UNIVERSIDADE ESTADUAL DO OESTE DO PARANÁ – CAMPUS DE CASCAVEL

CENTRO DE CIÊNCIAS BIOLÓGICAS E DA SAÚDE

PROGRAMA DE PÓS-GRADUAÇÃO EM BIOCIÊNCIAS E SAÚDE – MESTRADO

ANA CAROLINE BARBOSA RETAMEIRO

EFEITOS DO EXERCÍCIO RESISTIDO E DO LASER DE BAIXA POTÊNCIA NA ARTICULAÇÃO TALOCRURAL EM MODELO EXPERIMENTAL DE ARTRITE REUMATOIDE

CASCAVEL-PR

(Março/2020)

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Dissertação apresentada ao Programa De Pós-Graduação em Biociências e Saúde - Mestrado, do Centro de Ciências Biológicas e da Saúde, da Universidade Estadual do Oeste do Paraná, como requisito parcial para a obtenção do título de Mestre em Biociências e Saúde.

Área de Concentração: Fatores que influenciam a morfofisiologia orgânica.

ORIENTADOR: Gladson Ricardo Flor Bertolini.

COORIENTADOR: Lucinéia de Fátima Chasko Ribeiro e Taciane Stein Leal.

CASCAVEL-PR

(março/2020)

FICHA CATALOGRÁFICA

Ficha de identificação da obra elaborada através do Formulário de Geração Automática do Sistema de Bibliotecas da Unioeste.

Barbosa Retameiro, Ana Caroline
Efeitos do exercício resistido e do laser de baixa potência na articulação talocrural em modelo experimental de artrite reumatoide / Ana Caroline Barbosa Retameiro; orientador Gladson Ricardo Flor Bertolini; coorientadora Lucinéia de Fátima Chasko Ribeiro. -- Cascavel, 2020.
118 p.

Dissertação (Mestrado Acadêmico Campus de Cascavel) -- Universidade Estadual do Oeste do Paraná, Centro de Ciências Biológicas e da Saúde, Programa de Pós-Graduação em BioCiências e Saúde, 2020.

1. Artrite experimental. 2. Exercício. 3. Laser. 4. Articulação talocrural . I. Flor Bertolini, Gladson Ricardo, orient. II. Chasko Ribeiro, Lucinéia de Fátima, coorient. III. Título.

FOLHA DE APROVAÇÃO

ANA CAROLINE BARBOSA RETAMEIRO

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Esta dissertação foi julgada adequada para a obtenção do título de Mestre em Biociências e Saúde e aprovada em sua forma final pelo Orientador e pela Banca Examinadora.

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(fevereiro/2020)

AGRADECIMENTOS

Esse trabalho não é só meu, mas também de todas as pessoas que me deram suporte, não só na parte acadêmica, mas de várias formas ao longo da vida. Por isso, devo agradecer principalmente a meus pais: Joventina e Luiz Carlos e à minha irmã Bruna, por serem meus primeiros orientadores neste mundo e por seguirem dessa forma me dando suporte até hoje mesmo nas situações mais adversas.

Agradeço a todas minhas professoras e professores, que me apontaram o caminho até a graduação, às minhas orientadoras durante a graduação: Professoras Doutoras Márcia Miranda Torrejais, Célia Cristina Leme Beu e Rose Meire Costa Brancalhão; e, especialmente, à Professora Doutora Lucinéia de Fátima Chasko Ribeiro que me orientou durante todo o processo do mestrado: muito obrigada por toda paciência, dedicação, ensinamentos e por acreditarem que eu sou capaz.

Meus agradecimentos a meus coorientadores: Taciane Stein Leal e Gladson Ricardo Flor Bertolini por estarem sempre prontos para me auxiliar e sanar minhas dúvidas. Desejo, novamente, agradecer à Taciane juntamente com Morgana Neves, minhas queridas fisioterapeutas, por terem sido os pilares da projeção e do planejamento deste trabalho; pela companhia e suporte antes, durante e depois do período experimental, sempre me explicando coisas com as quais nunca havia tido contato com muita paciência e bom humor. Obrigada por dividir e ensinar um pouco da profissão de vocês para mim!

Estendo os agradecimentos a meus primeiros colegas de laboratório que se tornaram também grandes amigos: Suellen Scarton, Pâmela Buratti, Caroline Covatti, Matheus Felipe Zazula, Adriana Souza e Bruno Popik. E aos que foram chegando com o tempo e acrescentando ainda mais aprendizado, amizade, amor, companheirismo e felicidade em minha vida: Aldair Casagrande dos Santos, Bárbara Zanardini de Andrade, Ariadne Barbosa, Mylena Campos, Luiz Gustavo Vasconcelos Machado, Alana Ludemila Tavares, Juliana Souza, Ana Luiza Peretti, Maria Luiza Wutzke, Izabela Camilo, Pâmela Silva, Aline Reginato, Estefani Marin, Carol De Toni e Diego. E ainda aos amigos que não são do laboratório, mas estão sempre presentes em momentos bons e ruins me dando apoio, lembrando que tudo vai dar certo e deixando tudo mais leve: Aldair, Hellen Rambo, Fernanda Farfale e Giovanna Piovezan.

Além das pessoas, ainda devo agradecer aos meus animais de estimação, principalmente à Serafine, que sempre me ofereceu amor incondicional desde o início da graduação e sempre se sentou a meu lado

quando eu chegava em casa, muitas vezes cansada e desmotivada, e acabava por me fazer lembrar de alguns valores que, às vezes, seres humanos são incapazes de compreender.

Resta agradecer ao Programa de Pós-Graduação em Biociências e Saúde pela oportunidade de desenvolvimento da pesquisa, a CAPES pela bolsa concedida, e a todos que torceram, acreditaram e me ajudaram de alguma forma: MUITO OBRIGADA!

“Inacabado, sei que sou um ser condicionado, mas consciente do inacabamento, sei que posso ir além dele.”

Paulo Freire.

RESUMO

RETAMEIRO, A.C.B. **Efeitos do exercício resistido e do laser de baixa potência na articulação talocrural em modelo experimental de artrite reumatoide.** 118 páginas. Dissertação (Mestrado). Programa de Pós-Graduação em Biociências e Saúde, Centro de Ciências Biológicas e da Saúde, Campus de Cascavel, Unioeste, 2020.

A Artrite reumatoide (AR) é uma doença autoimune que afeta as articulações sinoviais e estruturas periarticulares, principalmente as presentes nos membros, ocasionando dor e limitações físicas aos portadores, o que repercute na qualidade de vida e traz reflexos em questões socioeconômicas devido aos afastamentos das atividades laborais e gastos com o tratamento. Na tentativa de minimizar seus efeitos, modalidades terapêuticas vêm sendo utilizadas, uma delas é o exercício físico e laser de baixa potência (LBP). No entanto, ainda existem lacunas quanto aos protocolos utilizados e quanto aos efeitos da associação das duas modalidades terapêuticas no tratamento da AR. Assim, este estudo analisou os efeitos do exercício resistido e do LBP em parâmetros funcionais e morfológicos da articulação talocrural em modelo de AR, promovido pelo completo adjuvante de Freund (CFA). Para tanto, nas avaliações funcionais foram utilizados 128 ratos *Wistar* e nas análises histomorfométricas, 80. Os animais foram submetidos ao protocolo de AR de fase aguda e de fase crônica da doença, para avaliação funcional foram 64 em cada fase e para histomorfometria foram 40 de cada fase, e cada uma delas subdividida em grupo controle (GC), grupo artrite (GA), controle laser (CL), controle exercício (CE), controle laser e exercício (CLE), artrite laser (AL), artrite exercício (AE), e artrite laser e exercício (ALE), todos com oito animais para os testes de funcionalidade e cinco para as análises histológicas. Nos grupos artrite, a indução ocorreu pela aplicação de CFA na base da cauda, para imunização e, posteriormente, na cavidade articular tíbio-femoral direita dos animais. Os tratamentos se iniciaram 24 horas após a injeção intra-articular, realizados em dias alternados com progressão de tempo e séries para o exercício. As avaliações funcionais de preensão, índice funcional do isquiático (IFC) e plano inclinado foram realizadas no dia da injeção intra-

articular, 24 horas depois e no 3º e 5º dias para o grupo agudo. Para o crônico, o intervalo entre as avaliações aumentou para sete dias, ocorrendo no 12º, 19º e 26º dias. Após o período experimental, as articulações talocrurais do membro pélvico experimental (direito) foram processadas e fotodocumentadas para as análises morfológicas da morfologia geral do tecido articular e histomorfométricas da altura da zona profunda e das demais zonas e da contagem de condrócitos. Todos os parâmetros analisados nas avaliações funcionais mostraram diferença significativa dos grupos artrite em relação aos controles nas duas fases da doença, com retorno de valores semelhantes ao controle nos grupos AL, AE e ALE, porém com maior eficácia na associação dos dois tratamentos. Na morfologia da articulação talocrural de GA foi possível observar angiogênese na subíntima da membrana sinovial e cartilagem articular com *pannus* e floculações nas duas fases da doença. Na fase crônica as alterações morfológicas da superfície articular foram mais expressivas do que na fase aguda, uma vez que 50% dos animais apresentaram tais características, e no agudo apenas em alguns destes e com maior evidência no tálus. Na fase aguda ainda foi observado angiogênese na subíntima de todos os demais grupos, porém com ausência de infiltrado inflamatório e com o grupo AE se assemelhando mais ao controle nesse aspecto. Nesta mesma fase foi possível notar maior presença de condrócitos em GA, CE e AE, o que foi comprovado na análise histomorfométrica, o que pode indicar uma fase de reparo tecidual mais avançada nestes grupos.

Palavras-Chaves: Angiogênese, Reparo tecidual, Artrite experimental, Adjuvante de Freund, Exercício, Laser, Articulação do Tornozelo.

ABSTRACT

RETAMEIRO, A.C.B. **Effects of resistant exercise and low level laser on ankle joint in a rheumatoid arthritis experimental model.** 118 páginas. Dissertação (Mestrado). Programa de Pós-Graduação em Biociências e Saúde, Centro de Ciências Biológicas e da Saúde, Campus de Cascavel, Unioeste, 2020.

Rheumatoid arthritis (RA) is an autoimmune disease that affects synovial joints and periarticular structures, especially those present in the limbs, causing pain and physical limitations for patients, which has an impact on life quality and reflects in socioeconomic issues, due to leave from work activities and treatment expenses. In an attempt to minimize its effects, therapeutic modalities have been used and, among then, physical exercise and low level laser therapy (LLLT). However, there are still gaps regarding the protocols used and the two therapeutic modalities association effects in the RA treatment. Thus, this study analyzed the resistance exercise and LLLT effects on ankle joint functional and morphological parameters in an RA model, promoted by complete Freund's adjuvant (CFA). For this, 128 Wistar rats were used in the functional evaluations and 80 in the histomorphometric analyzes. The animals were submitted to the RA protocol acute and chronic phase of the disease 64 each for the functional ones and 40 each for the histomorphometry and each subdivided in control group (GC), arthritis group (GA), laser control (CL), exercise control (CE), laser control and exercise (CLE), laser arthritis (AL), exercise arthritis (AE), and laser arthritis and exercise (ALE), all with eight animals for functionality tests and five for histological analysis. In the arthritis groups, the induction occurred by applying CFA at the tail base, for immunization and, after, in the animals' right tibiofemoral joint cavity. The treatments started 24 hours after the intra- articular injection, performed on alternated days with time and series progression for exercise. The grip, sciatic functional index (SFI) and inclined plane functional evaluations were performed on intra-articular injection day, 24 hours later and on 3^{rd} and 5^{th} days for acute group. For the chronic, the interval between evaluations increased to seven days, occurring on the 12^{th}, 19^{th} and 26^{th} days. After the experimental period, the right ankle joints were processed and photo-documented for morphological and histomorphometric analyzes. All the analyzed parameters in the functional evaluations showed significant difference in the arthritis group in relation to the controls in two disease phases, with values returning similar to the control in the AL, AE and ALE groups, but with greater efficiency in the two treatments association. In the GA ankle joint morphology it was possible to observe angiogenesis in synovial membrane

subintima and articular cartilage with pannus and flocculation, in both disease phases. The articular surface morphological changes were more expressive in chronic phase, since 50% of animals had such characteristics, and in acute only in some of them and with greater evidence in talus. In the acute phase, angiogenesis was also observed in the all other groups subintima, but with inflammatory infiltrate absence and with AE group being similar to the control in this aspect. In the same phase it was possible to notice a greater chondrocytes presence in GA, CE and AE, which was confirmed in the histomorphometric analysis which may indicate a more advanced tissue repair phase in these groups.

Key-words: Angiogenesis, Tissue Repair, Experimental arthritis, Freund's adjuvant, Exercise, Laser, Ankle Joint.

LISTA DE ILUSTRAÇÕES

LISTA DE ABREVIATURAS

μm – micrômetro

μL- microlitro

AMPc – AMP cíclico

AL - grupo atrite laser

AE - grupo artrite exercício

ALE- grupo artrite laser e exercício

AR - Artrite reumatoide

ATP – adenosina trifosfato

AV- Avaliação

Ca^{+}- Cálcio

Cl^{-} - Cloro

CE – controle exercício

CFA - Adjuvante Completo de Freund

CL – Controle Laser de Baixa potência

CLE – Controle laser de baixa potência mais exercício

cm -centímetros

Cox – Citocromo C oxidase

CRP- Proteína C reativa

DMARDs – Medicamentos modificadores do curso da doença

GAGs- Glicosaminoglicanos

GA- Grupo artrite

GC – grupo controle

K^{+}- Potássio

IFC – Índice Funcional do isquiático

IL-6 – Interleucina seis

J/cm^2- joules por centímetros quadrados

LBP- Laser de Baixa potência

LE- Lesão exercício

LLBP – Lesão laser de baixa potência

LLBPE – Lesão laser de baixa potência mais exercício

m- metros

Na^+- Sódio

PGs – Proteoglicanos

TCA – Ácido tricloroacético

TNFα – Fator de necrose tumoral α

Treg- Células T regulatórias

SUMÁRIO

1. INTRODUÇÃO

Artrite reumatoide (AR) é uma doença inflamatória autoimune, sistêmica e frequentemente progressiva (CAN et al., 2002; DE SOUZA et al., 2010; FIRESTEIN, 2003; KHURANA; BERNEY, 2005) que afeta cerca de 1 % da população em países desenvolvidos. No Brasil, são poucos os dados sobre a doença, com incidência entre 0,2 e 1% na população (MOTA et al., 2011; SENNA et al., 2004).

No entanto, dentre as doenças progressivas que acometem a saúde humana, a AR apresenta incidência crescente, principalmente por ser uma comorbidade relacionada a outras complicações clínicas e psicossociais contemporâneas, como o estresse e a obesidade (MARGARETTEN et al., 2011; NAGASE; KASHIWAGI, 2003). Suas manifestações clínicas incluem: pouco apetite, febre, anemia, edema (TORPY, 2011) e as que mais se destacam são as manifestações articulares (TORPY, 2011; TUFTS, 2012), principalmente nas articulações sinoviais, presentes em locais de grande amplitude de movimento, o que inclui a articulação talocrural ou tornozelo (DE CARVALHO et al., 2004).

A articulação talocrural está envolvida em uma série de movimentos que têm papel crucial na deambulação e na sustentação do corpo na posição ortostática. Compreende uma sindesmose distal entre a tíbia e a fíbula e um encaixe formado pelos terços distais dos ossos tíbia e fíbula com o tálus e seu acometimento, como acontece na AR, leva a alterações biomecânicas e funcionais que afetam de forma negativa a vida do portador (BOGART; ORT, 2008; BONO; BERBERIAN, 2001).

Normalmente é observado, nos indivíduos afetados pela AR, o desenvolvimento de sinovite persistente com edema e mobilidade articular reduzida, resultando no alongamento dos tendões, ligamentos e cápsulas

articulares, com subsequente instabilidade, o que leva ao imobilismo (GILES et al., 2008) e, consequentemente, à diminuição da massa e da força muscular, comprometendo a integridade da articulação e dos tecidos que a circundam, resultando em dor e alterações nos padrões de movimentos, o que limita a atividade diária (TORPY, 2011; TUFTS, 2012), as atividades laborais (ABELL et al., 2005; VLIELAND, 2003), trazendo consequências socioeconômicas (MOTA et al., 2011).

Pesquisas relacionadas à AR em humanos são difíceis de ser controladas, pois testes repetidos tornam-se extenuantes e, no processo inflamatório, interferem a ingestão de medicamentos e/ou hábitos diários do paciente. Com a proposta de estudar a etiopatogênese da AR e de buscar por tratamentos mais eficientes, muitos modelos experimentais foram desenvolvidos ao longo dos anos, e o modelo utilizando o adjuvante completo de Freund (CFA) se destaca por ter se mostrado eficaz em mimetizar os sintomas da AR humana (CAI et al., 2006; COOK; MOORE, 2006; FARIA et al., 2009; NAGAKURA et al., 2003; ZHANG et al., 2002).

Na prática clínica, a terapia padrão é a medicamentosa, que inclui glicocorticoides, metotrexato, anticorpos monoclonais, medicamentos modificadores da doença (DMARDS) e outros agentes farmacológicos relacionados a mecanismos inflamatórios (TAK et al., 2011). No entanto, estes medicamentos geralmente não possuem ação duradoura e podem levar à manifestação de diversos efeitos colaterais (BENUCCI et al., 2017; PASSOS,2016; PATEL et al., 2017; TORPY, 2011); portanto, outras abordagens terapêuticas têm sido buscadas.

Neste sentido, estudos demostram que o exercício físico tem apresentado benefícios na reabilitação de indivíduos com AR, dentre os quais estão o aumento da amplitude de movimento e da flexibilidade (FENTEM, 1994); diminuição significativa da dor e da atividade inflamatória, melhorando a marcha e a função geral (SHIH et al., 2006; KAPALE et al., 2017). No entanto, dados morfofuncionais estão concentrados em modelos anteriores, principalmente na articulação tibiofemoral, de forma que a sua ação na articulação talocrural ainda não foi estudada.

Além do exercício, o laser de baixa potência (LBP) vem sendo utilizado no tratamento de várias doenças musculoesqueléticas, incluindo a AR (ASADA et al., 1989; PALMGREN et al., 1989; WAYLONIS et al., 1988). Há evidências de que o LBP promove mudanças locais e sistêmicas o que pode levar à diminuição da dor, do edema e do enrijecimento articular. Também melhora a resposta inflamatória aguda, por redução de prostaglandinas e inibição de ciclooxigenase local, o que acarreta estímulo do processo de regeneração tecidual (BJORDAL et al., 2003; HENRIQUES et al., 2010; MORIYAMA et al., 2005).

Apesar de a literatura trazer os benefícios do exercício e do LBP, existem lacunas quanto aos protocolos utilizados e nos efeitos da associação das duas modalidades terapêuticas no tratamento da AR. E ainda, se o modelo induzido por CFA causa alterações nos aspectos morfofuncionais da articulação talocrural, assim como as modalidades de tratamentos. Sendo assim, o trabalho visou testar esta hipótese, a fim de obter informações sobre os protocolos de tratamento para a AR que auxilie os profissionais na busca da reabilitação de seus pacientes melhorando a qualidade de vida.

2. OBJETIVOS

2.1 Objetivo Geral

Analisar os efeitos do exercício resistido e de laser de baixa potência nos parâmetros morfofuncionais da articulação talocrural em modelo experimental de artrite reumatoide.

2.2 Objetivos Específicos

Avaliar as alterações nos tecidos da articulação talocrural em modelo de AR induzido por CFA e a ação dos tratamentos com exercício resistido e LBP.

- Analisar os efeitos dos tratamentos nos parâmetros funcionais de estado funcional do membro e força muscular na articulação do tornozelo em modelo de AR;
- Analisar os efeitos dos tratamentos nos aspectos morfológicos da membrana sinovial e da cartilagem articular da articulação talocrural. Foram observados: organização geral da membrana, vascularização e presença de infiltrado inflamatório nos vasos e na matriz extracelular. Na cartilagem foram observadas: presença de floculações, organização e disposição dos condrócitos, fissuras e degradação. Na análise histomorfométrica foram realizadas as medidas de: espessura total, espessura da zona superficial, espessura da zona profunda e contagem de condrócitos.

3. REVISÃO DE LITERATURA

3.1 ARTRITE REUMATOIDE

A AR é uma doença inflamatória sistêmica e frequentemente progressiva, crônica e autoimune (CAN et al., 2002; DE SOUZA et al., 2016; FIRESTEIN, 2003; KHURANA; BERNEY, 2005), que afeta cerca de 1 % da população em países desenvolvidos (MARIANO, 2011). No Brasil, um estudo realizado em 2004, mostrou incidência de 0,46%, o que representa quase um milhão de pessoas, entre os quais, os mais afetados eram pessoas entre a quarta e sexta décadas de vida, sendo uma faixa etária economicamente ativa (MOTA et al., 2011; SENNA et al., 2004).

A etiologia da doença ainda não é totalmente conhecida, no entanto, sabe-se que sua susceptibilidade é determinada por uma interação complexa entre fatores genéticos e ambientais (ADYUKOV et al., 2003; COSTENBADER et al., 2006; DI GIUSEPPE et al., 2014; SUGIYAMA et al., 2010). Como predisposições para o desenvolvimento da AR podem ser mencionadas: hereditariedade, poucos hábitos de higiene, exposição à agentes ambientais,

má nutrição, ferimentos, lactação prolongada, gravidez frequente, menopausa, transtornos psicológicos, ansiedade, tuberculose, ataques de reumatismo agudo (TUFTS, 2012) e infecção ambiental por bactéria ou vírus (KAPALE et al., 2017).

Devido à sua característica autoimune, sistêmica (TORPY, 2011) e simétrica (LLOPIS et al., 2017), diversos órgãos e tecidos do corpo podem ser afetados, como: coração, pele, pulmões, pleura e, principalmente, as articulações (TORPY, 2011). Dentre os sintomas que podem ser apresentados estão: pouco apetite, estado febril, anemia e edema (TORPY, 2011). No entanto, os que mais se destacam são as manifestações articulares (TORPY, 2011; TUFTS, 2012), principalmente nas sinoviais (DE CARVALHO et al., 2004), uma vez que os indivíduos afetados normalmente desenvolvem sinovite persistente com inchaço e mobilidade articular reduzida, o que pode resultar no alongamento dos tendões, ligamentos e cápsulas articulares, com subsequente instabilidade e diminuição da massa e força muscular e inibição direta da contração de grupos musculares adjacentes, comprometendo a integridade biomecânica da articulação e tecidos que a circundam, resultando em dor e padrões de movimentos que, geralmente, não oferecem energia suficiente e limitam as atividades físicas (TORPY, 2011; TUFTS, 2012).

Estas manifestações da AR levam a impactos significantes na qualidade de vida do indivíduo (GILES et al., 2008), podendo acometer atividades do dia a dia, bem como as laborais (ABELL et al., 2005; VLIELAND, 2003), o que pode levar a custos diretos e individuais ou indiretos e públicos, devido ao maior gasto com saúde, o que mostra sua grande importância social (MOTA et al., 2011; PINCUS et al., 1984;) e, assim, a necessidade de maiores investigações científicas relacionadas a maior compreensão e opções de tratamentos para esta enfermidade.

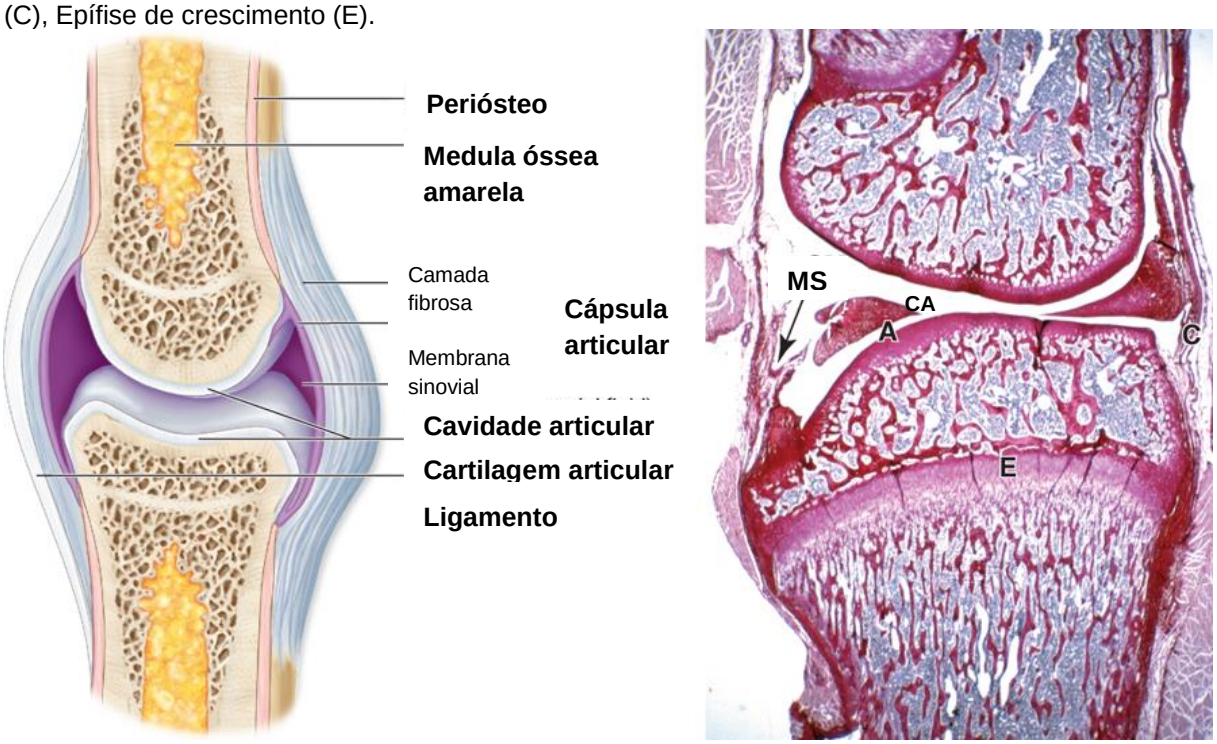
3.2 ARTICULAÇÕES SINOVIAIS

As articulações interligam o conjunto de ossos e cartilagens que formam o arcabouço do corpo, o que permite a mobilidade corporal. São divididas em três tipos, de acordo com a natureza do elemento que se interpõe entre as peças articuladas: tecido conjuntivo fibroso; articulações fibrosas, tecido cartilaginoso; articulações cartilagíneas, líquido sinovial; articulações sinoviais (DÂNGELO; FATTINI, 2007).

A articulação talocrural é uma dentre as articulações sinoviais que diferem dos outros tipos de articulações pelo movimento livre dos ossos, que estão relacionadas à amplitude de movimento, sendo chamadas de diartroses. Como característica, estas possuem várias estruturas e sistema biológico complexo, composto de células altamente especializadas, provenientes de vários tipos de tecidos, como hematopoiético e mesenquimal (HUI et al., 2012; IWANAGA et al., 2000).

A articulação sinovial saudável é constituída pela cápsula articular, membrana sinovial, líquido sinovial, cartilagem articular e elementos anexos associados, como os ligamentos capsulares (Figura 1) (HUI et al., 2012). A cápsula articular liga as extremidades ósseas, e delimita a cavidade articular. Esta cavidade contém o líquido sinovial; uma substância que, em humanos saudáveis, é caracterizada como: incolor, ou de aspecto claro, pálido-amarelada, transparente e viscosa que é um dialisado do plasma sanguíneo contendo elevado teor de ácido hialurônico, sintetizado pelos sinoviócitos, e liberado para o interior do espaço sinovial, com número relativamente pequeno de células (HUI et al., 2012; IWANAGA et al., 2000; JUNQUEIRA; CARNEIRO, 2013).

Figura 1. Representação dos componentes da articulação sinovial. Em A: Esquema de uma articulação sinovial típica. Em B: corte histológico de joelho de cobaia. Estão indicados: Membrana sinovial (MS), Cartilagem articular (A), Cavidade articular (CA), Cápsula articular (C), Epífise de crescimento (E).



Adaptado de JUNQUEIRA (2009).

3.2.1 Membrana e líquido sinovial

As paredes das capsulas articulares têm estruturas diferentes, conforme a articulação considerada, sendo em geral constituídas por duas camadas, uma externa, a fibrosa e uma interna, a camada ou membrana sinovial (JUNQUEIRA; CARNEIRO, 2013) cuja função primária é a produção de líquido sinovial, que nutre as cartilagens articulares, faz a remoção dos restos articulares e do tecido conjuntivo da cavidade articular (HUI et al., 2012; IWANAGA et al., 2000; OVALLE; NAHIRNEY, 2008).

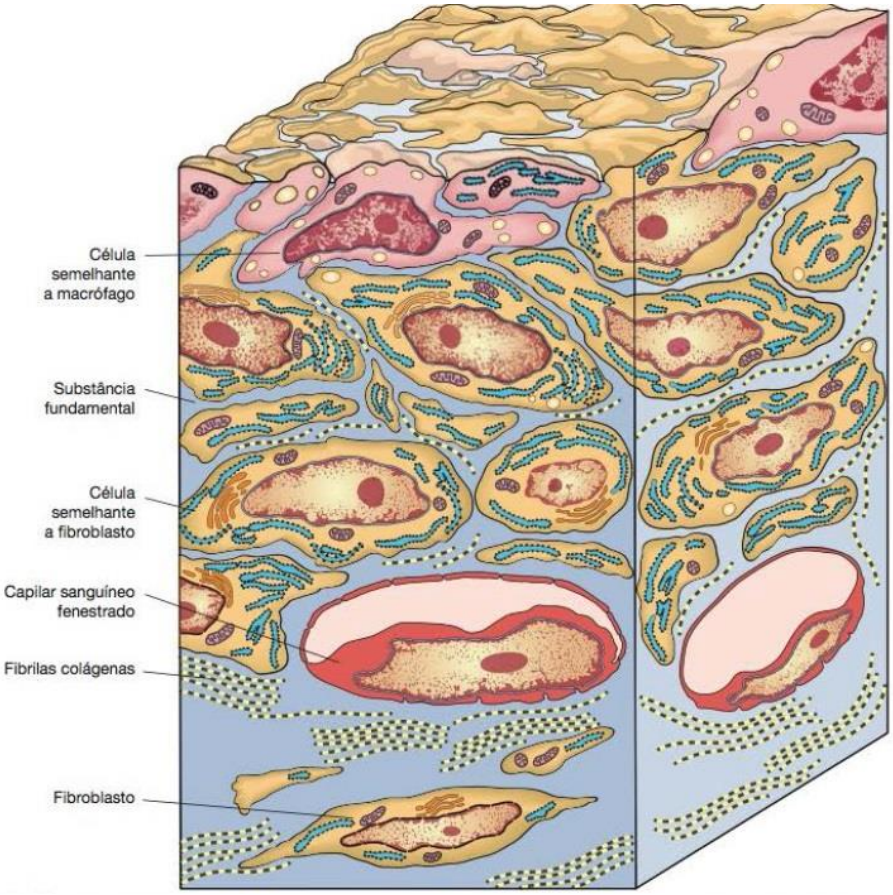
Ainda, o líquido sinovial facilita o deslizamento das superfícies articulares que são revestidas por cartilagem hialina, sem pericôndrio, através de vários lubrificantes, estes que são macromoléculas sintetizadas e secretadas pelas diferentes células sinoviais dos quais destaca-se: o ácido

hialurônico, um glicosaminoglicano (GAG) não sulfatado, responsável pela característica viscosa do líquido sinovial com propriedades lubrificantes tanto em condições estáticas quanto dinâmicas (HUI et al., 2012; IWANAGA et al., 2000; JUNQUEIRA; CARNEIRO, 2013). É também é uma via transportadora de substâncias entre a cartilagem articular (avascular) e o sangue dos capilares da membrana sinovial; desta forma, nutrientes e oxigênio passam do sangue para a cartilagem articular e gás carbônico difunde-se no sentido contrário (JUNQUEIRA; CARNEIRO, 2013).

A membrana sinovial contém, na sua região, superficial uma camada única que possui de uma a três camadas celulares de profundidade e é chamada de íntima sinovial na qual encontram-se várias células modificadas do tecido conjuntivo denominadas sinoviócitos (BARLAND; NOVIKOFF; HAMERMAN, 1962; IWANAGA et al., 2000; OVALLE; NAHIRNEY, 2008; SHIKICHI et al., 1999). As células do tipo A têm aspecto e a atividade funcional semelhante a macrófagos, sua função é a remoção de materiais particulados e correspondem de 20 a 30% das células de revestimento, as do tipo B são semelhantes a fibroblastos (Figura 2). São responsáveis pela secreção de GAGs e glicoproteínas e correspondem de 75 a 80% da contagem total de células (JUNQUEIRA; CARNEIRO, 2013; IWANAGA et al., 2000; OVALLE; NAHIRNEY, 2008).

A camada subíntima está localizada subjacente à íntima e, ao contrário desta, que é relativamente espessa, é composta por tecido conjuntivo frouxo e muito vascularizado, podendo ser do tipo areolar, adiposa ou fibrosa. Na adiposa, há uma camada íntima única e achatada enquanto a matriz contém células adiposas. A camada fibrosa da cápsula articular possui fina camada de sinoviócitos e matriz formada por tecido conjuntivo denso (LEACH et al., 1988; SHIVELY; VAN SICKLE, 1977). Numerosos capilares fenestrados são encontrados em todos os tipos de subíntima sinovial, de forma que o sangue que extravasa pode, rapidamente, interagir com o líquido sinovial em casos de lesões articulares fazendo a remoção de artefatos intracelulares e regulação de eventos imunológicos (IWANAGA et al., 2000; OVALLE; NAHIRNEY, 2008).

Figura 2: Representação esquemática tridimensional da ultraestrutura da membrana sinovial e seus diferentes tipos celulares.



Adaptado de JUNQUEIRA; CARNEIRO (2013).

A membrana sinovial de rato observada por MURASHIGE (1971) contém duas camadas de célula distintas compreendendo uma mais próxima do lúmen, que consiste em maior parte de células do tipo A e uma camada mais profunda consistindo em células do tipo B, com uma linha celular maior do que uma camada de células (CUTLIP; CHEVILLE, 1973; LEACH et al, 1988).

Sinoviócitos do tipo A, células normalmente circulares e localizadas na parte superior da íntima, são imunorreativas a vários anticorpos monoclonais contra macrófagos ou substâncias derivadas destes (BURMESTER et al., 1983; HOGG et al., 1985; IZUMI et., 1995). Eles também expressam antígeno IA, o qual desempenha ação na apresentação de antígenos nos estágios iniciais da resposta imunológica (ATHANASOU, 1995; NOZAWA-INOUE et al., 1988).

A superfície das células do tipo A é coberta por microvilosidades, estrutura única de macrófagos típicos (figura 3 A) que são ativados na captação de substâncias estranhas injetadas no interior da cavidade articular. Fisiologicamente, absorvem e degradam constituintes extracelulares, debris celulares, microrganismos e antígenos no fluido sinovial e matriz íntima, com o uso de um sistema vesicular e lisossomal bem desenvolvido. Como as células sinoviais assumem significativa quantidade de proteínas, elas podem ser capazes de modificar a composição proteica do fluído sinovial (SOUTHWICK; BENSCH, 1971).

As células do tipo B possuem núcleo relativamente grande, geralmente profundamente recuado, comparado com a pequena quantidade de citoplasma circundante (figura 3 B) (JILANI; GHADIALLY, 1986) e é sugerido que sua função seja secretória. Uma vez que, células do tipo B secretam colágeno, fibronectina (MAPP; REVEL, 1985), ácido hialurônico (ROY; GHADIALLY, 1976) e outras GAGs no interstício da íntima e na cavidade articular. E por isso sendo, também, consideradas envolvidas, direta ou indiretamente, no controle da composição proteica do fluído sinovial.

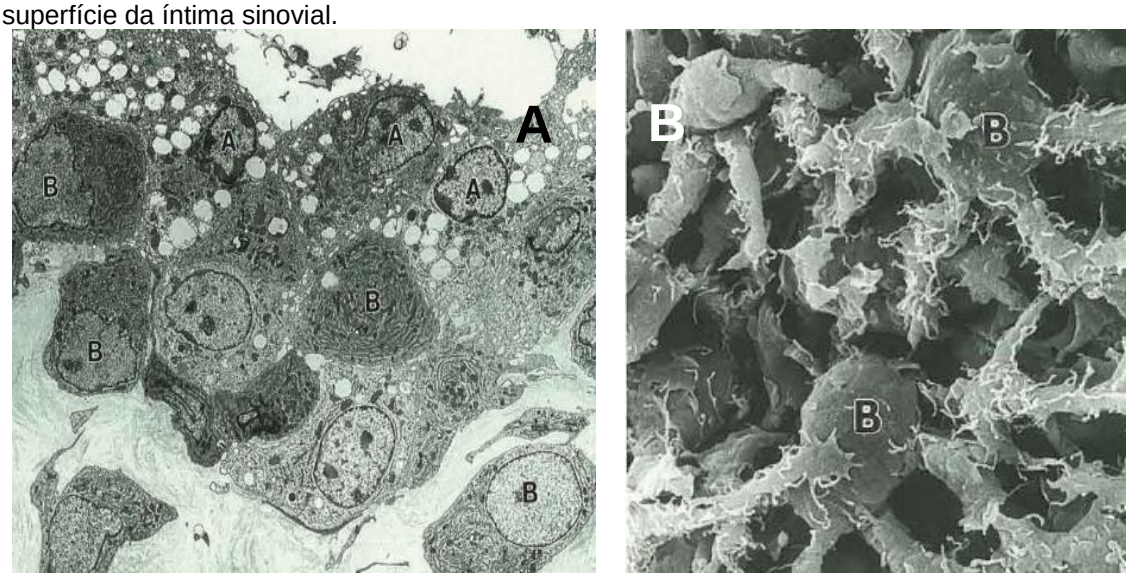
Outra atividade que mostra o envolvimento de sinoviócitos é a de barreira, pois estabelecem, parcialmente, permeabilidade preferencial das substâncias da base da íntima sinovial. Esta barreira, denominada hematoarticular, oferece resistência à troca passiva e livre de algumas substâncias (NISHIJIMA, 1981).

Esta seleção de substâncias ocorre para manter a concentração relativa de proteínas com peso molecular maior diferente entre o plasma e o fluído sinovial, mantendo-as geralmente com fluidez menor em relação ao sérum. Já as proteínas plasmáticas de peso molecular menor, são mantidas no fluido sinovial em concentrações maiores (SHANNON; GRAHAM, 1971).

Estudos anteriores de ultraestrutura dos sinoviócitos de roedores indicaram a natureza endócrina do tipo B (GRAABAEK, 1982). Em ratos, estes apresentam várias membranas circundadas de grânulos eletrodensos, os quais se parecem com células endócrinas pituitárias. A formação de grupos de

sinoviócitos com granulações próximas a capilares fenestradas e sua rápida degranulação em resposta a estímulos nocivos à membrana sinovial (LINCK; PORTE, 1988) sugerem a sua função como células receptoras e secretórias, uma característica semelhante a membros paraneurais (FUJITA et al., 1988). Uma vez que as células do tipo B sempre estão perto de capilares fenestrados, eles podem também confrontar a possibilidade da função hormonal (LINCK; PORTE, 1988).

Figura 3: Ultraestrutura dos dois tipos de sinoviócitos na articulação temporomandibular de rato. Células do tipo A (A), ocupando a porção superficial da camada íntima. Células do tipo B (B), distantes da cavidade articular (CA). Em B: Eletromicrografia de sinoviócitos em amostras maceradas de articulação de cavalos. Células do tipo B (B) estendendo radialmente vários processos primários espessos que formam uma ampla rede de processos para cobrir a superfície da íntima sinovial.



Adaptado de: INAWAGA (2000)

3.2.4 CARTILAGEM ARTICULAR

A cartilagem articular é uma forma especializada de tecido conjuntivo de consistência rígida que cobre a superfície epifisária dos ossos articulados a ela (MOW et al., 1992; OVALLE; NAHIRNEY, 2008). Desta forma, atua como suporte para tecidos moles, absorvendo choques e facilitando o deslizamento dos ossos nas articulações de forma a minimizar a concentração de força ao transmitir a carga de um osso para o seguinte dentro dessa complexa estrutura (BRANDT, 2003). Trata-se de um tecido altamente hidratado, aneural e

desprovido de suprimento sanguíneo, nervoso e linfático (MOW et al., 1992) sendo que, os responsáveis por sua nutrição são os capilares localizados no pericôndrio ou, como já explanado anteriormente, o líquido sinovial (OVALLE; NAHIRNEY, 2008).

Na diartrose, a cartilagem articular atua juntamente com o osso subcondral como tecido especializado dinâmico que permite a distribuição de carga entre os ossos adjacentes (PAN et al., 2009) com elasticidade para suportar cargas cíclicas durante o tempo de vida do organismo sem falhar. A anisotropia mecânica descreve o comportamento da cartilagem articular como um material visco-elástico que, graças à sua composição e organização, enrijece devido à permeabilidade dependente da tensão (JURVELIN et al., 2003). É um material bifásico com as propriedades mecânicas da sua fase líquida interagindo com as propriedades da sua fase sólida (ZHU et al., 1993).

A fase sólida é composta de colágeno, principalmente do tipo II, este que está presente como uma rede de fibras responsáveis pela forma geral do tecido. Essa rede é preenchida com GAGs, proteoglicanos e, em menor parte com glicoproteínas. A fase líquida apresenta composição de água e eletrólitos (Ca^{2+}, K^{+}, Na^{+} e Cl^{-}) na qual todos os componentes sólidos estão imersos (LAI et al., 1991). As duas, em conjunto, constituem a chamada matriz extracelular e são responsáveis pelas propriedades biomecânicas da cartilagem combinando rigidez compressiva e resiliência ao cisalhamento, o que mantém a estabilidade enquanto existem forças atuando sobre a estrutura e provocando seu deslocamento em planos diferentes (ARMIENTO et al., 2018).

Além da sua função como distribuidora de carga, a cartilagem articular permite o movimento da articulação sinovial com um coeficiente muito baixo de atrito (UNSWORTH et al., 1975). O mecanismo de lubrificação na articulação sinovial é complexo e dependente das condições de carga, sendo o resultado da combinação da lubrificação do filme fluido em alta carga (efeito newtoniano) e o limite de lubrificação em cargas baixas (RADIN et al., 1972). O fluido sinovial que preenche a cavidade articular tem viscosidade diretamente proporcional ao seu teor de ácido hialurônico (OGSTON; STANIER, 1953).

Ao longo da matriz extracelular, os proteoglicanos polianiônicos atraem o fluido intersticial e sua propensão a se espalhar em grande volume é reduzida pela rede de colágeno. Quando a cartilagem articular é comprimida, o líquido intersticial se move proporcionando a rigidez do tecido (MAROUDAS et al., 1991). Este módulo de compressão muda de acordo com a profundidade e depende da localização anatômica (ATHANASIOU et al. 1995). A rigidez compressiva da cartilagem é reforçada pela resistência à tração fornecida pela rede de colágeno existente (RESPONTE et al., 2007).

A produção da matriz extracelular ocorre por um tipo de célula madura e altamente especializada: o condrócito (BUCKWALTER; MANKIN, 1997). Nos tecidos vivos ocupam totalmente as lacunas, distribuídos em grande volume de matriz que é composta de 70 a 75% por água e 15 a 20% por colágeno, principalmente. Embora sua superfície pareça regular em microscópio óptico, a microscopia eletrônica mostra reentrâncias e saliências que aumentam a superfície de contato facilitando as trocas com o meio extracelular, o que é importante para sua nutrição, uma vez que as cartilagens são desprovidas de capilares sanguíneos, a oxigenação dos condrócitos é deficiente, de forma que estas vivem sobre baixas tensões de oxigênio (JUNQUEIRA; CARNEIRO, 2013).

Apesar da sua classificação como um único tipo de célula, devido à sua derivação comum a partir de células tronco mesenquimais, os condrócitos articulares mostram diferenças significativas em morfologia, densidade e organização em toda a profundidade da cartilagem em que se encontra. Esta heterogeneidade da cartilagem está relacionada com uma orientação diferencial das fibras colágenas, distribuição de proteoglicanos (PG) e tensão nos quatro níveis de organização, desde a superfície articular até a cavidade onde está presente a medula óssea (figura 4) (ARMIENTO et al., 2018) são estes:

1) Zona superficial: onde a densidade celular é maior e as células são dispostas em aglomerados horizontais, estando as lacunas achatadas no sentido paralelo à porção superior (SCHUMACHER et al., 2002), possui fibras

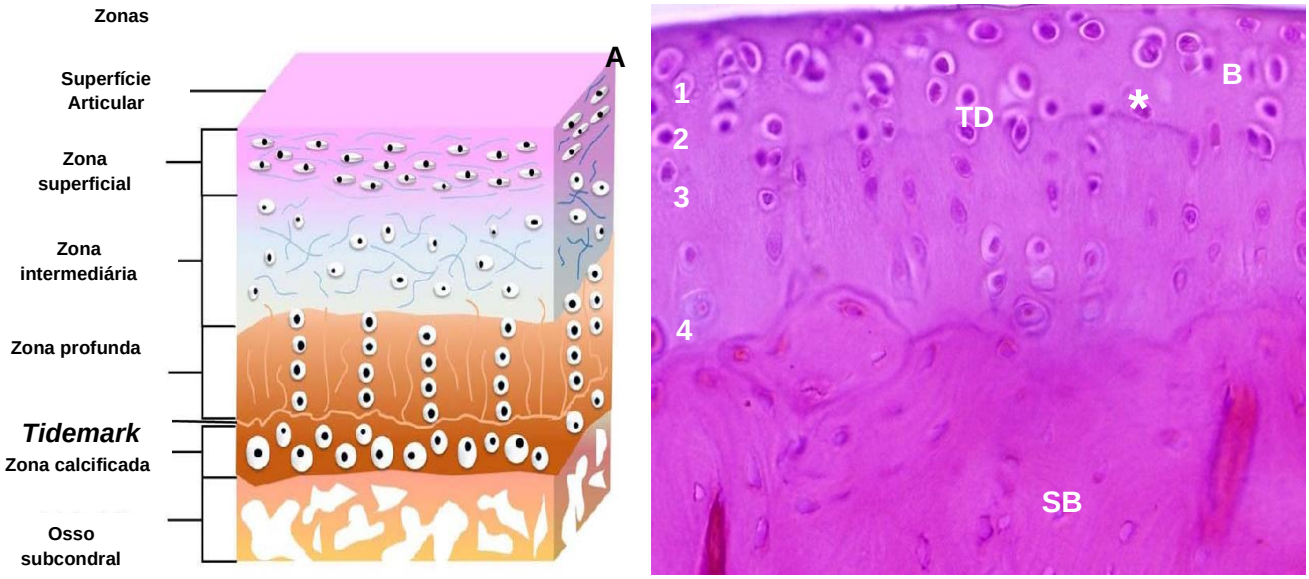
de colágeno tipo II orientadas tangencialmente à superfície (SIMON; JACKSON, 2006; WATRIN et al., 2001) o que proporciona resistência às tensões e desempenha papel vital na manutenção da saúde da cartilagem articular por funcionar como superfície de baixo atrito que resiste à tração e distribui as cargas de forma ideal (SCHUMACHER et al., 2002).

2) Zona de transição ou intermediária: Preenchida por condrócitos esféricos no interior de uma rede desorganizada de colágeno, composto por fibras mais espessas onde ocorrem forças de compressão, com o colágeno distribuído obliquamente e com grande quantidade de PGs (CLARK, 1991).

3) Zona profunda ou radial: onde os condrócitos encontram-se em menor densidade e geralmente isolados em lacunas mais arredondadas (GENESER, 2003; SCHUMACHER et al., 2002), devido à esta mudança de formato ocorrer de forma correspondente no núcleo, as fibras de colágeno se distribuem de forma vertical e a carga é distribuída para resistir a compressão.

4) Zona de cartilagem calcificada: Comparada a outras zonas, a interface entre a cartilagem e o osso subcondral tem uma composição peculiar em GAGs e glicoproteínas (LYONS et al., 2005) e é conhecida como cartilagem calcificada devido a presença de uma frente de mineralização (ZHANG et al., 2012), visível como uma linha fina (*tidemark*) que é corada fortemente em hematoxilina (FAWNS; LANDELLS, 1953) e demarca a fronteira entre a cartilagem calcificada e não calcificada (SIMON; JACKSON, 2006).

Figura 4: Zonas da cartilagem articular. Em A: Esquema representando as zonas da cartilagem articular com a disposição dos condrócitos. Em B: Fotomicrografia da tíbia na articulação talocrural de rato *Wistar*, estão indicados: Zona superficial (1), Zona intermediária (2), Zona profunda (3), *Tidemark* (TD), osso subcondral (SB) e condrócito (*) (H/E).



Adaptado de HSHD Endocrine / Autora.

A composição bioquímica da cartilagem articular varia entre as espécies, e sua espessura está relacionada de forma proporcional à massa corporal, enquanto a densidade celular reduz em relação a esta (MALDA et al., 2013).

O colágeno do tipo II fornece a força tênsil e os PGs que correspondem de 2 a 10% e são responsáveis pela resistência a pressões (OVALLE; NAHIRNEY, 2008; WATRIN et al., 2001). Sendo assim, além da função de secreção de colágeno, PGs e glicoproteínas, como a condronectina, realizada pelos condrócitos, a cartilagem hialina degrada glicose, principalmente por mecanismo anaeróbio com formação de ácido lático como produto final e os nutrientes transportados pelo sangue atravessam o pericôndrio, penetram na matriz da cartilagem e alcançam os condrócitos mais profundos. A movimentação de moléculas ocorre, principalmente, através da água de solvatação das macromoléculas e o bombeamento promovido pelas forças de compressão e descompressão exercidas sobre as cartilagens (JUNQUEIRA; CARNEIRO, 2013).

O metabolismo da matriz extracelular, a densidade e a organização de condrócitos sofrem importantes alterações durante o processo de crescimento e maturação (JADIN et al., 2005). A absorção e redistribuição de forças compressivas são adaptações frente às demandas funcionais e por isso são responsáveis pelo desenvolvimento morfológico e manutenção da homeostase da cartilagem articular (VANWANSEELE; LUCCHINETTI; STUSSI, 2002).

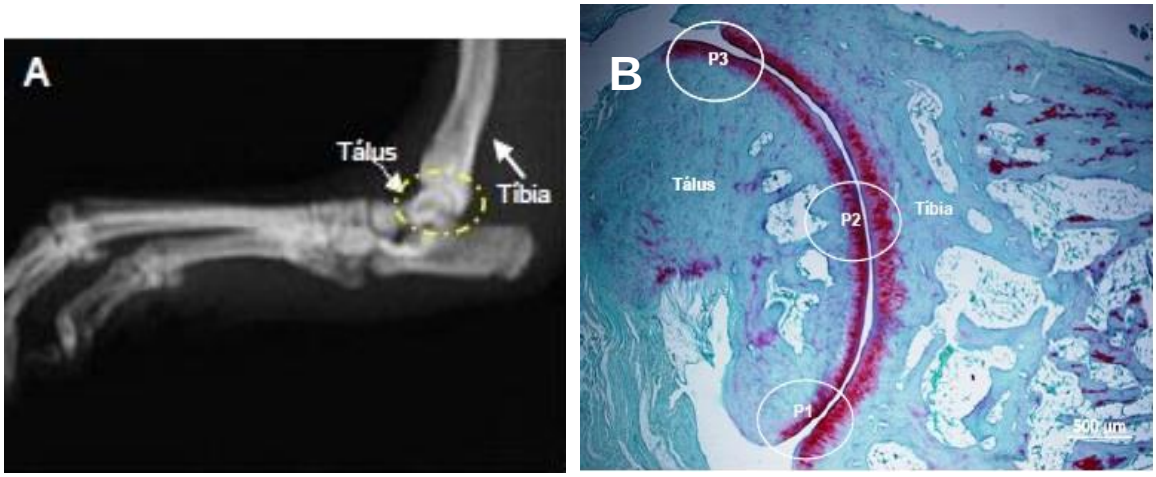
Em suma, as articulações sinoviais possuem diversos componentes que desempenham as mais variadas funções, estas que permitem a amplitude adequada de movimentos e alterações nestes componentes podem comprometer a integridade biomecânica da articulação e tecidos que a circundam, resultando em padrões de movimentos que, geralmente, não oferecem energia suficiente e limitam as atividades físicas do indivíduo afetado. (TORPY, 2011; TUFTS, 2012).

3.2.5 Articulação Talocrural

O membro inferior, como um todo, faz o suporte do peso corporal na posição vertical onde se destacam as articulações do quadril, joelho e talocrural ou do tornozelo. A articulação talocrural (Figura 5) é classificada como gínglimo, compreende uma sindesmose distal entre a tíbia e a fíbula e um encaixe formado pelos terços distais dos ossos tíbia e fíbula com o tálus e seus movimentos primários são os de: flexão plantar, extensão (flexão dorsal), inversão e eversão. (BOGART; ORT, 2008; BONO; BERBERIAN, 2001).

É a articulação que possui a maior área de superfície dentre as que suportam o peso do corpo, atividades como a deambulação podem produzir tensões que correspondem ao dobro da força suportada pelo joelho ou quadril em humanos (MANDI, 2012). A estabilidade desta articulação é mantida pela anatomia óssea incrementada pelas cargas compressivas provenientes da manutenção da posição do corpo. Durante a movimentação os ligamentos, que constituem as partes moles da articulação, passam a desenvolver o papel de estabilizadores (RENSTRÖM; LYNCH, 1999).

Figura 5: Articulação talocrural de rato. Em A, radiografia em perfil lateral da articulação e, em B, fotomicrografia em corte sagital, coloração em Safranina O *Fast Green.*



Adaptado de TSAI et al. (2007); KUNZ (2014).

3.2.6 Ação da AR nas articulações

Artrite é uma doença autoimune, inflamatória e degenerativa com desenvolvimento gradual que pode progredir para articulações dos pulsos, joelhos ou ombros, no entanto, afeta, primeiramente, articulações das mãos e dos pés (LLOPIS et al, 2017), como é o caso da articulação talocrural. As principais manifestações clínicas são crônicas e simétricas (LLOPIS et al, 2017), como: dores severas nas articulações, força muscular reduzida, enrijecimento e fadiga em uma ou mais das pequenas articulações e movimentações físicas limitadas (MCINNES; O'DELL, 2010) são geralmente seguidas por inchaço e calor, acompanhados por dores musculares que podem piorar e persistir por semanas ou meses (MCINNES; O'DELL, 2010).

Embora a causa precisa do desenvolvimento doença continue desconhecida, acredita-se que as deformações das articulações ocorrem devido a sua caraterística autoimune, ou seja, células do sistema imunológico que deveriam atacar bactérias e elementos externos, atacam tecidos saudáveis do próprio corpo, como se fossem células invasoras, liberando substâncias que provocam inflamação (KAPALE et al., 2017; PASSOS, 2016), o que ocasiona sinovites erosivas que podem levar a alto nível de destruição articular (DE

CARVALHO et al., 2004). Monócitos, neutrófilos e macrófagos são descritos como os maiores responsáveis por mediar a destruição tecidual (DAVIGNON et al., 2013; KAPALE et al., 2017; KINNE et al., 2000) e além destes, a presença de citocinas inflamatórias, como o fator de necrose tumoral alfa (TNFα) (KINNE et al., 2000; MCINNES; SCHETT, 2011), aumento de concentrações de exoglicosidases e agentes degenerativos mediados por NF-k beta (SOFAT et al., 2012).

Essas células do sistema imunológico formam uma camada fibrosa de tecidos anormais, chamada *pannus,* que libera substâncias que aceleram a erosão óssea e destruição da cartilagem, de forma que o seu formato e alinhamento são perdidos resultando em deformações, e danos aos ligamentos adjacentes (FIRESTEIN, 2003; KAPALE et al., 2017). Os componentes da matriz extracelular da cartilagem são enzimaticamente degradados pelas metaloproteinases, hialuronidases e agrecanases, respectivamente (NAGASE; KASHIWAGI, 2003). No entanto, o principal alvo é a membrana sinovial, e seu espessamento causa danos irreversíveis à cápsula e à cartilagem articular (Figura 6), uma vez que essas estruturas são repostas pelo *pannus* e, abaixo deste arranjo, a cartilagem fica corroída e destruída e as articulações se tornam fixas devido a estrutura espessa e endurecida, o que resulta no edema, modificação do tecido articular e, devido ao desuso, na atrofia de estruturas adjacentes, como: pele, ossos, músculos, nervos e, ainda, podem acometer o tecido conectivo de vasos sanguíneos (FIRESTEIN, 2003).

O maior acometimento desta estrutura ocorre devido a hiperplasia no processo inflamatório, que configura sinovite (FIRESTEIN, 2003), pois o grande número de células do sistema imune que invadem esta estrutura, levam a proliferação celular, neovascularização e formação de folículos linfoides germinativos. Os mecanismos envolvidos no recrutamento das células inflamatórias para o interior da membrana sinovial têm sido extensivamente estudados (BARTOK; FIRESTEIN, 2010) mostrando hiperemia sinovial (CAROTTI et al., 2002), proliferação de sinoviócitos invasivos semelhantes a fibroblastos que resistem à apoptose e aumento na propriedade de aderência e invasão (LEFÈVRE et al., 2009), infiltração de leucócitos, resposta

neoangiogênica associada (BOTTINI, 2013; MCINNES; SCHETT, 2011) alterações na função dos linfócitos T e B, produção anormal de citocinas e anticorpos (BUGATTI et al., 2007; FEKETE et al., 2007).

Além disso, ocorre a elevação de três a 100 vezes de citocinas pró-inflamatórias, bem como fator de necrose tumoral α (TNFα), interleucina seis (IL-6), interleucina beta (IL 1β) e proteína C reativa (CRP) (KAPALE et al., 2017) que proporcionam a destruição das camadas superficiais da cartilagem articular (RANNOU et al., 2006).

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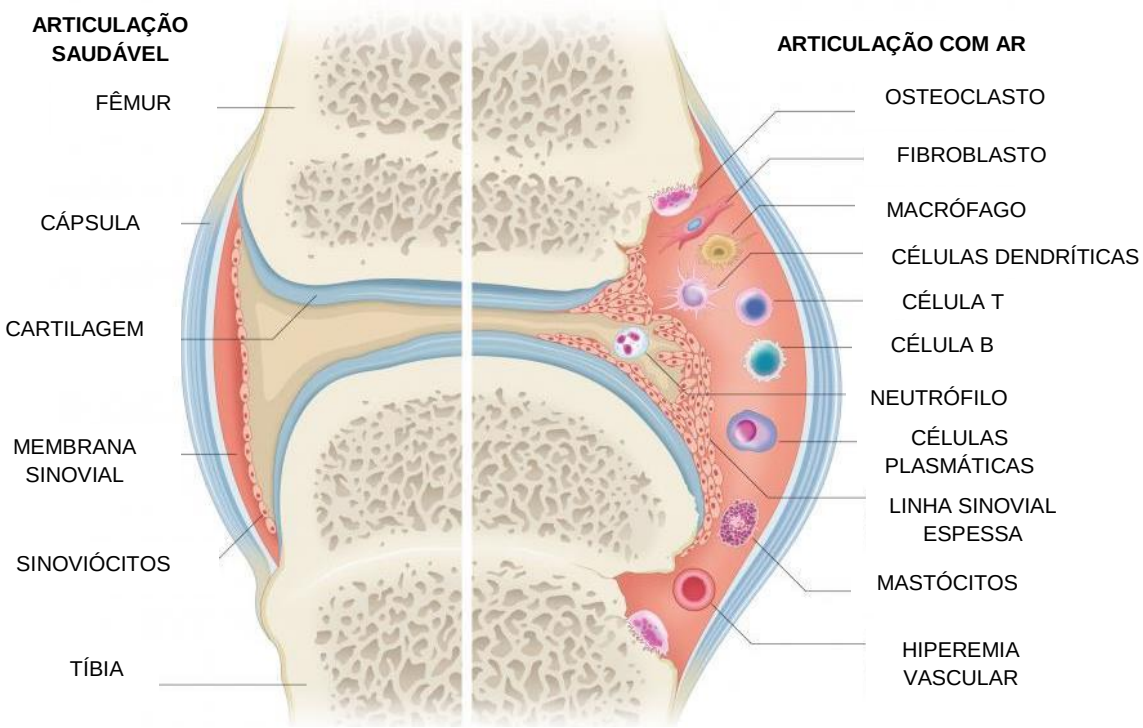
Figura 6: Esquema representando a anatomia de uma articulação. A esquerda, articulação saudável. A direita, articulação afetada pela AR com as diversas células da resposta imune que causam inflamação e danos aos ossos e a cartilagem.

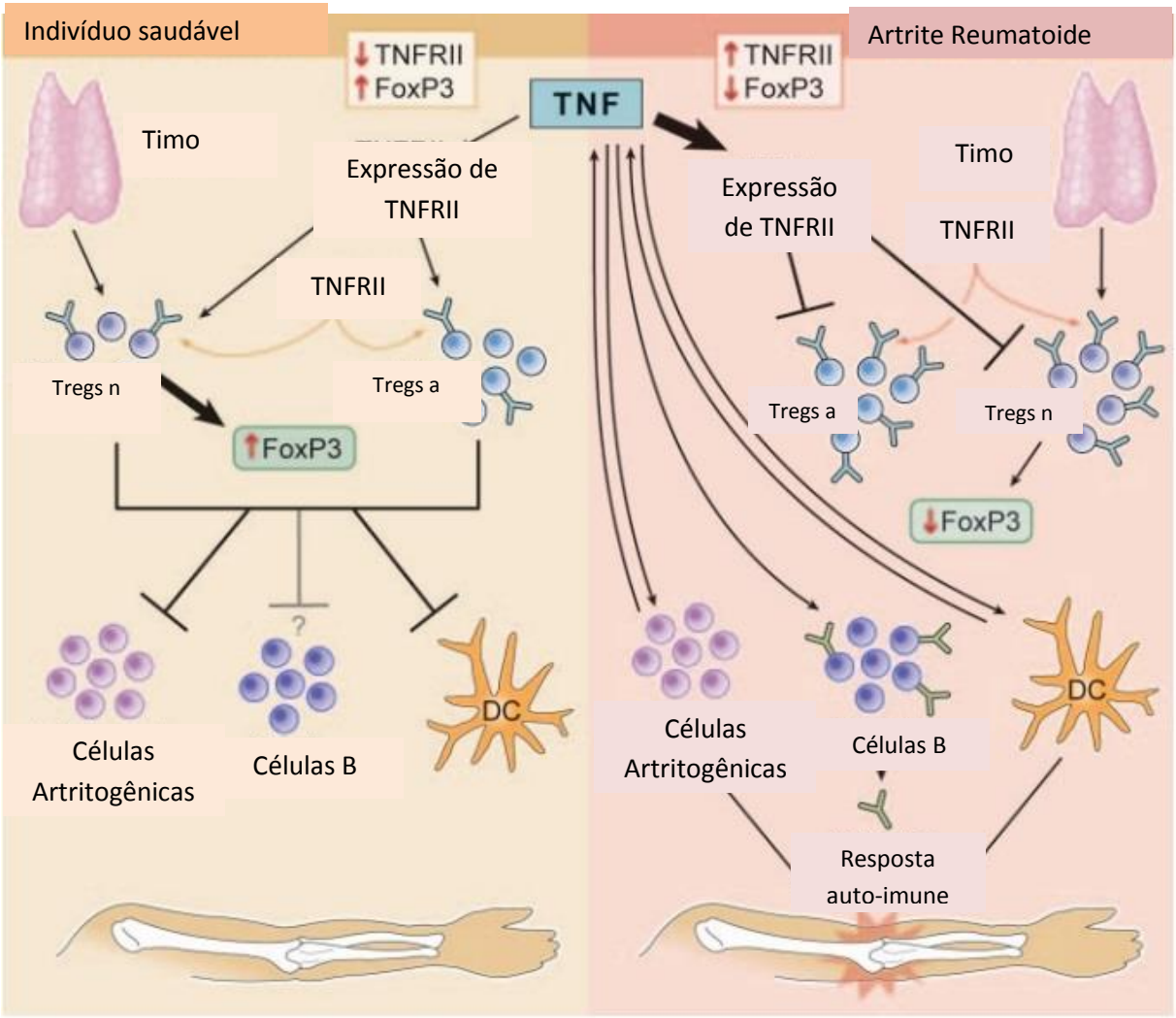


Adaptado de: SMOLEN et al.(2016).

Um estudo publicado por Valencia et al. (2006), mostrou que pacientes com AR possuem alterações nas células T regulatórias (Treg) CD25+ associados ao aumento da expressão da proteína Fox P23 e TNFα. As Treg podem ser agrupadas em dois grandes gêneros: um intrínseco, gerado pelo

timo (caraterizadas por expressão de CD25+ e Fox P3) (FEHERVAZI et al., 2004); e Tregs geradas depois de imunização ou exposição a antígenos ou autoantígenos (HOMANN et al., 1999). *In vivo*, as funções das Tregs são baseadas na indução de citocinas como IL-10, *Trasforming growth factor* beta (TGF beta) e IL 4 e 5; modulação das células apresentadoras de antígeno; e, algumas vezes, têm efeitos supressores diretos em células T patogênicas. Além disso, todas as Tregs podem agir como “supressoras expectadoras” por suprimir outras células T efetoras de antígenos específicos diferentes e esta habilidade faz com que sejam consideradas células imunossupressoras fisiológicas e sítio-específicas de alto interesse terapêutico. Como ilustrado na figura 7, na AR, ocorre efeito negativo direto do TNFα na regulação das funções efetivas das Tregs CD4+, CD25+, o que inicia um ciclo vicioso de aumento do processo patogênico da doença (EHRENSTEIN et al., 2004).

Figura 7: Ciclos de feedback operacional envolvendo efeitos diretos do TNF nos Tregs CD25 aumentando o status de doença da AR



. Adaptado de: FOUSTERI, G., VON HERRATH (2006).

Em suma, todos esses fatores levam a um acometimento geral da articulação, levando a limitação funcional e incapacitação (ANANDARAJAH; SCHWARZ, 2004), o que leva a impacto significante na qualidade de vida do indivíduo (GILES et al., 2008) podendo afetar atividades do dia a dia, bem como profissionais (ABELL et al., 2005; VLIELAND, 2003), o que impulsiona a procura de novos tratamentos e estudos nesta área.

3.3 TRATAMENTO PARA ARTRITE REUMATOIDE

O tratamento se inicia com o diagnóstico prematuro e monitoramento da progressão da doença (BÉRTOLO, 2007). Na prática clínica, a terapia padrão inclui glicocorticoides, anti-inflamatórios, analgésicos e DMARDs (FERRAZ-AMARO et al., 2009; TORPY, 2011) que conferem apenas alívio temporário, não interferindo na progressão da doença. Os analgésicos disponíveis no Brasil são paracetamol e dipirona (BENUCCI et al., 2017; PASSOS, 2016; PATEL et al., 2017; TORPY, 2011). Analgésicos opioides, como codeína e tramadol podem ser administrados, porém com muita cautela, pois em doenças crônicas trazem risco de sonolência, depressão sensorial e dependência (BENUCCI et al., 2017; PASSOS, 2016; PATEL et al., 2017; TORPY, 2011).

Além dos efeitos adversos, que fazem com que estes medicamentos tenham que ser administrados com muita cautela, sendo evitados sempre que possível ou empregados em doses mínimas, pelo menor tempo possível, em forma decrescente e quando realmente necessários (PASSOS, 2016), muitos pacientes não respondem positivamente, pois alguns indivíduos podem continuar sofrendo com limitações funcionais (PINCUS et al., 1999). Com a saúde debilitada, menor expectativa de vida, deficiências na qualidade de vida que afetam as atividades laborais e relações sociais (CHORUS et al., 2003; MCINNES; SCHETT, 2011). Desta forma, permanece a grande necessidade de abordagens terapêuticas alternativas ou associadas ao uso dos medicamentos.

3.3.1. Exercício físico como tratamento

Atividade física é definida como movimento corporal produzido por músculos esqueléticos que requer gasto de energia, o que influencia no desenvolvimento e na saúde em geral ao longo de toda a vida (BAMMAN et al., 2014; COLPANI et al., 2013; JUONALA et al., 2013). Dentro do seu mecanismo de ação, há evidências crescentes de que o treinamento físico ativa mobilização de células progenitoras endoteliais da medula óssea para facilitar a regeneração celular (MOEBIUS-WINKLER et al., 2011), células progenitoras neurais para induzir a hipertrofia fisiológica (XIAO et al., 2014) e de que os tecidos: muscular, adiposo e ósseo podem funcionar como órgãos endócrinos autênticos, produzindo e liberando proteínas na circulação que modulam respostas fisiológicas durante e após o exercício (CATOIRE et al., 2014; PEDERSEN; FEBBRAIO, 2008; YOU; NICKLAS, 2008).

Estes fatores promovem bem-estar cardiometabólico, melhoram o desempenho cognitivo e ajudam efetivamente na prevenção e tratamento de várias doenças, incluindo as cardiovasculares, neurológicas, sarcopenia, osteoporose e câncer (STRANAHAN; MATTSON, 2012). E ainda, programas de exercícios orientados de forma correta são essenciais para aperfeiçoar a saúde de pessoas em uma ampla variedade de deficiências (PETERSON et al., 2012).

Em seu estudo, Benhamou (2007) sugeriu que a atividade física era prejudicial em pacientes com AR e que os profissionais da saúde orientassem os portadores a evitar exercícios e manter o repouso. No entanto, estudos publicados sugerem que os indivíduos acometidos podem se beneficiar com segurança de atividades físicas (VLIELAND, 2003) de forma que, além de trazer benefícios para a população geral, oferece melhor qualidade de vida entre indivíduos com a doença, podendo ser considerada atividade fundamental para atenuar sintomas, conforme demonstrado em várias pesquisas, incluindo achados de estudos randomizados controlados (BAILLET, et al., 2009; LEMMEY et al., 2009; NEUBERGER et al., 2007)

O exercício físico provoca uma série de respostas fisiológicas, resultantes de adaptações autonômicas e hemodinâmicas (MONTEIRO;

FILHO, 2004) que geram tensão, os tendões transmitem forças do músculo para o osso, a musculatura é comprimida e, por consequência, os vasos arteriais periféricos, elevando drasticamente a resistência periférica total e reduzindo a perfusão muscular. Para restabelecer o fluxo sanguíneo, ocorre aumento na atividade do sistema nervoso simpático, no débito cardíaco e na pressão arterial média (MCARDLE et a., 2001).

Em indivíduos com AR, ocorre inflamação sinovial em seus revestimentos, o que leva à hipertrofia sinovial e algumas vezes à infiltração nos tecidos tendinosos. O aumento de citocinas inflamatórias circulantes também afeta o colágeno, levando a danos e desorganização de sua estrutura, sob cargas mecânicas, estes se tornam mais rígidos, provendo produção menos eficiente de força (REEVES et al., 2006; ONAMBELE et al., 2006).

A função principal da cartilagem na articulação sinovial é proteger o osso de danos por meio da redução da fricção entre os ossos adjacentes durante o movimento (MILNER, 2008). Na AR, as camadas articulares superficiais são destruídas (RANNOU et al., 2006) e esta degeneração leva à falha da articulação, limitação funcional e incapacitação (ANANDARAJAH; SCHWARZ, 2004) e, depois de repousar por longos períodos, o fluido sinovial é expelido para a cavidade articular resultando em contato entre as diferentes áreas da cartilagem. Quando o movimento é retomado o mecanismo do fluido de lubrificação é reativado, promovendo melhora na lubrificação e da saúde articular (SCHOLES; UNSWORTH, 2004). Durante os períodos de compressão e descompressão, os quais podem ser alcançados através de forças mecânicas com exercícios regulares cíclicos em série, a cartilagem responde de forma sítio-específica, o que previne a fragilidade e disfunção deste tecido (AROKOSKI et al., 2004)

Todas estas respostas fisiológicas podem levar a benefícios, estes que já foram demostrados por estudos de reabilitação com exercício físico em indivíduos com AR, dentre os quais estão: aumento da amplitude de movimento, flexibilidade, (FENTEM, 1994), diminuição significativa da dor,

melhorando a marcha e a função geral (SHIH et al., 2006; KAPALE et al., 2017), redução da atividade inflamatória e seus sintomas, limitando a destruição da articulação e incapacitação (KAPALE et al., 2017), melhora cardiorrespiratória e saúde cardiovascular, aumento da massa muscular, redução da adiposidade (incluindo gordura atenuada no tronco), melhora a força muscular e condicionamento físico, bem estar psicológico (LEMMEY et al., 2009), melhora na fadiga (NEILL et al., 2006) e na qualidade de vida geral (ABELL et al., 2005).

Dentre as diversas modalidades de exercícios existentes, o treinamento resistido passou a ganhar visibilidade como sendo uma das melhores formas de treinamento físico visando saúde e qualidade de vida, é uma modalidade que visa oferecer resistência contra a ação muscular por meio da utilização de pesos, que podem ser os do próprio corpo (SANTARÉM, 1999) e que, se trabalhada de forma responsável e adequada, pode trazer benefícios como: aumento de força muscular, melhora da coordenação motora, manutenção e aumento de flexibilidade, até o fortalecimento ósseo e articular, que leva a uma melhora do desempenho das atividades de vida diária, e assim, à funcionalidade e à autossuficiência (BERMUDES et al., 2003; POSNER et al., 1995). Além da melhora dos sintomas anteriormente citados, ocorre também diminuição da dor. Uma das explicações para a referida analgesia é a liberação endógena de opioides pelo sistema nervoso central e periférico onde a ativação de receptores centrais e periféricos resulta nos efeitos antinociceptivos, bem como na expressão em células imunes para produzir significativa antinocicepção (HUA; CABOT, 2010; LESNIAK; LIPKOWSKI, 2011)

Em suma, o papel do exercício na promoção da saúde articular na AR pode ser de grande importância (MAINI; FELDMANN, 2004). Considerando ser uma intervenção de bom custo-benefício (DE JONG et al., 2003; STAVROPOULOS et al., 2013; METSIOS et al., 2009) e com poucos efeitos colaterais. Porém, como as informações dos protocolos utilizados na AR ainda são contraditórias (BENHAMOU, 2007; SCHOLES; UNSWORTH, 2004), tornam-se necessárias mais pesquisas que mostrem os efeitos funcionais e morfofisiológicos desta prática como tratamento desta condição inflamatória.

3.3.2. LBP como tratamento

O termo Laser vem do inglês: *Light amplification by stimulated emission of radiation*, ou seja, amplificação de luz por emissão estimulada de radiação. Trata-se de dispositivos que normalmente geram radiação eletromagnética relativamente uniforme em comprimento de onda, fase e polarização, originalmente descrita por Theodore Maiman em 1960 (MAIMAN, 1960) no qual é utilizada uma fonte de luz ou energia de radiação (VERMA et al., 2012).

Uma das modalidades utilizadas é o laser de baixa potência, este afeta os sistemas biológicos por meios não termais (LIN et al., 2010), por meio da absorção da radiação vermelha e infravermelha que possui a capacidade de promover analgesia, regeneração tecidual e reduzir processos inflamatórios (SILVA et al., 2007).

A absorção de fótons leva a célula a estados eletronicamente excitados e, consequentemente, à aceleração de reações de transferências de elétrons (YU et al., 1997), o que ocasiona maior produção de adenosina trifosfato (ATP) (PASSARELLA, 1989), e ao aumento de atividade de antiportes Na^+/H^+ e Ca^{2+}/Na^+, e todos os transportadores acionados por ATP para íons, como Na^+/K^+ ATPase e bombas de Ca^{2+}.

Como o ATP é um substrato da adenilciclase, sua concentração também controla a produção de AMP cíclico (AMPc). Ambos Ca^{2+} e AMPc são mensageiros secundários muito importantes, uma vez que os íons Ca^{2+} regulam quase todos os processos do corpo humano (contração muscular, coagulação sanguínea, transferência de sinais em nervos, expressão de genes, entre outros) (HAMBLIN, 2009). Além disso, a foto ativação de enzimas terminais, como Cox, desempenha papel vital na ativação de cascatas biológicas diversas subsequentes à irradiação do laser, controlando a proliferação celular, síntese proteica, depósito e organização do colágeno (KARU et al., 2004).

As evidências de que o LBP proporciona ao organismo uma melhor resposta a quadros inflamatórios, por meio da redução do edema, redução do quadro álgico e bioestimulação celular (KHOZEIMEH et al., 2015; KARU 2004) tornam o LBP um dos recursos de escolha no tratamento de inúmeras doenças musculoesqueléticas, incluindo a AR (HAKER; LUNDEBERG, 1990; KLEIN; EEK, 1990), para a qual este tratamento tem demonstrado tanto mudanças locais quanto sistêmicas, incluindo diminuição da dor, edema e enrijecimento articular, e melhora da resposta inflamatória aguda por redução de prostaglandinas e inibição de ciclooxigenase local, o que acarreta estímulo do processo de regeneração tecidual (HENRIQUES et al., 2010; MORIYAMA et al., 2005).

Além destes benefícios, o LBP é uma terapia relativamente simples e de baixo custo que tem se mostrado como ideal para dar suporte a tratamentos convencionais (MORIYAMA et al., 2009), o que torna relevante pesquisas sobre seus efeitos em indivíduos acometidos pela AR em associação com outra modalidade terapêutica, que também tem se mostrado eficaz na reabilitação da doença.

3.4. ESTUDOS EXPERIMENTAIS COM CFA

Pesquisas relacionadas à AR realizadas em humanos são difíceis de ser controladas, pois testes repetidos tornam-se extenuantes e tanto a ingestão de medicamentos, quanto hábitos diários de cada indivíduo podem interferir no processo inflamatório. Estudos *in vivo* realizados em animais, utilizando modelos experimentais de indução da artrite, podem gerar maiores informações sobre essa problemática. Com a proposta de estudar a etiopatogênese da artrite e na busca de diferentes tratamentos, muitos modelos experimentais foram desenvolvidos ao longo dos anos para representar a artrite em seres humanos (CAI et al., 2006; COOK; MOORE, 2006). Dentre os modelos experimentais de AR desenvolvidos (CAI et al., 2006; COOK; MOORE, 2006; NAGAKURA et al., 2003; YU et al., 2002), o CFA se destaca (CAI et al., 2006; COOK; MOORE, 2006; NAGAKURA et al., 2003).

Em modelos animais, a manifestação e a gravidade dos sintomas podem variar de acordo com o gênero do animal usado, forma e local da administração das espécies de *Mycobacterium*, e o número e intervalo de inoculações (CAI et al., 2006; COOK; MOORE, 2006; NAGAKURA et al., 2003). A artrite induzida por CFA é um modelo adequado, pois mimetiza os sinais e sintomas da AR humana, incluindo as mudanças histopatológicas, a infiltração celular, a hipersensibilidade e o edema da articulação afetada (BARTON et al., 2013).

ARTIGO 1

Physical exercise and low-level laser therapy effects on ankle joint in experimental rheumatoid arthritis model.

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Physical exercise and low-level laser therapy effects on ankle joint in experimental rheumatoid arthritis model.

ABSTRACT

Background. Rheumatoid Arthritis (RA) is an autoimmune and inflammatory disease that affects, mainly, body members which includes the ankle joint. Treatment involves physical exercises and low level laser therapy (LLLT) to modulate the inflammatory process and maintain physical capacity. In this way, the propose of this is study was analyze physical exercise and LLLT effects on functional and morphological parameters of the ankle joint submitted to AR model promoted by complete Freund's adjuvant (CFA). **Methods.** For functional analysis sample was composed by 64 male Wistar rats were randomly divided into two major groups: control (n=32) and arthritis (n=32) and each one was subdivided into four groups: treated and untreated (n=8) and for histomorphometrical 40 male Wistar rats were used in the same way, but control and arthritis (n=20) and treated and untreated (n=5). The RA promotion occurred first by an immunization with CFA injection at the tail base and, after seven days, by intra-articular injection for arthritis groups. For control groups the same protocol was used, but with saline injection. Treatments with LLLT and/or resisted stair climbing exercise, started 24 hours after the intra-articular injection, performed on alternating days with progressive time and series for exercise. Joint disability was evaluated by Sciatic Functional Index (SFI), hold force and incline plane evaluation and ankle joint morphological aspects by morphological and morphometrical analysis. The generalized mixed model test was applied for all parameters, but with Fischer (LSD) post test for functional and Sidak for morphometry (p=0, 05). **Results.** The association between

treatments showed positive effects on the functional measures once the member functionality recovery the initial values through the evaluations; on histological analysis the morphology presented differences in the arthritis group that had no treatment. The synovial membrane presented subintimal with angiogenesis revealing a large amount of blood vessels, which were turgid of blood cells and inflammatory cells inside the vessels. The cartilage presented smaller extracellular matrix and, consequently, higher chondrocyte density. Therefore, the cartilage was degraded with pannus in some animals. **Conclusions.** The arthritis model was effective in mimicking the disease in a systemic way and the combination of the two treatments was able to help in peripheral functionality.

Keywords

Experimental arthritis, Freund’s adjuvant, exercise, Laser, ankle joint.

INTRODUCTION

Rheumatoid arthritis (RA) is characterized as a systemic and frequently progressive inflammatory disease [1] that affects about 1% of the population in developed countries. In Brazil, there are few data about the disease, with an incidence between 0,2 and 1% over the population [2].

Among progressive diseases that affect human health, RA has increasing incidence, mainly because it is comorbidity related to other clinical and psychosocial complications [3]. Its clinical manifestations can include: poor appetite, fever, anemia and edema [4] and the most prominent are the articular manifestations [5], especially in synovial joints, present in large movement amplitude, which includes the ankle joint [6].

The ankle joint is involved in a lot of body movements, which play a key role in walking activity and supporting the body in the standing position. It comprises a distal syndesmosis between the tibia and fibula bones and a socket formed by the tibia and fibula distal thirds bones with talus and its involvement, as happens in RA, leads to biomechanical and functional changes that negatively affect the patient's life [7]. In individuals affected by RA is usually observed persistent synovitis development with edema and reduced joint mobility, resulting in tendons stretching and ligaments and joint capsules instability, leading to immobility [8] and consequently, in muscle mass and

strength decrease, compromising the joint and surrounding tissues integrity, resulting in pain and changes in movement patterns that limit daily activities [5] as well as work activities [9], what show their great socioeconomic relevance.

Research related to RA in humans is sometimes difficult to control, once the repeated tests become strenuous and medications ingestion or daily habits may interfere in the inflammatory process. In this way, many experimental models has been developed over the years, and the Freund's complete adjuvant (CFA) model stands out for being effective in mimicking human RA symptoms [10].

Standard therapy is made with drugs, using pharmacological agents related to inflammatory mechanisms [11]. However, these drugs usually have no lasting action and may lead to several side effects manifestations [12]; Therefore, other therapeutic approaches have been sought.

There are studies showing physical exercise as positive RA individual's rehabilitation, showing increase in motion range and flexibility [13]; significant decrease in pain and inflammatory activity, improving gait and general functions [14,15] . However, morphofunctional data are generally concentrated in previous models, mainly in the tibiofemoral joint, so that its action in the ankle joint hasn't been studied yet.

In addition to exercise, low level laser therapy (LLLT) has been applied to treat a lot of musculoskeletal disorders, including RA [16, 17]. There is evidence that LLLT promotes local and systemic changes which may lead to decreasing pain, edema and joint stiffness. It also improves inflammatory

response reducing prostaglandins and inhibiting local cyclooxygenase, which leads to tissue regeneration process stimulation [18, 19].

Although the literature brings the exercise and LLLT benefits, there are gaps regarding the protocols used and the association effects of both therapeutic modalities in RA treatment. Also, the CFA model causes changes in the ankle joint morphofunctional aspects, as well as the treatment modalities. Thus, the objective was test the exercise and LLLT effects on the ankle joint functionality and morphological aspects in order to obtain information about the RA treatment protocols that help professionals in their patients rehabilitation, improving their life quality.

METHODS

Animals and experimental groups

The study is an experimental research and randomized into groups using Microsoft Excel 2010, composed by 80 Wistar male rats, aged 15 weeks; kept in plastic polypropylene boxes, with access to water and feed at will; and controlled temperature at 21ºC ±1ºC, 12 hours light/dark photoperiod. The study was approved by the Ethics Committee for Animal Use (CEUA) of the State University of Western Paraná (Unioeste).

The animals were randomly separated into two big groups: arthritis (n=40) and control (n=40). And subdivided into eight groups (n=5) as follows: control group (CG), control laser (CL), control exercise (CE), control laser + exercise (CLE), arthritis group (AG), arthritis laser (AL), arthritis exercise (AE) and arthritis laser + exercise (ALE) (Figure 1).

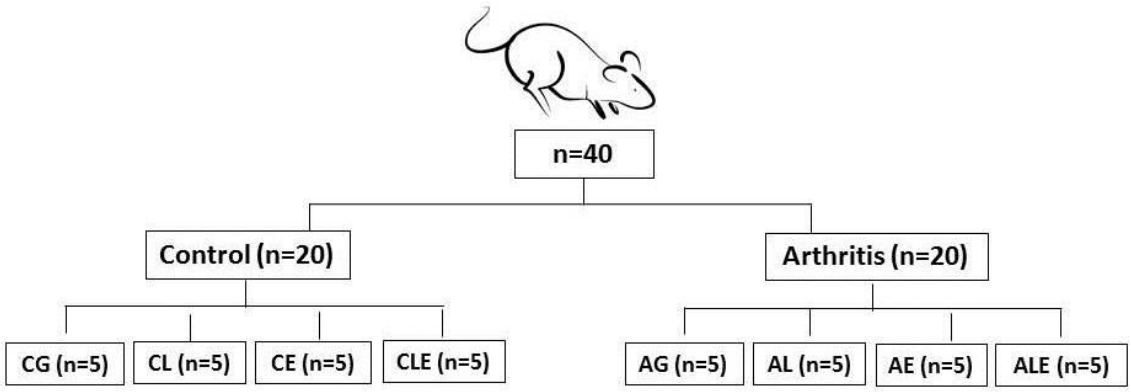


Figure 1: Experimental groups. Are indicated: CG: Control Group; CL: Control Laser; CE; Control Exercise; CLE: Control Laser and Exercise; AG: Arthritis Group; AL; Arthritis Laser; AE: Arthritis Exercise and ALE: Arthritis Laser and Exercise.

CFA-induced disability

The arthritis model was induced by a pre-sensitized intradermal injection at the tail base with 50 µL of CFA (0.5 mg/mL, *Mycobacterium butirycum*) in AG, AE, AL and ALE animals. The CG, CL, CE and CLE groups received saline solution (sodium chloride 0.9%) instead CFA. Thus, the administration area where occurred the substances injection was trichotomized and submitted to asepsis with iodinated alcohol (1%). Seven days after the first stimulus in the tail, an intra-articular injection with 50 µL (0.5 mg/mL) of CFA *Mycobacterium butirycum* or saline solution (according to the subgroups mentioned above) was performed in the tibiofemoral joint, in the animals right pelvic limb.

Evaluations

Animals

All the animals passed by functional evaluations. In this way, before the experiment starts they were trained and adapted to the equipment. After that, they were submitted to seven evaluations. A basal evaluation was made seven days after the tail injection and before the intra-articular application. After 24 hours, and before the treatment starts, evaluation 1 (EV1) was performed. On the third, fifth, 12^{th}, 19^{th} and 26^{th} days after intra-articular injection EV2, EV3, EV4, EV5 and EV6, respectively, were performed. On the 28^{th} day, the animals were euthanized (Figure 2).

The muscle strength evaluation was performed using a grip strength meter (Insight®, Ribeirão Preto, São Paulo), described by Bertelli and Mira [20]. The animal was positioned so that it grasped on a grid connected to a force transducer, with the right pelvic limb, then pulled by the tail with increasing force until the grip is lost, at which time. The device provides the force exerted by the animal. The left pelvic limb was immobilized by the evaluator's hand so as not to interfere in the evaluation.

Motor function was evaluated using the inclined plane (Insight®, Ribeirão Preto, São Paulo). The test measured the animal's ability to remain in top of a ramp whose inclination increased five degrees every five seconds. The maximum angle which the animal remained on the ramp without slipping for at

least five seconds defined the inclined plane test score. The measurements were performed with the animal positioned in the transverse plane (vertical position).

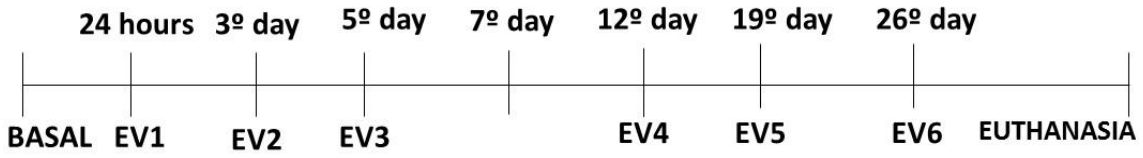


Figure 2: Evaluations schedule.

Treatment Protocol

For CE, CLE, AE and ALE the treatment was made through resisted climbing exercise in stairs use. For this, a vertical wooden ladder with 67 iron steps, high of 1.18 m, width 20.5 cm and 60º inclination was used. At the top of the stairs was located a box, with 20X20 cm in height and width, in which the animals rested between one series and another with a 60 s interval. The treatment starts 24 hours after the intra-articular injection. For this, was used a 100g overload coupled to the tail. In the first week the animals performed four series of five climbs on the stairs and in the second week, with the same overload, the series changed for four with seven ascents, and in the last week for four series of 10 ascents completing 14 days of intercalated treatment.

In the CL, CLE, AL and ALE treatment the animals were submitted to LLLT (Laserpulse Ibramed®). The other groups' animals received the pen contact, but without beam emission, in the sensitized pelvic limb in the knee region. The application points were anterior to the patella, medial face in the

tibiofemoral joint, and posterior in the popliteal region. The treatment parameters were point technique, in four points, with a 660 nm wavelength; 30 mW of power; spot area; 0.06 cm^2; energy density, 5 J/cm^2 per point; time per point, 10 s; and total energy per point, 0.003 J.

Ankle joint histological processing

After being collected, the right pelvic limb ankle joints were fixed in Metacarn, and packed in 70% alcohol after two days. For the descaling procedure, they were rinsed in running water for 24 hours to be descaled using 5% trichloroacetic acid (TCA). After decalcification, the pieces were dehydrated in alcohol upward series (70%, 80%, 90%, 100%I,100%II and 100%III), diaphanized through n-butyl alcohol I, II and III and emblocked in histological paraffin. Subsequently, microtomy was performed by obtaining seven um sagittal sections on Olympus R CUT 4055® microtome. The slides were stained with hematoxylin and eosin for morphologic and histomorphometrical analysis in light microscopic (Olympus®).

In the general morphological analysis, the synovial membrane morphological characteristics were observed. For this, the anterior joint region was standardized as a point of analysis. The characteristics observed were: membrane general organization (intima and subintima layer) and its cells (synoviocytes), vascularization and inflammatory infiltrate presence inside the vessels and in the extracellular matrix. In the joint cartilage the following characteristics were observed: presence of flocculations in the articular surface, chondrocytes organization and disposition, fissures and degradation. In the joint cavity synovial infiltrate presence and pannus formation was analyzed.

For histomorphometric analysis, the interest regions were photomicrographed using Olympus® DP71 microscope. The protocol for measuring articular cartilage thickness and chondrocyte number was adapted from Ando et al. and Hagiwara et al. For this, three distinct regions were defined: P1, anterior end (near the phalanges); P2, joint middle region and P3, posterior articular end.

The tibia and talus middle or P2 regions were selected as analysis regions, since they are the site of highest joint weight discharge (Kunz et al., 2014). Photomicrographs were performed at 400X magnification for the following measures performing: total cartilage thickness, defined as the distance between the cartilaginous surface and the osteochondral junction; the surface zone thickness comprising the space between the cartilage surface and tidemark; and the deep zone thickness which is bounded superiorly by the tidemark and inferiorly by the subchondral bone. The chondrocytes number was counted using an 300um X 300um area where only the chondrocytes that was fully within the borders limits were considered.

Statistical analysis

Statistical analyzes were performed using the BioStat program through the generalized mixed models test. For the functional analyzes, the LSD post-test was used and for the histomorphometric analyzes, the Sidak post-test was used. The significance adopted was $p<0.05$.

RESULTS

In muscle strength there was significant difference in the member functionality evaluation between the evaluations ($F(4.3)= 9.9$, $p<0.0002$), between the groups ($F=(7.56)=5.8$ $p< 0.00004$), and there was interaction between groups and evaluations ($F(32.3)=3.4$, $p<0.0001$) (Table 1).

Within groups, there was significant difference between control groups (CG, CL, CE and CLE) and the arthritis groups that were treated (AL, AE and ALE) ($p<0.0002$) and values returns close to control values in all the treated groups. Therefore, the CE group showed a positive difference in relation to all the other groups (Table 1).

Between groups, the control groups were different from all the arthritis groups ($p< 0.00004$), and all the treated groups (AL, AE and ALE) had values returned close to control values.

The motor function evaluation showed differences between the evaluations ($F(7.4)=23.6$, $p<0.0001$), between the groups ($F(7.56)=5.4$, $p<0.0001$) and there was interaction between groups and evaluations ($F(49,4)=5.9$, $p<0.0002$) (Table 2).

Within groups, there was significant difference between groups with arthritis that were treated (AL, AE and ALE) and groups without arthritis (CG, CL, CE and CLE) ($p<0.0001$). In EV4 the AL group returned to the initial values and in AV5 AE and AEL restored the initial values to (Table 2).

Between groups the animals of the control groups (CG, CL, CE and CLE) had significant differences ($p<0.0001$) when compared with all arthritis

groups. In EV4 all the arthritis groups (AG, AE, Al and ALE) restored the initial values (Table 2)

Table 1: Grip strength exercised throughout the evaluations within the same group and between groups

	AV0	*AV1*	*AV2*	*AV3*	*AV4*	*AV5*	*AV6*
GC	64,5±13^{aA}	61,9±9,4 ^{abAB}	85,7±22,5^{acA}	77,0±14,2$^{a\ AC}$	76,9±19,5$^{a\ A}$	83,4±21,2^{a} AC	78,6±19,9^{aA}
GA	62,1±13,4$^{a\ A}$	39,5±19$^{a\ AB}$	43,6±15,7^{aB}	46,7±11,6 $^{a\ B}$	49,3±9,3$^{a\ B}$	51,4±9,8^{aB}	50,1±3, 7 ^{aB}
CL	66,5±14,4 $^{a\ A}$	73,7±21,7 $^{a\ A}$	80,6±14,4 ^{aA}	75,4±13,7 $^{a\ AC}$	73,0±11,8 $^{a\ A}$	77,2±16,2^{a} AC	78,5±16,8 $^{a\ A}$
AL	60,9±16,1 ^{abA}	50,8±20,5 ^{abAB}	48,9±17,9 ^{abB}	48±15,7 $^{a\ B}$	58,4±12,0 ^{abAB}	60,0±15,5 abABC	63,7±15,3 abAB
CE	71,8±14,1$^{abc\ A}$	66,3±12,4^{abcAB}	66,4±24,8^{abcAB}	75,9±13,4^{abcAC}	85,2±12 ^{abA}	89,9±15,3^{a} AC	83,7±14,7^{abA}
AE	62,9±6 ^{abA}	48,9±18,4 ^{abAB}	46,7±16,6 ^{aB}	48,5±14 ^{aB}	51,2±17,8 ^{aB}	56,9±17,8 abBC	65,8±16,5 abAB
CLE	64,0±17,8^{aA}	73,8±22,3^{aA}	76,9±14,8^{aA}	79,6±16,8$^{a\ AC}$	79,5±16,1$^{a\ A}$	77,0±19,5^{a} AC	79,9±10,4^{aA}
ALE	65,6±12,8^{abA}	48,2±23,5^{aAB}	46,6±16,5^{aB}	56,8±16,6^{abAB}	63,8±14,4^{abAB}	61,0±14,6^{ab} AC	69,2±20,3^{abAB}

Values are expressed in mean ± standard error. Different letters represents different values. In the lines, the lowercase letters, comparison within the same group and in columns, with capital letters, comparison between groups throughout the evaluations.

Table 2: Maximum angle reached by animals during the evaluations within the same group and between groups.

	EV0	*EV1*	*EV2*	*EV3*	*EV4*	*EV5*	*EV6*
CG	59.8±0.7 ^{aA}	58.8±1^{aA}	59,6±1 $^{a\ ACD}$	59.8±1 ^{aAC}	59.4±1 ^{aA}	59.4±0.8 $^{a\ A}$	60.2±0.8 $^{a\ A}$
AG	59.0±0.7 $^{a\ A}$	58.3±1$^{a\ A}$	58,1±1^{aABCD}	59±1^{aAC}	57.7±1 $^{a\ A}$	58.8±0.8 $^{a\ A}$	57.7±0.8 $^{a\ ABC}$
CL	58.3±0.7 ^{aA}	57.3±1$^{a\ A}$	57,7±1 ^{aACD}	57.1±1$^{a\ AC}$	55.4±1 $^{a\ A}$	55.6±0.8 $^{a\ A}$	55.2±0.8 ^{aBC}
AL	58.8±0.7 ^{aA}	48.8±1.0^{bcB}	53,5±1$^{b\ BD}$	54.6±1,1$^{b\ BC}$	56.9±1^{aA}	58.3±0.8^{aA}	57.9±0.8^{aAC}
CE	58.3±0.7 ^{aA}	58.3±1 $^{a\ A}$	59,0±1 ^{aACD}	58.6±1 ^{aAC}	58.8±1 ^{aA}	58.5±0.8 $^{a\ A}$	57.9±0.8 ^{aAC}
AE	58.3±0.7 $^{a\ A}$	50.0±1.0^{bcB}	54,4±1$^{bd\ BCD}$	55.6±1 ^{adAC}	57.3±1 ^{adA}	58.1±0.8 $^{a\ A}$	58.1±0.8 $^{a\ AC}$
CLE	60.8±0.7 $^{a\ A}$	59±1 $^{a\ A}$	58,5±1 $^{a\ ACD}$	58.8±1 $^{a\ AC}$	58.6±1 $^{a\ A}$	59.2±0.8 $^{a\ A}$	59.6±0.8 $^{a\ A}$
ALE	57.9±0.7$^{ac\ A}$	47.5±1. ^{bB}	54,6±1$^{ac\ BCD}$	56.2±1$^{ac\ AC}$	57.7±1 $^{ac\ A}$	58.8±0.8 $^{a\ A}$	59.0±0.8 $^{a\ A}$

Values are expressed in mean ± standard error. Different letters represents different values. In the lines, the lowercase letters, comparison within the same group and in columns, with capital letters, comparison between groups throughout the evaluations.

Histomorphometrical analysis

Articular cartilage morphometry

The histomorphometical analysis of P2 articular cartilage of both tibia and talus showed no significant difference in any of the analyzed parameters (Table 3 and 4).

Table 3: Tibia histomorphometrical analysis

TIBIA	*Total Thickness*	*Superficial Zone*	*Deep Zone*	*Chondrocytes*
CG	154±11.7 [a]	70.6±7.4 [a]	82.7±8.3 [a]	14.9±1.1 [a]
AG	142.8±11.7 [a]	55.7±8 [a]	85.1±9.3 [a]	21.5±2 [a]
CL	162.5±11.5 [a]	72.5±7 [a]	89±8.4 [a]	16.5±1.2 [a]
AL	156.6±12 [a]	71±7.4 [a]	84.7±8.5 [a]	19±1.5 [a]
CE	171.2±11.5 [a]	75.7±6.5 [a]	94.7±8.4 [a]	18.3±1.3 [a]
AE	161.5±11.5 [a]	75.8±7 [a]	84.6±8 [a]	18.4±1.3 [a]
CLE	164±11.6 [a]	74±7 [a]	87.1±8.2 [a]	18.1±1.3 [a]
ALE	153.7±12 [a]	66.3±7 [a]	87.4±8.2 [a]	17±1.2 [a]

Values are expressed in mean ± standard error. Different letters represents different values.

Table 4: Talus histomorphometrical analysis

TALUS	*Total Thickness*	*Superficial Zone*	*Deep Zone*	*Chondrocytes*
CG	140 ±10.6[a]	44.1±5 [a]	95.6±8.3 [a]	17.1±1.1 [a]
AG	139.3±11.5 [a]	52.7±6.4 [a]	84.8±8.9 [a]	21.7±1.7 [a]
CL	124.1±10 [a]	48.1±5.1 [a]	75.1±7.7 [a]	17.6±1.1 [a]
AL	118.3±10.6 [a]	40±4.4 [a]	80±8.3	19.4±1.3 [a]
CE	122.9±9.4 [a]	43.1±4.3 [a]	80.3±7.3 [a]	18.2±1.1 [a]
AE	118.5±10 [a]	45±4.7 [a]	72.7±7.7 [a]	16±1 [a]
CLE	126.7±10 [a]	48±5 [a]	78.2±7.7 [a]	19±1.2 [a]
ALE	131.4±10 [a]	45.1±4.7 [a]	87.5±7.7 [a]	19.7±1.2 [a]

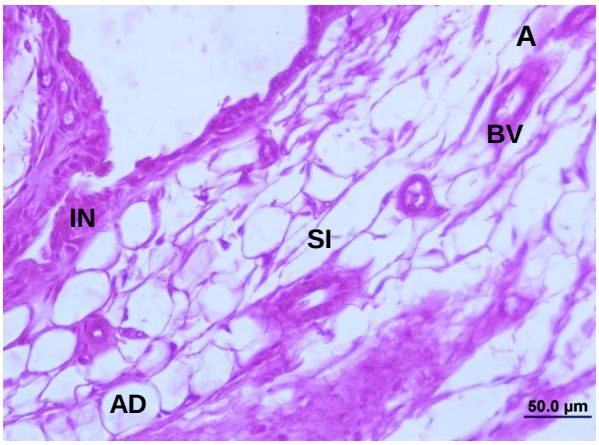
Values are expressed in mean ± standard error. Different letters represents different values.

Morphological analysis

Synovial Membrane

The control group presented characteristic morphology with two to three cell layers, types A and B synoviocytes in the synovial intima, and subintima with adipose cells predominance (Figure 3A). The arthritis group (AG) showed subintima with angiogenesis, revealing a large blood vessels number, which were turgid with red blood cells with inflammatory cells inside the vessels (Figure 3B). The laser groups (CL and AL), exercise (CE and AE) and groups where the association of the two treatments was performed (CLE and ALE), had

the same characteristics of angiogenesis and red blood cells congestion, however, was not observed the presence of inflammatory cells.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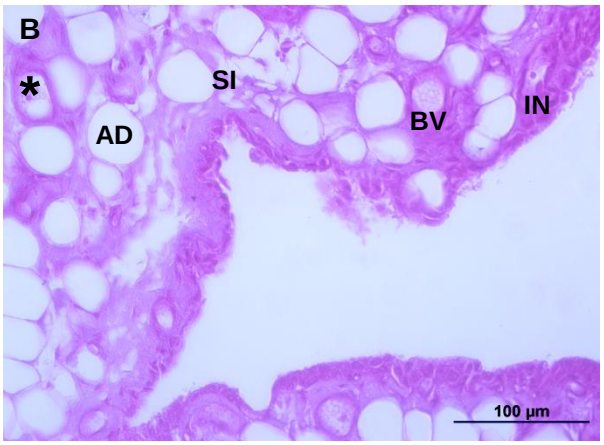
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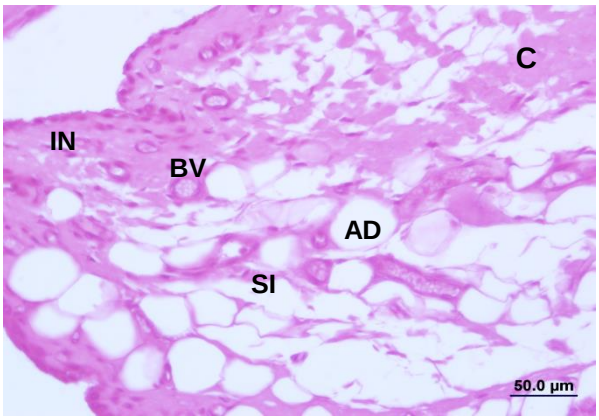
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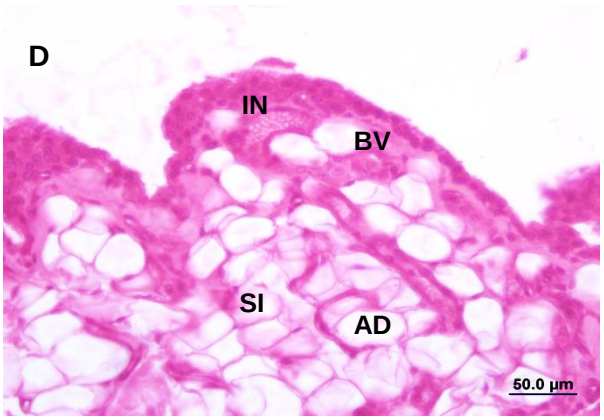
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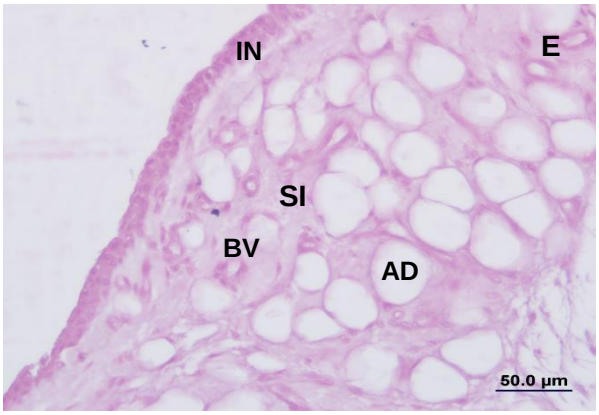
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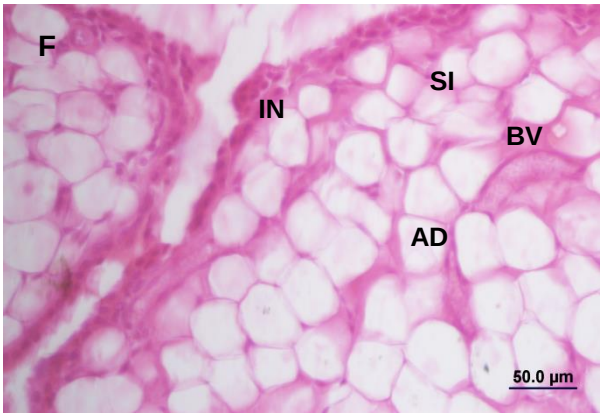
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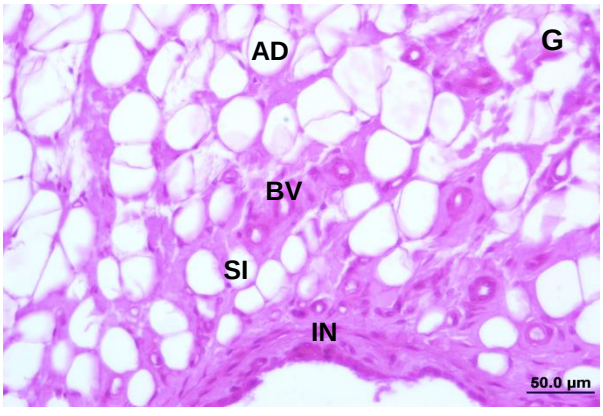
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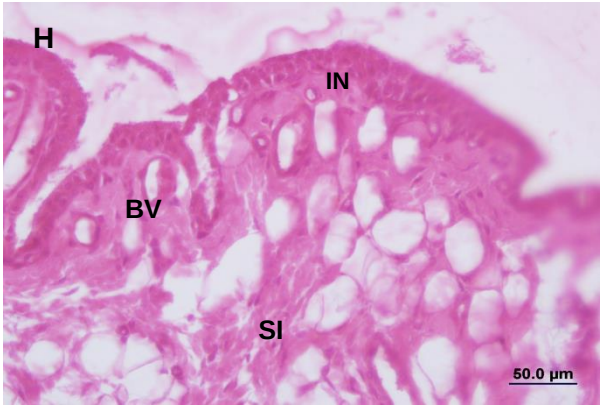
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Figure 3: Wistar rats talocrural joint synovial membrane photomicrographs, 28 days after induction of knee arthritis by CFA, sagittal section, hematoxylin and eosin staining. A: Control group; B: Arthritis group; C: Control Laser; D: Arthritis Laser; E: Control exercise; F: Arthritis exercise; G: Control laser and exercise and H: Arthritis laser and exercise. Are indicated: Intimate layer (IN), Subintimate layer (SI), Blood vessels (BV) filled by red blood cells; adipocytes (AD) and inflammatory infiltrate (*).

Articular cartilage

The CG group showed, in tibia and talus, characteristic morphology, that is, smooth articular surface and chondrocytes organized in four cell layers. In the superficial zone was identified a higher cell density arranged in horizontal with flattened clusters. In the intermediate zone the chondrocytes assumed rounded aspect and were arranged alone or in isogenic groups. The deep zone showed chondrocytes organized in gaps, and it was separated from the calcified zone by a basophilic line called *tidemark*. The subcondral bone also had a normal appearance (Figure 4A).

In the GA group, the cartilage presented a smaller extracellular matrix amount and the chondrocytes, consequently, with higher density. In addition, the superficial zone was totally disorganized with cartilage degradation and pannus in 50% of the analyzed animals, one animal only in the talus and the other in both talus and tibia (Figure 4B).

The CL, CE, CLE, AL and AE groups showed no alterations, morpholologically resembling the CG group.

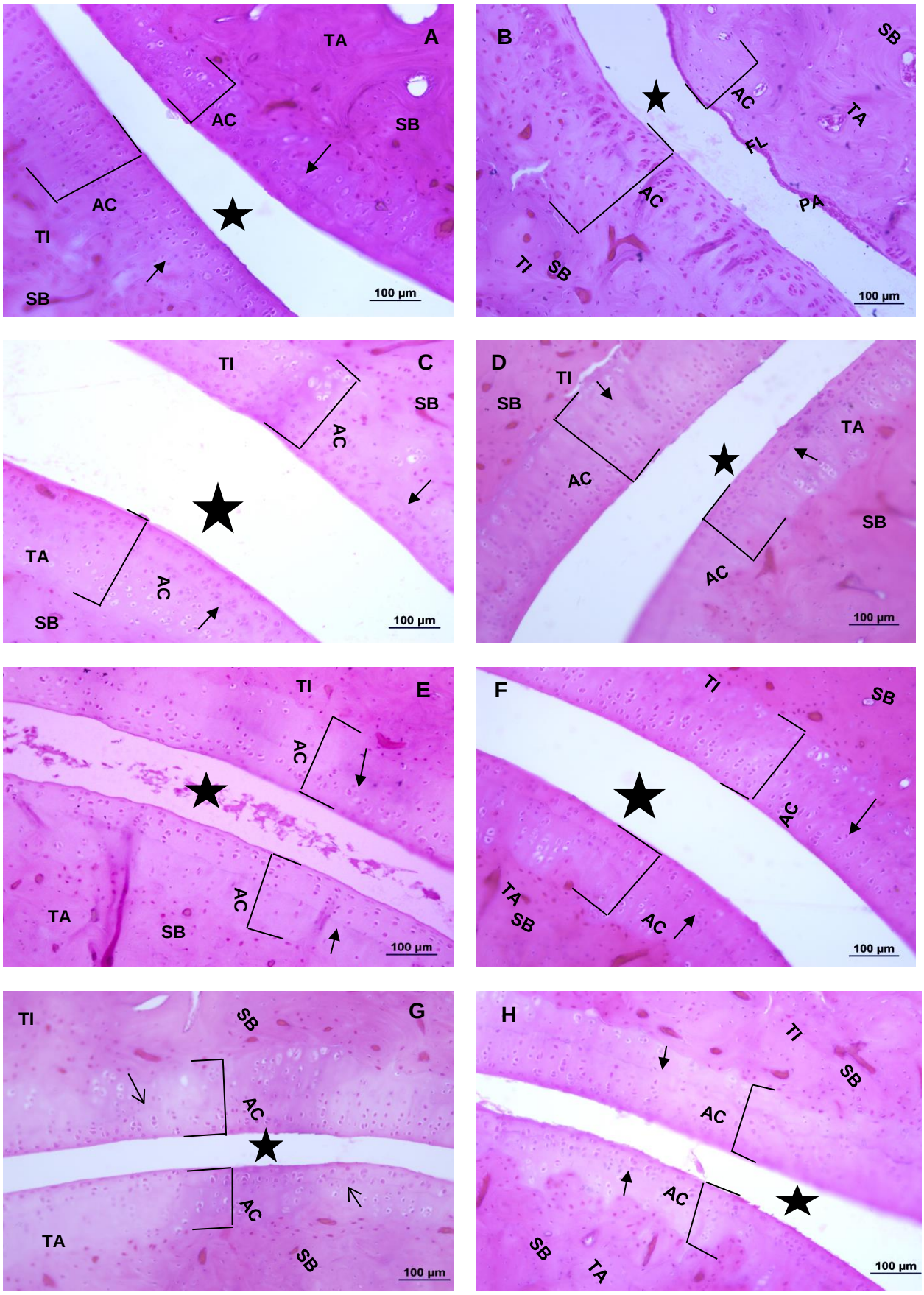
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Figure 4: Wistar rats talocrural joint cartilage photomicrographs, 28 days after arthritis induction in the knee by CFA, sagittal section, hematoxylin and eosin staining. A: Control group; B: Arthritis group; C: Control Laser; D: Arthritis Laser; E: Control exercise; F: Arthritis exercise; G: Control laser and exercise and H: Arthritis laser and exercise. Are indicated: Tibia (TI), Talus (TA) Articular cartilage (AC), Subchondral bone (SB), Tidemark (arrow), Articular cavity (star); Flocculation (FL) and *pannus* (PA).

DISCUSSION

Both treatments used in this study showed positive effects in functional evaluations (muscle strength and motor function). Many studies showed that physical activity promotes pain reduction [21-26]. Exercise is a form of stressful stimulus capable of promoting changes in homeostasis with responses reorganizations mainly from the neuroendocrine system, probably generating an increase of endogenous glucocorticoids serum [27].

All of these physiological responses can lead to benefits, which have already been demonstrated by rehabilitation studies with physical exercise in individuals with RA, among which are: increased range of motion, flexibility [13] significant decrease in pain, improving gait and general function [14, 15], cardiorespiratory improvement and cardiovascular health, increased muscle mass, reduced adiposity, improves muscle strength and physical fitness, psychological well-being [28], improves fatigue [39] and overall life quality [21].

Hurkmans et al. inferred in a systematic review that physical exercises and behavioral education in patients with RA are recommended for clinical use and LLLT therapy is among the proposed guidelines, but the recommendation parameters are not well clarified [30]. In this study, LLLT also showed improvements in the functional parameters. Brosseau et al. made a systematic

review relating the LLLT efficacy in RA treatment and concluded that laser therapy has a beneficial effect when used at least four weeks, with effects on pain reduction and morning stiffness [31].

A study made by Khozeimeh et al., 2015 showed that LLLT therapy provides the body a better response to inflammatory conditions, by reducing edema, reducing pain and provides cellular biostimulation [32]. In addition to these benefits, LLLT is relatively simple and low-cost therapy that has been shown to be ideal for supporting conventional treatments.

Hsieh et al.showed that inflammatory action and functionality recovery using LLLT therapy is related actin in modulation of inflammatory mediators, indicating beneficial effects using 4.5 J/cm2 with inflammation reduction [33] what corroborates with the results of the present study.

The morphometrical analysis in this study didn’t show significant differences in any tested parameters, but the morphological analyses showed in AG a subintima with angiogenesis, revealing a large amount of blood vessels, turgid with red blood cells, with inflammatory cells inside of this on synovial membrane.

Firestein, 2003 showed on his study that the greater involvement of synovial membrane structure occurs due to hyperplasia in the inflammatory process which configures synovitis [34], since the large number of immune system cells that invade this structure, lead to cell proliferation, neovascularization and germinal formation of germinal lymphoid follicles. The mechanisms involved in the inflammatory cells recruitment into synovial

membrane have been extensively studied [35] showing synovial hyperemia [36], fibroblast-like invasive synoviocytes proliferation that resist apoptosis and increased in the property of adherence and invasion[38], leukocyte infiltration, associated neoangiogenic response [37, 38](; changes in the function of T and B lymphocytes, abnormal production of cytokines and antibodies [39, 40].

On this study the treated arthritis groups (AL, AE and ALE) also presented the same characteristics of angiogenesis and red blood cells congestion, however inflammatory cells presence was not observed. Scholes et al. demonstrated that the degeneration caused by RA in joints causes immobility what leads the synovial fluid to rest for long periods and when the movement returned, the lubrication fluid mechanism is reactivated, promoting improvement in lubrication and joint health [41].

Khozeimeh et al. brings evidence that LLLT treatment provides the body with a better response to inflammatory conditions reducing edema, pain and making cellular biostimulation [32]. In addition, other studies had point LLLT as a therapy that has demonstrated both local and systemic changes, including decrease in pain, edema and joint stiffness, reduction in prostaglandins and inhibition in local cyclooxygenase, what leads to tissue stimulation and regeneration process [42, 19, 20].

In the present study, the AG articular cartilage morphology showed a lower extracellular matrix amount and higher chondrocytes density. In addition, the superficial zone was totally disorganized with cartilage degradation and pannus presence in 50% of the analyzed animals. These results corroborate with the studies of Kapale et al., where the immune system cells are described

as forming a fibrous abnormal layer of tissues, called as pannus, which releases substances that accelerate bone erosion and cartilage destruction, what leads to loss of its shape and alignment, resulting in deformations [15]. These deformations leads the joints to become fixed due to the tick and hardened structure, which results in edema, articular tissue modification and, due to disuse, in adjacent structures atrophy, such as: skin, bones, muscles, nerves and even blood vessels connective tissues [34].

In the present study, the CFA Application occurred in the knee joint, we analyzed the ankle joint and the morphological analysis showed characteristic arthritis morphology in GA group. These results may show successful systematic characteristic of the sickness [1, 34, 43] in CFA experimental model.

CONCLUSION

Both treatments (LLLT and exercise) and the combination of treatments had positive effect on the functional evaluations and the morphology showed efficacy in systematic arthritis induction.

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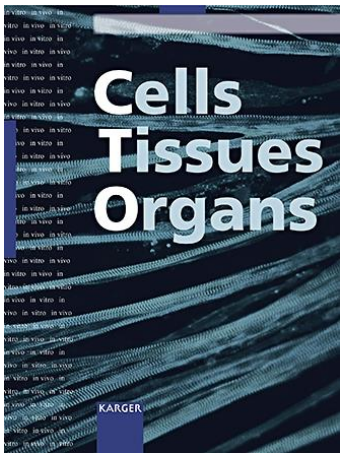

ARTIGO 2

Physical exercise and low-level laser therapy systemic effects on ankle joint in acute experimental rheumatoid arthritis model.

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Keywords: Experimental arthritis, Freund’s adjuvant, exercise, Laser, ankle joint

ABSTRACT

Background. Rheumatoid arthritis (RA) is a chronic, autoimmune, and systemic inflammatory disease characterized by symmetrical synovitis of peripheral joints, which includes the ankle joint and, if untreated, may lead to joint destruction and a loss of functional capacity. Treatment may involve physical exercises and low level laser therapy (LLLT) to reduce pain, disability and modulation of inflammatory process, what can improve functionality. In this way, the propose of this is study was analyze physical exercise and LLLT effects on functional and morphological parameters of the ankle joint submitted to AR acute model promoted by complete Freund's adjuvant (CFA). **Methods.** For functional analysis 64 male Wistar rats were randomly divided into two major groups: control (n=32) and arthritis (n=32) and each one was subdivided into four groups: treated and untreated (n=8) and for histomorphometrical 40 male Wistar rats were used in the same way, but control and arthritis (n=20) and treated and untreated (n=5). The RA promotion occurred first by an immunization with CFA injection at the tail base and, after seven days, by intra-articular injection for arthritis groups. For control groups the same protocol was used, but with saline injection. The treatments with LLLT and/or resisted stair climbing exercise, started 24 hours after the intra-articular injection, performed on alternated days with progressive time and series for exercise. Joint disability was evaluated by Sciatic Functional Index (SFI), hold force and incline plane evaluation and ankle joint morphological aspects by morphological and morphometrical analysis. The mixed generalized model test was applied for all parameters, but with Fischer (LSD) post test for functional and Sidak for morphometry (p=0, 05). **Results.** The association between LLLT and exercises presented positive changes in functional parameters; in the histological analysis the arthritis group synovial membrane showed subintimal with angiogenesis revealing large amount of blood vessels, which were turgid of blood cells and inflammatory cells inside the vessels. The arthritis articular cartilage presented flocculation on the joint surface, chondrocytes disorganization and degeneration of the superficial layers with subchondral bone exposure and *pannus*. The arthritis groups treated only with LLLT and both treatments had disorganized chondrocytes and tidemark absence in some animals. The arthritis group treated with exercise showed greater amount of chondrocytes, as confirmed in the histomorphometric analysis. **Conclusion.** The arthritis model was effective in mimicking the disease in a systemic way, the combination of the two treatments was able to help in peripheral functionality and exercise helped in morphological recovery of the ankle joint.

INTRODUCTION

Rheumatoid arthritis (RA) is a chronic, autoimmune, and systemic inflammatory disease that occurs in 0.24% to 1% of the population [1]. Due to its autoimmune, systemic [2] and symmetrical [3] characteristics, many organs and body tissues can be affected. However, the ones that stand out most are articular manifestations [4], mainly in the synovial ones, which includes the ankle joint [5].

Ankle joint comprises a distal syndesmosis between the tibia and fibula bones and a socket formed by the by tibia and fibula distal thirds bones with talus that is involved in a lot of body movements, which play a crucial role in walking activity and supporting the body in the standing position and its commitment, as happen in RA, leads to biomechanical and functional changes that causes limitations that negatively affects the patient's life [6].

The main clinical manifestations includes: severe joint pain, reduced muscle strength, stiffness and fatigue and limited physical movements that are usually followed by edema and heat, accompanied by muscle pain that can worsen and persist for weeks or months [7] what limits the individual's daily activities [4] as well as work activities [8] which shows the great socioeconomic relevance in RA study.

The standard therapy to RA is usually made with drugs, with pharmacological agents related to inflammatory mechanisms [9]. However, these drugs usually have no lasting action and may lead to several side effects manifestations [10]; In this way, other therapeutic approaches have been researched. The research is related to RA in humans is difficult to control, once the repeated tests become strenuous and medications ingestion or daily habits may interfere in the inflammatory process. Therefore, many experimental models have been developed over the years, and the Freund's complete adjuvant (CFA) model stands out for being effective in mimicking human RA symptoms [11].

Although the literature brings the exercise and LLLT benefits, there are gaps regarding the protocols used and the association effects of both therapeutic modalities in RA treatment. Studies show that if worked in a responsible and adequate way, exercises can bring benefits such as: increase in muscular strength, improvement in motor coordination, maintenance and increase of flexibility, bone and joint strengthening, which leads to an improvement in the activities performance of daily living activities, and thus, functionality and self –sufficiency [12]. In addition, there is also a decrease in pain with opioides endogenous release by the central and peripheral nervous system, where the central and peripheral receptors results in antinociceptive effects, as well as in immune cells expression [13].

In addition to exercise, there are evidences that low level laser therapy (LLT) provides the body with a better response to inflammatory conditions, by reducing edema, pain and making cellular bio stimulation [14]. This treatment has demonstrated both local and systemic changes including decreased pain, swelling and joint stiffness, and improved acute inflammatory response by reduction in prostaglandins and inhibition of local cyclooxygenase, which leads to stimulating the tissue regeneration process [15].

Thus, the study aimed to test the systemic effects of both and association of the two treatments on ankle joint functional and histomorphometrical aspects in a CFA-induced arthritis model in order to obtain information about the RA treatment protocols that helps professionals in their patients rehabilitation, improving their life quality.

METHODS

The study is an experimental research and animals were randomized into groups using Microsoft Excel 2010, the sample was composed by 80 Wistar male rats, aged 15 weeks; kept in plastic polypropylene boxes, with access to water and feed at will; and controlled temperature at 21ºC ±1ºC, 12 hours light/dark photoperiod. The study was approved by the Ethics Committee for Animal Use (CEUA) of the State University of Western Paraná (Unioeste).

For functional analysis 64 male Wistar rats were randomly separated into two big groups: arthritis (n=32) and control (n=32). And subdivided into eight groups (n=8) as follows: control group (CG), control laser (CL), control exercise (CE), control laser + exercise (CLE), arthritis group (AG), arthritis laser (AL), arthritis exercise (AE) and arthritis laser + exercise (ALE). For histomorphometrical analysis 40 male Wistar rats were used, divided in the same way but with arthritis (n=20) and control (n=20). And subdivided into the same eight groups (n=5).

Systemic RA induction through CFA

The systemic arthritis model were induced by a pre-sensitized intradermal injection at the tail base with 50 µl of CFA (0,5 mg/mL, *Mycobacterium butirycum*) in AG, AE, AL and ALE animals. The CG, CL, CE and CLE groups received saline solution (sodium chloride 0,9%) instead CFA. Seven days after the first stimulus in the tail an intra-articular injection with 50 µL (0,5 mg/ml) of CFA *Mycobacterium butirycum* or saline solution (according to the subgroups mentioned above) was performed, to test the systemic response in the ankle, in the tibiofemoral joint, after tricomization and asepsis with iodinated alcohol (1%) of the area, in the animals right pelvic limb.

Functional evaluations

All the animals passed by functional evaluations (Figure 1). In this way, before the experiment starts they were trained and adapted to the equipment. After that, they were submitted to seven evaluations. A basal evaluation was made seven days after the tail injection and before the intra-articular application. After 24 hours, and before the treatment starts, evaluation 1 (EV1)

was performed. On the third and fifth days after intra-articular injection EV2, and EV3 respectively, were performed. On the seventh day, the animals were euthanized.

The member functionality was evaluated by the sciatic functional index (SFI), a reproducible quantitative method of the rat sciatic nerve functional condition that uses analyzing data characteristic of animal's printed footprint (De Medinaceli et al., 1993). Footprints measurements (Figure 2 A) are made and used in a formula (Figure 2 B), by which the SFI is calculated.

This formula uses measurements on the non-injured pelvic limb (N) and the one that passed by experimental procedures (E) by obtained the measurements: footprint length, total toe spread (or distance from first to fifth toes) and intermediate fingers spreading (or distance between the second and fourth fingers) as shown in figure 2A. In this work, to obtain footprint impressions, the Bain et al. [16] method was used. In this way, the animals were placed on a clear acrylic catwalk enclosed at the top to make them walk their full length into a small dark environment that they used for shelter. This catwalk had a mirror attached on the underside, which allowed the filming of the rats' gait in video for further analysis. The measurements were performed using the Image-Pro Plus® 6.0 software.

Motor function was evaluated using the inclined plane (Insight®, Ribeirão Preto, São Paulo). The test measured the animal's ability to stay on top of a ramp whose inclination increased five degrees every five seconds. The maximum angle which the animal remained on the ramp without slipping for at least five seconds defined the inclined plane test score. The measurements were made with the animal positioned with in the transverse plane (vertical position).

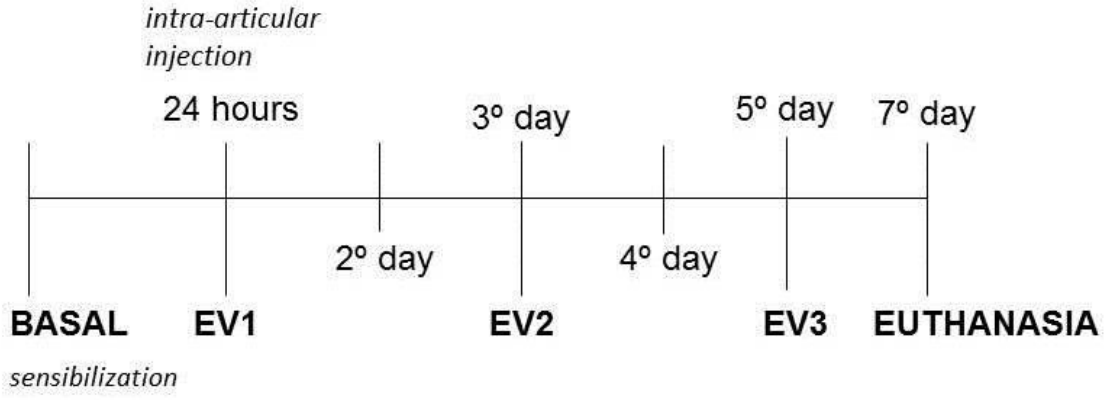
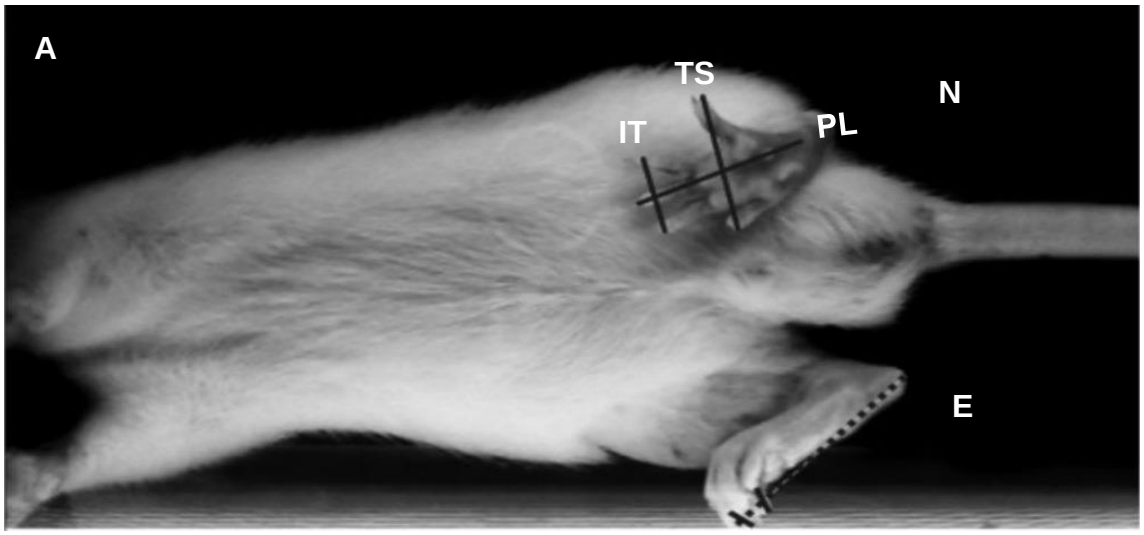


Figure 1: Functional evaluations schematization.



B

$$SFI = -38.3 \times \left(\frac{EPL - NPL}{NPL} \right) + 109.5 \times \left(\frac{ETS - NTS}{NTS} \right) + 13.3 \times \left(\frac{EIT - NIT}{NIT} \right) - 8.8$$

Figure 2: In A: Wistar rat footprint. Are indicated N: non-experimental member; E: experimental member; PL: Print Length (footprint length); TS: Total Toe Spread (total fingers spread); IT: Intermediate Toe Spread (middle fingers spread). In B formula used for calculate the Sciatic Functional Index. Are indicated: Sciatic Functional Index (SFI); N: normal, or not operated; E: experimental or operated; PL: Print Length (Footprint Length); TS: total toe spread; IT: Intermediate Toe Spread. [17]

Treatment Protocol

For CE, CLE, AE and ALE the treatment was made through resisted climbing exercise in stairs use. For this, a vertical wooden ladder with 67 iron steps, high of 1,18 m, width 20,5 cm and 60º inclination was used. At the top of the stairs was placed a box, with 20X20 cm in height and width, in which the animals rested between one series and another with a 60 s interval. The treatment starts 24 hours after the intra-articular injection. For this, was used a 100g overload coupled to the tail. The animals performed four series of five climbs on the stairs completing seven days of intercalated treatment.

In the CL, CLE, AL and ALE treatment the animals were submitted to LLLT (Laserpulse Ibramed®). The other groups' animals received the pen contact, but without beam emission, in the sensitized pelvic limb in the knee region. The application points were anterior to the patella, medial face in the tibiofemoral joint, and posterior in the popliteal region. The treatment parameters were point technique, in four points, with a 660 nm wavelength; 30 mW of power; spot area; 0,06 cm^2; energy density, 5 J/cm^2 per point; time per point, 10 s; and total energy per point, 0,003 J.

Ankle joint histological processing

After being collected, the right pelvic limb ankle joints were fixed in Metacarn, and packed in 70% alcohol after two days. For the descaling

procedure, they were rinsed in running water for 24 hours to be descaled using 5% trichloroacetic acid (TCA). After decalcification, the pieces were dehydrated in alcohol upward series (70%, 80%, 90%, 100%I,100%II and 100%III), diaphanized through n-butyl alcohol I, II and III and emblocked in histological paraffin. Subsequently, microtomy was performed by obtaining seven um sagittal sections on Olympus R CUT 4055® microtome. The slides were stained with hematoxylin and eosin for morphologic and histomorphometrical analysis in light microscopic (Olympus®).

In the general morphological analysis, the synovial membrane morphological characteristics were observed. For this, the anterior joint region was standardized as a point of analysis. The characteristics observed were: membrane general organization (intima and subintima layer) and its cells (synoviocytes), vascularization and inflammatory infiltrate presence inside the vessels and in the extracellular matrix. In the joint cartilage the following characteristics were observed: presence of flocculations in the articular surface, chondrocytes organization and disposition, fissures and degradation. In the joint cavity synovial infiltrate presence and pannus formation was analyzed.

For histomorphometric analysis, the interest regions were photomicrographed using Olympus® DP71 microscope. The protocol for measuring articular cartilage thickness and chondrocyte number was adapted from Ando et al. and Hagiwara et al. For this, three distinct regions were identified: P1, anterior end (near the phalanges); P2, joint middle region and P3, posterior articular end.

The tibia and talus middle or P2 regions were selected as analysis regions, since they are the site of highest joint weight discharge (Kunz et al., 2014). Photomicrographs were performed at 400X magnification for the following measures performing: total cartilage thickness, defined as the distance between the cartilaginous surface and the osteochondral junction; the surface zone thickness comprising the space between the cartilage surface and tidemark; and the deep zone thickness which is bounded superiorly by the tidemark and inferiorly by the subcondral bone. The chondrocytes number was counted using an 300um X 300 um area where only the chondrocytes that was fully within the borders limits were considered.

Statistical analysis

Statistical analyzes were performed using the BioStat program through the mixed generalized models test. For the functional analyzes, the LSD post-test was used and for the histomorphometric analyzes, the Sidak post-test was used. The significance adopted was $p<0,05$.

RESULTS

There was significant difference in the member functionality evaluation between the evaluations (F (3.96)=11.3; p<0.0001), between the groups (F(7.32)=9.1; p<0.0001), and there was interaction between groups and evaluations (F=(22)=2,3; p=0,003) (Table 1).

When comparing within groups, there was significant difference between groups with and without arthritis (p<0.00001), and in AV3 all the treated groups (AE, AL and ALE) completely restored the initial values (Table 1).

Between groups the animals of the control groups (CG, CL, CE and CLE) had significant differences (p<0,00001) when compared with all the arthritis groups. In AV3 all the arthritis groups (AG, AE, AL and ALE) returned to the initial values (Table 1).

The motor function evaluation showed differences between the evaluations (F(3.4)=36.1, p<0.00001), between the groups (F(7.52)=4.1, p=0.001) and there was interaction between groups and evaluations (F(23.9)=4.7, p<0.0001) (Table 2).

Within groups, there was significant difference between all arthritis groups (AG, AL, AE and ALE) and groups without arthritis (CG, CL, CE and CLE) (p<0,0001). In AV3 the ALE group returned to the initial values (Table 2).

Between groups the animals of the control groups (CG, CL, CE and CLE) had significant differences (p<0,0001) when compared with all arthritis groups. In AV3 all the arthritis groups (AG, AE, AL and ALE) restored the initial values (Table 2).

Table 1: SFI throughout evaluations within the same group and between groups

	EV0	*EV1*	*EV2*	*EV3*
CG	-0.6 ± 7.4^{a,A}	-9.2 ± 5.7^{a,A}	-0.2 ± 7.3$^{a,\ AC}$	-8.6 ± 7.1$^{a,\ A}$
AG	-7.4 ± 7.4^{a,A}	-23.8 ± 5.7^{a,A}	-45.6 ± 7.3^{b,B}	-34 ± 7.1^{a,b,A}
CL	-8.4 ± 7.4^{a,A}	-13.2 ± 5.7^{a,A}	-0.4 ± 7.3^{a,AC}	-9 ± 7.1$^{a,\ A}$
AL	-9.2 ± 7.4^{a,A}	-51.6 ± 5.7^{b,B}	-40.2 ± 7.3^{b,c,BC}	-27.8 ± 7.1^{a,c,A}
CE	-11.2 ± 7.4^{a,A}	-8.6 ± 5.7^{a,A}	-10.4 ± 7.3$^{a,\ AC}$	-19.4 ± 7.1^{aA}
AE	-15.6 ± 7.4^{a,A}	-49.2 ± 5.7^{b,B}	-42.6 ± 7.3^{b,BC}	-18.8 ± 7.1^{a,A}
CLE	-2.6 ± 7.4^{a,A}	-6 ± 5.7^{a,A}	-13.8±7.3^{a,ABC}	-8 ± 7.1^{a,A}
ALE	-13.8 ± 7.4^{a,A}	-44 ± 5.7^{b,B}	-39.2 ± 7.3^{a,b,BC}	-24.2 ± 7.1$^{a,\ A}$

Values expressed in mean ± standard error. Different letters represents different values. In the lines, the lowercase letters, comparison within the same group and in columns, with capital letters, comparison between groups throughout the evaluations.

Table 2: Maximum angle reached by animals during the evaluations within the same group and between groups.

	AV0	*AV1*	*AV2*	*AV3*
CG	58.8±0.9 $^{a, A}$	57.4±1.0 ^{a,A}	58.3±1.4 $^{a, AB}$	57.9±1.6 ^{a,A}
AG	58.5±0.8 ^{a,A}	50.2±1.0 ^{b,c,B}	52.5±1.3 $^{b,c, AC}$	54.4±1.5 ^{d,A}
CL	57.4±0.9 ^{a,A}	56.9±1.0 ^{a,A}	57.4±1.4 ^{a,AB}	57.4±1.6 ^{a,A}
AL	58.3±0.8 ^{a,A}	49.0±1.0 ^{b,B}	53.1±1.3 ^{c,AC}	55.0±1.5 ^{c,A}
CE	58.8±0.9 ^{a,A}	58.6±1.0 ^{a,A}	58.3±1.4 ^{a,AB}	56.9±1.6 ^{a,A}
AE	57.7±0.8 ^{a,A}	47.7±1.0 ^{b,B}	54.2±1.3 ^{c,AB}	55.0±1.5 ^{c,A}
CLE	59.3±0.9 ^{a,A}	56.9±1.0 ^{b,A}	59.5±1.4 $^{a\ c,AB}$	57.6±1.6 ^{a,b,c,A}
ALEA	57.3±0.8 ^{a,A}	48.3±1.0 ^{b,B}	52.7±1.3 ^{c,AC}	56.3±1.5 ^{a,A}

Values expressed in mean ± standard error. Different letters represents different values. In the lines, the lowercase letters, comparison within the same group and in columns, with capital letters, comparison between groups throughout the evaluations.

Histomorphometrical analysis

Articular cartilage morphometry

The histomorphometric analysis of the articular cartilage P2 region showed no significant difference between the groups both in the total cartilage thickness and in the superficial and deep zones. In the chondrocyte count, there was a significant difference between the groups (Wald (1; 6605.6) = 25.2, p=0.001). AE showed a higher chondrocytes number when compared to the other groups and CL when compared with CG (table 3).

Table 3: Tibia histomorphometrical analysis

TIBIA	*Total Thickness*	*Superficial Zone*	*Deep Zone*	*Chondrocytes*
CG	145±9.1 [a]	76.1±9 [a]	70±6,3 [a]	12,7±1,2[a]
AG	163±9.1 [a]	88±10.1 [a]	72,7±6,5 [a]	17,7±1,6[acd]
CL	166±10.8 [a]	79±10.7 [a]	84,4±9 [a]	17,4±1,9[acd]
AL	141±10 [a]	65.5±8.1 [a]	70±6,8 [a]	15,3±1,5[acd]
CE	143,4±9.1 [a]	79±9 [a]	64,1±5,8 [a]	15,3±1,4[acd]
AE	138,6±10.8 [a]	61.2±8.3 [a]	72,2±7,7 [a]	25,2±2,8[bcd]
CLE	137,3±9.1 [a]	65.7±7.6 [a]	69±6,2 [a]	16±1,5[acd]
ALE	145±8.5 [a]	83±9 [a]	59±5 [a]	15,5±1,3[acd]

Values expressed in mean ± standard error. Different letters represents different values.

In the P2 talus cartilage, the histomorphometric analysis of both cartilage total thickness and the superficial and deep zones, did not show any significant difference between groups. In the chondrocyte count, there was a significant difference between the groups (Wald (1; 5958.6)=19.8, p=0.006). AE showed a higher chondrocytes number when compared to AL, CLE and GC. ALE and CE showed the same characteristic, but ALE only when compared with GC and CE with CLE and GC (table 4).

Table 4: Talus histomorphometrical analysis

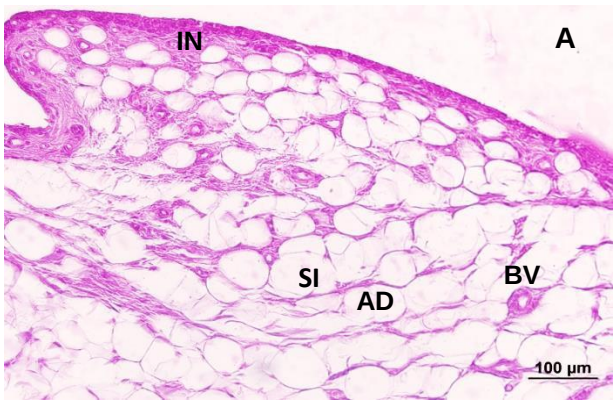
TÁLUS	*Total Thickness*	*Superficial Zone*	*Deep Zone*	*Chondrocytes*
CG	140.6±9.7 [a]	48.3±4.5 [a]	91.7±9 [a]	13.4±1.3[bc]
AG	151.2±113 [a]	60.5±6.1 [a]	92±9.8 [a]	19.7±2.1[acde]
CL	118±9.7 [a]	51.2±5.7 [a]	66.4±7.8 [a]	15.4±1.8[bde]
AL	139.3±11.4 [a]	47.7±4.8 [a]	92.5±10 [a]	15.5±1.6[bcde]
CE	133.4±9.2 [a]	54±5 [a]	78.3±7.7 [a]	20.3±2[ade]
AE	137.2±11.3 [a]	54±6 [a]	82.6±9.7 [a]	22.4±2.6[ade]
CLE	126.3±8.8 [a]	54.7±5.1 [a]	71.3±7 [a]	15±1.5[bacd]
ALE	131.4±8.5 [a]	45.1±3.9 [a]	87.5±8.1 [a]	17.9±1.6[acde]

Values expressed in mean ± standard error. Different letters represents different values.

Morphological analysis

Synovial membrane

The control group (CG) ankle joints, after seven days of systemic experimental arthritis, showed characteristic morphology, that is, the synovial membrane with normal aspects, containing two to three cell layers, type A and B synoviocytes in the synovial intima, and subintima with adipose cells predominance (Figure 3 A). The arthritis group (AG) presented subintima with angiogenesis, revealing a large amount of blood vessels, which were turged of red blood cells, configuring vascular congestion and also with inflammatory cells inside the vessels and, in some animals, inflammatory cells diapedesis has already occurred, that is, moving towards the articular tissue (Figure 3 B). The laser groups (CL and AL), exercise (CE and AE) and groups where the combination of the two treatments was performed (CLE and ALE) had the same angiogenesis and congestion of red blood cells characteristics, however, the inflammatory cells presence was not observed.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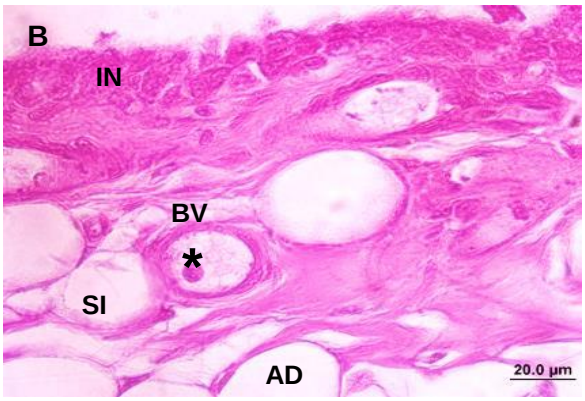
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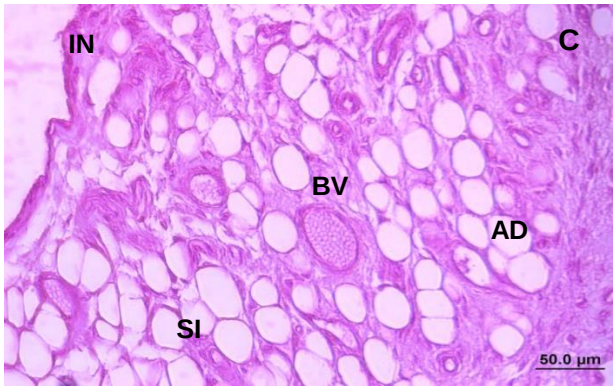
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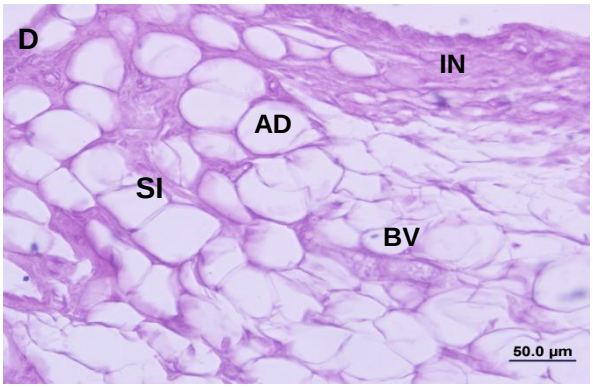
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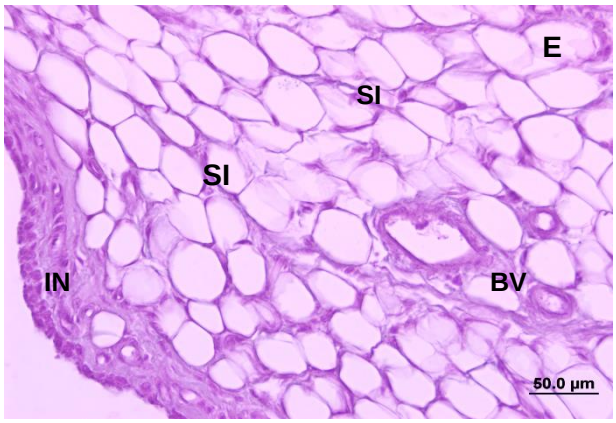
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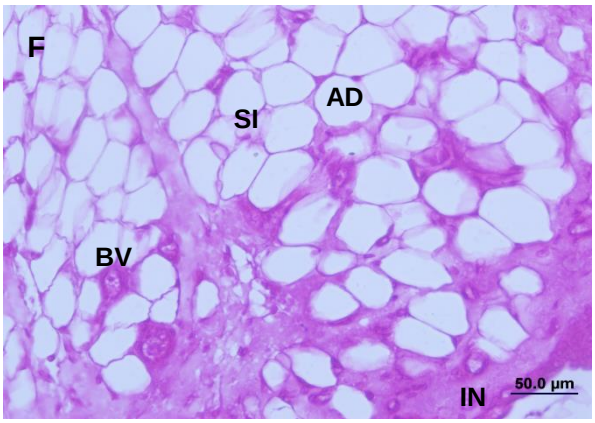
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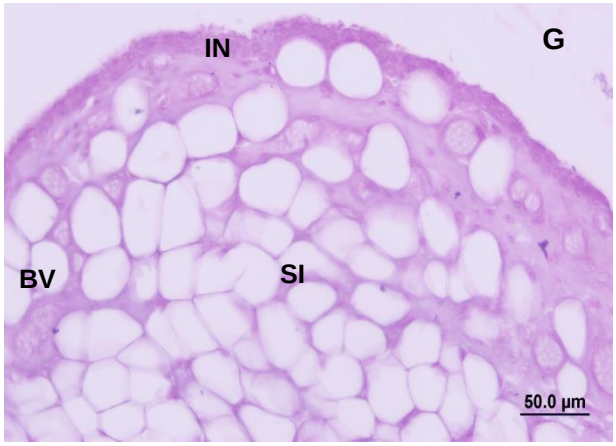
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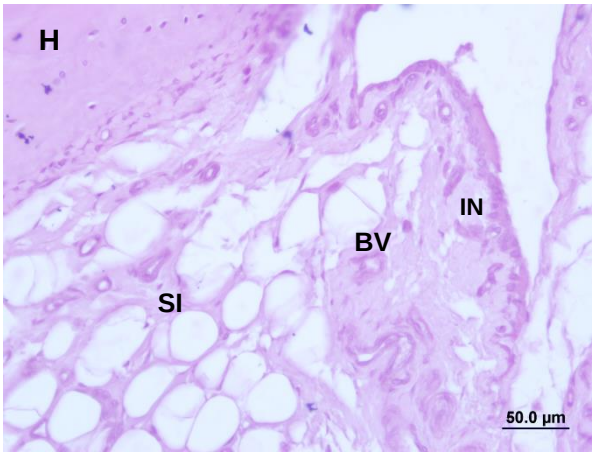
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Figure 3: Wistar rats talocrural joint synovial membrane photomicrographs, seven days after induction of systemic arthritis by CFA, sagittal section, hematoxylin and eosin staining. A: Control group; B: Arthritis group; C: Control Laser; D: Arthritis Laser; E: Control Exercise; F: Arthritis exercise; G: Control laser and exercise; F: Arthritis laser and exercise. Are indicated: Intimate layer (IN), Subintimate layer (SI), Blood vessels (BV) filled by red blood cells; adipocytes (AD) and inflammatory infiltrate (*).

Articular Cartilage

The CG group articular cartilage, of both tibia and talus bones showed a smooth surface and the chondrocytes were organized in the four characteristic cell layers. In the superficial zone, a higher cell density was observed, with the cells arranged in horizontal clusters, with a flattened aspect. In the intermediate zone, the cells assumed a rounded appearance and were arranged isolated or isogenous groups. Soon after, the chondrocytes were organized in gaps, corresponding to the deep zone, separated from the calcified zone, by a basophilic line, the tidemark, with the normal-looking subchondral bone (Figure 5A).

In AG group, in some animals, there was a predominance of changes in the articular cartilage, especially in talus, where in addition to flocculation on the articular surface and chondrocytes disorganization, it was possible to observe superficial layers degeneration with consequent subchondral bone exposure and pannus. In the tibia, flocculations were found on the joint surface and chondrocyte disorganization (Figure 5B). The CL, CE and CLE articular cartilage did not show any changes, being morphologically similar to the CG. In the AL group, chondrocyte disorganization and tidemark absence were observed in some animals (Figure 5C). The AE group had a higher amount of chondrocytes (Figure 5D), as already showed in the histomorphometric analysis (TABLES). In the cartilage, the CLE group was shown to be similar to CG, while in ALE group, was possible to observe chondrocytes disorganization, flocculation and tidemark absence.

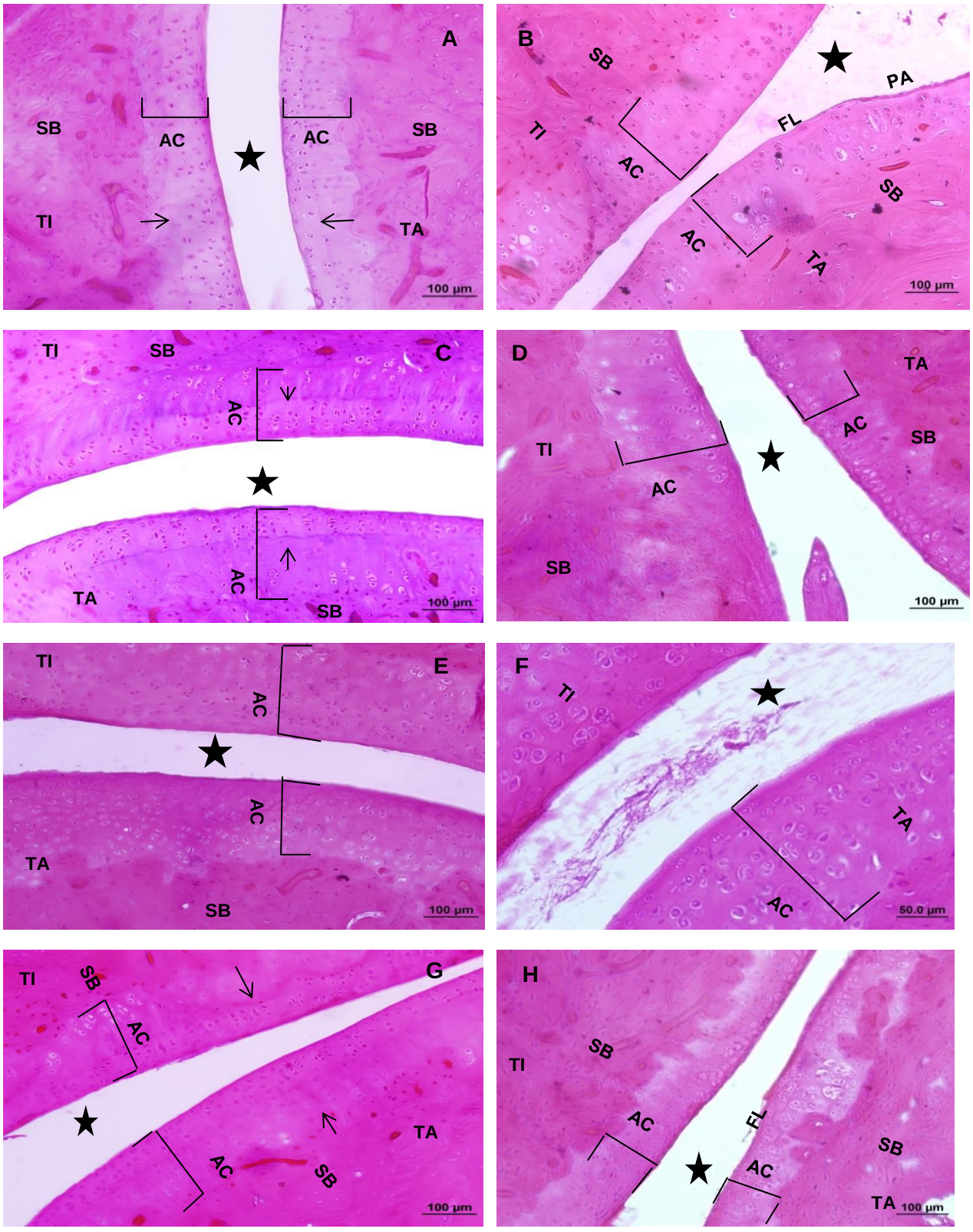

Figure 4: Wistar rats talocrural joint cartilage photomicrographs, seven days after arthritis induction in the knee by CFA, sagittal section, hematoxylin and eosin staining. A: Control group; B: Arthritis group; C: Control Laser; D: Arthritis Laser; E: Control Exercise; F: Arthritis Exercise; G: Control Laser and Exercise; F: Arthritis Laser and Exercise. Are indicated: Tibia (TI), Talus (TA) Articular cartilage (AC), Subchondral bone (SB), Tidemark (arrow), Articular cavity (star); Flocculation (FL) and *pannus* (PA).

DISCUSSION

RA is commonly associated with pain and functional disability [18]. In the present study, in the functional parameters tested, it was observed that the limb functionality was affected by RA. In the SFI, both separate and associated treatments showed return to the initial values. Already the motor function evaluation by the inclined plane showed that the association of the two treatments was effective in reversing the disablement caused by the disease.

The AR usually presents symmetrical, persistent and destructive polyarthritis affecting particularly the hand and feet small joints, like the ankle joint. The disease main clinical features include joint pain, stiffness and swelling, joint damage, fatigue, functional loss what leads to disability [19].

As synovitis comprises the main RA clinical feature, its therapy has been focused on inflammation modulation and consequently disease degenerative progression deceleration by anti-inflammatory drugs administration like: corticosteroids, drugs modifying the course of the disease synthetic immunosuppressive drugs, used alone or in combination [20]. However, this treatment displays several important side effects, which sometimes does not show clinical improvement and disease remission [21]. Therefore, other treatment strategy which presents an anti-inflammatory effect, but do not suppress the entire immune system has been investigated [22]. LLLT has shown a promising non pharmacological alternative to RA treatment due to its local healing stimulation, inflammatory process modulation and pain relief effects [23-26] and it may help with the functional aspects.

The LLLT photobiological effects consists in light photons absorption by chomophores related to mitochondrial respiratory chain on cytochrome c, which produces a photosignal, subsequently transduced into the cell. This photosignaling involves transient free radical production, increases adenosine triphosphate (ATP), and modulates cellular redox potential, which could induces redox-sensitive transcription factor. LLLT could also generate singlet oxygen stimulating processes, such as RNA and DNA synthesis. Therewith, photobiological effects of LLLT are observed, as increase of cell migration, cytokine levels, growth factors, inflammatory mediator modulation, and increase of tissue oxygenation [27,28].

Hurkmans et al. inferred in a systematic review that physical exercises and behavioral education in patients with RA are recommended for clinical use and LLLT therapy is among the proposed guidelines, but the recommendation parameters are not well clarified [29]. In this study, LLLT also showed improvements in the functional parameters. Brosseau et al. made a systematic review relating the LLLT efficacy in RA treatment and concluded that laser therapy has a beneficial effect when used ate least four weeks, with effects on pain reduction and morning stiffness [30].

A study made by Khozeimeh et al. showed that LLLT therapy provides the body a better response to inflammatory conditions, by reducing edema, reducing pain and provides cellular bio stimulation [31]. In addition to these

benefits, LLLT is relatively simple and low-cost therapy that has been shown to be ideal for supporting conventional treatments.

Hsieh et al., showed that inflammatory action and functionality recovery using LLLT therapy is related actin in modulation of inflammatory mediators, indicating beneficial effects using 4,5 J/cm2 with inflammation reduction [32] what corroborates with the results of the present study.

Physical inactivity is common in patients with RA and has been associated with muscle and bone loss. In this way, physical activity and exercises are also important components of no pharmacology therapy in RA. Metsios et al. showed that exercise has been shown to reduce inflammation [33].

A study made by Cooney et al. recommended that RA patients should be taught and supported in an effective exercise program of moderate exercise 3-5 days/week for 30-60 minutes/session, combined with moderate strengthening) [34]. Forestier et al. brings evidences that when the disease is very active or there is severe involvement of the joints of the lower limbs, activities with low impact on the joints or with load alleviation are recommended [35].

On this study, the synovial membrane morphological analysis the AG group presented subintima with angiogenesis, revealing a large amount of blood vessels, which were turged of red blood cells, configuring vascular congestion and also with inflammatory cells inside the vessels and, in some animals, inflammatory cells diapedesis has already occurred, that is, moving towards the articular tissue. As already stated above, synovitis comprises the main RA clinical feature and this is due to the fact that it is an inflammatory autoimmune disease characterized by chronic and symmetrical degeneration of synovial joints [3]. Tending to worsen over time as joint architecture is modified by synovits, that is, synovial membrane inflammation [18].

Although the disease development precise cause remains unknown, it is believed that joints deformities occur due to their autoimmune characteristic, that is, immune system cells that should attack bacteria and external elements, attack healthy body tissues, as if they were invasive cells, releasing substances that cause inflammation [36]. Choy, described that the triggering event seems to be innate immune response activation, which includes arthritis-associated antigen presentation, by exogenous material and autologous antigens, through major histocompatibility complex and costimulatory proteins by dendritic cells, macrophages and activated B cells to T cells, which promote its differentiation mainly into T helper [22]. McInness inferred that T and B cell activation mediate effector function RA through cytokines and chemokines release, leukocyte activation, macrophages, fibroblast and endothelial cells, as well as B cell produced autoantibodies [7].

Additionally, Elshabrawy et al. showed that polymorphonuclear cells, mobilized by chemokines and cytokines, infiltrate the synovial compartment and produce a wide range of pro-inflammatory cytokines, leading to increase in cell proliferation, vasodilatation, vascular permeability, and proteolytic enzyme

secretion, from synovium stromal cells and from chondrocytes [37]. Pap at al. described that the metalloproteinases promotes collagen type II degradation and alter glycosaminoglycan composition and water retention capacity of joint cartilage, which result in joint biochemical and mechanical dysfunctions [38].

The present study synovial membrane morphological analysis also showed that the laser groups (CL and AL), exercise (CE and AE) and groups where the combination of the two treatments was performed (CLE and ALE) had the same angiogenesis and congestion of red blood cells characteristics, however, the inflammatory cells presence were not observed.

Angiogenesis is important for normal physiological growth [39]. Abnormal vessels growth can lead to chronic diseases and angiogenesis plays an important role in the healing process after conditions such as inflammatory disorders [40]. Physical activity has shown as a cardiac and skeletal muscle adaptation important stimulus for adaptation and physiological remodeling process to increase capillary density [41] and mitochondrial enzyme activity [42].

Hudlicka et al. showed some possible stimuli for exercise-induced angiogenesis, including increase in blood flow, also described as hyperemia, movement stress, muscle stretch, hypoxia and metabolic disturbance [43].

The LLLT anti-inflammatory effect has been widely described in literature [30, 44-45] and has been associated to prostaglandin levels alteration and vascular permeability [46]. Dos Anjos et al. showed LLLT decreased inflammatory area, as well as the number of infiltrate cells [25].

Despite well-known LLLT anti-inflammatory effect is extensive clinical use in treatment on inflammatory conditions, the impact of this treatment on immune response, especially on immune cells behavior, is still poorly exploited [47].

On this study, the articular cartilage morphological analysis showed that in AG group, in some animals, there was a predominance of changes in the articular cartilage, especially in talus, where in addition to flocculation on the articular surface and chondrocytes disorganization, it was possible to observe superficial layers degeneration with consequent subchondral bone exposure and pannus. In the tibia, flocculation was found on the joint surface and chondrocyte disorganization.

RA is an inflammatory autoimmune disease characterized by synovial joints chronic degeneration. Rosa et al. says that joints space inflammation can lead to the cartilage destruction and ultimately the bone [48]. Due to its autoimmune characteristic immune system cells attack healthy body tissues, as if they were invasive cells, releasing substances that cause inflammation [36], which causes erosive synovitis that can lead to a high level of joint destruction [5]. Monocytes, neutrophils and macrophages are described as the most responsible for mediating tissue destruction [36] and, in addition, McInes and Schett, showed inflammatory cytokines presence, such as tumor necrosis factor

alpha (TNF α) [49] and Sofat et al. showed increased oxoglycosidases concentrations and degenerative agents mediated by NF-k beta [50].

The immune system cells form a fibrous and abnormal tissue, called pannus, which releases substances that accelerate bone erosion and cartilage destruction, losing their shape and alignment resulting in deformations and damage to adjacent ligaments [36]. The extracellular matrix cartilage components are enzymatically degraded by metalloproteinases, hyaluronidases and agrecanases, respectively [51]. In addition, as showed by Firestein, the synovial membrane thickening causes irreversible damage to the capsule and articular cartilage, since the structures are replaced by the pannus and, bellow this arrangement, the cartilage became corroded and destroyed and the joints become fixed due to the thick and hardened structure, which results in edema [52].

The present study morphological analysis also showed that CL, CE and CLE articular cartilage did not show any changes, being morphologically similar to the CG. But, in the AL group, chondrocyte disorganization and tidemark absence were observed in some animals and the AE group had a higher amount of chondrocytes, what was confirmed by the histomorphometric analysis. In the cartilage, the CLE group was shown to be similar to CG, while in ALE group, was possible to observe chondrocytes disorganization, flocculation and tidemark absence.

Gonçalves et al. described these reactions as a possible reflect of the articular cartilage damaged restoration, due to the chondrocytes proliferative capacity and subchondral bone repair, with increased nutritional support in the region [53].

In this study was possible to observe that the groups treated with exercise presented morphology closest to the control group. As already said above, the joint affected by RA become fixed and, as showed by Simas et al., Immobilization leads to articular cartilage degeneration, decreased thickness, decreased cartilage matrix synthesis, irregular cartilage surface and lower total cartilage mass and volume [54].

The histological changes that occur after immobilization of the synovial joint are progressive resulting in the matrix structural macromolecules loss, but, when remobilized, the chondrocytes starts to synthesize the macromolecules quickly enough again, and the cartilage can be successfully synthesized, making it reversible changes [55].

Moore et al. showed that because of cartilage's biphasic nature, purely biomechanical benefits of exercise on tissue health are possible as well [56]. Cartilage is highly-hydrated (70-80% water), thus mechanical loading drives pressurization of its interstitial fluid. This pressurization allows for the fluid-born support of compressive stresses, stiffening of the cartilage, and shielding of the extra cellular matrix from stresses, together, these outcomes, through the mechanisms of interstitial lubrication and nutrition [56] Nonetheless, under sustained loads, cartilage thins/strains due to pressure-induced fluid exudation,

driving a loss of interstitial fluid pressure and the defeat of numerous biomechanical functions, including lubricity [57].

In his study, Narmoneva et al. showed that articular cartilage requires some load and movement regime to maintain its physical nature and biochemical properties. Joint load reduction and immobilization in experimental in vivo models induce joint degeneration, including reduced hydration, altered structure and reduced proteoglycan synthesis and decrease in cartilage thickness [58].

Sandell and Aigner, brings on their study that chondrocyte proliferation, as happened in the present study exercise groups, may result from the articular instability possibly modifying the intensity and distribution of forces in the articular cartilage, leading to the proteoglycans and collagens breakdown and, consequently, to the loss of matrix structure and to an increase in water content and these injuries to the structure of the cartilage produced chondrocytes death and initiated a repair response, resulting in chondrocyte proliferation [59]. In this way, the tissue can be passing through a repair phase, characterized by the proliferation of the chondrocytes and the increase of chondrocyte metabolic activity [59]. Thus, the cartilage lesions in the treated groups were less severe and less frequent suggesting the predominance of articular cartilage in the repair phase.

CONCLUSION

The CFA model showed to be effective in reproduce the RA systemic characteristic, since even with the intra-articular CFA application occurring in the tibio femoral joint, classic RA manifestations occurred in the ankle joint synovial membrane and cartilage. Both treatments (LLLT and exercise) and the combination of treatments had positive effect on the functional evaluations, and the morphology and morphometry showed that the synovial membrane inflammatory aspects and the cartilage lesions in the treated groups were less severe, and the groups treated with exercise presented histology more similar to the control group. Thus, the higher chondrocyte number on this exercise groups, may indicate a more advanced repair phase.

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CONSIDERAÇÕES FINAIS

O modelo de indução da artrite por CFA foi eficaz em induzir a doença de forma sistêmica, uma vez que, mesmo com a injeção intra-articular do adjuvante ocorrendo na articulação tíbio-femoral, foi possível observar manifestações características da artrite na articulação talocrural em ambas as fases da doença. O tratamento com exercício resistido e LBP se mostrou eficaz quando usado separadamente e em associação nos aspectos funcionais testados. No período agudo os parâmetros histomorfométricos avaliados mostraram que os aspectos inflamatórios da membrana sinovial e as lesões da cartilagem articular dos grupos tratados se apresentaram de forma menos severa. Além disso, o maior número de condrócitos nos grupos exercício pode indicar uma fase de reparo tecidual mais avançada.

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ANEXO I

unioeste

Universidade Estadual do Oeste do Paraná

Pró-Reitoria de Pesquisa e Pós-Graduação

Comitê de Ética no Uso de Animais

Parecer de Protocolo

Autorização

O protocolo intitulado **"Efeitos da eletroterapia em modelos animais de patologia – Análises funcionais e morfológicas."**, sob a responsabilidade de **Gladson Ricardo Flor Bertolini** que envolve a produção, manutenção ou utilização de animais pertencentes ao filo *Chordata*, subfilo *Vertebrata*, para fins de pesquisa científica encontra-se **Aprovado** para execução, está de acordo com as Normas editadas pelo Conselho Nacional de Controle de Experimentação Animal (CONCEA) e foi aprovado pelo Comitê de Ética no Uso de Animais (CEUA) do UNIOESTE em reunião de 27/10/2017.

FINALIDADE	Pesquisa Científica
Vigência da autorização	01/12/2017 - 01/06/2019
Espécie/linhagem/raça	Rato heterogênico Wistar
N. de animais	160
Peso/Idade	250-350g/3 meses
Sexo	Masculino
Origem	Biotério Central da UNIESTE campus Cascavel.

Cascavel, 30/10/2017

Prof. Dra. Luciana Oliveira de Fariña
Coordenadora do CEUA
Portaria nº 3730/2016 - GRE

ANEXO II

Advances in Rheumatology – Preparing your manuscript

Research

Criteria

Research articles should report on original primary research.

Advances in Rheumatology strongly encourages that all datasets on which the conclusions of the paper rely should be available to readers. We encourage authors to ensure that their datasets are either deposited in publicly available repositories (where available and appropriate) or presented in the main manuscript or additional supporting files whenever possible. Please see Springer Nature's information on recommended repositories. Where a widely established research community expectation for data archiving in public repositories exists, submission to a community-endorsed, public repository is mandatory. A list of data where deposition is required, with the appropriate repositories, can be found on the Editorial Policies Page.

Double-blind peer review

Please note: Advances in Rheumatology operates double-blind peer review. The following information should not be included in the main manuscript file, but should instead be uploaded as part of the covering letter:

Title page

Competing interests

Authors’ contributions

Acknowledgements

Authors’ information

Preparing your manuscript

The information below details the section headings that you should include in your manuscript and what information should be within each section.

Please note that your manuscript must include a 'Declarations' section including all of the subheadings (please see below for more information).

Title page

The title page should:

present a title that includes, if appropriate, the study design e.g.:

"A versus B in the treatment of C: a randomized controlled trial", "X is a risk factor for Y: a case control study", "What is the impact of factor X on subject Y: A systematic review"

or for non-clinical or non-research studies a description of what the article reports

list the full names and institutional addresses for all authors

if a collaboration group should be listed as an author, please list the Group name as an author. If you would like the names of the individual members of the Group to be searchable through their individual PubMed records, please include this information in the “Acknowledgements” section in accordance with the instructions below

indicate the corresponding author

Abstract

The Abstract should not exceed 350 words. Please minimize the use of abbreviations and do not cite references in the abstract. Reports of randomized controlled trials should follow the CONSORT extension for abstracts. The abstract must include the following separate sections:

Background: the context and purpose of the study

Methods: how the study was performed and statistical tests used

Results: the main findings

Conclusions: brief summary and potential implications

Trial registration: If your article reports the results of a health care intervention on human participants, it must be registered in an appropriate registry and the registration number and date of registration should be in stated in this section. If it was not registered prospectively (before enrollment of the first participant), you should include the words 'retrospectively registered'. See our editorial policies for more information on trial registration

Keywords

Three to ten keywords representing the main content of the article.

Background

The Background section should explain the background to the study, its aims, a summary of the existing literature and why this study was necessary or its contribution to the field.

Methods

The methods section should include:

The aim, design and setting of the study

The characteristics of participants or description of materials

a clear description of all processes, interventions and comparisons. Generic drug names should generally be used. When proprietary brands are used in research, include the brand names in parentheses

the type of statistical analysis used, including a power calculation if appropriate

Results

This should include the findings of the study including, if appropriate, results of statistical analysis which must be included either in the text or as tables and figures.

Discussion

This section should discuss the implications of the findings in context of existing research and highlight limitations of the study.

Conclusions

This should state clearly the main conclusions and provide an explanation of the importance and relevance of the study reported.

List of abbreviations

If abbreviations are used in the text they should be defined in the text at first use, and a list of abbreviations should be provided.

ANEXO III

Cells Tissues Organs Guidelines

Research Article

Title

First Name(s) Surname1, First Name(s) Surname1, First Name(s) Surname2*, First Name(s) Surname3, First Name(s) Surname1

1 Department, Institute/University/Hospital, City, (State,) Country

2 Department, Institute/University/Hospital, City, (State,) Country

3Department, Institute/University/Hospital, City, (State,) Country

Short Title: to be used as running head

*Corresponding Author

Full name

Department

Institute/University/Hospital

Street Name & Number

City, State, Postal code, Country

Tel:

Fax:

E-mail:

Keywords: Please provide 3–5 keywords highlighting the most important points of your paper.

Abstract

A short Abstract should summarize the main points and reflect the content of the article. It should be written in a clear and concise way and be unstructured, set in 1 paragraph. Abbreviations used in the main text may be introduced and used. Use neither bibliographic references nor references to figures or tables in the Abstract.

Refer to the Author Guidelines for more information about the maximum accepted length (word count) of an Abstract in your chosen journal.

Introduction

The Introduction should provide a summary of the background to the relevant field of research and the specific problems addressed and should state the hypotheses being explored as well as the main goal(s) of the study. Conclusions or findings should not appear in the Introduction.

Materials and Methods

The Materials and Methods section should clearly list all inclusion and exclusion criteria, methods of research, and variables evaluated and should state how

outcomes were assessed. All terms should be adequately defined and statistical information should be sufficiently detailed so that a study can be repeated.

Results

The Results section should describe the most important findings of the study, analysis, or experiment. The most important results should be indicated, and relevant trends and patterns should be described.

Discussion/Conclusion

The Discussion/Conclusion should provide an evaluation of the results. There should be a clear discussion of the implications, significance, and novelty of the results presented and whether the data support or contradict previous studies.

Statements

All papers must contain the following statements after the main body of the text and before the reference list:

Acknowledgement

In the Acknowledgement section, authors must include individuals and organizations that have made substantive contributions to the research or the manuscript. An exception is where funding was provided, which should be included in Funding Sources. Please refer to the Guidelines issued by the ICMJE to determine non-author contributors that should be included in the Acknowledgement section.

Statement of Ethics

Published research must comply with the guidelines for human studies and should include evidence that the research was conducted ethically in accordance with the World Medical Association Declaration of Helsinki. In the manuscript, authors should state that subjects (or their parents or guardians) have given their written informed consent and that the study protocol was approved by the institute’s committee on human research. If ethical approval was not required or obtained, please state why.

Disclosure Statement

Authors are required to disclose any possible conflicts of interest. All forms of support and financial involvement (e.g. employment, consultancies, honoraria, stock ownership and options, expert testimony, grants or patents received or pending, royalties) which took place in the previous three years should be listed, regardless of their potential relevance to the paper. Also the nonfinancial relationships (personal, political, or professional) that may potentially influence the writing of the manuscript should be declared. If there is no conflict of interest, please state: “The authors have no conflicts of interest to declare.”

Funding Sources

Authors must give full details about the funding of any research relevant to their study, including sponsor names and explanations of the roles of these sources in the preparation of data or the manuscript.

Author Contributions

In the Author Contributions section, a short statement detailing the contributions of each person named as an author should be included. Contributors to the paper who do not fulfil the ICMJE Criteria for Authorship should be credited in the Acknowledgement section.