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**EFEITOS DO EXERCÍCIO FÍSICO EM CAMUNDONGOS COM
INFLAMAÇÃO ARTICULAR INDUZIDA POR DIÓXIDO DE
TITÂNIO**

Londrina
2020

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Defesa de Mestrado apresentada ao Curso de Pós-Graduação em Ciências da Saúde da Universidade Estadual de Londrina, como requisito parcial à obtenção do título de Mestre em Ciências da Saúde.

Orientador: Prof. Dr. Rafael Deminice

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Londrina, 17 de fevereiro de 2020.

DEDICO

Dedico este trabalho aos meu pais. Serei sempre grato pela educação, formação, conhecimento e oportunidades que me proporcionaram.

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RESUMO

Artrite é uma doença que afeta milhões de pessoas ao redor do mundo, se manifestando de várias maneiras e conseqüentemente afetando a qualidade de vida e função muscular das pessoas portadoras da doença. Estudos mostram o potencial do exercício físico como uma ferramenta no tratamento da doença e melhora na função muscular. Foram utilizados sessenta camundongos swiss fêmeas durante sessenta dias para 1) estudar a ocorrência da sarcopenia associada à artrite induzida por dióxido de titânio (TiO₂) e 2) verificar o efeito da corrida voluntária sobre a sarcopenia induzida por TiO₂. Para tanto foram feitos dois experimentos. O primeiro, para determinar a dose adequada de dióxido de titânio (TiO₂) e ocorrência de inflamação, utilizou dezenove animais, divididos em grupo controle (C, n=7), 0,3mg TiO₂ (n=6) e 3mg TiO₂ (n=6). O TiO₂ foi injetado na articulação femorotibial. No segundo experimento, quarenta e um animais foram alocados em gaiolas individuais, divididos em quatro grupos: controle (C, n=10), artrite (A, n=11), exercício (E, n=10) e artrite exercício (AE, n=10). Os grupos A e AE foram injetados com TiO₂, os grupos E e AE tinham uma roda instalada em suas respectivas gaiolas para a prática de corrida voluntária. Análises de peso corporal, força e morfologia muscular, histologia e bioquímica foram realizadas para constatar a artrite e sarcopenia induzidas pelo TiO₂. A injeção de TiO₂ causou dano ao tecido articular, redução da área de secção transversa muscular, menor força de preensão, promoveu maiores níveis de dor e inflamação e aumento do TNF- α na articulação. O exercício foi eficaz para mitigar o dano tecidual e contrariar os efeitos decorrentes da injeção de TiO₂ na articulação femorotibial. Foi capaz de promover hipertrofia, aumento de força de pressão e diminuir o número de leucócitos na região inflamada. Assim, os resultados obtidos sugerem que o exercício tem efeitos protetores e pode ser usado como uma ferramenta para diminuir o grau das alterações causadas pelo TiO₂ no tecido muscular e articular.

Palavras chave: Artrite. Sarcopenia. Exercício físico.

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ABSTRACT

Arthritis is a disease that affects millions of people around the world, manifesting itself in various ways and consequently affecting the quality of life and muscle function of people with it. Studies show the potential of physical exercise as a tool in the treatment of this disease and improvement in muscle function. Sixty female Swiss mice were used for 60 days to study the occurrence of sarcopenia associated with titanium dioxide-induced arthritis and to verify the effect of resistance exercise on titanium dioxide-induced sarcopenia. For this two experiments were done. The first, to determine the appropriate titanium dioxide (TiO₂) dose and the occurrence of inflammation, used nineteen animals divided into control group (C, n=7), 0.3mg TiO₂ (n=6) and 3mg TiO₂ (n=6). In the second experiment, forty-one animals were housed in individual cages, divided into four groups: control (C, n=10), arthritis (A, n=11), exercise (E, n=10) and exercise arthritis (AE, n=10). Groups A and AE received a titanium dioxide injection in the femur-tibial joint and had a running wheel installed in their respective cages for voluntary wheel running. Analysis of body weight, muscle strength, morphology, histology and biochemistry were made to verify whether arthritis and sarcopenia were induced by titanium dioxide. The injection caused damage to joint tissue, reduced muscle cross sectional area, lowered grip strength, increased pain and inflammation, and increased TNF- α in the joint. Exercise was effective in mitigating tissue damage and counteracting the effects of TiO₂ injection into the femur-tibial joint. It was able to promote muscle hypertrophy, increase grip strength and decrease the number of leukocytes in the knee joint. Thus, the results suggest exercise has protective effects and can be used as a tool to decrease the degree of changes caused by TiO₂ in muscle and joint tissue.

Key words: Arthritis. Sarcopenia. Physical exercise.

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1 INTRODUÇÃO

1.1 ARTRITE

A artrite é uma doença autoimune e inflamatória que afeta principalmente as articulações[1] e apresenta mais de 100 tipos[2]. O diagnóstico da doença é muito amplo e considera qualquer doença articular ou sintomas que levem à essa conclusão, sendo que as principais manifestações se enquadram como artrite inflamatória ou artrite reumatoide[3]. Os tipos mais comuns podem ser divididos em três grupos principais: artrite degenerativa, artrite inflamatória e artrite metabólica[4]. A abordagem terapêutica é baseada no tipo de artrite, sendo a osteoartrite considerada uma condição degenerativa decorrente do envelhecimento enquanto a artrite inflamatória é vista geralmente como doença autoimune[5]. A condição de artrite metabólica, conhecida como gota é uma das formas mais dolorosas da doença[6]. Essa condição é causada por altos níveis de ácido úrico no sangue, formando cristais nos tecidos articulares que resultam em inflamação e dor severa.

Um dos tipos mais abordados e populares na literatura é a artrite reumatoide. O principal sintoma é a dor crônica que se manifesta em diferentes intensidades e locais, outros fatores relacionados às articulações incluem inchaço, rigidez e aumento de sensibilidade[7–9]. A artrite do joelho geralmente está ligada à perda de cartilagem articular e pode ser observada nos exames de raio-x. Essa condição pode causar níveis mais altos de dor e rigidez na articulação do joelho. Características como peso corporal, nível de atividade física, anatomia individual do joelho e lesões podem influenciar o desenvolvimento da artrite.

A causa fundamental da artrite é desconhecida mas o processo que leva à doença inclui reações autoimunes baseadas em uma combinação de suscetibilidade genética e fatores ambientais[10]. A artrite apresenta fatores capazes de aumentar o risco de sarcopenia[11], incluindo diminuição do nível de atividade física[12], maiores níveis de TNF- α e interleucina 1 β (IL-1 β), altos níveis de proteína C-reativa (PCR), imobilidade causada por rigidez e dor[13].

Devido à dor e rigidez, as pessoas com osteoartrite do joelho apresentam níveis mais baixos de atividades da vida diária e também de atividade física, tornando os músculos das pernas mais fracos. Portanto, os desdobramentos da osteoartrite do joelho levam ao ganho de peso, estilo de vida sedentário e suas comorbidades.

1.1.2 Epidemiologia Da Artrite

Um estudo aponta que a prevalência estimada da artrite para os Estados Unidos da América (EUA) e Europa é de 0,5 a 1% da população adulta[14]. A doença pode ocorrer em qualquer idade mas comumente acontece em pessoas entre 45 e 65 anos[15], com uma taxa de prevalência três vezes maior para o sexo feminino comparado ao masculino[14]. Dados mostram incidência de 25-50 novos casos por ano a cada 100,000 habitantes[16].

Em 2013 nos EUA custos médicos somados com as perdas salariais causadas pela artrite somaram 304 bilhões de dólares, valor equivalente a 1% do PIB do país no mesmo ano[17]. Tal dado faz com que a artrite seja a causa principal de perdas salariais e incapacidade física no país[18].

1.2 SARCOPENIA

Inicialmente, sarcopenia era um termo utilizado para definir a perda de músculo associada ao envelhecimento, porém, atualmente é usado para indicar qualquer perda de tecido e função muscular devido ao envelhecimento, doenças crônicas, baixa ingestão proteica e inatividade física[19,20]. A sarcopenia é uma condição comum que exige uma grande atenção dos sistemas de saúde, porém, não há consenso em uma definição amplamente aceita[21].

Abordagens sobre o conceito de sarcopenia geralmente abrangem dados de massa muscular, força e função muscular[22]. Duas definições comumente aceitas são provenientes do *European Working Group on Sarcopenia on Older People* (EWGSOP) e do *International Working Group on Sarcopenia* (IWGS)[20,23]. O EWGSOP leva em consideração a avaliação da massa magra acompanhada de baixa força de preensão manual e/ou velocidade de marcha, enquanto a definição do IWGS utiliza apenas a massa magra e velocidade de marcha[20,23]. Apesar de serem mais comuns, essas definições apresentadas não vão enquadrar os mesmos sujeitos em todos os casos[21].

A perda de massa e função muscular é um fenômeno natural decorrente do envelhecimento. Pelo fato de naturalmente homens apresentarem maior força muscular que mulheres, a perda da mesma é maior no sexo masculino frente ao feminino[22]. Portanto, para que a sarcopenia seja diagnosticada, é necessária a avaliação da massa muscular, força muscular e performance física[24].

A sarcopenia está estritamente correlacionada com incapacidade física, baixa qualidade de vida e morte[25], além disso, é associada a grandes comorbidades como obesidade, osteoporose, diabetes tipo 2 e resistência à insulina[26,27].

É conhecido que as causas da sarcopenia incluem inflamação crônica decorrente da doença, declínio nos níveis de atividade física e aumento do gasto calórico em estado de repouso[25,28]. Níveis elevados de citocinas pró-inflamatórias, como fator de necrose tumoral alfa (TNF- α) e interleucina-6 (IL-6), podem promover perda de massa muscular e estão associados com a redução de massa e força muscular em pessoas mais velhas[29].

Em situações onde há inflamação crônica e inatividade, como a sarcopenia, é observado desequilíbrio entre síntese e degradação muscular[30], levando a reduções na massa e função muscular que são associadas com um aumento de mortalidade e quedas[31], osteoporose[32] e, conseqüentemente, fragilidade.

1.2.1 Edpidemiologia Da Sarcopenia

Ao redor do mundo, em 2014, mais de 50 milhões de pessoas eram afetadas pela sarcopenia e para os próximos 40 anos a estimativa é de mais de 200 milhões[25]. Em pessoas que apresentam alguma outra condição de saúde irregular, a prevalência de sarcopenia é maior[33]. A presença de outras doenças, como doença pulmonar obstrutiva crônica, doenças cardiovasculares e câncer tem sido associadas com a perda de massa muscular[34].

1.3. EXERCÍCIOS E SEUS BENEFÍCIOS EM PESSOAS COM ARTRITE E SARCOPENIA

Estudos mostram que o exercício físico, tanto aeróbio quanto anaeróbio, proporcionam melhorias nas capacidades funcionais de pessoas com artrite [35,36]. Em outra publicação[37] foi demonstrado que o exercício resistido progressivo pode combater a sarcopenia e dinapenia(que é a perda de força e potência muscular relacionada à idade)[38]. Além disso, a atividade física pode reduzir a dor e melhorar a função muscular de pessoas com artrite em 40%[39].

Os benefícios da atividade física também se aplicam às pessoas com sarcopenia. Estudos prévios, realizados com adultos em meia idade e idade avançada, mostram que os indivíduos com maior nível de atividade física apresentam maiores níveis função física[40–43]. Recente estudo por Tourndare e colaboradores[44] abordou o gerenciamento da sarcopenia. De acordo com os autores isso deve ser feito via combinação que engloba aspectos nutricionais, exercício e medicamentos anti-inflamatórios e anabólicos. Via de regra treinamentos de exercício devem combinar exercício aeróbio e anaeróbio, visando melhorar a função cardiovascular e aumento de massa magra[45]. Todavia, programas de

atividades e exercícios para pessoas com sarcopenia devem ser feitos sob medida para o paciente.

Há um grande número de pessoas afetadas pela artrite, suas consequências diminuem a qualidade de vida dos pacientes, levando ao desenvolvimento da sarcopenia.

2. OBJETIVO

Os objetivos desse estudo foram (1) verificar os efeitos da artrite induzida por TiO_2 na massa e força muscular (2) averiguar o efeito do exercício voluntário sobre a perda de massa e função muscular induzida em animais que receberam injeção de TiO_2 .

3. MATERIAL E MÉTODOS

Foram utilizados sessenta camundongos Swiss fêmeas provenientes do Biotério Central da Universidade Estadual de Londrina. Os animais foram mantidos durante todo o experimento no Biotério do Centro de Educação Física e Esporte da mesma instituição. Os animais foram acomodados em gaiolas individuais com ração (Nuvilab CR-1, Nuvital) e água *ad libitum*, ciclo claro/escuro de 12h/12h e temperatura controlada ($25\pm 2^\circ\text{C}$). Todos os procedimentos foram feitos de acordo com os preceitos da Lei nº 11.794, de 8 de outubro de 2008, do Decreto nº 6.899, de 15 de julho de 2009, e com as normas editadas pelo Conselho Nacional de Controle da Experimentação Animal (CONCEA). A pesquisa foi aprovada pela Comissão de Ética no Uso de Animais da Universidade Estadual de Londrina (CEUA/UEL), em 08/05/2018 (Anexo 1).

Experimento 1: Dezenove animais pesando inicialmente 20-25g, com 4 semanas de vida, foram divididos aleatoriamente em grupo controle (C, n=7), injeção intra-articular de dióxido de titânio (TiO_2) de 0,3mg (n=6) e injeção intra-articular de TiO_2 de 3mg (n=6). Dióxido de titânio, um pó branco e inodoro, além de ser utilizado para produção de próteses ortopédicas, pode ser utilizado como pigmentação branca em tintas, corantes alimentícios, protetores solares e cremes cosméticos[46]. O TiO_2 (mantido em solução salina de 0.9%) proveniente do Laboratório de Dor, Inflamação, Neuropatia e Câncer do Centro de Ciências Biológicas da Universidade Estadual de Londrina, Londrina – PR) foi aplicado em dose única no joelho direito (articulação femorotibial) da pata traseira. Os animais foram sacrificados sessenta dias após a injeção. O período foi estipulado para a ocorrência, escolha da dosagem adequada de TiO_2 e detecção de inflamação no local da injeção[47].

Experimento 2: Quarenta e um animais pesando inicialmente 20-25g, com 4 semanas de vida, foram divididos aleatoriamente em grupo controle (C, n=10), artrite (A, n=11), exercício (E, n=10) e artrite exercício (AE, n=10). Os grupos A e AE foram submetidos a uma única injeção de 3 mg TiO_2 mg. A dose e tempo escolhidos foram baseados nos resultados do experimento 1. Os animais dos grupos C e E não foram expostos à injeção de TiO_2 . Os animais dos grupos E e AE foram submetidos a um protocolo de exercício voluntário através de uma roda de corrida instalada dentro de suas respectivas gaiolas. A distância percorrida pelos animais (km/dia) foi monitorada por um odômetro acoplado às gaiolas. O tempo de duração do experimento foi suficiente para que ocorressem adaptações robustas no músculo esquelético[48]. Os animais submetidos ao protocolo de exercício foram acomodados nas gaiolas com roda de corrida no dia da injeção de TiO_2 .

3.1 FORÇA E FUNÇÃO MUSCULAR

Para a avaliação da força de tração das patas foi utilizado o aparelho Grip Strength Meter (Insight Instrumentos, Ribeirão Preto, Brasil), seguindo protocolo previamente descrito[49].

A avaliação da hiperalgesia mecânica e formação de edema foi realizada com base no protocolo existente[50].

A espessura articular foi avaliada por medidas transversais utilizando um paquímetro digital[50]. Após uma semana de adaptação ao aparelho de teste rota rod [51], os animais foram submetidos a testes com aumento progressivo de rotações por minuto em um período de 2 minutos, onde o melhor resultado foi utilizado para análise[52].

As análises acima foram feitas três vezes por semana, a partir do dia onde foi aplicada a injeção de TiO_2 .

3.2 EUTANÁSIA E COLETA DE TECIDOS

A eutanásia dos animais ocorreu no sexagésimo dia de experimento. A articulação da pata traseira direita e os músculos sóleo e extensor longo dos dedos (EDL) dos animais foram coletados para análise histológica. O músculo tibial anterior foi coletado e utilizado para a análise de interleucinas (TNF- α e IL-6). O sangue foi coletado, centrifugado e o plasma utilizado para determinação de interleucinas circulantes.

3.3 HISTOLOGIA

As amostras de tecido muscular foram fixadas durante 24 horas em paraformaldeído a 4%, desidratadas em soluções de álcool, lavadas em xilol e por fim inclusas em parafina[53]. Cortes transversais de 5 μ m foram feitos para a análise da área de secção transversa (AST) das fibras musculares. Para esta análise os cortes foram corados com hematoxilina e eosina, fotos foram capturadas usando uma câmera acoplada à um microscópio óptico com aumento de 100x. A mensuração da AST foi feita através do software ImageJ, 20 fibras por imagem de um total de 365 imagens.

As amostras de articulação dos animais foram coletadas e imersas em uma solução de ácido nítrico (HNO_3 , 5%) por 14 dias para descalcificação. Durante este período a solução onde as amostras estavam imersas era renovada a cada 2 dias. Após a descalcificação as amostras foram desidratadas em soluções de álcool, lavadas em xilol e inclusas em parafina. Cortes transversais de 5 μ m foram feitos e corados por hematoxilina e eosina para observação da ocorrência de alterações no tecido articular. Fotos foram

capturadas usando uma câmera acoplada à um microscópio óptico com aumento de 100x.

3.4 ANÁLISES BIOQUÍMICAS

As amostras das articulações foram homogeneizadas em 500 μ L de solução tampão [54,55] contendo inibidores de protease, níveis de TNF α e IL-6 foram determinados por ELISA usando kits de eBioscience.

3.5 ANÁLISE ESTATÍSTICA

A normalidade da distribuição dos dados foi analisada com o teste de Shapiro-Wilk. Os resultados foram apresentados em valores de média e desvio padrão. Na comparação dos resultados intergrupais e ao longo do tempo foi utilizado ANOVA Two-way e post-hoc de Tukey. Na ausência de normalidade, foi utilizado o teste de Kruskal-Wallis seguido de post-hoc de Dunn. Em todos os casos, os resultados foram considerados significativos quando $p < 0,05$. O programa GraphPad Prism 6 foi utilizado para as análises estatísticas.

4 RESULTADOS

4.1 ARTIGO

Effects of physical exercise in mice with titanium dioxide- induced articular inflammation

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ABSTRACT: Arthritis is a disease that affects millions of people around the world, manifesting itself in various ways and consequently affecting the quality of life and muscle function of people with it. Studies show the potential of physical exercise as a tool in the treatment of this disease and improvement in muscle function. Sixty female Swiss mice were used for 60 days to study the occurrence of sarcopenia associated with titanium dioxide-induced arthritis and to verify the effect of resistance exercise on titanium dioxide-induced sarcopenia. For this two experiments were done. The first, to determine the appropriate titanium dioxide (TiO₂) dose and the occurrence of inflammation, used nineteen animals divided into control group (C, n=7), 0.3mg TiO₂ (n=6) and 3mg TiO₂ (n=6). In the second experiment, forty-one animals were housed in individual cages, divided into four groups: control (C, n=10), arthritis (A, n=11), exercise (E, n=10) and exercise arthritis (AE, n=10). Groups A and AE received a titanium dioxide injection in the femur-tibial joint and had a running wheel installed in their respective cages for voluntary wheel running. Analysis of body weight, muscle strength, morphology, histology and biochemistry were made to verify whether arthritis and sarcopenia were induced by titanium dioxide. The injection caused damage to joint tissue, reduced muscle cross sectional area, lowered grip strength, increased pain and inflammation, and increased TNF- α in the joint. Exercise was effective in mitigating the effects of TiO₂ injection into the femur-tibial joint. It was able to promote muscle hypertrophy, increase grip strength and decrease the number of leukocytes in the knee joint. Thus, the results suggest exercise has protective effects and can be used as a tool to decrease the degree of changes caused by TiO₂ in muscle and joint tissue.

Key words: arthritis, sarcopenia, physical exercise.

INTRODUCTION

Arthritis is a chronic, autoimmune, inflammatory systemic disease that affects primarily the joints[1] having more than 100 types[2]. The main symptom is chronic pain that manifests at different intensities and locations, other factors related to joints include swelling, stiffness and increased sensitivity[3–5]. Arthritis of the knee is usually linked to loss of articular cartilage and can be seen on x-ray examination. This condition may cause higher levels of pain and stiffness of the knee joint. Characteristics such as body weight, physical activity level, individual knee anatomy and previous injuries can influence the development of arthritis.

Treatment of knee osteoarthritis (OA) includes surgical procedures such as osteotomy, more common for young individuals, and knee replacement. Total knee arthroplasty (TKA) is strongly recommended as an effective and cost-effective treatment for patients with OA who have severe symptoms or functional limitations[6]. A knee replacement surgery replaces the bone with a prosthesis which is usually made with metal components. The concern with metal components is that their byproducts can cause bone and soft tissue loss due to local inflammation and necrosis to a lesser or higher degree[7–9].

Data from a study points out that the estimated prevalence of arthritis for the United States and Europe is 0.5 to 1% of the adult population[10]. The disease can occur at any age but it commonly happens in people between 45 and 65 years old[11], with a prevalence rate three times higher for females compared to males[10]. Data show an incidence of 25-50 new cases per year per 100,000 population[12].

Initially, sarcopenia was a term used to define muscle loss associated with aging, but it is currently used to indicate any loss of muscle tissue and function due to aging, chronic disease, low protein intake and physical inactivity[13,14]. In 2014 over 50 million people were affected by sarcopenia and for the next 40 years it is estimated that this number reaches over 200 million people[15]. Sarcopenia is closely correlated with physical disability, poor quality of life and death[16]; in addition, it is associated with major comorbidities such as obesity, osteoporosis, type 2 diabetes and insulin resistance[15,17].

Causes of sarcopenia include chronic inflammation from chronic pain, decline in physical activity levels, and increased resting caloric expenditure[15,18]. The effects of sarcopenia lead to increased falls, osteoporosis[19] and, consequently, frailty. Decrease in muscle mass and function are associated with increased mortality and higher risk of fall[20]. Higher levels of pro-inflammatory cytokines, tumor necrosis factor alpha (TNF- α) and interleukin-6 (IL-6), may promote muscle loss and are associated with reduced muscle mass and strength in older people[21]. In situations where there is chronic inflammation and

physical inactivity, such as sarcopenia, imbalance between muscle synthesis and muscle breakdown is observed[22].

Arthritis has some characteristics that increase the risk of sarcopenia[23], including decreased physical activity [24], higher levels of TNF- α and interleukin 1 β (IL-1 β), high levels of C-reactive protein (CRP), stiffness and pain[25]. Due to the pain inflammation and stiffness, people with OA show lower levels of daily life activities and physical activity as well. In addition, this condition leads to weaker leg muscles. Altogether, the outcomes of knee OA lead to weight gain and a sedentary lifestyle and its comorbidities.

Studies show that both aerobic and anaerobic exercise provides improvements in the functional abilities of people with arthritis[26,27]. In another publication[28] it was shown that progressive resistance exercise can combat age-related sarcopenia and dynapenia, loss of strength and muscle power[29], in addition, physical activity can reduce pain and improve muscle function by 40%[30]. The benefits of exercise and physical activity go beyond improving the quality of life of people with arthritis. A study found that in 2013 in the United States the total cost of medical costs plus salary losses totaled \$ 304 billion, equivalent to 1% of the nation's GDP in the same year[31].

Many people are affected by arthritis and its consequences decrease the quality of life of patients, leading to the development of sarcopenia. Exercise has the potential to be an important and viable tool for reducing the effects of arthritis. Nevertheless, the effects of exercise on people with arthritis and sarcopenia have not been observed. Thus, the aims of this study were (1) to verify the effects of titanium dioxide (TiO₂) induced arthritis on muscle mass and strength, and (2) to investigate the effect of voluntary exercise on muscle loss and muscle function induced in animals that received TiO₂ injection.

METHODS

Sixty female Swiss mice from the Central Animal Facility of Londrina State University were used. The animals were housed in individual cages with food (Nuvilab CR-1, Nuvital) and water ad libitum, 12h/12h light/dark cycle and controlled temperature ($25\pm 2^{\circ}\text{C}$). All procedures were performed in accordance with the Brazilian law and the National Council for Control of Animal Experimentation (CONCEA). The research was approved by the Animal Use Ethics Committee of Londrina State University. Two experiments were conducted as follows.

Experiment 1: Nineteen animals, initially weighing 20-25g, 4 weeks old, were randomly divided into control group (C, $n = 7$), intra-articular titanium dioxide injection (TiO_2) of 0.3mg ($n = 6$) and injection intra-articular 3 mg TiO_2 ($n = 6$). Titanium dioxide, a white and odorless powder, besides being used in the production of orthopedic prosthesis, can be used as white pigment in paint, food colorant, sunscreen and cosmetic creams[32]. TiO_2 (suspended in 0.9% saline solution) from the Pain, Inflammation, Neuropathy and Cancer Laboratory of the Center for Biological Sciences of Londrina State University, Londrina - PR) was injected in the right knee (femorotibial joint) of the hindpaw. The time period was stipulated for occurrence, choice of appropriate TiO_2 dosage and detection of injection site inflammation. Animals were sacrificed sixty days after injection. The time period was stipulated to choose the appropriate TiO_2 dosage and to check the occurrence of inflammation[33].

Experiment 2: Forty-one animals, initially weighing 20-25g, 4 weeks old, were randomly divided into control group (C, $n = 10$), arthritis (A, $n = 11$), exercise (E, $n = 10$) and arthritis exercised group (AE, $n = 10$). Groups A and AE were submitted to a single injection of 3 mg TiO_2 mg. The TiO_2 dosage and time chosen were based on results from Experiment 1. Animals in groups C and E were not exposed to the TiO_2 injection. On the day of TiO_2 injection, animals from groups E and AE were housed in individual cages with access to a running wheel. The distance traveled by the animals was monitored daily by an odometer coupled to the cages. The duration of the experiment was beyond the minimum necessary for robust skeletal muscle adaptations to occur[34].

STRENGTH AND MUSCLE FUNCTION

Grip strength was assessed with a Grip Strength Meter device (Insight Instrumentos, Ribeirão Preto, Brazil) as previously described[35].

Evaluation of mechanical hyperalgesia and edema formation was performed based on existing protocol[36].

Joint thickness was assessed by transverse measurements using a digital caliper[36]. After one week of familiarization with the rota rod test apparatus, animals were submitted to an increased rpm protocol which lasted for 2 minutes[37]. The highest time result in 3 attempts was used for analysis.

These analysis mentioned previously were performed three times a week starting from the day the TiO₂ injection took place.

EUTHANASIA AND TISSUE COLLECTION

The animals were euthanized on the 60th day of the experiment. The right hind paw joint, and soleus and extensor digitorum longus (EDL) muscles of the animals were collected for histological analysis. The tibialis anterior muscle was collected and used for interleukin analysis (TNF α and IL-6). Blood was collected, centrifuged and plasma was used to determine circulating interleukins.

HISTOLOGICAL ANALYSIS

Soleus and EDL tissue samples were fixed for 24 hours in 4% paraformaldehyde, dehydrated in alcohol solutions, washed in xylol and finally embedded in paraffin[38]. Cross sections (5 μ m thick) were made for the analysis of cross-sectional area (CSA) of muscle fibers. For this analysis the sections were stained with hematoxylin and eosin, and photos were captured using a camera attached to an optical microscope at 100x magnification. CSA was measured using Image J software (National Institute of Health, Bethesda, MD, USA.).

Joint samples were collected and immersed in a nitric acid solution (HNO₃, 5%) for 14 days for decalcification. During this period the solution where the samples were immersed was renewed every 2 days. After decalcification the samples were dehydrated in alcohol solutions, washed in xylol and embedded in paraffin. Cross sections (5 μ m thick) were made for the analysis to observe the occurrence of changes in joint tissue. Photos were captured using a camera attached to an optical microscope at 100x magnification.

BIOCHEMICAL ANALYSIS

Joint samples were homogenized in a 500 μ L buffer[39,40] containing protease inhibitors and levels of TNF α and IL-6 were determined by ELISA using eBioscience kits.

STATISTICAL ANALYSIS

Data normality was analyzed by the Shapiro-Wilk test. Results were presented as mean and standard deviation. Comparisons between groups were made by Two-way ANOVA followed by Tukey's post-hoc. In the absence of normality, the Kruskal-Wallis test was used followed by Dunn's post-hoc test. In all cases, the results were considered significant when $p < 0.05$. The software GraphPad Prism 6 (GraphPad Software, San Diego, CA, U.S.A.) was used for statistical analysis.

RESULTS

In the first experiment, no change was demonstrated in total weight gain and muscle mass between groups, as shown in Figure 1.

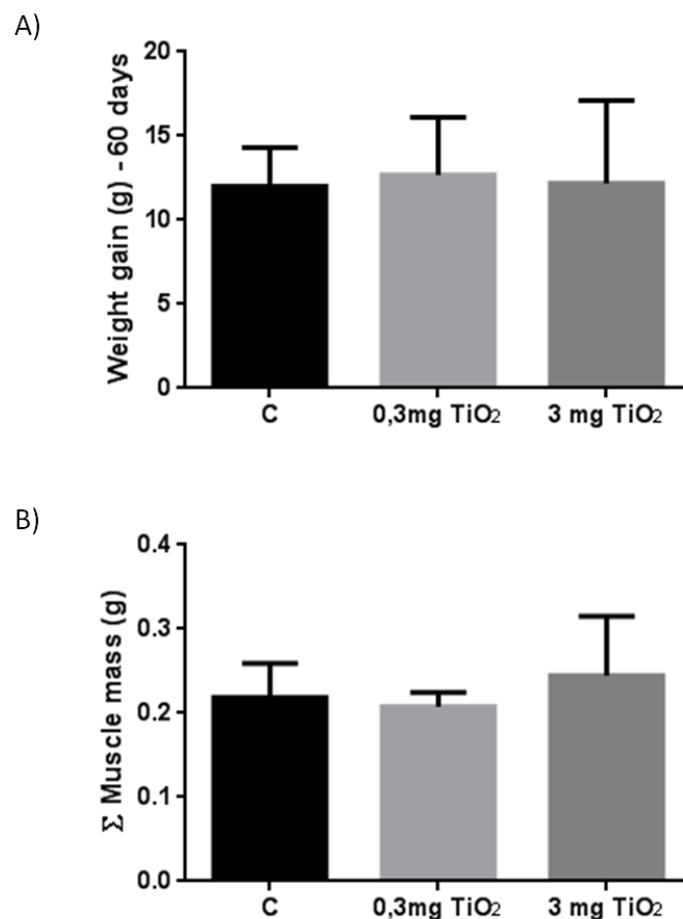


Figure 1. (A) Total body weight gain. **(B)** Sum of muscle weight collected on euthanasia.

The animals which received the 0,3 or 3 mg dose of TiO₂ have shown signs of articular inflammation being successfully induced by the proposed model. The pictures in Figure 2 demonstrate histopathological changes in the knee joint, which are dose dependent.

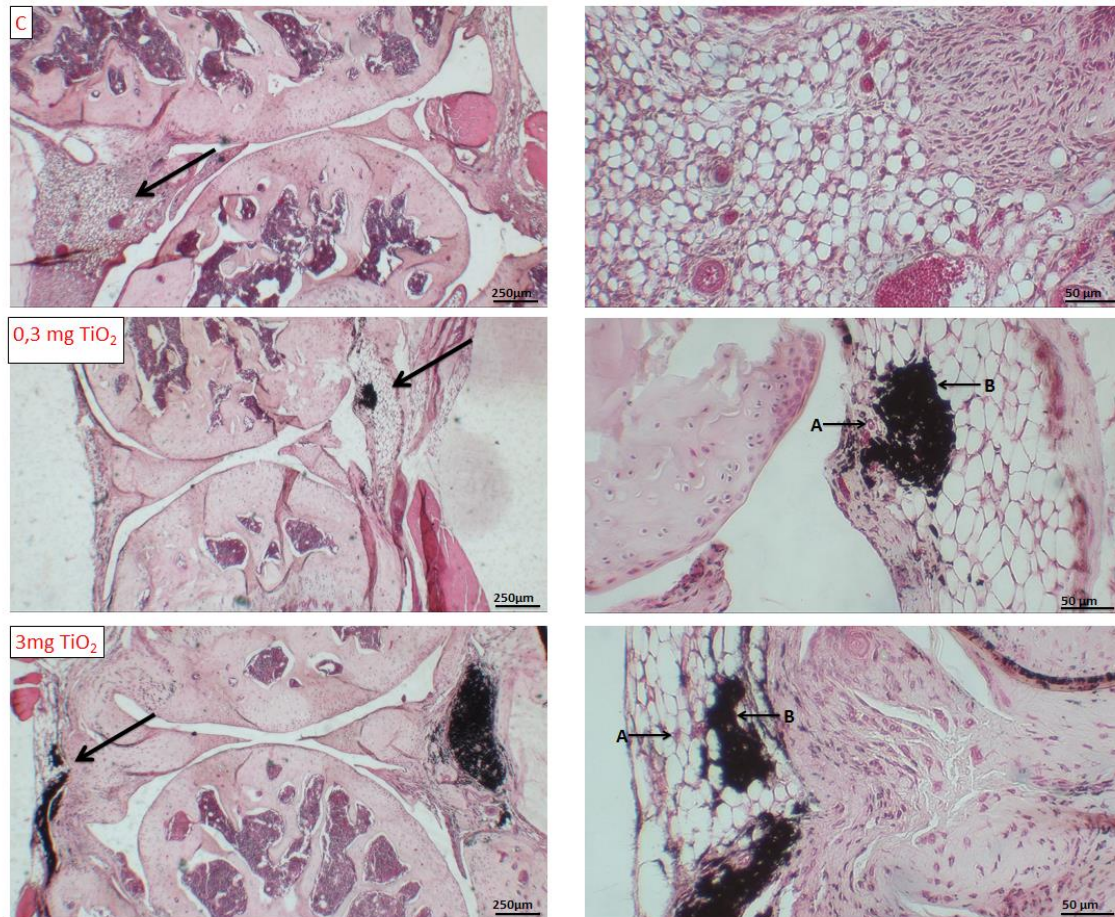
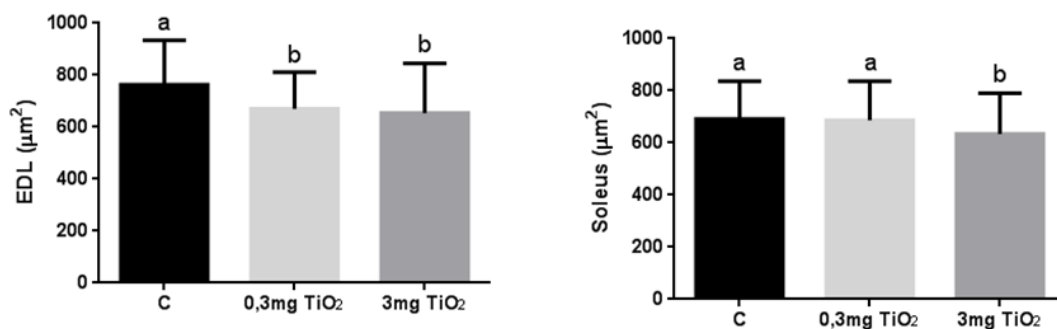


Figure 2. Representative pictures of H & E staining from each group. Pictures were taken using a microscope at 100x magnification. Arrows without letter point to area which is shown in greater magnification. Arrow (A) indicates vascular proliferation. Arrow (B) indicates leukocyte infiltration.

Data regarding muscle cross-sectional area (CSA) demonstrated a reduction occurred on both TiO_2 groups. The 0,3 mg TiO_2 dose caused a reduction only in the EDL muscle compared to control animals, while a dose of 3 mg TiO_2 reduced the CSA in the EDL and soleus muscles. For overall strength gain there were no changes between groups. As the experiment progressed there were only two specific times where differences in group average strength were noticed. The data mentioned above can be seen in Figure 3.

A)



B)

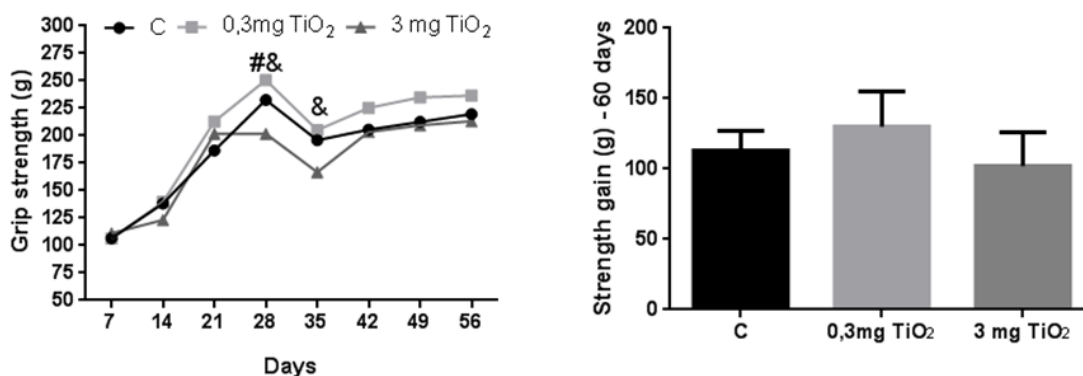


Figure 3. (A) Muscle cross-sectional area. Different letters mean $p < 0,05$. Two-way Anova followed by Tukey's post-hoc. **(B)** Grip strength progression and total grip strength gain. The symbol (&) represents difference between groups C and 0,3 mg TiO₂, $p < 0,05$. The symbol (#) represents difference between groups C and 3 mg TiO₂, $p < 0,05$. One-way Anova followed by Tukey's post-hoc. Values on panels A and B are shown as mean and standard deviation.

Based on data presented for experiment 1, the 3 mg dose of TiO₂ was chosen because of the articular inflammation and muscle atrophy presented. Experiment two presented similar results for total weight gain and muscle mass than those presented on experiment one. Thus, the results obtained indicate muscle mass does not seem to be affected by the articular injection of TiO₂, regardless of the level of physical activity, as shown in Figure 4.

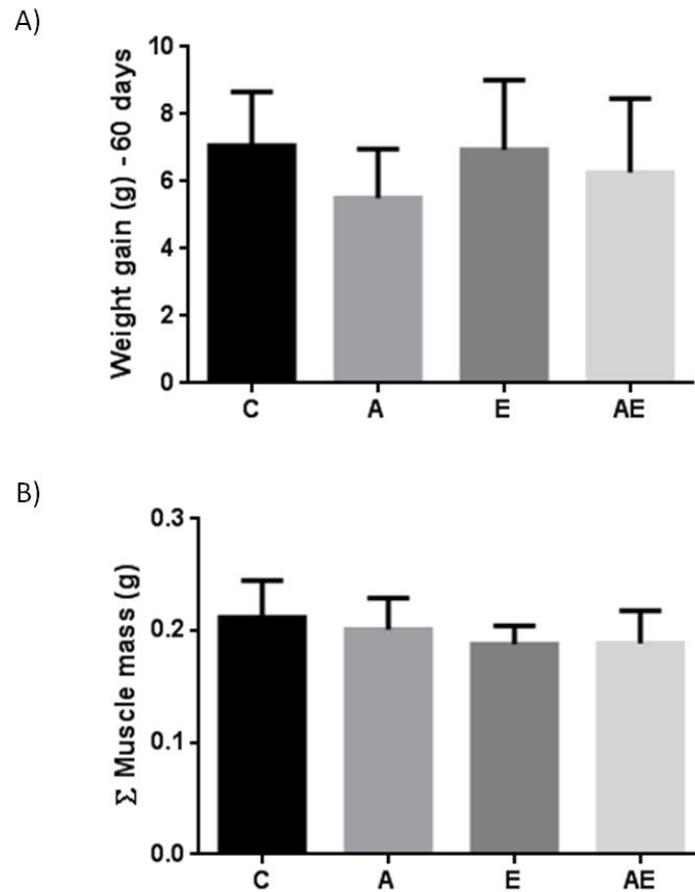


Figure 4. (A) Total body weight gain. **(B)** Muscle weight on euthanasia. b.w. = body weight. Different letters mean $p < 0,05$. One-way Anova followed by Tukey's post-hoc. Values on panels A and B are shown as mean and standard deviation.

Analysis of variables related to muscle morphology and function are on Figure 5. Exercise was effective on both groups which were exposed to the voluntary exercise protocol. Histological data revealed both soleus and E.D.L. had higher CSA when compared to their control groups and exercised animals had higher grip strength as well. Neither the rota rod balance test nor the running wheel test results indicated any difference between groups over time.

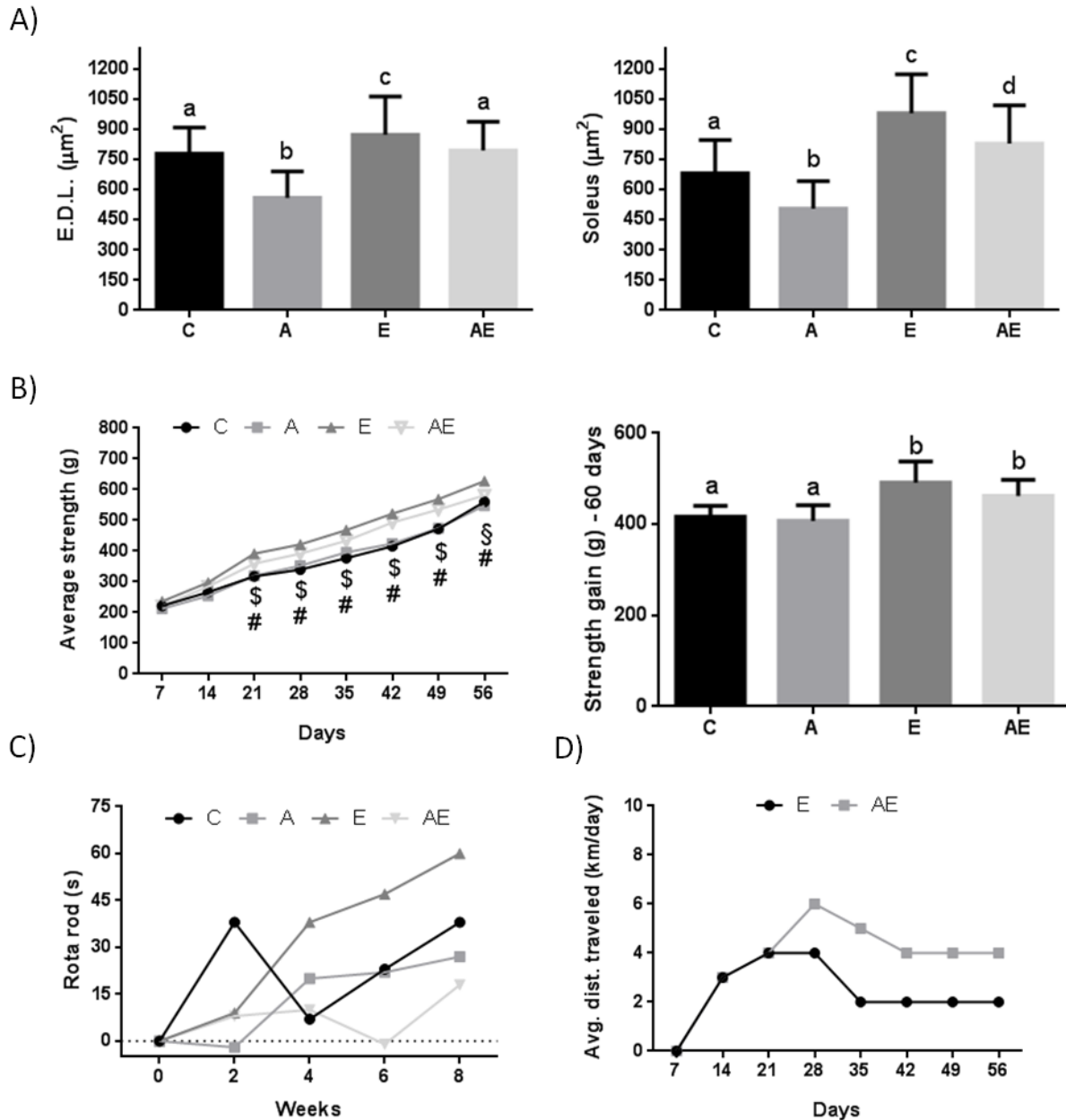


Figure 5. (A) Muscle cross-sectional area. Different letters mean $p < 0,05$. Two-way Anova followed by Tukey's post-hoc. **(B)** Grip strength progression and total grip strength gain. The (#) symbol represents difference between groups C and E, $p < 0,05$. The symbol (\$) symbol represents difference between groups A and AE, $p < 0,05$. The symbol (§) represents difference between groups E and AE, $p < 0,05$. One-way Anova followed by Tukey's post-hoc. **(C)** Rota rod balance test. Two-way Anova followed by Tukey's post-hoc. **(D)** Average distance traveled. Two-way Anova followed by Tukey's post-hoc. Values on panels A, B, C and D are shown as mean and standard deviation.

In this experiment the animals' mechanical articular nociception was evaluated. The results show lower threshold values for both groups, indicating these animals were more uncomfortable toward the pressure applied to their paw during the test, due to the pain caused by articular inflammation. The femur-tibial joint was collected and assessed for

pro-inflammatory cytokine levels. Groups C and E had the lowest levels of TNF- α while groups A and AE had the highest. Exercise prevented elevated TNF- α , resulting in a significantly lower result for group AE, which was not different from group C, when compared to group A. While there were differences on levels of TNF- α none were found between groups for articular levels of IL-6. Figure 6 displays information on histological analysis of femur-tibial joint, cytokine levels, mechanical hiperalgesia and articular edema.

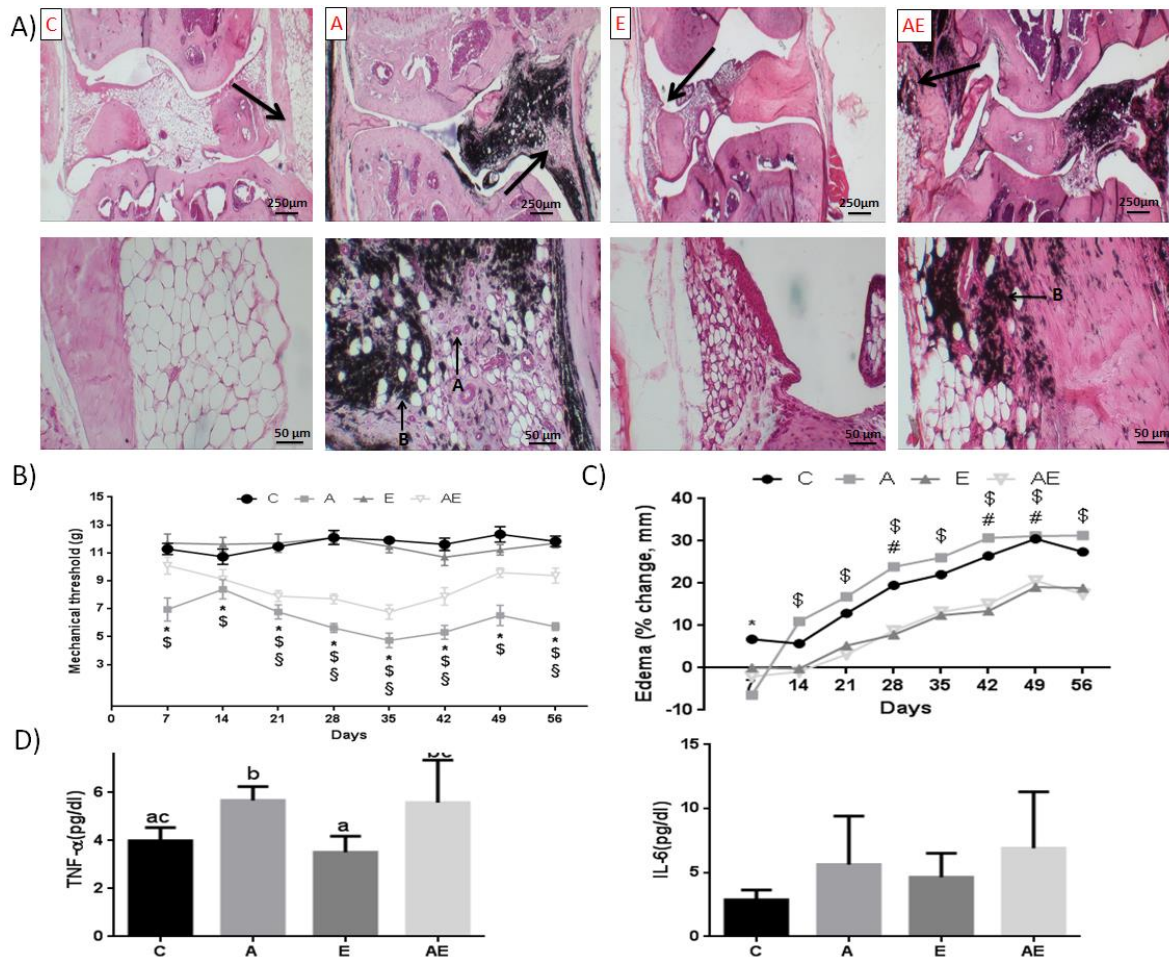


Figure 6. A) Representative pictures of H & E staining from each group. Pictures were taken using a microscope at 100x magnification. Arrows without letter point to area which is shown in greater magnification. Arrow (A) indicates vascular proliferation. Arrow (B) indicates leukocyte infiltration. **B)** Mechanical hiperalgesia. The symbol (*) represents difference between groups C and A, $p < 0,05$. The symbol (#) represents difference between groups C and E, $p < 0,05$. The symbol (\$) represents difference between groups A and AE, $p < 0,05$. The symbol (§) represents difference between groups E and AE, $p < 0,05$. One-way Anova followed by Tukey's post-hoc. Data are shown as mean and SEM. **C)** The symbol (\$) represents difference between groups A and AE, $p < 0,05$. **D)** Cytokine analysis. Different letters mean $p < 0,05$. One-way Anova followed by Tukey's post-hoc. Panel B's results are shown as mean and SEM. Panels C and D results are shown as mean and standard deviation.

DISCUSSION

Our data indicate that TiO₂ inoculation successfully promoted joint tissue inflammation and damage, muscle atrophy, decreased strength and increased nociception. Conversely, exercise was effective to mitigate tissue damage and counter act the effects ensued by the TiO₂ injection in the femur-tibial joint. Thus, the results obtained suggest that exercise has protective effects and can be used as a tool to lessen the degree of the alterations caused by TiO₂ on muscle and joint tissue.

Our data demonstrated that TiO₂ caused vascular proliferation, leukocyte infiltration and TiO₂ accumulation, which indicates inflammation, following the same inflammation induction protocol from previous studies[33]. Titanium accumulation in human tissues, such as lung and skin, is characterized by deposition of black pigmentation, followed by necrosis and granulomatous reactions[32]. The TiO₂ deposition observed in Figure 2 is in accordance with studies showing that it mimicks prosthesis inflammation[41–43]. Therefore the inflammatory response caused by prosthesis can be studied through the inflammation model of the present study[33]. The data obtained from this part of the experiment can be a representation of what happens with human prosthesis as its compounds start to wear out, leading to joint inflammation and pain.

It is known that exercise has beneficial effects on muscle mass and strength and that sarcopenia is associated with skeletal muscle biology[44–46]. The loss of muscle fiber number and atrophy, in particular type 2 fibers, is the main cause of sarcopenia[47]. Considering the muscles collected for CSA analysis, soleus has predominantly type 1 fibers while EDL has predominantly type 2 fibers[48]. Our data demonstrated that animals from group AE presented higher soleus CSA compared to group C and for EDL the CSA of group AE was similar to group C. Regarding grip strength, as Figure 5 shows, from day 21 to the end of experiment two exercised animals presented more strength than their control groups. These findings indicate exercise caused muscle hypertrophy and, therefore, was able to reduce the impact of inflammation on the skeletal muscle tissue.

There were no changes in the average distance traveled between exercised groups. It seems inflammation and pain were not a barrier for animals to voluntarily exercise. Data from a recent review on running wheel studies shows distance traveled by female mice is higher than males, although it is not quite clear how the animal's sex affects adaptive responses to running[49]. The same study states that during the first 3 weeks of access to the running wheel running activity increases and another study showed a pattern of reduction of running activity beginning at 8 weeks of age[50], and both running patterns happened during experiment two. Despite the occurrence of these patterns, our data alone

could be insufficient to present an argument explaining why animals from group AE maintained a higher, yet not significant, distance traveled average compared to group E.

A recent study has shown that there are harmful effects to the knee joint of animals exposed to the model used in this study, resulting in pain and alterations in tissues where inflammation is present, and higher levels of TNF- α , IL-1 β , IL-6 and IL-10[51]. Our data demonstrated that exercised animals had lower TNF- α levels, which is correlated to lower joint inflammatory parameters[52], this is a good outcome since it is both a remarkable event and common to have increased production of TNF- α in arthritis contributing to the pathophysiology of articular inflammation and pain[53]. Higher levels of TNF- α , which were observed on both TiO₂ groups, amplify the inflammatory response, leading to recruitment of other inflammatory cells and the development of sarcopenia[18,43].

In conclusion, results indicate that an induction of articular inflammation proposed by the TiO₂ model was successful. TiO₂ exposition caused joint tissue damage, articular inflammation and pain which promoted a reduction of muscle CSA, lower grip strength. On the other hand, exercise was effective to mitigate tissue damage and counter act the effects ensued by the TiO₂ injection in the femur-tibial joint. Thus, the results obtained suggest that exercise has protective effects and can be used as a tool to lessen the degree of the alterations caused by TiO₂ on muscle and joint tissue.

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5. CONCLUSÃO

Os resultados indicam que a proposta de modelo de artrite foi bem sucedida , pois a injeção com TiO_2 causou dano ao tecido articular, inflamação e dor. Como consequência, foi demonstrada atrofia e redução da força muscular. Em contrapartida, o exercício foi eficaz para mitigar o dano tecidual e proteger contra os efeitos deletérios da artrite induzida por injeção de TiO_2 . Assim, os resultados obtidos sugerem que o exercício tem efeitos protetores e pode ser usado como uma ferramenta para diminuir o grau das alterações causadas pelo TiO_2 no tecido muscular e articular.

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ANEXOS

ANEXO 1 - APROVAÇÃO DO COMITÊ DE ÉTICA NO USO DE ANIMAIS DA
UNIVERSIDADE ESTADUAL DE LONDRINA - UELA



COMISSÃO DE ÉTICA NO USO DE ANIMAIS

OF. CIRC. CEUA Nº 66/2018

Londrina, 09 de Maio de 2018.

Prezado (a) professor (a)

Certificamos que o projeto intitulado: "Efeitos do exercício físico em camundongos com artrite e sarcopenia induzida por dióxido de titânio." protocolo nº1582.2018.94 sob a responsabilidade de **Rafael Deminice**, que envolve a produção, manutenção e/ou utilização de animais pertencentes ao filo Chordata, subfilo Vertebrata (exceto o homem) para fins de pesquisa científica, encontra-se de acordo com os preceitos da Lei nº 11.794, de 8 de outubro de 2008, do Decreto nº 6.899, de 15 de julho de 2009, e com as normas editadas pelo Conselho Nacional de Controle da Experimentação Animal (CONCEA) e foi **aprovado** pela Comissão de Ética no Uso de Animais da Universidade Estadual de Londrina (CEUA/UEL) em **08/05/2018**.

Os objetivos do projeto são estudar a ocorrência da sarcopenia associada à artrite induzida por dióxido de titânio e verificar o efeito do exercício resistido sobre sarcopenia induzida. Grau de invasividade=3

Vigência do Projeto	06/2018 a 05/2020
Espécie/linhagem	Camundongo isogênico / Swiss
Nº de animais	72
Peso/Idade	20-25 g
Sexo	Machos
Origem	Biotério Central - Universidade Estadual de Londrina
Amostras a serem coletadas	Sangue, articulação femurotibial

Cumpra orientar que caso pretendam-se quaisquer alterações no protocolo experimental aprovado, deve-se submeter o novo protocolo à apreciação da CEUA/UEL anteriormente à execução das modificações.

Coloco-me à disposição para quaisquer esclarecimentos que se fizerem necessários. Sem mais para o momento, subscrevo, cordialmente.

Maria Fernanda R. Graciano
Prof. Dra. Maria Fernanda Rodrigues Graciano
Coordenadora da CEUA/UEL

Ilmo.(a) Sr.(a)
Prof. (a) Dr. (a) Rafael Deminice
Responsável pelo projeto
Departamento de Educação Física - CEFE

C/C para o Biotério Central do CCB
C/C para a Chefia do Depto. de Educação Física - CEFE
C/C para a Direção do CEFE