plus exercise); FES (Fibromyalgia

induced plus exercise and TRP supplementation) and FS (Fibromyalgia induced plus TRP supplementation). During the experiment, animals were kept in individual cages in a temperature-controlled room (22 ± 2 ℃), with a light/dark cycle of 12/1 2 hours and had free access to water and diet.

After FM induction, the rats of the exercised groups were submitted to three weeks of low-intensity aerobic exercise, while those of the supplemented groups received a TRP supplemented diet. The body weight of the animals was measured once a week (on Fridays) during the experiment.

Fibromyalgia Induction

Repeated intramuscular injections of acidic saline are known to mimic the conditions of chronic and widespread pain [28]. The first injection produces transient hyperalgesia that decreases after 24h, and the second, administered 3 days later, promotes bilateral hyperalgesia for more than 4 weeks [28, 29]. Thus, the rats from F, FE, FES and FS groups received two unilateral intramuscular injections containing 20 µL of acidic saline (pH 4.0) in the right gastrocnemius muscle, while animals from CON groups received injections containing 20 µL of neutral saline (pH 7.4), with a 3-day interval between injections. For these fibromyalgia induction procedures, the animals were maintained under surgical sedation with isoflurane, sprayed with 100% FiO_2, in an avalvular circuit and spontaneous breathing.

Measurement of mechanical hyperalgesia

The mechanical hyperalgesia was measured following a protocol described previously [30]. In brief, three animals at a time were housed in individual boxes with wire-mesh floor and acrylic walls, which were placed on a raised platform. After 30 minutes of adaptation, a mechanical stimulus of increasing pressure (expressed in grams) was applied to the plantar surface of the right and left hind paws of each rat,

using an electronic Von Frey apparatus (Insight, Ribeirão Preto - SP , Brazil). The mechanical stimulus was applied alternate 5 times to each hind paw of each animal in the pre-injection period and 3 times in the post-injection. Such stimuli were applied alternate to the left paw of each of the 3 rats and then to the left paw in the same order, so that each animal had and interval of approximately 30 seconds to receive the next stimulus to the contralateral hind paws. Pressure values were recorded by observing behaviors in nociceptive responses such as paw withdrawal, paw licking or jumping with all four legs. The withdrawal threshold in the pre-injection period was determined by calculating the median of the 5 measurements and then calculating how much each value deviated from the median. The 3 values that deviated less from the median were used to determine the mean and obtain the threshold value. The withdrawal thresholds in the post-injection period were determined by the mean of 3 consecutive pressure measurements. All measurements were taken in a quiet, temperature-controlled room at the same time of the day (8 to 10 a.m.), once a week (on Wednesdays). The same evaluator did all the tests blind for the treatments and the recorded pressure values. Mechanical hyperalgesia was measured before FM induction (pre-injection), 24 hours after the second injection of both acidic or neutral saline, and once a week for the following three weeks.

Running velocity test and aerobic exercise training protocols

To determine the running speed (i.e. exercise intensity) during the exercise training sessions, the animals were submitted to a maximal running velocity (MRV) test on a treadmill (AVS, São Paulo-SP, Brazil), based on the total exercise time until fatigue (TTF) test, as previously described [31]. In summary, one week before the test the animals were adapted to the treadmill, on five consecutive days (5 min/day, 5% tilt up) and daily increases in treadmill velocity (8, 10, 11, 12 m/min). After the adaptation week,

each animal performed three tests of progressive exercise until fatigue on three alternate days. During the tests, the initial velocity of the treadmill was 10 m/min (5% tilt up), being increased by 1 m/min every 3 minutes until fatigue which was defined when the animal could no longer keep the treadmill pace, when the test was interrupted. The mean MRV obtained by the animals in the TTF tests was calculated and established as a reference (i.e. 100 % of MRV) to determine the exercise intensity during the exercise training sessions.

The aerobic exercise training was conducted on an electric-driven treadmill five times a week (Monday to Friday) for three consecutive weeks (Adapted from Sharma et al. [32]). The duration and intensity of exercise were gradually increased over the 3-week period. During the first week, the animals ran at 10 m/min (50 % of the MRV), for 30 minutes. Then, on the second week, the animals ran at 11 m/min (55 % of the MRV), for 40 minutes. And during the third week the running speed was 12 m/min (60 % of the MRV), for 45 minutes. The exercise protocol included 2 minutes of warm-up (5 to 8 m/min) and 2 minutes of cooling down (5 to 8 m/min) within the total time of the running session. No incentive was used during the running.

Diets and supplementation

The control and supplemented diets were purified and packed in pellets (RHOSTER, São Paulo, Brazil). The animals had free access to diet and water being the consumption ad libitum during the experimental period (3 weeks). Diets (210 g/week) were supplied twice a week (on Tuesdays' and Thursdays' in the morning). The animal home cage was cleaned every 2 days. On these days the leftovers in the feeder of each animal were separated and weighed to calculate weight gain, food consumption and food efficiency coefficient (FEC) individually.

The composition and profile of the amino acids of the diets are described in Table 1. The composition of the diets was based on the American Institute of Nutrition (AIN-93M) adult rodent maintenance diet [33] and adapted from the increase in concentration of TRP to the typical amino acid profile of casein (RHOSTER, São Paulo, Brazil).

Table 1. Composition and typical amino acid profile of control and experimental diets.

Nutrient	AIN-93M Diet	Experimental Diet
Composition	**g/Kg diet**	**g/Kg diet**
Casein (>85%protein)	140	140
Sucrose	100	100
Cornstarch	465,6	465,6
Dextrinized cornstarch	155	155
Fiber	50	50
Mineral mix	35	35
Vitamin mix	10	10
L-cystine	1,8	1,8
Choline bitartrate	2,5	2,5
Soybean oil	4	4
Amino acid profile	**g/Kg diet**	**g/Kg diet**
Isoleucine	9,0	8,2
Leucine	10,9	10,6
Phenylalanine	8,5	8,0
Tyrosine	7,4	7,5
Valine	10,5	9,9
Tryptophan	2,5	7,6
TRP/LNAA (%)	5,4	15,32

TRP**/**LNAA, the ratio of tryptophan to the sum of the other large neutral amino acids. Reeves et al. (1993).

The typical amino acid profile estimated for the diets was analyzed by HPLC (CBO Análises Laboratoriais, Valinhos-SP, Brazil). The used TRP concentration was based on previous studies on protein supplementation enriched with alpha-lactalbumin, and its repercussion on plasma and brain concentrations of TRP and serotonin, respectively [20,21,22].

Sample collection

At the end of the experimental period, 48 hours after the last exercise session, the animals were euthanized by decapitation in a clean room without strange noises. Euthanasia occurred on different days for different groups, but always occurred in the morning (8 am to 10 am). Immediately after decapitation, blood was collected by total exsanguination in separator gel tubes, which were subsequently centrifuged at 704 g (model Z216MK, Hermle, Germany) for 10 minutes. Serum was withdrawn and stored at -80 ℃ for cortisol analyzes. The gastrocnemius muscle of the right limb was dissected, washed in cooled saline, frozen on dry ice and stored at -80 ℃ for the quantification of IL-6 and TNF cytokine concentrations.

Body weight and weight gain determination, food consumption, food efficiency coefficient (FEC)

The animals were weighed (Mettler Toledo, Brazil) once a week (Thursday's mornings) during the experiment. The weight gain of each animal was calculated based on the equation: final weight - initial weight. The food consumption of each rat was evaluated weekly based on the amount of diet added minus the rest in the feeder, as described above. Food efficiency coefficient of each rat was calculated based on the ratio between animal weight gain and dietary intake.

Determination of serum cortisol concentrations

Analysis of serum cortisol concentrations was performed in duplicate by ELISA (Enzyme-linked Immunosorbent Assay) using a commercial kit (Kit Cat. # EIA1887-CTS-Lot EIA 1887, MARBURG-German), as recommended by the manufacturer.

Determination of muscle concentrations of IL-6 and TNF

The analyses of the muscle concentrations of IL-6 and TNF were performed using the multiplex immunoassay at the Specialized Laboratory in Scientific Analyzes (LEAC, São Paulo - Brazil). Muscle tissue (100 mg; gastrocnemius) was homogenized with 50 mM phosphate buffer (pH 7.0) and added with Tween-20 (0.05%) and aprotinin (5 mg/ml). The samples were then centrifuged at 8000 g (model Z216MK, Hermle, Germany) at 4 ℃ for 5 minutes and the supernatants were removed. The xMAP (MAP = Multiple Analyte Profiling) technique was used, in which magnetic microspheres are stained with two different spectral fluorochromes. Each sphere has a "signature" based on "color code". Thus, the analyte binds, by means of non-reversible covalent bonds, to the capture antibodies located on the surface of the microspheres. Detection is done by means of a third fluorescent marker (phycoerythrin), bound to the detection antibody. The RECYTMAG-65K-02 kit (IL-6 and TNF, Millipore, USA) was used that uses these microspheres as the base of the multiplex immunoassay and doses all cytokines simultaneously. In this way, each bead is conjugated to a specific analyte antibody and read on the Luminex (manufacturer) equipment using a dual lasers system. A laser beam detects the microsphere (test specific color code) and the other laser quantifies the reporter signal in each microsphere. The minimum detection concentrations of this kit are 30.6 pg/mL for IL-6 and 1.9 pg/mL for TNF.

Statistical analysis

Data were submitted to the Kolmogorov-Smirnov normality test. Data for body weight, weight gain, food consumption, FEC and cortisol, IL-6 and TNF concentrations were compared using one-way analysis of variance (ANOVA), followed by Tukey's post-hoc test for multiple comparisons. Data for mechanical hyperalgesia were compared using two-way ANOVA repeated measures, followed Bonferroni's post-hoc

tests for multiple comparisons. Data were analyzed using SPSS (IBM SPSS Statistics for Windows, Version 19.0. Armonk, NY: IBM Corp) and a p value of < 0.05 was considered significant. Results are expressed as means ± standard deviation (SD).

Results

Body weight, weight gain, food consumption and food efficiency coefficient (FEC)

The initial body weight was not different between the experimental groups (Table 2). At the end of three weeks of interventions, body weight did not differ between groups. Likewise, the FEC was not different between the experimental groups.

Table 2. Body weight, weight gain, food consumption and coefficient of food efficacy.

Parameters	CON	F	FE	FES	FS	p value
Initial weight (g)	310,12 ± 12,96	298,25 ± 20,88	318,29 ± 19,72	293,14 ± 16,41	307,0 ± 20,17	>0,05
Final weight (g)	318,62 ± 17,18	310,75 ± 23,81	324,57 ± 24,50	298,28 ± 21,73	318,11± 24,63	>0,05
Weight gain (g)	8,5 ± 11,91	12,5 ± 11,75	6,28 ± 9,10	5,14 ± 14,78	11,11± 14,86	>0,05
Food consumption (g)	278,0 ± 42,44	279,75 ± 33,49	289,0 ± 47,51	236,66 ± 42,03	239,50± 40,31	>0,05
CFE (%)	2,90 ± 3,80	4,50 ± 3,90	1,90 ± 2,60	2,0 ± 6,40	3,11 ± 4,50	>0,05

FEC: food efficacy coefficient; CON, control; F, fibromyalgia induced; FE, fibromyalgia induced plus exercise; FES, fibromyalgia induced plus exercise and TRP supplementation; FS, fibromyalgia induced plus TRP supplementation. Data are means ± SD of 8 animals in each group. ANOVA one-way followed by Tukey.

Mechanical hyperalgesia

Data presented Fig 1 refers to effects of interventions (i.e. aerobic exercise and supplementation) either alone or in combination on mechanical hyperalgesia at different moments.

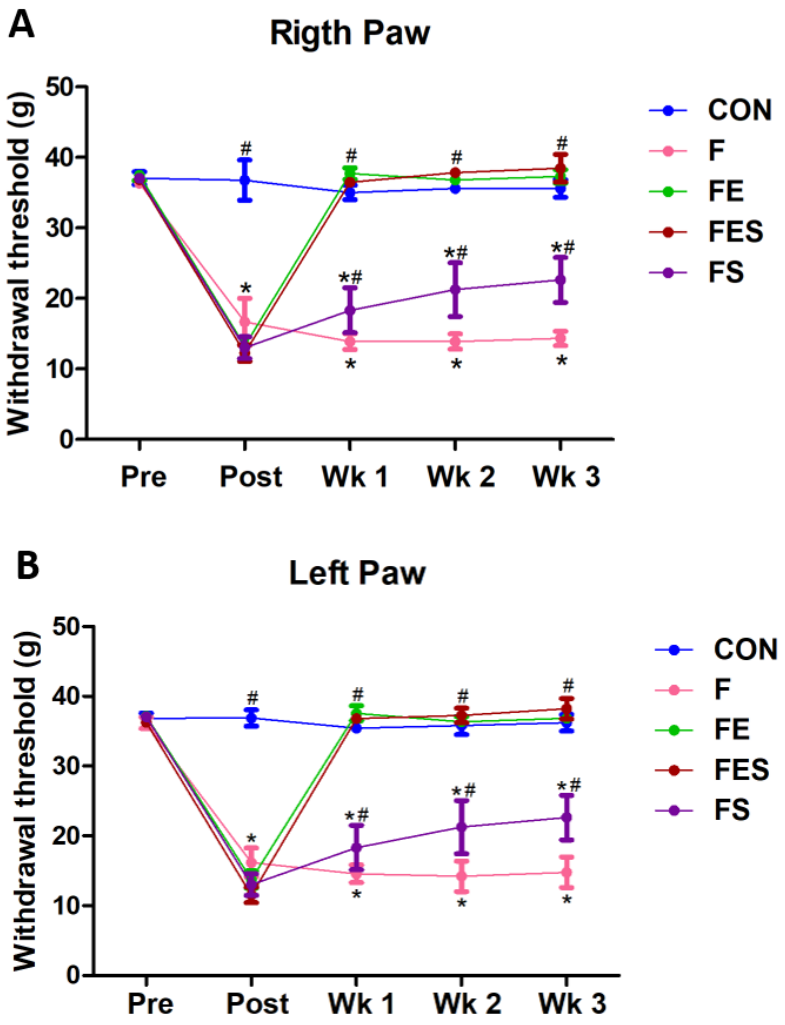

Fig 1. Mechanical hyperalgesia.

(A) Withdrawal threshold of right paw. (B) Withdrawal threshold of left paw. CON, control; F, fibromyalgia induced; FE, fibromyalgia induced plus exercise; FES, fibromyalgia induced plus exercise and TRP supplementation; FS, fibromyalgia induced plus TRP supplementation; PRE, pre-injection; POST, post-injection. The data are means ± SD from 8 animals in each group. * $p<0.05$ vs CON group. $^{\#}$ $p<0.05$ vs F group (ANOVA two-way repeated measures, followed by Bonferroni post hoc test).

The withdrawal thresholds remained unchanged ($p > 0.05$) in both right and left paws in the CON group for all measurements (Fig 1A and Fig 1B), which indicates that neutral saline did not influence the mechanical sensitivity of these animals. However, at the post-injection moment the animals from F, FE, FES and FS groups exhibited decreased withdrawal threshold in both right and left paws ($p <0.05$), when compared to those from CON group, which demonstrates the effectiveness of acidic saline in inducing bilateral hyperalgesia.

Concerning the effects of interventions (Fig 1A and Fig 1B), exercise training (F vs FE) increased ($p < 0.05$) withdrawal threshold on both hind paws (i.e. reduced bilateral mechanical hyperalgesia) to control levels at weeks 1 (~ 162 %), 2 (~ 157 %) and 3 (~ 151 %), while TRP supplementation (F vs FS) also reduced ($p < 0.05$) bilateral hyperalgesia to lesser extents at weeks 1 (~ 26 %), 2 (~ 49 %) and 3 (~ 67 %). However, when treatments were combined (F vs FES) the bilateral hyperalgesia returned to control levels being the reductions respectively 156 %, 166 % and 162 % at weeks 1, 2 and 3.

Serum cortisol concentrations

The results presented in Fig 2 refer to the cumulative effects of interventions (i.e. aerobic exercise and supplementation) either alone or in combination over three weeks on serum cortisol. Serum cortisol concentrations were higher ($p <0.05$) in animals from F group when compared to those from CON group, which indicates that the stress induced by acidic saline was sufficient to increase serum concentrations of serum cortisol in rats.

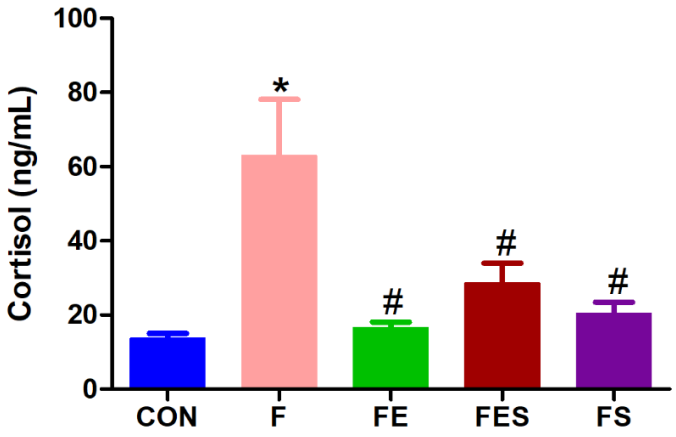

Fig 2. Serum cortisol concentrations.

CON, control; F, fibromyalgia induced; FE, fibromyalgia induced plus exercise; FES, fibromyalgia induced plus exercise and TRP supplementation; FS, fibromyalgia induced plus TRP supplementation. Data are means ± SD from 6 to 8 animals in each group. * $p<0.05$ vs CON group. # $p<0.05$ vs F group (ANOVA one-way followed by Tukey's post hoc test).

Regarding the effects of interventions, exercise training alone reduced ($p <0.05$) serum cortisol concentrations by 72 % (F vs FE), while TRP supplementation itself reduced ($p <0.05$) it by 67 % (F vs FS). The combination of treatments, however, reduced ($p <0.05$) it by 54 % (F vs FES) only. This indicates that both interventions either alone or in combination play a role in reducing serum cortisol concentrations in this model.

Muscle concentrations of IL-6 and TNF

The results presented in Fig 3 also refer to the cumulative effects of interventions (i.e. aerobic exercise and supplementation) either alone or in combination over three weeks on muscle cytokines. Animals from F group showed higher ($p <0.05$) muscle IL-6 concentrations than those from CON group (Fig 3A). This indicates that neutral saline

did not affect the muscle concentrations of this cytokine, whereas acidic saline promoted the increase in IL-6 concentrations.

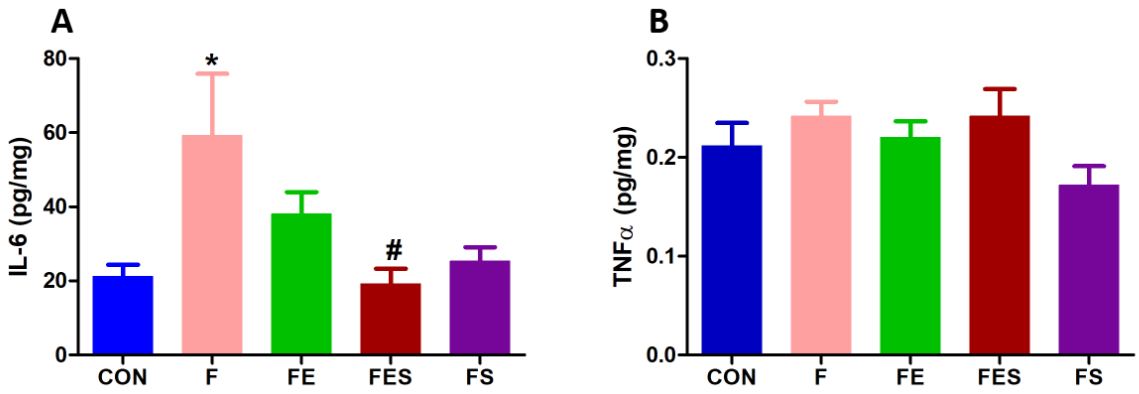

Fig 3. Concentrations of inflammatory cytokines in muscle tissue.
(A) Interleukin-6 (IL-6). (B) Tumor necrosis factor (TNF). CON, control; F, fibromyalgia induced; FE, fibromyalgia induced plus exercise; FES, fibromyalgia induced plus exercise and TRP supplementation; FS, fibromyalgia induced plus TRP supplementation. Data are means ± SD from 6 to 8 animals in each group). * $p<0.05$ vs CON group. $^{\#}$ $p<0.05$ vs F group (ANOVA one-way followed by Tukey's post hoc test).

Respecting the effects of interventions, the isolated effects of exercise training (F vs FE) and TRP supplementation (F vs FS) were not sufficient to reduce ($p > 0.05$) the muscle concentrations of IL-6. The combination of treatments (F vs FES), nevertheless, reduced ($p < 0.05$) the muscle concentrations of IL-6 by 68 %.

With reference to muscle TNF concentrations (Fig 3B), no differences were observed between the experimental groups.

Discussion

The aim of this study was to verify the effects of aerobic exercise associated with TRP supplementation on hyperalgesia, as well as on cortisol, IL-6 and TNF

concentrations in 12-month-old female rats with experimental FM. It was found that by the end of 3 weeks of intervention LIAE alone reduced bilateral hyperalgesia (~ 151 %) and serum cortisol concentrations (72 %), while TRP supplementation itself diminished bilateral hyperalgesia (~ 57 %) and cortisol concentrations (67 %). Although, muscle TNF concentrations were not affected, the association of LIAE with TRP supplementation further reduced bilateral hyperalgesia (~ 162 %), being an important decrease (68 %) in muscle IL-6 concentrations observed, though the serum cortisol concentrations backed off to 45 %.

The rat model used in the present study showed bilateral hind paw mechanical hyperalgesia that persisted over three weeks. We also observed elevated serum cortisol and muscle IL-6 concentrations in these animals over this period. There are evidences on that stress associated with high serum cortisol concentrations may exacerbate musculoskeletal pain in FM patients [34], and potentiate the pronociceptive effects of inflammatory cytokines such as IL-6 and TNF [8, 35]. Although the FM induction model used in this study is considered non-inflammatory [30], previous studies [35,36] on non-inflammatory stress models (i.e. sleep deprivation and sound stress) have observed that stress induces the persistent elevation of pro-inflammatory cytokines such as IL-6 and TNF. Despite that, in the present study no significant differences in muscle TNF concentrations between control and FM animals were observed. Considering that the influence of HPA axis activity and systemic hypercortisolemia on the pathophysiology of FM is controversial [5, 8, 37, 38, 39], which leads to the existence of a considerable heterogeneity of FM patients, it is possible that the elevated cortisol shown here in the model of repeated acidic saline is associated with only a sub-population of FM patients.

Concerning the effects of exercise, we observed that the LIAE program applied was efficient in returning bilateral mechanical hyperalgesia to control levels on the first week of the intervention, which persisted until the end of intervention (i.e. week 3). Such exercise benefit was followed by a reduction in serum cortisol concentrations. However, the observed reduction in muscle concentrations of IL-6 did not reach statistical significance. Musculoskeletal exercises release neurotransmitters, such as noradrenaline and serotonin, and activates specific receptors that helps to reduce stress-indicative scores [40, 41, 42]. Long-term aerobic activities increase the plasma concentrations of free tryptophan (TRP-F), whereas the concentrations of large neutral amino acid (LNAA) are reduced as a result of its increased uptake and oxidation by the exercised muscles [43]. Thus, the TRP-F/LNAA ratio decreased which augments the locomotion of TRP-F to the brain, thereby increasing the cerebral serotonin concentrations [44, 45]. Since serotonin is responsible for stimulating the HPA axis in response to stress [23], it is possible that our LIAE program has increased the concentrations of cerebral serotonin and thus reduced the release of cortisol in this model of FM. Such possibility warrants further investigations.

Regarding the results of TRP supplementation alone, it diminished bilateral hyperalgesia and serum cortisol concentrations to lesser extensions as compared to exercise. Tryptophan supplementation has been shown to improve the function of cerebral serotonin, which helps reducing the release of cortisol in stressful situations [24]. Such effect is thought to occur because the consumption of supplements rich in TRP increases their plasma proportion over the sum of the other LNAA giving TRP the advantage in the competition for accessing to the brain [22]. In this study, the TRP concentration in the control diet was 2.5g/kg while in the TRP supplemented it was 7.6g/kg. Thus, TRP/LNAA ratio in the TRP diet was approximately three times higher

than that in the control diet (TRP/LNAA=15.32 and TRP/LNAA=5.4, respectively). In addition, weight gain and food consumption did not differ significantly between groups, ensuring that similar amounts of diet were consumed by the animals throughout the study period. Therefore, the higher TRP/LNAA ratio of the TRP supplemented diet appears to have favored the increase of brain TRP which would result in reduced serum cortisol and muscle IL-6 concentrations in the TRP supplemented group. Despite that, taking into consideration that tryptophan is a precursor of serotonin and is closely linked with psychiatric disorders, which consequently affects stress the absence of other behavioral tests (i.e. anxiety, stress) is a limitation of the present study.

More important, concerning the combination of treatments, we found that the association of TRP supplementation with LIAE did not further reduced bilateral hyperalgesia significantly (i.e. 11% vs exercise), being an important decrease in muscle IL-6 concentrations observed (~ 68 %), though the serum cortisol concentrations backed down (i.e. 18% vs exercise). These results indicate that combination of treatments generated a synergy of the isolated effects on a biomarker indicative of inflammation, but not on that of stress and on hyperalgesia.

In this sense, the suggestion of whether is necessary or not adding TRP supplementation to LIAE in FM therapy warrants cost-benefit analyzes of the patient health conditions. On this wise, it has to be considered that little is known about the ideal amounts of TRP consumption. Although, TRP supplementation is related to improvements of symptoms such as mood, cognitive status and fatigue in patients undergoing chronic stress [20,21], its chronic and excessive consumption may be associated with increases in oxidative stress and in the risk of cardiovascular diseases [46,47]. Therefore, further studies on the safety of TRP supplementation in the management of FM patients are needed to determine accurate amounts and time of

TRP consumption to achieve the benefits of supplementation. Moreover, since we observed that serum cortisol concentrations pulled back, behavioral assessments (i.e. fatigue, stress, anxiety and depression) should also be performed.

Finally, these results have clinical relevance as it gives insights into the potential of combining non-pharmacological therapies in the management of FM, specially it brings about the need for new researches to investigate the mechanisms involved in the behavioral and biochemical effects observed here.

In conclusion, the association of TRP supplementation to LIAE does not potentiate significantly the reduction of bilateral mechanical hyperalgesia promoted by LIAE in 12-month old female rats with experimental FM, however an important decrease in IL-6 is evident.

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ARTIGO 2

Effects of aerobic exercise associated with tryptophan supplementation on hyperalgesia and oxidative stress in female rats with fibromyalgia

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Abstract

The objective of this study was to verify the effects of aerobic exercise associated with tryptophan supplementation on hyperalgesia and oxidative stress in female rats with fibromyalgia. Female Wistar rats (initial body weight: ~ 350 g; age: 12 months) were randomly divided into 5 groups: CON (Control); F (Fibromyalgia); FE (Fibromyalgia plus exercise); FES (Fibromyalgia plus exercise and tryptophan supplementation) and FS (Fibromyalgia plus tryptophan supplementation). Fibromyalgia was induced with two injections (20 µL) of acidic saline solution (pH 4.0) in the right gastrocnemius muscle with a 3-day-interval. The control animals received the same doses of neutral saline solution (pH 7.4). The exercised animals underwent progressive low-intensity aerobic exercise (LIAE) on a treadmill (10-12 m/min, 30-45 min/day, 5 days/week) for three weeks. During this period, the supplemented animals received a tryptophan supplemented diet (210 g / week), while the others received a control diet. Mechanical hyperalgesia was evaluated pre-injection, post-injection and at the end of the third week. The enzymatic activity of superoxide dismutase (SOD) and catalase (CAT) as well as the hepatic malondealdehyde (MDA) concentrations were evaluated after three weeks of interventions. Experimental fibromyalgia caused bilateral hyperalgesia and reduced SOD (37.88 %) and CAT (30.50 %) in the liver. After three weeks of intervention, both LIAE alone and combined with tryptophan supplementation reduced hyperalgesia (151 % and 162 %, respectively), though it did not affect SOD and CAT activities or MDA concentrations in animals with fibromyalgia. However, tryptophan supplementation itself reduced hyperalgesia (~ 67%) and further reduced MDA (24 %) concentrations attenuating the effects of oxidative stress among animals with fibromyalgia. In conclusion, the association between LIAE and tryptophan

supplementation does not potentiate significantly their benefits to mechanical hyperalgesia and has no effect on oxidative stress in female rats with fibromyalgia.

Keywords: Low-intensity exercise; Mechanical hyperalgesia; Acid saline.

Introduction

Fibromyalgia is a painful chronic syndrome that has a complex and multifactorial pathophysiology. However, there is evidence that increased oxidative stress and generation of free radicals may play an important role in the etiology of fibromyalgia[1-3].

Under normal conditions there is a balance between reactive oxygen species (ROS) and antioxidants inside the cell, in the membranes and in the extracellular space. On the other hand, in situations of oxidative / antioxidant imbalance, the ROS attacks to polyunsaturated fatty acids in membrane lipids leads to lipid peroxidation, loss of membrane fluidity, changes in membrane potentials and, eventually, rupture that results in release of cellular content and organelles[4]. Such oxidative damages may lead to cellular dysfunctions that contribute to the pathophysiology of various diseases, including fibromyalgia[5].

In this context, studies have shown that patients with fibromyalgia present increased plasma concentrations of oxidative damage biomarkers, mainly MDA, as well as reduced concentrations of antioxidant enzymes such as superoxide dismutase (SOD) and catalase (CAT)[6,7]. Moreover, these oxidative stress markers have been associated with the etiology of musculoskeletal pain, one of the main symptoms associated with fibromyalgia [1,8,9].

Regarding fibromyalgia treatment, exercise has been recommended as a non-pharmacological therapy for pain diseases[10-13]. In this context, low to moderate intensity

aerobic exercises are recommended[14] and are reported to reduce the systemic concentration of oxidative stress biomarkers in patients with fibromyalgia [15,16]. In addition, concerning diet, tryptophan supplementation has also been used in the treatment of fibromyalgia and other pain-associated syndromes, since low-serum tryptophan levels have been associated with conditions such as chronic fatigue syndrome and fibromyalgia [17,18]. Previous studies have shown that tryptophan supplementation can reduce MDA concentrations and increase antioxidant activity, thus attenuating the effects of oxidative stress[19,20].

Despite these isolated benefits of exercise and diet reported, the possible positive effects of associating aerobic exercise with tryptophan supplementation to individuals with fibromyalgia are not well understood. Therefore, this study was designed to verify the effects of aerobic exercise associated with tryptophan supplementation on hyperalgesia and oxidative stress in female rats with fibromyalgia.

Materials and Methods

Animals and experimental procedures

The experimental procedures were approved by the Ethics Committee for Animal Use of the Federal University of Viçosa (process number 21/2015) and were conducted according to the Guide for the Care and Use of Laboratory Animals (2011), National Academy of Sciences (USA).

Twelve-month old female adult Wistar rats (initial body weight: ~ 350 g) from the Central Biotherm of the Biological Center for Health Sciences at the Federal University of Viçosa (UFV) were randomly assigned to groups of 8 rats each: CON (Control); F (Fibromyalgia); FE (Fibromyalgia plus exercise); FES (Fibromyalgia plus exercise and tryptophan supplementation) and FS (Fibromyalgia plus tryptophan supplementation).

During the experiment, animals were kept in individual cages in a temperature-controlled room (22 ± 2 ℃), with a light/dark cycle of 12/12 hours and had free acce ss to water and diet.

After fibromyalgia induction, the rats of the exercised groups were submitted to three weeks of low-intensity aerobic exercise (LIAE), while those of the supplemented groups received a tryptophan supplemented diet. The body weight of the animals was measured once a week (on Fridays) during the experiment.

Fibromyalgia Induction

Repeated intramuscular injections of acidic saline are known to mimic the conditions of chronic and widespread pain[21]. The first injection produces transient hyperalgesia that decreases after 24h, and the second, administered 3 days later, promotes bilateral hyperalgesia for more than 4 weeks[21,22]. Thus, the rats from F, FE, FES and FS groups received two unilateral intramuscular injections containing 20 µL of acidic saline (pH 4.0) in the right gastrocnemius muscle, while animals from CON groups received injections containing 20 µL of neutral saline (pH 7.4), with a 3-day interval between injections. For these fibromyalgia induction procedures, the animals were maintained under surgical sedation with isoflurane, sprayed with 100% FiO_2, in an avalvular circuit and spontaneous breathing.

Measurement of mechanical hyperalgesia

The mechanical hyperalgesia was measured following a protocol described previously[23]. In brief, three animals at a time were housed in individual boxes with wire-mesh floor and acrylic walls, which were placed on a raised platform. After 30 minutes of adaptation, a mechanical stimulus of increasing pressure (expressed in grams) was applied to the plantar surface of the right and left hind paws of each rat, using an electronic Von Frey apparatus (Insight, Ribeirão Preto - SP , Brazil). The mechanical

stimulus was applied alternate 5 times to each hind paw of each animal in the pre-injection period and 3 times in the post-injection. Such stimuli were applied alternate to the left paw of each of the 3 rats and then to the left paw in the same order, so that each animal had and interval of approximately 30 seconds to receive the next stimulus to the contralateral hind paws. Pressure values were recorded by observing behaviors in nociceptive responses such as paw withdrawal, paw licking or jumping with all four legs. The withdrawal threshold in the pre-injection period was determined by calculating the median of the 5 measurements and then calculating how much each value deviated from the median. The 3 values that deviated less from the median were used to determine the mean and obtain the threshold value. The withdrawal thresholds in the post-injection period were determined by the mean of 3 consecutive pressure measurements. All measurements were taken in a quiet, temperature-controlled room at the same time of the day (8 to 10 a.m.), once a week (on Wednesdays). The same evaluator did all the tests blind for the treatments and the recorded pressure values. Mechanical hyperalgesia was measured before fibromyalgia induction (pre-injection), 24 hours after the second injection of both acidic or neutral saline, and at the end of the third week. The mean withdrawal threshold between the hind paws was calculated and established as a reference for determining the mechanical sensitivity of the animals in each intervention period.

Running velocity test and aerobic exercise training protocols

To determine the running speed (i.e. exercise intensity) during the exercise training sessions, the animals were submitted to a maximal running velocity (MRV) test on a treadmill (AVS, São Paulo-SP, Brazil), based on the total exercise time until fatigue (TTF) test, as previously described[24]. In summary, one week before the test the animals were adapted to the treadmill, on five consecutive days (5 min/day, 5% tilt up) and daily

increases in treadmill velocity (8, 10, 11, 12 m/min). After the adaptation week, each animal performed three tests of progressive exercise until fatigue on three alternate days. During the tests, the initial velocity of the treadmill was 10 m/min (5% tilt up), being increased by 1 m/min every 3 minutes until fatigue which was defined when the animal could no longer keep the treadmill pace, when the test was interrupted. The mean MRV obtained by the animals in the TTF tests was calculated and established as a reference (i.e. 100 % of MRV) to determine the exercise intensity during the exercise training sessions.

The aerobic exercise training was conducted on an electric-driven treadmill five times a week (Monday to Friday) for three consecutive weeks [Adapted from Sharma et al.[25]]. The duration and intensity of exercise were gradually increased over the 3-week period. During the first week, the animals ran at 10 m/min (50 % of the MRV), for 30 minutes. Then, on the second week, the animals ran at 11 m/min (55 % of the MRV), for 40 minutes. And during the third week the running speed was 12 m/min (60 % of the MRV), for 45 minutes. The exercise protocol included 2 minutes of warm-up (5 to 8 m/min) and 2 minutes of cooling down (5 to 8 m/min) within the total time of the running session. No incentive was used during the running.

Diets and supplementation

The control and supplemented diets were purified and packed in pellets (RHOSTER, São Paulo, Brazil). The animals had free access to diet and water being the consumption ad libitum during the experimental period (3 weeks). Diets (210 g/week) were supplied twice a week (on Tuesdays' and Thursdays' in the morning). The animal home cage was cleaned every 2 days. On these days the leftovers in the feeder of each animal were separated and weighed to calculate weight gain, food consumption and food efficiency coefficient (FEC) individually.

The composition and profile of the amino acids of the diets are described in Table 1. The composition of the diets was based on the American Institute of Nutrition (AIN-93M) adult rodent maintenance diet[26] and adapted from the increase in concentration of TRP to the typical amino acid profile of casein (RHOSTER, São Paulo, Brazil).

Table 1. Composition and typical amino acid profile of control and experimental diets.

Nutrient	AIN-93M Diet	Experimental Diet
Composition	**g/Kg diet**	**g/Kg diet**
Casein (>85%protein)	140	140
Sucrose	100	100
Cornstarch	465,6	465,6
Dextrinized cornstarch	155	155
Fiber	50	50
Mineral mix	35	35
Vitamin mix	10	10
L-cystine	1,8	1,8
Choline bitartrate	2,5	2,5
Soybean oil	4	4
Amino acid profile	**g/Kg diet**	**g/Kg diet**
Isoleucine	9,0	8,2
Leucine	10,9	10,6
Phenylalanine	8,5	8,0
Tyrosine	7,4	7,5
Valine	10,5	9,9
Tryptophan	2,5	7,6

Reeves et al. (1993).

The typical amino acid profile estimated for the diets was analyzed by HPLC (CBO Análises Laboratoriais, Valinhos-SP, Brazil). The used tryptophan concentration was based on previous studies on protein supplementation enriched with alpha-lactalbumin, and its repercussion on plasma and brain concentrations of tryptophan and serotonin, respectively[27,28,29].

Sample collection

At the end of the experimental period, 48 hours after the last exercise session, the animals were euthanized by decapitation in a clean room without strange noises. Euthanasia occurred on different days for different groups, but always occurred in the morning (8 am to 10 am). Immediately after decapitation, blood was collected by total exsanguination in separator gel tubes, which were subsequently centrifuged at 704 g (model Z216MK, Hermle, Germany) for 10 minutes. Serum was separated and stored at -80 ℃ for urea and creatinine analyzes. The hepatic tissue was dissected, we ighed, washed in cooled saline, frozen on dry ice and stored at -80 ℃ for the determination of SOD and CAT antioxidant activity and MDA concentrations.

Determination of body weight, weight gain and food consumption.

The animals were weighed (Mettler Toledo, Brazil) once a week (Thursday's mornings) during the experiment. The weight gain of each animal was calculated based on the equation: final weight - initial weight. The food consumption of each rat was evaluated weekly based on the amount of diet added minus the rest in the feeder, as described above.

Determination of lipid peroxidation in hepatic tissue

Lipid peroxidation was evaluated according to the methodology proposed by Buege and Aust[30], by determining the ability of malondialdehyde (MDA), a secondary product of lipid peroxidation, to react with thiobarbituric acid (TBARS) (Merck, Germany). In order to do so, spectrophotometric measurement (Multiskan GO, Thermo Scientific®, Finland) was performed at 535 nm.

Determination of SOD and CAT activity in hepatic tissue

The activity of the CAT and SOD enzymes in the hepatic tissue were measured according to Lowry et al[31] methodology with spectrophotometer readings (Multiskan GO, Thermo Scientific®, Finland) at a wavelength of 700nm, so that the results could be normalized by the protein concentrations.

Determination of the serum concentrations of urea and creatinine

The urea and creatinine serum concentrations were determined in order to evaluate deleterious effects of tryptophan supplementation. The collected blood was placed in a tube containing 3.13% sodium citrate (Citratelblood = 1/9). Plasma was obtained via centrifugation of the blood at 3000Rpm for 15 min (temp 4 °C). The urea and creatinine levels were measured using commercial kits (Bioclin) in the biochemical analyzer (BS-200, Mindray, China), according to the manufacturer instructions.

Statistical analysis

Data were submitted to the Kolmogorov-Smirnov normality test. Data for body weight, weight gain, food consumption, FEC, SOD and CAT antioxidant activity, urea, creatinine and MDA concentrations were compared using one-way analysis of variance (ANOVA), followed by Tukey's post-hoc test for multiple comparisons. Data for mechanical hyperalgesia were compared using ANOVA with repeated measures, followed Bonferroni's post-hoc tests for multiple comparisons. Data were analyzed using SPSS (IBM SPSS Statistics for Windows, Version 19.0. Armonk, NY: IBM Corp) and a p value of < 0.05 was considered significant. Results are expressed as means ± standard deviation (SD).

Results

Body weight, weight gain and food consumption.

The initial body weight was not different between the experimental groups (p>0,05). At the end of three weeks of interventions, body weight did not differ between groups (p>0,05). Likewise, the weight gain and food consumption were not different between the experimental groups over the weeks (Figure 1).

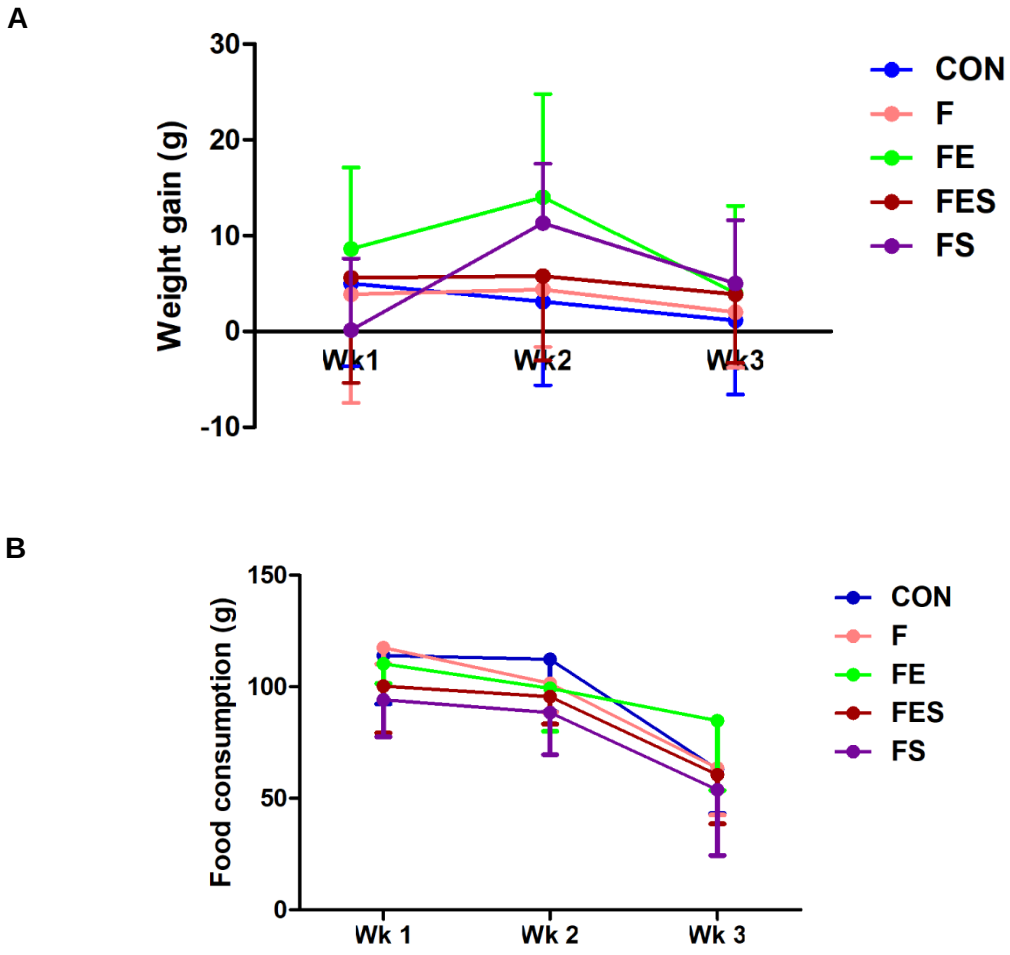

Figure 1. Food consumption and weight gain.
CON, control; F, fibromyalgia; FE, fibromyalgia plus exercise; FES, fibromyalgia plus exercise and tryptophan supplementation; FS, fibromyalgia plus tryptophan supplementation. Data are means ± SD of 8 animals in each group. ANOVA with repeat measures followed by Bonferroni.

Mechanical hyperalgesia

The withdrawal threshold remained relatively unchanged ($p > 0.05$) in the CON group for all measurements (Table 2), which indicates that neutral saline did not influence the mechanical sensitivity of these animals. However, at the post-injection moment the animals from F, FE, FES and FS groups exhibited lower withdrawal threshold (i.e. increased mechanical hyperalgesia) ($p < 0.05$), compared to those from CON group, which demonstrates the effectiveness of acidic saline in inducing hyperalgesia.

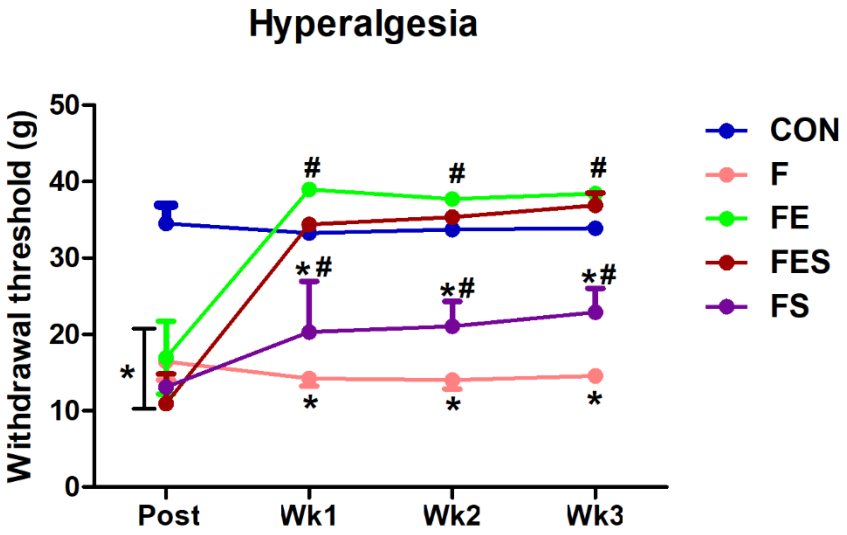

Figure 2. Withdrawal threshold of the hind paws.

CON, control; F, fibromyalgia; FE, fibromyalgia plus exercise; FES, fibromyalgia plus exercise and tryptophan supplementation; FS, fibromyalgia plus tryptophan supplementation; PRE: pre-injection; POS: pos-injection. The data are means ± SD from 8 animals in each group. * $p<0.05$ vs CON group. # $p<0.05$ vs F group (ANOVA with repeated measures, followed by Bonferroni).

Concerning the effects of the interventions (Table 2), at the end of the third week, exercised animals (FE) presented greater (~ 151%) withdrawal thresholds (i.e. reduced mechanical hyperalgesia), compared to those from F group. Animals supplemented with tryptophan (FS) also presented a higher degree (~ 67%) of withdrawal thresholds (i.e. reduced mechanical hyperalgesia) than those from F group ($p< 0.05$). Animals from the combined treatment group (FES) also exhibited higher withdrawal thresholds (i.e. reduced mechanical hyperalgesia) than those from F group (~ 162%).

Activity of antioxidant enzymes and lipid peroxidation in the liver

SOD and CAT activities were higher in the CON group compared to the groups with experimental fibromyalgia ($p <0.05$) (Table 2), indicating a reduction in the antioxidant defense capacity and the presence of an oxidative disorder in these animals. However, it was not possible to observe an increase in MDA concentrations at the end of the three weeks of experiment (Table 2).

Table 2 – Activity of antioxidant enzymes (SOD and CAT) and MDA concentrations.

Parameters	CON	F	FE	FES	FS
U SOD/mg prt	2,6 ± 1,1	1,6 ± 0,2*	1,5 ± 0,2*	1,5 ± 0,2*	1,5 ± 0,2*
U CAT/mg prt	2,6 ±1,0	1,8 ±20,4*	1,7 ±0,7*	1,4 ±0,2*	1,7±0,2*
MDA nm/g prt	0,1± 0,1	0,1 ± 0,1*	0,1 ± 0,1*	0,7 ± 0,1*	0,1±0,1*#

CAT: catalase: SOD: superoxide dismutase; MDA: malondealdehyde; CON, control; F, fibromyalgia; FE, fibromyalgia plus exercise; FES, fibromyalgia plus exercise and tryptophan supplementation; FS, fibromyalgia plus tryptophan supplementation. The data are means ± SD from 8 animals in each group. * $p<0.05$ vs CON group. # $p<0.05$ vs F group (ANOVA one-way followed by Tukey).

However, tryptophan supplementation, LIAE and its association did not affect the SOD and CAT activities ($p >0.05$). In relation to lipid peroxidation, tryptophan

supplementation alone further reduced the MDA concentrations (F vs FS; p <0.05), indicating a reduction of oxidative stress in animals with fibromyalgia. Despite that, no effect of LIAE or LIAE associated with tryptophan supplementation in MDA concentrations was found (Table 2).

Serum urea and creatinine

Serum concentrations of urea and creatinine (Table 3) did not differ between groups, demonstrating that the interventions did not affect the metabolism of these substances. In addition, all groups presented values considered normal for plasma urea concentrations (between 16 and 40 mg / dl) and creatinine (0.6 to 1.2 mg / dl), demonstrating that none of the interventions had a deleterious effect on renal function.

Table 3 – Urea and cretinine concentrations.

Parameters	CON	F	FE	FES	FS
Urea (mg/dl)	22,4 ± 9,0	24,9 ±3,9	26,9 ±3,4	28,1 ± 4,3	30,6±7,2
Creatinine (mg/dl)	0,4 ± 0,1	0,3 ± 0,1	0,4 ± 0,4	0,2 ± 0,1	0,3 ±0,1

CON, control; F, fibromyalgia; FE, fibromyalgia plus exercise; FES, fibromyalgia plus exercise and tryptophan supplementation; FS, fibromyalgia plus tryptophan supplementation. The data are means ± SD from 8 animals in each group. * p<0.05 vs CON group. (ANOVA one-way followed by Tukey).

Discussion

In this study, 12-month-old female rats with fibromyalgia were submitted to both LIAE and tryptophan supplementation, exclusively and in combination for a period of three weeks. The main findings after three weeks of intervention were: 1) LIAE alone and the combination of LIAE with tryptophan supplementation reduced mechanical hyperalgesia (~ 151 % and ~ 162 %, respectively), but did not influence the hepatic

SOD and CAT activities or MDA concentrations; and 2) TRP supplementation alone reduced mechanical hyperalgesia (~ 67%) and further reduced hepatic MDA (24%) concentrations attenuating the effects of oxidative stress among fibromyalgia animals.

The animal fibromyalgia model used in the present study showed hyperalgesia in the hind paw that persisted over three weeks. Additionally, animals with fibromyalgia had a reduction in SOD and CAT activities, indicating a reduction in the antioxidant defense capacity in these animals. There is evidence that a deficiency of antioxidant enzymes in fibromyalgia patients may contribute to the low capacity of cellular protection against oxidative damage, supporting the hypothesis that the fibromyalgia symptoms are linked to oxidative disorder[3]. According to Altindag and Celik[32], symptoms such as pain and morning stiffness are negatively correlated with the total antioxidant capacity of fibromyalgia patients. Recent studies have shown that increased oxidative stress appears to be directly involved in peripheral and central nervous system sensitization, altering muscle nociception and inducing musculoskeletal hyperalgesia in patients with fibromyalgia [33-36]. Nevertheless, in the present study MDA concentrations were higher in the CON group when compared to the other experimental groups. This contradictory fact may be related to the technique (TBARS test) used for lipid peroxidation analysis. The fact is that the TBA can reacts with a variety of compounds such as sugars, amino acids, bilirubin and albumin, producing interference in MDA measurement, particularly in situations such as initial stages of diseases, where lipid peroxidation can be smaller when compared to the non-specific background reaction between TBA and products not derived from lipid peroxidation [37]. Additionally, MDA is unstable for a long period of time, because its oxidation yields organic alcohols and acid, not determined by the TBARS test[38].

Regarding the effects of the LIAE, we observed that the LIAE program was efficient to return the mechanical hyperalgesia to the control levels at the end of the third week. However, LIAE by itself was not able to increase SOD and CAT activities or to alter MDA concentrations in animals with fibromyalgia. There are few studies relating aerobic exercise to oxidative stress, and the results are conflicting. According to Ihan et al[39], repeated exercise reduces oxidative stress and increases antioxidant capacity. Differently, Finauld et al[40] and Nazıroğlu et al[41] reported that during exercise, free radicals can increase in response to the body's natural defenses. Despite that, although exercise may cause increased lipid peroxidation in the cells, low to moderate intensity aerobic exercises seem to improve the metabolic control of oxidative stress products in patients with fibromyalgia in the long term[15,16]. In a previous study[42] we showed that LIAE reduces mechanical hyperalgesia and plasma cortisol concentrations in female rats with fibromyalgia. Such finding could help to explain the reduction in hyperalgesia even without a significant reduction of oxidative stress markers observed here.

Regarding the isolated effects of tryptophan supplementation, we found a significant improvement in mechanical hyperalgesia in animals from FS group, in relation to those from F group. These FS animals also showed a reduction in MDA concentrations, compared to animals from F group. This suggests a role of TRP supplementation in the reduction of oxidative stress and mechanical hyperalgesia. Previous studies have shown that some tryptophan metabolites (i.e. 3-hydroxyanthranilic acid and 3-hydroxyquinurenine) and tryptophan metabolic enzymes, such as tryptophan 2,3-dioxygenase (TDO), have antioxidant activity[20,43,44]. According to Mao et al[19], increased dietary levels of tryptophan efficiently alleviate oxidative stress by regulating the TDO activity and other non-enzymatic antioxidants in the liver,

suggesting the involvement of the kynurenines pathway in the modulation of oxidative stress and mechanical hyperalgesia in animals with fibromyalgia. Nonetheless, such possibility warrants further investigations.

The combination of treatments, however, did not significantly reduce hyperalgesia (11% - FES vs FE) and did not affect SOD and CAT activities or MDA concentration. These results indicate that the combination of tryptophan supplementation with LIAE does not potentiate the isolated effects of LIAE on mechanical hyperalgesia and has no effect on oxidative stress. Thus, the decision to associate tryptophan supplementation with LIAE depends on a cost-benefit analysis as well as the patient's health. Tryptophan seems to play an important role in reducing hyperalgesia and oxidative stress. In addition, as previously shown[42], tryptophan acts on stress modulation by reducing plasma cortisol concentration in female rats with fibromyalgia, and is thought to be an important clinical resource in the management of patients with chronic pain syndromes such as fibromyalgia. However, further research is needed to determine the sufficient amounts of tryptophan supplementation to obtain its therapeutic effects as well as the possible health risks to the patient when consuming TRP enriched supplements. In this study, the tryptophan concentration in the control diet was 2.5 g / kg while it was 7.6 g / kg in the tryptophan supplemented diet. Weight gain and food intake did not differ significantly between groups, ensuring that similar amounts of diet were consumed by all animals during the study period. However, supplemented animals showed no increase in plasma urea or creatinine concentrations, or increased oxidative stress, indicating that the amounts of supplemented tryptophan were not deleterious to the animal’s health even in amounts three times higher than the control diet.

In conclusion, the association between LIAE and tryptophan supplementation does not potentiate significantly their isolated benefits to mechanical hyperalgesia and has no effect on oxidative stress in female rats with fibromyalgia.

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ARTIGO 3

Modulação serotoninérgica da hiperalgesia no modelo de fibromialgia com salina ácida: efeitos do exercício aeróbico associado à suplementação com triptofano

Serotoninergic modulation of hyperalgesia in the acidic saline model of fibromyalgia: effects of aerobic exercise associated with tryptophan supplementation

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Resumo

O objetivo deste estudo foi avaliar os efeitos do exercício aeróbico de intensidade baixa (EAIB) associado à suplementação com triptofano na modulação serotoninérgica da hiperalgesia em ratas com fibromialgia induzida por salina ácida. Ratas Wistar (peso corporal inicial: ~ 350 g; idade: 12 meses) foram divididas aleatoriamente em 5 grupos: CON (Controle); F (Fibromialgia); FE (Fibromialgia mais exercício); FES (Fibromialgia mais exercício e suplementação com triptofano) e FS (Fibromialgia mais suplementação com triptofano). A fibromialgia foi induzida com duas injeções (20 µL) de solução salina ácida (pH 4,0) no músculo gastrocnêmio direito, com intervalo de 3 dias. Os animais controles receberam as mesmas doses de solução salina neutra (pH 7,4). Os animais exercitados foram submetidos a um programa EAIB em esteira rolante elétrica (10-12 m / min, 30-45 min / dia, 5 dias / semana) por três semanas. Durante esse período, os animais suplementados receberam dieta suplementada com triptofano (210 g / semana), enquanto os demais receberam dieta controle. A hiperalgesia mecânica foi avaliada pré-injeção, pós-injeção e ao final da terceira semana. As concentrações cerebrais de triptofano, serotonina (5-HT) e quinureninas (QUINU), bem como as concentrações séricas de substância P foram avaliadas após três semanas de intervenções. A fibromialgia experimental causou hiperalgesia, mas não influenciou o metabolismo de triptofano e a formação de 5-HT e QUINU. Após três semanas de intervenção, o EAIB e a combinação de EAIB com a suplementação com triptofano reduziram a hiperalgesia mecânica bilateral (~151% e ~162% respectivamente), mas não alteraram as concentrações cerebrais de triptofano e 5-HT entre os animais com fibromialgia experimental. A suplementação com triptofano reduziu a hiperalgesia mecânica bilateral (~67%) e aumentou a concentração cerebral de triptofano entre os animais com fibromialgia. A suplementação com triptofano

aumentou as concentrações cerebrais de QUINU, a atividade inferida da indolamina 2,3 dioxigenase (IDO) e a redução da razão 5-HT/QUINU nos grupos FS e FES. As intervenções não alteraram as concentrações séricas de substância P ao final das três semanas de experimento. Em conclusão, o modelo de fibromialgia induzida por salina ácida provoca hiperalgesia sem alterar sistema serotoninérgico de modulação da dor. A associação da suplementação com triptofano ao EAIB não potencializa a redução da hiperalgesia mecânica promovida pelo exercício aeróbico neste modelo.

Palavras-chave: Fibromialgia; triptofano; exercício aeróbico; serotonina; quinureninas; substância P.

Introdução

A fibromialgia é uma síndrome dolorosa crônica que afeta 2% a 5% da população mundial, sendo de prevalência predominante em mulheres[1,2]. Sua patofisiologia, ainda não completamente elucidada, tem sido relacionada a “déficits” nos sistemas endógenos de modulação da dor[3-5]. Baixos níveis de serotonina e elevados níveis de substância P no fluído cérebro-espinhal de pacientes com fibromialgia sugerem alterações em neurotransmissores inibitórios e excitatórios do sistema nervoso central que resultam em alterações da resposta central à dor[6-9]

Distúrbios no metabolismo do triptofano parecem ter um papel importante na patofisiologia da fibromialgia. Existem duas vias principais do metabolismo do triptofano que resultam na formação de dois metabólitos diferentes, a serotonina (5-HT) e quinurenina (QUINU). A degradação do 5-HT forma o ácido 5-hidroxiindolacético (5-HIAA), enquanto a QUINU é posteriormente convertida em metabólitos neuroativos,

como o ácido quinolínico[10]. Evidências suportam a existência de uma relação inversa entre os níveis de triptofano, 5-HT ou 5-HIAA e as medidas clínicas de dor, bem como uma correlação positiva entre os níveis de QUINU e a percepção da dor[11-13]

Neste contexto, modelos animais de indução à fibromialgia têm sido utilizados para desenvolver terapias profiláticas e curativas, assim como para investigar os possíveis mecanismos fisiopatológicos ligados à etiologia da fibromialgia. Estudos anteriores mostram que animais com fibromialgia induzida por injeções intramusculares de salina ácida apresentam melhora da hiperalgesia mecânica após a realização de programas de exercícios aeróbicos de intensidades baixa e média[14-16]. O exercício aeróbico parece ativar mecanismos inibitórios endógenos que incluem opioides e serotonina, bem como reduzir a concentração sistêmica de biomarcadores indicativos de estresse (Ex.: Cortisol e noradrenalina) e inflamação (Ex.: Citocinas). Adicionalmente, a suplementação com triptofano também tem sido testada em modelos experimentais de fibromialgia, com o objetivo de aumentar sua disponibilidade cerebral, o que potencializaria as ações moduladoras do sistema serotoninérgico de controle da dor[17-21]. A suplementação com triptofano tem mostrado melhorar a função da serotonina cerebral, o que ajuda a reduzir a liberação de cortisol em situações de estresse[22].

Neste sentido, apesar das evidências dos efeitos isolados do exercício aeróbico e da suplementação com triptofano no sistema serotoninérgico, pouco se conhece sobre a influência da associação destas intervenções. Deste modo, este estudo tem como objetivo avaliar os efeitos do exercício aeróbico de baixa intensidade associado à suplementação com triptofano na modulação serotoninérgica da hiperalgesia em ratas com fibromialgia induzida por salina ácida.

Materiais e Métodos

Animais e procedimentos experimentais

Os procedimentos experimentais foram aprovados pela Comissão de Ética no Uso de Animais da Universidade Federal de Viçosa (processo número 21/2015) e foram conduzidos de acordo com o Guide for the Care and Use of Laboratory Animals (2011), National Academy of Sciences (US).

Ratas Wistar adultas com 12 meses de idade (peso inicial: ~ 350 g), provenientes do Biotério Central do Centro de Ciências Biológicas da Saúde da Universidade Federal de Viçosa (UFV), foram alocadas randomicamente em grupos de 8 ratas cada: CON (Controle); F (Fibromialgia induzida); FE (Fibromialgia induzida mais exercício); FES (Fibromialgia induzida mais exercício e suplementação com triptofano); e FS (Fibromialgia mais suplementação com triptofano). Durante o experimento, os animais foram mantidos em gaiolas individuais numa sala com temperatura controlada (22 ± 2ºC), ciclo claro/escuro de 12/12 horas e livre acesso à água e dieta. Após a indução à fibromialgia, as ratas dos grupos exercitados foram submetidas a três semanas de exercício aeróbico de baixa intensidade, enquanto as dos grupos suplementados receberam uma dieta suplementada com triptofano (Tabela 1). A massa corporal dos animais foi mensurada uma vez por semana (às sextas-feiras) durante o experimento.

Indução à fibromialgia

Foi utilizado o modelo de injeções intramusculares repetidas de ácido para mimetizar as condições de dor crônica e difusa[23]. A primeira injeção produz hiperalgesia transitória que diminui após 24h, e a segunda, administrada 3 dias após, promove hiperalgesia bilateral por mais de 4 semanas[23,24]. Assim, as ratas dos grupos F, FE, FES e FS receberam duas injeções intramusculares unilaterais contendo 20 µL de salina ácida (pH 4,0) no músculo gastrocnêmio direito, enquanto as ratas do grupo CON receberam duas injeções intramusculares unilaterais contendo 20 µL de salina neutra (pH 7.4). Para estes procedimentos de indução à fibromialgia, os animais foram mantidos em sedação cirúrgica, com isofluorano, vaporizado com FiO_2 100%, em circuito avalvular e respiração espontânea.

Mensuração da hiperalgesia mecânica

A hiperalgesia mecânica foi mensurada seguindo um protocolo descrito previamente[25]. Os animais foram acomodados em uma caixa com piso de arame e paredes de acrílico, sobre uma plataforma elevada. Após 30 minutos de adaptação, foi aplicado estímulo mecânico com pressão crescente (expresso em gramas) na região plantar das patas traseiras direita e esquerda, sobre uma plataforma elevada, utilizando-se um Von Frey automático (Insight Pesquisa e Ensino, Ribeirão Preto – SP, Brasil). Tais estímulos foram aplicados alternadamente à pata esquerda de cada uma das 3 ratas e depois à pata esquerda na mesma ordem, de modo que cada animal tenha um intervalo de aproximadamente 30 segundos para receber o próximo estímulo às patas traseiras contralaterais. Os valores de pressão foram registrados mediante a observação de comportamentos em resposta nociceptiva como a retirada da pata, lambedura da pata ou salto com as quatro patas. Para cada pressão exercida, o teste foi repetido 5 vezes consecutivas em cada pata pré-injeção e 3 vezes consecutivas no

experimento. O limiar de retirada pré-injeção foi determinado pelo cálculo da mediana das 5 medições e, em seguida, calculando-se quanto cada valor se desviou da mediana. Os 3 valores que menos desviaram da mediana foram utilizados para determinar a média e obter o valor do limiar. Os limiares de retirada pós-injeção e após a terceira semana de intervenção foram determinados pela média de 3 medidas de pressão consecutivas [28]. Todas as medições foram realizadas em uma sala silenciosa, com temperatura controlada e na mesma hora do dia (8 às 10 a.m.), às quartas-feiras. O avaliador fez o procedimento cego para os tratamentos e para os valores de pressão registrados. O mesmo indivíduo aplicou os testes na linha de base e no experimento. A hiperalgesia mecânica foi mensurada antes da indução à fibromialgia (pré-injeção), 24h após a segunda injeção de salina ácida, e uma vez por semana durante 3 semanas.

Protocolos do teste de velocidade de corrida e do treinamento aeróbico

Para determinação da velocidade/intensidade da corrida nos treinamentos, durante os treinamentos, os animais foram submetidos a um teste de velocidade máxima de corrida (VMC), baseado no teste do tempo total de exercício até a fadiga (TTF), conforme descrito previamente[26]. Resumidamente, uma semana antes do teste os animais tiveram um período de adaptação à esteira (AVS, São Paulo-SP, Brasil), em cinco dias consecutivos (5 min/dia; 5% de inclinação para cima) e com aumentos diários da velocidade da esteira (8, 10, 11, 12 m/min). Após a semana de adaptação, cada animal realizou, em três dias alternados, três testes de exercício progressivo até a fadiga. Durante os testes, a velocidade inicial da esteira foi de 10m/min (5% de inclinação para cima), sendo aumentada em 1 m/min a cada 3 min até a fadiga a qual foi definida quando o animal não conseguia mais manter a corrida de acordo com a velocidade da esteira, quando o teste foi interrompido. A média da VMC obtida no TTF

pelos animais foi calculada e estabelecida como referência (ex., 100% da VMC) para a determinação da velocidade de corrida no protocolo de treinamento.

O treinamento aeróbico foi realizado em uma esteira elétrica cinco vezes por semana (segunda a sexta-feira), durante três semanas consecutivas [Adapted from Sharma et al.[27]]. A velocidade e a duração do exercício foram gradualmente aumentadas ao longo do período de 3 semanas. Na primeira semana, os animais correram a 10m/min (50% da VMC), por 30 minutos. Na segunda semana, os animais correram a 11m/min (55% da VMC), por 40 minutos. E na terceira semana a velocidade de corrida foi de 12 m/min (60% da VMC), por 45 minutos. O protocolo de exercício incluía 2 minutos de aquecimento (5 a 8m/min) e 2 minutos de resfriamento (5 a 8m/min). Não foi utilizado qualquer incentivo à corrida.

Dietas e suplementação

As dietas, controle e suplementada, foram purificadas e acondicionadas em pellets (RHOSTER, São Paulo, Brasil). Os animais tiveram livre acesso à dieta e a água sendo o consumo ad libitum durante o período experimental (3 semanas). As dietas (210g/semana) foram administradas duas vezes por semana (às terças e quintas-feiras, no período da manhã). As gaiolas dos animais foram higienizadas a cada 2 dias. Nestes dias, as sobras nos comedores de cada animal foram separadas e pesadas para calcular o ganho de peso, o consumo alimentar e o coeficiente de eficiência alimentar individualmente.

A composição e o perfil dos aminoácidos das dietas estão descritos na tabela 1. A composição das dietas foi baseada na dieta para manutenção de roedores adultos do Instituto Americano de Nutrição (AIN-93M)[28], e adaptada a partir do aumento da concentração de triptofano ao perfil típico de aminoácidos da caseína (RHOSTER, São Paulo, Brasil).

Tabela 1. Composição e perfil típico de aminoácidos das dietas controle e experimental.

Nutrient	AIN-93M Diet	Experimental Diet
Composition	**g/Kg diet**	**g/Kg diet**
Casein (>85%protein)	140	140
Sucrose	100	100
Cornstarch	465,6	465,6
Dextrinized cornstarch	155	155
Fiber	50	50
Mineral mix	35	35
Vitamin mix	10	10
L-cystine	1,8	1,8
Choline bitartrate	2,5	2,5
Soybean oil	4	4
Amino acid profile	**g/Kg diet**	**g/Kg diet**
Isoleucine	9,0	8,2
Leucine	10,9	10,6
Phenylalanine	8,5	8,0
Tyrosine	7,4	7,5
Valine	10,5	9,9
Tryptophan	2,5	7,6
TRP/LNAA (%)	5,4	15,32

TRP**/**ANG, razão do triptofano à soma de outros aminoácidos neutros grandes.

Reeves et al. (1993).

O perfil típico de aminoácidos estimado para as dietas foi analisado por HPLC (CBO Análises Laboratoriais, Valinhos-SP, Brasil). A concentração de triptofano usada foi baseada em estudos prévios sobre a suplementação proteica enriquecida com alpha-lactalbumina e sua repercussão nas concentrações plasmáticas e cerebrais de triptofano e serotonina, respectivamente[18-20].

Coleta de amostras

Ao final do período experimental, 48 horas após a última sessão de exercício, os animais sofreram eutanásia, por decapitação, em local limpo e sem ruídos estranhos ao ambiente dos experimentos. A eutanásia ocorreu em dias diferentes para os grupos, mas sempre no período da manhã (8h às 10h a.m). Imediatamente após a decapitação, o sangue foi coletado por exsanguinação total em tubos com gel separador que, posteriormente, foram centrifugados a 704g (modelo Z216MK, Hermle®, Alemanha), por 10 minutos. O soro foi retirado e armazenado a -80 ºC para as análises de substância P. O cérebro foi rapidamente removido, lavado em salina resfriada, imediatamente congelado em gelo seco e armazenado à -80 ℃ até as análises de triptofano, 5-HT e QUINU pela cromatografia líquida de alta pressão (HPLC).

Determinação do peso corporal e do consumo alimentar

Os animais foram pesados (Mettler Toledo®, Brazil) uma vez por semana (às terças-feiras no período da manhã) durante o experimento. O consumo alimentar dos animais foi avaliado semanalmente com base na quantidade de dieta adicionada menos o resto nos comedouros.

Determinação das concentrações cerebrais de triptofano, serotonina e quinureninas

A preparação das amostras e a determinação das concentrações cerebrais de triptofano, 5-HT e da QUINU foram realizadas no Instituto René Rachou (FIOCRUZ, Minas Gerais, Brasil). Três volumes de metanol gelado foram adicionados às amostras (50 µ + 150 µL), submetidos ao vórtice e centrifugados a 13.000 g por 10 min (4 °C). Os compostos hidrofóbicos (ácidos graxos, proteínas) foram removidos do sobrenadante utilizando resina C18 ativada. A resina C18 ativada foi adicionada às

amostras na proporção de 1/5 da massa da amostra (10 mg), submetida a vórtice e centrifugada a 13.000 g durante 10 min (4 °C). O sobrenadante foi transferido para um novo tubo e o solvente removido em um SpeedVac. As amostras foram reconstituídas no mesmo volume de água MilliQ e transferidas para frascos de polipropileno de 100 µL e colocados em um amostrador automático resfriado (5 °C). Os padrões triptofano, 5-HT e QUINU foram adquiridos à Sigma Aldrich (St. Louis, MO) com 96% de pureza. As análises de cromatografia líquida e de espectrometria de massa foram realizadas no Nexera Ultra High Performance Liquid Chromatografhy (UHPLC) (Shimadzu, Kyoto, Japão) equipado com uma coluna Shimadzu Shim-Pack XR-ODSIII (C18, 2,2 µm, 80 Å, 2,2 × 200 mm) de acordo com o protocolo descrito por Danielski et al[29].

As razões QUINU/TRP, 5-HT/QUINU e 5-HT/TRP foram calculadas com o objetivo de avaliar a participação da via da quinurenina no metabolismo de triptofano cerebral, inferido pela atividade enzimática da indolamina 2,3 dioxigenase (IDO), bem como analisar a participação da via da serotonina e a síntese de 5-HT e QUINU no cérebro dos animais com fibromialgia experimental[30-32].

Determinação da concentração sérica de substância P

A dosagem da concentração sérica de substância P foi determinada por técnica de imunoensaio enzimático (ELISA) no Laboratório Especializado em Análises Científicas (LEAC, São Paulo). Neste ensaio, um anticorpo monoclonal específico para substância P foi pré-revestido em uma microplaca. Uma reação de inibição competitiva foi lançada com a substância P marcada com biotina e substância P não marcada (padrões ou amostras) com o anticorpo pré-revestido específico para substância P. Após a incubação, o conjugado não ligado foi lavado. Em seguida, a avidina conjugada com Peroxidase de Horseradish foi adicionada a cada microplaca e incubada. A quantidade de conjugado Peroxidase de Horseradish ligado é inversamente

proporcional à concentração de SP na amostra. Após a adição da solução de substrato, a intensidade da cor desenvolvida é inversamente proporcional à concentração de substância P na amostra. O kit comercial utilizado foi o CEA393RA (Substância P, Uscn®, China), com a leitura sendo realizada no equipamento Stat Fax modelo 2100 (Awareness Technology, USA) e a concentração expressa como unidades pg/mL.

Análise estatística

Os dados foram submetidos ao teste de normalidade Kolmogorov-Smirnov. Todos os dados obtiveram distribuição normal e foram comparados usando-se a análise de variância ANOVA one-way ou ANOVA com medidas repetidas, seguidas dos testes post hoc de Tukey e Bonferroni para as múltiplas comparações. Os dados foram analisados utilizando-se SPSS (IBM SPSS Statistics for Windows, Version 19.0. Armonk, NY: IBM Corp) e foi adotado o nível de significância de até 5%. Os resultados são expressos como média ± desvio padrão.

Resultados

Peso corporal e consumo alimentar

O peso corporal inicial não foi diferente entre os grupos experimentais (Tabela 2). Ao final das 3 semanas de intervenções, o peso corporal e o consumo alimentar também não diferiram entre os grupos experimentais.

Tabela 2. Peso corporal e consumo alimentar.

PARÂMETROS	CON	F	FE	FES	FS
PI (g)	310,1 ±12,9	298,2 ±20,9	318,3 ±19,7	293,1 ±16,4	307,0 ±20,2
PF (g)	318,6 ±17,2	310,7 ±23,8	324,6 ±24,5	298,3 ±21,7	318,1±24,6
CA (g)	278,0 ± 42,4	279,7 ±33,5	289,0 ±47,5	236,7 ±42,0	239,5±40,3

PI: peso inicial; PF: peso final; CA: consumo alimentar; CON: controle; F: fibromialgia; FE: fibromialgia mais exercício; FES: fibromialgia mais exercício e suplementação com triptofano; FS: fibromialgia mais suplementação com triptofano. Os dados são apresentados em média ± DP de 8 animais em cada grupo. ANOVA one-way seguido de Tukey.

Hiperalgesia mecânica

Não houve diferença significativa dos limiares de retirada bilateralmente no período pré-injeção ($p>0,05$) entre os grupos experimentais. O limiar de retirada permaneceu relativamente inalterado no grupo CON em todas as medidas (Tabela 3), indicando que a injeção de salina neutra não influenciou a sensibilidade mecânica destes animais. Entretanto, no período pós-injeções de salina ácida nos animais dos grupos F, FE, FES e FS, o limiar de retirada diminuiu ($p<0,05$), quando comparados aos animais do grupo CON, demonstrando a efetividade do modelo animal de indução à hiperalgesia mecânica bilateral.

Tabela 3. Limiar de retirada das patas traseiras (g).

GRUPOS	PATA DIREITA			PATA ESQUERDA		
	PRE	POS	SEMANA 3	PRE	POS	SEMANA 3
CON	37,05±0,91	36,78±2,86	35,60±1,25	36,87±0,72	36,89±1,17	36,21±1,18
F	36,36±0,42	16,68±3,33*	14,34±1,02*	36,20±0,80	16,20±2,09*	14,78±2,18*
FE	36,47±0,73	12,27±0,32*	36,31±0,97#	37,15±0,64	13,75±1,21*	36,87±0,40#
FES	36,34±0,46	11,76±1,17*	37,98±1,97*#	36,29±0,44	11,54±1,10*	38,21±1,46*#
FS	36,67±0,49	13,27±0,89*	23,20±3,17*#	37,06±0,41	13,01±1,55*	22,63±3,20*#

CON: controle; F: fibromialgia; FE: fibromialgia mais exercício; FES: fibromialgia mais exercício e suplementação com triptofano; FS: fibromialgia mais suplementação com triptofano; POS: pós-injeção; PRE: pré-injeção. Os dados são apresentados em média ± DP de 8 animais em cada grupo. * $p<0.05$ vs CON. # $p<0.05$ vs F (ANOVA com medidas repetidas seguida pelo teste post hoc de Bonferroni).

Em relação aos efeitos da intervenção (Figura 1), ao final da terceira semana, os animais exercitados (F vs FE) apresentaram aumento (~ 151 %) nos limiares de retirada bilateralmente, enquanto os animais suplementados com triptofano (F vs FS) também reduziram em menor grau (~ 67 %) a hiperalgesia bilateral ($p>0,05$). Na combinação dos tratamentos (F vs FES), a hiperalgesia também apresentou redução significante ($p<0,05$) ao final da terceira semana (~162 %).

Concentrações cerebrais de triptofano, quinureninas e serotonina

Os resultados apresentados na figura 1 mostram os efeitos cumulativos das intervenções (FE, FES, FS) durante três semanas nas concentrações cerebrais de triptofano, QUINU e 5-HT. As concentrações cerebrais de triptofano, QUINU e 5-HT não diferiram entre o grupo CON e o grupo F, sugerindo que o modelo de salina ácida parece não influenciar o metabolismo cerebral do triptofano e as vias de formação de 5-HT e QUINU.

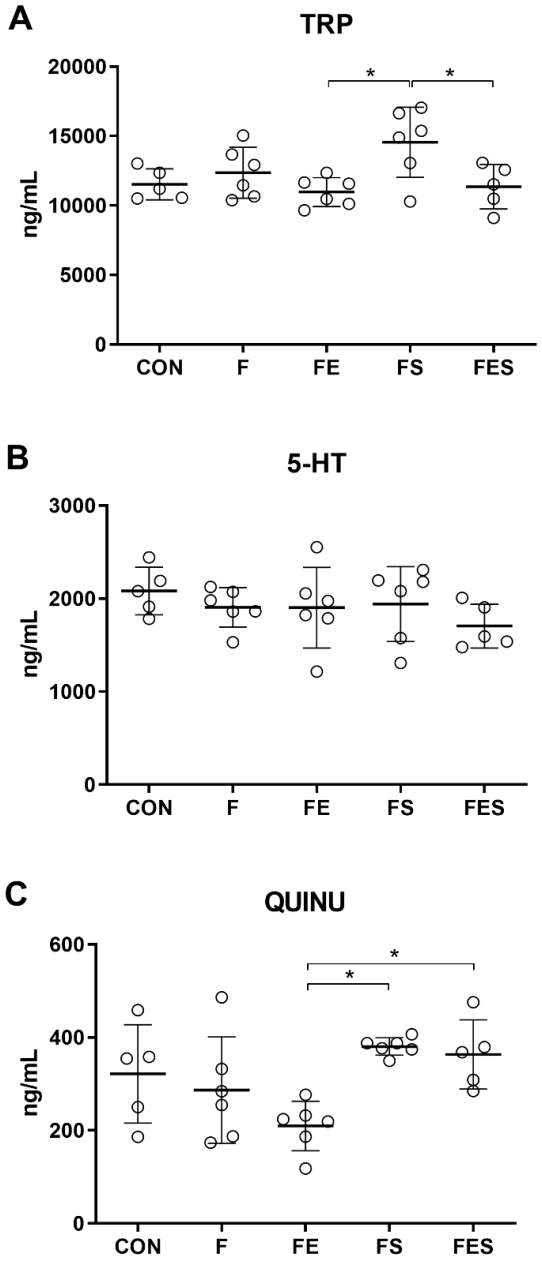

Figura 1. Concentrações cerebrais de triptofano (A), serotonina (B) e quinureninas (C). CON: controle; F: fibromialgia; FE: fibromialgia mais exercício; FES: fibromialgia mais exercício e suplementação com triptofano; FS: fibromialgia mais suplementação com triptofano. Os dados são apresentados em média ± DP de 8 animais em cada grupo. ANOVA one-way seguida pelo teste post hoc de Tukey (* $p<0.05$).

Com relação aos efeitos da suplementação com triptofano, do exercício aeróbico, e da associação de ambos sobre as concentrações cerebrais de triptofano e

seus metabólitos, foi possível observar que os animais do grupo FS apresentaram aumento significativo ($p<0,05$) de triptofano, quando comparados aos grupos FE e FES (Fig. 1A). Isto indica que a suplementação isolada de triptofano favorece o aumento da concentração cerebral deste aminoácido nos animais com fibromialgia não submetidos ao exercício físico (FS).

Não foi possível observar diferença nas concentrações cerebrais de 5-HT entre os grupos experimentais (Fig. 1B). Entretanto, a diluição das estruturas cerebrais, ricas neste neurotransmissor, pela massa cerebral total usada na produção dos homogenatos pode ter mascarado alguma diferença entre os grupos.

Com referência às concentrações cerebrais de QUINU (Fig. 1C), os grupos FS e FES apresentaram aumento significativo quando comparados aos animais apenas exercitados, indicando que a suplementação com triptofano contribuiu com o metabolismo do triptofano pela via das quinureninas.

Apesar das diferenças nas concentrações cerebrais de triptofano, curiosamente, a atividade da IDO inferida pela razão QUINU/TRP (Fig. 2A) e a razão entre 5-HT/QUINU (Fig. 2B) não diferiram entre os grupos com fibromialgia suplementados com triptofano (FS), ou exercitados e suplementados com triptofano (FES). Estes dois grupos tiveram concentrações cerebrais mais elevadas de QUINU (Fig. 1C), maior atividade inferida da IDO (Fig. 2A), e menor razão 5-HT/QUINU do que o grupo com fibromialgia submetidos apenas ao exercício aeróbico (FE) (Fig. 2B). Isto sugere que o excesso de triptofano advindo da suplementação parece ativar a IDO no cérebro contribuindo para que o triptofano seja preferencialmente metabolizado pela via da quinurenina, e não pela via da serotonina.

Com relação à razão de 5-HT/TRP, também não foi possível observar diferença entre os grupos experimentais (Fig. 2C), indicando que tanto a suplementação com

triptofano isolada quanto associada ao exercício aeróbico não parecem influenciar o aumento da atividade metabólica da via serotoninérgica nos grupos experimentais.

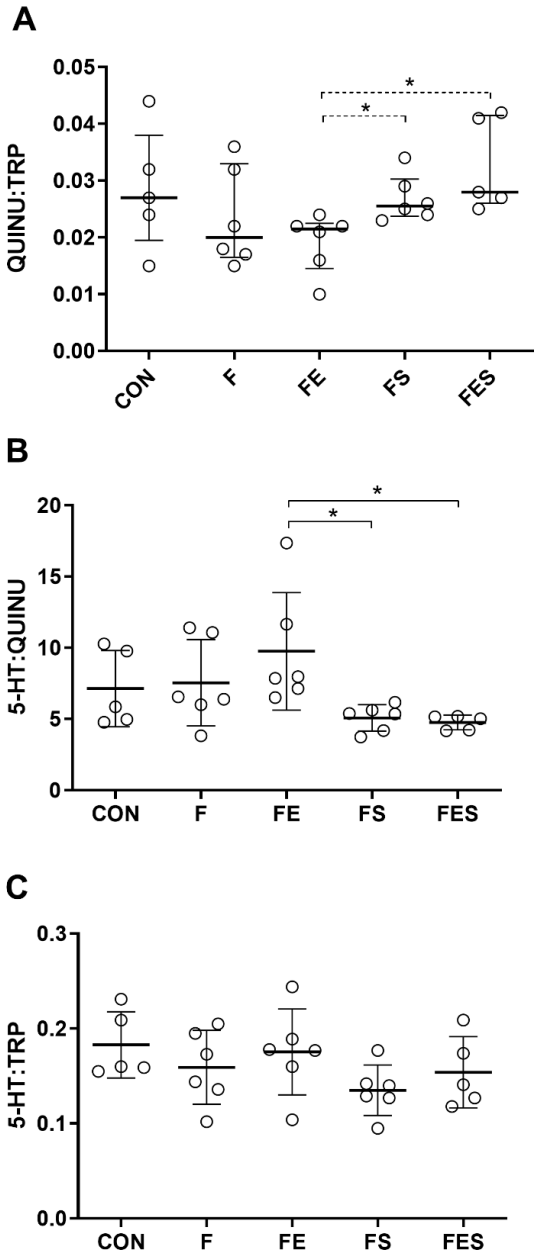

Figura 2. Razão entre quinureninas e triptofano (A), razão entre serotonina e quinureninas (B) e razão entre serotonina e triptofano (C). CON: controle; F: fibromialgia; FE: fibromialgia mais exercício; FES: fibromialgia mais exercício e suplementação com triptofano; FS: fibromialgia mais suplementação com triptofano. Os dados são apresentados em média ± DP de 8 animais em cada grupo. ANOVA one-way seguida pelo teste post hoc de Tukey (* $p<0.05$).

Concentrações séricas de substância P

Os resultados apresentados na figura 3 mostram os efeitos cumulativos das intervenções (FE, FES, FS) durante três semanas nas concentrações séricas de substância P. Observa-se que não houve diferença entre os animais do grupo CON, quando comparados aos do grupo F, sugerindo que o modelo de salina ácida parece não influenciar o aumento das concentrações séricas de substância P em ratas.

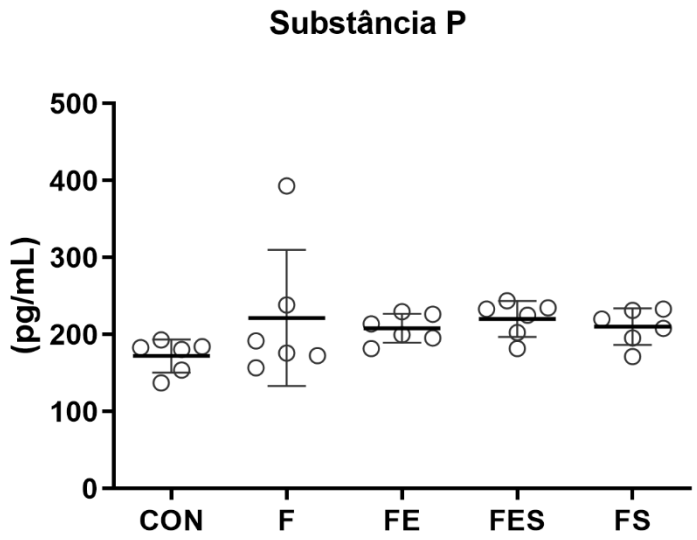

Figura 3. Concentrações séricas de substância P.
CON: controle; F: fibromialgia; FE: fibromialgia mais exercício; FES: fibromialgia mais exercício e suplementação com triptofano; FS: fibromialgia mais suplementação com triptofano. PRE: pre-injeção; POS: pós-injeção. Os dados são apresentados em média ± DP de 8 animais em cada grupo. * $p<0.05$ vs CON. $^{\#}$ $p<0.05$ vs F (ANOVA one-way seguida pelo teste post hoc de Tukey).

Com relação aos efeitos das intervenções, os grupos com fibromialgia experimental não apresentaram diferenças significativas, quando comparados ao grupo CON. Isto indica que, tanto o exercício aeróbico, a associação deste à

suplementação com triptofano, quanto a suplementação isolada com triptofano não influenciaram as concentrações séricas de substância P.

Discussão

Neste estudo, ratas com fibromialgia induzida por salina ácida foram submetidas ao exercício aeróbico de intensidade baixa e à suplementação com triptofano, tanto isoladamente quanto combinados por três semanas. Os principais achados, após três semanas de intervenção, foram: 1) o exercício aeróbico e a combinação deste com a suplementação com triptofano reduziram a hiperalgesia mecânica bilateral (~151% e 162% respectivamente), mas não influenciaram o aumento das concentrações cerebrais de triptofano e 5-HTentre os animais com fibromialgia experimental; 2) a suplementação exclusiva de triptofano reduziu a hiperalgesia mecânica bilateral (~67%) e aumentou as concentrações cerebrais de triptofano e QUINU nos animais com fibromialgia; 3) a suplementação com triptofano aumentou as concentrações cerebrais de QUINU, a atividade inferida da IDO e a redução da razão 5-HT/QUINU nos grupos FS e FES; 4) as intervenções não alteraram as concentrações séricas de substância P ao final das três semanas de intervenção.

O modelo de fibromialgia utilizado no presente estudo mostrou uma hiperalgesia bilateral nas patas traseiras, que persistiu ao longo de três semanas. Entretanto, as concentrações cerebrais de triptofano, 5-HT e QUINU não diferiram entre o grupo CON e grupo F, indicando que a hiperalgesia pode não estar relacionada a distúrbios no metabolismo cerebral do triptofano e na formação de seus metabólitos, neste modelo. Estes achados não confirmam a hipótese de que o modelo de dor crônica induzida por salina ácida influencia a redução das concentrações centrais de serotonina, bem como o aumento das concentrações de QUINU[15,7]. Entretanto, em estudo realizado anteriormente[14] observamos que este modelo de estresse não inflamatório parece

influenciar, predominantemente, alterações no eixo hipotalâmico-pituitário-adrenal (HPA), levando ao aumento das concentrações de cortisol sérico e IL-6 muscular. Há evidências de que o estresse associado a altas concentrações séricas de cortisol pode exacerbar a dor musculoesquelética em pacientes com fibromialgia[33] e potencializar os efeitos pronociceptivos das citocinas inflamatórias, como IL-6 e TNF[34,35]. Adicionalmente, as concentrações séricas de substância P não diferiram entre o grupo CON e o grupo F, indicando que este modelo de indução parece não alterar o equilíbrio do sistema de modulação serotoninérgico, uma vez que as concentrações de substância P são moduladas negativamente por neurônios serotoninérgicos eferentes[9,36] e este efeito não foi observado nos animais com fibromialgia do presente estudo.

Com relação aos efeitos do exercício aeróbico, este foi eficiente em retornar a hiperalgesia mecânica bilateral aos níveis do grupo controle, ao final da terceira semana. Há evidências que os exercícios aeróbicos de longa duração aumentam as concentrações plasmáticas de triptofano livre (TRP-F), enquanto as concentrações dos aminoácidos neutros grandes (ANG) são reduzidas, como resultado de sua maior captação e oxidação pelos músculos exercitados[37]. Assim, a relação TRP-F / ANG diminuiu, o que aumenta a locomoção de TRP-F para o cérebro, aumentando, assim, as concentrações de serotonina cerebral[38,39]. Estudos sugerem que opioides endógenos e a serotonina possuem um papel importante na analgesia produzida pelo exercício em humanos e animais com dor[40,41]. Entretanto, não foi possível observar um incremento das concentrações cerebrais de triptofano e 5-HT em resposta ao protocolo de exercício utilizado no presente estudo. Evidências sugerem que a melhora do quadro de hiperalgesia observada poderia estar relacionada ao efeito anti-inflamatório associado ao exercício aeróbico, tendo como consequência a

normalização da atividade neuroendócrina do eixo HPA, bem como a redução de biomarcadores de estresse (cortisol) e de inflamação (citocinas inflamatórias como IL-6)[14,17].

A respeito aos efeitos da suplementação isolada de triptofano, observou-se diminuição da hiperalgesia bilateral no grupo FS, em relação ao grupo F, assim como aumento significativo da concentração cerebral de triptofano, quando comparada aos demais grupos experimentais. Isto indica que a suplementação com dieta rica em triptofano aumenta sua proporção plasmática sobre a soma dos outros ANG, dando ao triptofano a vantagem na competição pelo acesso ao cérebro[21]. No presente estudo, a concentração de triptofano na dieta controle foi de 2,5g / kg enquanto no TRP suplementado foi de 7,6g / kg. Assim, a relação TRP / ANG na dieta triptofano foi aproximadamente três vezes maior do que na dieta controle (TRP / ANG = 15,32 e TRP / ANG = 5,4, respectivamente). Como o ganho de peso e o consumo de alimentos não diferiram significativamente entre os grupos, a maior relação TRP / ANG da dieta suplementada com triptofano parece ter favorecido o aumento do triptofano cerebral. Apesar disso, não foi observado aumento das concentrações cerebrais totais de 5-HT no grupo FS, quando comparado aos demais grupos experimentais, diferente do que foi observado em outros estudos[42,43]. Isto sugere que o aumento de 5-HT parece ocorrer apenas em regiões específicas de ação do sistema serotoninérgico de modulação da dor (ex., neurônios serotoninérgicos do bulbo ventromedial rostral e núcleo magno da rafe no tronco cerebral; e em regiões hipotalâmicas)[44,45], o que justificaria a melhora da hiperalgesia nestes animais. Assim, a análise no cérebro como um todo é considerada uma limitação do presente estudo, o que justifica investigações futuras.

Na análise dos efeitos da combinação dos tratamentos, observou-se que a associação do exercício aeróbico com a suplementação com triptofano não reduziu significativamente a hiperalgesia bilateral (11% vs exercício) e também não influenciou o aumento de triptofano e 5-HT entre os animais com fibromialgia. Estes resultados indicam que a combinação dos tratamentos não potencializa os efeitos do EAIB na redução da hiperalgesia e sugerem que o programa de exercício parece anular (por mecanismos ainda desconhecidos) os efeitos da suplementação com triptofano.

No entanto, apesar das diferenças nos níveis cerebrais de triptofano encontradas entre FS e os demais grupos com fibromialgia experimental, curiosamente, os níveis de QUINU, a atividade da IDO inferida pela razão QUINU:TRP e a razão entre 5-HT:QUINU não diferiram entre FS e FES. Estes dois grupos tiveram concentrações cerebrais mais elevadas de QUINU, maior atividade inferida da IDO, e menor razão entre as vias da serotonina e da quinurenina (5-HT:QUINU) do que o grupo FE. Neste modelo experimental, o excesso de triptofano advindo da suplementação parece ativar a IDO no cérebro contribuindo para que o triptofano seja preferencialmente metabolizado pela via da quinurenina, e não pela via da serotonina. Esta sugestão encontra suporte na literatura, onde já foi relatado que cerca de 95 % do triptofano da dieta é metabolizado pela via da quinurenina, enquanto apenas 1-2% serão processados pela via da serotonina[46].

Deste modo, no que se refere à melhora na hiperalgesia, tanto o exercício aeróbico aplicado, quanto a suplementação com triptofano, isoladamente, e a combinação dos tratamentos produziram a melhora do quadro de hiperalgesia dos animais com fibromialgia. Não obstante, a melhora da hiperalgesia nos grupos exercitados parece estar ligada, principalmente, aos efeitos anti-inflamatórios produzidos pelo exercício, enquanto a diminuição da hiperalgesia nos animais suplementados com triptofano

parece estar associada ao aumento das concentrações cerebrais de triptofano e a uma possível melhora na função moduladora da serotonina em regiões específicas do sistema nervoso central[20-22]. Estudos futuros devem priorizar a análise das concentrações de serotonina em regiões hipotalâmicas e do tronco cerebral relacionadas à modulação serotoninérgica da dor, bem como prováveis mecanismos pelos quais a combinação exercício aeróbico e suplementação com triptofano restringe o aumento das concentrações cerebrais de triptofano.

Em conclusão, o modelo de fibromialgia induzida por salina ácida promove hiperalgesia bilateral em ratas, sem, no entanto, alterar o metabolismo cerebral do triptofano e a formação de seus metabólitos (5-HT e QUINU) nas vias de serotonina e quinurenina. A associação da suplementação com triptofano ao exercício aeróbico de intensidade baixa não potencializa a redução da hiperalgesia mecânica bilateral promovida pelo exercício neste modelo de fibromialgia. Entretanto, a suplementação com triptofano, isoladamente, reduz a hiperalgesia e aumenta as concentrações cerebrais de triptofano neste modelo de fibromialgia.

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5. CONCLUSÕES GERAIS

O modelo de fibromialgia induzida por salina ácida promove hiperalgesia bilateral em ratas, sem, no entanto, alterar o metabolismo cerebral do triptofano e a formação de seus metabólitos (5-HT e QUINU) nas vias de serotonina e quinureninas. Entretanto, há aumento das concentrações séricas de cortisol e musculares IL-6, bem como redução da atividade antioxidante de SOD e CAT nos animais com fibromialgia, sugerindo que tanto alterações no eixo HPA quanto o aumento do estresse oxidativo podem ter um papel importante na fisiopatologia da fibromialgia.

O exercício aeróbico de intensidade baixa reduz a hiperalgesia mecânica bilateral (~151%) e as concentrações séricas de cortisol (72%), mas não altera a atividade de SOD, CAT e MDA, bem como as concentrações cerebrais de triptofano e 5-HT entre os animais com FM experimental, o que indica que a melhora da dor pode estar relacionada à redução da sensibilização central modulada por uma normalização das funções neuroendócrinas do eixo HPA.

A suplementação com triptofano reduz a hiperalgesia mecânica bilateral (~67%) e as concentrações séricas de cortisol (67%), reduz as concentrações hepáticas de MDA, além de aumentar as concentrações cerebrais de triptofano entre os animais com fibromialgia. Isto indica que o triptofano parece ter um papel fundamental na regulação das principais vias envolvidas com a etiologia da dor crônica musculoesquelética e pode ser utilizada como uma alternativa ao tratamento de subpopulações com fibromialgia. Futuros estudos devem considerar o uso de suplementos ricos em triptofano no tratamento da fibromialgia, bem como estabelecer os riscos, as quantidades e o tempo de consumo do triptofano necessário à obtenção dos benefícios em seres humanos.

A combinação do exercício aeróbico de intensidade baixa com a suplementação com triptofano reduz a hiperalgesia bilateral (162%), as concentrações séricas de cortisol (54%) e as concentrações musculares de IL-6 (68%), mas não afeta a atividade de SOD e CAT, bem como as concentrações hepáticas de MDA e o metabolismo cerebral do triptofano. As intervenções também não alteram as concentrações cerebrais de QUINU e as concentrações séricas de substância P ao final das três semanas de intervenções.

Em conjunto, os achados deste estudo indicam que a associação da suplementação com triptofano ao exercício aeróbico de intensidade baixa não potencializa os efeitos do exercício sobre os parâmetros avaliados neste modelo de fibromialgia experimental, entretanto uma importante redução em IL-6 é evidente.

ANEXO

Aprovação pela Comissão de Ética no Uso de Animais (CEUA) da Universidade Federal de Viçosa (UFV)

CERTIFICADO

A Comissão de Ética no Uso de Animais - CEUA/UFV certifica que o processo nº 21/2015, intitulado "Influência do exercício físico associado a dieta rica em triptofano no controle da dor em ratos com fibromalgia experimental", coordenado pelo professor Antônio José Natali do Departamento de Educação Física, está de acordo com a Legislação vigente (Lei Nº 11.794, de 08 de outubro de 2008), as Resoluções Normativas editadas pelo CONCEA/MCTI, a DBCA (Diretriz Brasileira de Prática para o Cuidado e a Utilização de Animais para Fins Científicos e Didáticos) e as Diretrizes da Prática de Eutanásia preconizadas pelo CONCEA/MCTI, portanto sendo aprovado por esta Comissão em 12/06/2016, com validade de 12 meses.

CERTIFICATE

The Ethic Committee in Animal Use/UFV certify that the process number 21/2015, named "Influence of exercise associated with tryptophan rich diet in pain control in rats with experimental fibromyalgia", is in agreement with the a ctual Brazilian legislation (Lei Nº 11.794, 2008), Normative Resolutions edited by CONCEA/MCTI, the DBCA (Brazilian Practice Guideline for the Care and Use of Animals for Scientific Purposes and Teaching) and the Guidelines of Practice the Euthanasia recommended by CONCEA/MCTI therefore being approved by the Committee on July 12, 2016 valid for 12 months.

Profª. Átima Clemente Alves Zuanon
Presidente
Comissão de Ética no Uso de Animais – CEUA/UFV