UNIVERSIDADE ESTADUAL PAULISTA
"JÚLIO DE MESQUITA FILHO"
INSTITUTO DE BIOCIÊNCIAS – RIO CLARO

PROGRAMA DE PÓS-GRADUAÇÃO EM CIÊNCIAS BIOLÓGICAS
(BIOLOGIA CELULAR E MOLECULAR)

DIAGNÓSTICO DA ARTICULAÇÃO ARTRÍTICA DE RATOS WISTAR SUBMETIDOS A TREINOS DE NATAÇÃO

Juan Parente Santos

Tese apresentada ao Instituto de Biociências do Câmpus de Rio Claro, Universidade Estadual Paulista, como parte dos requisitos para obtenção do título de Doutor em Ciências Biológicas (Biologia Celular e Molecular).

NOVEMBRO/2017

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Doutorando: Juan Parente Santos
Orientadora: Profa. Dra. Maria Izabel Camargo Mathias
Co-orientador: Dr. André Arnosti e Dra. Maria José Misael Morsoleto

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UNIVERSIDADE ESTADUAL PAULISTA

Câmpus de Rio Claro

CERTIFICADO DE APROVAÇÃO

TÍTULO DA TESE: DIAGNÓSTICO DA ARTICULAÇÃO ARTRÍTICA DE RATOS WISTAR SUBMETIDOS À TREINOS DE NATAÇÃO

AUTOR: JUAN PARENTE SANTOS
ORIENTADORA: MARIA IZABEL SOUZA CAMARGO
COORIENTADORA: MARIA JOSÉ MISAEL DA SILVA MORSOLETO
COORIENTADOR: ANDRÉ ARNOSTI

Aprovado como parte das exigências para obtenção do Título de Doutor em CIÊNCIAS BIOLÓGICAS (BIOLOGIA CELULAR E MOLECULAR), pela Comissão Examinadora:

Profa. Dra. MARIA IZABEL SOUZA CAMARGO
Departamento de Biologia / IB Rio Claro

Profa. Dra. FABIANA ALONSO ROCHA
x / x

Profa. Dra. RUSLEYD MARIA MAGALHÃES DE ABREU
Centro de Ciências Biológicas e da Natureza / Universidade Federal do Acre

Prof. Dr. FRANCISCO SALVIANO SALES NOBRE
x / Instituto Federal de Educação, Ciência e Tecnologia do Ceará

Prof. Dr. CARLOS ALBERTO ANARUMA
Departamento de Educação Física / IB Rio Claro

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Instituto de Biociências - Câmpus de Rio Claro -
Avenida 24-A no. 1515, 13506900, Rio Claro - São Paulo
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SUMÁRIO

RESUMO

RESUMO:

A Artrite Reumatóide (AR) é uma doença autoimune de etiologia desconhecida, caracterizada pela poliartrite periférica e simétrica, que causa deformidade e destruição das articulações (grandes e pequenas) por erosão da cartilagem e do osso. O presente estudo teve como objetivo principal avaliar, por meio de radiografias, medidas de espessura articular, carga critica, técnicas histológicas, e de microscopia eletrônica de varredura, os efeitos da natação sobre a articulação artrítica, tendo como modelo ratos Wistar machos submetidos a 2 protocolos de natação distintos (com e sem sobrecarga). Foram utilizados 60 indivíduos com 150 dias, e peso médio de 450 g, divididos em grupos: Controle (**GC**) (10 indivíduos saudáveis); (**GT1**) (10 indivíduos com AR induzida sedentários); (**GT2**) (10 indivíduos sem AR induzida e submetidos ao treinamento de natação com sobrecarga); (**GT3**) (10 indivíduos com AR induzida e submetidos ao treinamento de natação com sobrecarga); (**GT4**) (10 indivíduos sem AR induzida e submetidos ao treinamento de natação sem sobrecarga); (**GT5**) (10 indivíduos com AR induzida e submetidos ao treinamento de natação sem sobrecarga). Os resultados mostraram que os animais do grupo (**GT5**) obtiveram rendimentos favoráveis no quesito de estabilizar ou retardar os sintomas da artrite reumatoide, assim como os animais dos grupos (**GT2**) e (**GT4**) mantiveram suas articulações preservadas. Os indivíduos do grupo (**GT1**) confirmaram a instalação de processos inflamatórios na articulação que foram potencializados pelo comportamento de sedentarismo e no grupo (**GT3**) a alta intensidade do exercício físico provocou a evolução do quadro inflamatório da articulação, com consequente espessamento da membrana sinovial e redução do cavidade articular, comprovando que o excesso ou a falta de acompanhamento na prática do exercício físico em portadores de AR pode ser tão prejudicial quanto a não prática da mesma.

Palavras-chave: natação, articulação, ratos, artrite, inflamação, morfologia, Zymosan

ABSTRACT

ABSTRACT:

Rheumatoid arthritis (RA) is an autoimmune disease of unknown etiology, characterized by peripheral and symmetrical polyarthritis, which causes deformity and destruction of the joints (large and small) by erosion of the cartilage and bone. The objective of the present study was to evaluate the effects of swimming on the arthritic joint by means of radiographs, measures of joint thickness, critical load, histological techniques, and scanning electron microscopy, using as model male Wistar rats submitted to 2 swimming protocols (with and without overload). Sixty individuals with 150 days and mean weight of 450 g were divided into groups: Control (**GC**) (10 healthy subjects); (**GT1**) (10 individuals with sedentary induced RA); (**GT2**) (10 subjects without RA induced and submitted to swimming training with overload); (**GT3**) (10 individuals with RA induced and submitted to swimming training with overload); (**GT4**) (10 subjects without RA induced and submitted to swimming training without overload); (**GT5**) (10 subjects with RA induced and submitted to swimming training without overload). The results showed that the animals in the (**GT5**) group had favorable yields in terms of stabilizing or delaying the symptoms of rheumatoid arthritis, just as the animals in the (**GT2**) and (**GT4**) groups maintained their joints preserved. The individuals of the (**GT1**) group confirmed the installation of inflammatory processes in the joint that were potentiated by the behavior of sedentarism and in the (**GT3**) group the high intensity of the physical exercise caused the evolution of the inflammatory picture of the joint, with consequent thickening of the synovial membrane and reduction of the inter -articular, proving that the excess or lack of follow-up in the practice of physical exercise in patients with RA can be as harmful as the non-practice of it.

Keywords: swimming, joint, rats, arthritis, inflammation, morphology, Zymosan

INTRODUÇÃO

1.Introdução

Distúrbios osteoarticulares foram registrados por Eder, em 1500 a.C. No entanto, no decorrer da história, têm sido encontrados relatos de dores articulares que sugerem a presença de várias formas de lesões. Nesse sentido, Hipócrates, por volta do ano de 400 a.C já havia descrito com exatidão as alterações osteo cartilaginosas hoje conhecidas como Artrite. Atualmente são reconhecidas mais de 100 doenças reumáticas, porém, acredita-se que a artrite é uma das mais relevantes no contexto mundial, devido sua alta prevalência (OSLER, 2012; JONES, 2016, REHMAN et al., 2017).

De acordo com Lorenz et al. (2005) a artrite é caracterizada por ser uma degeneração progressiva da cartilagem articular. Já a artrite reumatóide (AR) é uma doença autoimune de etiologia desconhecida, caracterizada por poliartrite periférica e simétrica, que leva à deformidade e à destruição das articulações por meio da ocorrência de erosão cartilaginosa e óssea. Afeta duas vezes mais as mulheres do que os homens e atualmente sabe-se que há uma tendência de incidência maior na medida em que o indivíduo vai envelhecendo (PEETERS et al., 2015; THYSEN et al.,2015). Com a idade ocorre a progressão da doença, e com isso os seus portadores ficam limitados ou impossibilitados de realizar suas atividades cotidianas, o que provoca grande impacto econômico tanto para o portador quanto para a sociedade (LAURINDO et al., 2002).

O primeiro sinal da presença desta doença é a inflamação da membrana sinovial, estrutura que reveste a parede interna da cápsula fibrosa que envolve as articulações sinoviais, responsáveis pelos movimentos, ou seja, joelhos e dedos das

mãos (ERNSTGÅRD, 2017). É na membrana sinovial que é produzido o líquido sinovial, que nutre a cartilagem e lubrifica a sua superfície, permitindo assim a perfeita diminuição do atrito na superfície articular. Quando a membrana sinovial é lesada esta sofre um processo inflamatório que a torna mais espessa, além de provocar um aumento de seu volume. Uma outra consequência dessa inflamação é a diminuição ou interrupção da produção do líquido sinovial. Nessa última situação começa então a ser produzido um líquido inflamatório que destrói progressivamente as cartilagens articulares, prejudicando a sua função, limitando os movimentos e causando fortes dores (DEWIRE; EINHORN, 2001; MOGIL, 2009). A persistência desses estímulos e/ou a incapacidade do sistema imunológico em controlar a inflamação levam à cronicidade da doença (BASBAUM, et al., 2009; PEDERSEN, 2000, 2007).

Um dos maiores problemas encontrados pelos pesquisadores com relação a essa doença quando se analisa os motivos de sua existência e da sua prevalência é a dificuldade de identificá-la nos indivíduos quando essa ainda se encontra no início do processo de instalação, porém, é de consenso que estresses biomecânicos que são capazes de atingir a cartilagem articular e o osso subcondral, além de alterações bioquímicas na cartilagem e na membrana sinovial, são fatores que contribuem para a patogenicidade (BLANCO, 2014). Vale ainda ressaltar a manifestação de fatores genéticos, os quais também devem ser considerados nesse tipo de avaliação (AMERICAN COLLEGE OF RHEUMATOLOGY SUBCOMMITTEE ON OSTEOARTHRITIS GUIDELINES, 2002; JORDAN et al., 2003; DIAZ-PENÃ, 2016, ERNSTGÅRD, 2017).

Nas últimas décadas estudos para melhor se entender os efeitos dos exercícios físicos sobre pacientes portadores de AR tem sido frequentemente

realizados. A literatura tem mostrado que a prática de exercícios físicos proporciona benefícios capazes de controlar a velocidade das alterações morfológicas decorrentes da AR, como regularidade da produção de liquido sinovial, nutrindo a cartilagem, o que proporciona ganhos à saúde e reflete diretamente na maior longevidade do indivíduo (UTHMAN et al., 2013; WITHALL et al., 2015). Cabe salientar que, além das alterações morfológicas, muitas são aquelas fisiológicas detectadas durante o exercício físico. De forma geral, a intensidade dos exercícios pode ser avaliada por critérios como: percepção do esforço dos indivíduos, medida da frequência cardíaca ou ainda estimativa do gasto energético, sendo que a frequência cardíaca máxima, o gasto energético e o VO_2 máximo podem ser estimados via sistema de monitoramento cardíaco (SANTOS et al., 2013).

Existe também na literatura relatos que mostram a ocorrência de variações fisiológicas como: frequência cardíaca; consumo de oxigênio; percepção de esforço, quando comparados indivíduos que praticam exercícios físicos no solo com aqueles que os praticam na água (SANTOS, 2005). Nos exercícios aquáticos, não só o fluxo de sangue e a termo regulação são afetados, mas também o metabolismo geral, por exigir maior demanda de gasto calórico para manter a ativação do sistema orgânico, do sistema nervoso, da composição sanguínea e da psique, fatores que sofrem alterações devido ao processo de imersão (SANTOS, 2005).

No caso específico da prática de exercícios na água, a resistência natural desta multiplica o esforço exigido em um movimento corporal, por mais simples que ele seja. Por outro lado, segundo as leis da física, a água responde na mesma intensidade a uma força aplicada sobre ela, ou seja, a resistência oferecida pela água é proporcional à força do movimento, permitindo assim que qualquer indivíduo

possa (e deva) se exercitar, independentemente do seu nível de condicionamento físico, ou seja: jovens, crianças, idosos, obesos, magros, gestantes, entre outros (SANTOS, 2005). Atrelado a isso, uma das principais vantagens da atividade física aquática, seja ela estática ou em deslocamento, é a redução da frequência cardíaca de repouso, como efeito crônico (KANITZ et al., 2010). Durante a imersão, há o incremento do peso hidrostático que pode ser definido como a diferença da massa corporal e o empuxo que devido à ação de forças que agem simultaneamente retardam o movimento e consequentemente exige maior gasto calórico, proporcional a área projetada, o que leva a ocorrência de menor propensão a impactos durante a prática. (SANTOS, 2005).

Nos estudos que abordam a temática exercício físico praticado por indivíduos portadores de AR têm sido observados incontáveis benefícios para os mesmos (AMERICAN COLLEGE OF RHEUMATOLOGY SUBCOMMITTEE ON OSTEOARTHRITIS GUIDELINES, 2002; BUCKWALTER et al., 2004; COIMBRA et al., 2002; F. VERHOEVEN et al., 2016) os quais incluem: melhora do fluxo sanguíneo, maior síntese de endorfina, melhor lubrificação articular, incremento no desempenho da mecânica da contração muscular, que juntos melhoram a capacidade funcional do indivíduo. Exercícios praticados especificamente em ambiente aquático promovem maior redução das forças que comprimem as articulações inferiores quando comparados àqueles praticados no solo (BECKER, 1998; BEMENT, 2005; FOSS, 2000; DUARTE, 2013). Como consequência, o impacto sobre o sistema musculoesquelético torna-se menor na água, causando também danos menores e elegendo esse tipo de exercício físico como aquele ideal para ser praticado por pacientes obesos, idosos e portadores de AR.

Sendo assim, a prática da indução da AR intra-articular pelo Zymosan vem se mostrando ser uma importante estratégia em diversos estudos em laboratório, pois favorece a instalação do processo inflamatório que remete a AR; nessa condição pode-se estudar como o exercício físico nas suas formas sistemática e terapêutica age sobre os locais inflamados (no caso articulações) permitindo auxiliar na melhora da qualidade de vida dos indivíduos.

OBJETIVOS

2. OBJETIVOS

2.1. Objetivo Geral

O presente trabalho teve como objetivo demonstrar comparativamente quais são as alterações morfológicas que ocorrem na organização da cartilagem articular do joelho de ratos Wistar (*Rattus norvegicus albinus*) com 150 dias de idade e submetidos à indução de AR por meio de injeção intra-articular de Zymosan (*Saccharomyces cerevisae*), nas situações de sedentarismo e da prática de exercícios físicos (natação) com e sem cargas adicionais.

2.2. Objetivos Específicos

- Analisar comparativamente por meio de imagens radiográficas (raios-X) os perfis ântero-posterior e látero-lateral da articulação do joelho direito dos indivíduos sadios e com AR induzida;

- Analisar no nível da ultramorfologia (MEV) a ocorrência de alterações: na cápsula articular, membrana sinovial, côndilos do fêmur e da tíbia, osso subcondral, cartilagem articular e zonas cartilagíneas.

- Analisar histologicamente (HE) a ocorrência de alterações estruturais no tecido dos componentes da articulação sinovial do joelho.

MATERIAL E MÉTODOS

3. MATERIAL E MÉTODOS

Para a realização deste trabalho foram utilizados 60 ratos machos Wistar, com peso médio de 450 gramas (balança padrão digital - Marte Balanças e Aparelhos de Precisão LTDA, modelo LC 1 N° 27885, carga máxima de 1000g e mínima de 5g; menor divisão de 0,2g e pré-aquecimento de 20 min, selo do INMETRO/DIMEL n° 152/94) e com 90 dias de idade os quais foram obtidos do Biotério Central da UNESP de Botucatu, SP, Brasil.

Todos os procedimentos foram devidamente aprovados pelo Comitê de Ética em Uso de Animais do Instituto de Biociências da Universidade Estadual Paulista de Rio Claro, SP, sob número: 5957 na decisão N° 05/2015 em sua 23° reunião ordinária.

Os indivíduos do estudo foram alocados e mantidos em gaiolas coletivas de polipropileno (15 gaiolas com 4 (quatro) animais/gaiola), (medindo 40x34x16cm) em sala do Biotério do Laboratório de Biodinâmica do Departamento de Educação Física do IB da UNESP de Rio Claro, SP, com temperatura (22°C±2°C) e ciclo de luminosidade claro/escuro (12/12 horas) controlados, sendo que ficaram sob iluminação no período das 6:00 as 18:00 horas, além de terem recebido água *ad libitum.* Todos os animais foram alimentados com ração padrão para ratos **Presence (**INVIVO NUTRIÇÃO E SAÚDE ANIMAL LTDA*.)* e a troca da maravalha das caixas coletivas foi realizada diariamente.

Para melhor visualização e identificação dos animais, estes foram marcados no dorso com números (01 a 60) com tinta preta, atóxica e resistente a água.

3.1. Indução da artrite reumatóide (AR)

Para indução da artrite reumatoide (AR) nos ratos, foi utilizado o Zymosan (*Saccharomyces cerevisae*), referencia Z4250 – 250 mg Envase/lote : BCBL6140V, adquirido da SIGMA – ALDRICH Brasil LTDA, o qual foi foi injetado com seringa para insulina BD Ultra-Fine™,Cód: 328325 Fabricante: Becton Dickinson – agulha fixa (integrada) com 8mm (5/16'') de comprimento por 0,30mm (30G) de diâmetro, sem espaço residual e com capacidade para até 50 unidades de insulina.

3.2. Grupos de estudo

Os animais aqui estudados foram primeiramente alocados aleatoriamente em seis grupos (10 indivíduos/grupo) que consistiram de:

GC=CONTROLE (10 ratos sedentários e sem indução de AR)

GT1= TRATAMENTO 1(10 ratos sedentários e com AR induzida)

GT2=TRATAMENTO 2 (10 ratos sem AR e treinados com sobrecarga por 30 dias).

GT3=TRATAMENTO 3 (10 ratos com AR induzida e treinados com sobrecarga por 30 dias).

GT4=TRATAMENTO 4 (10 ratos sem AR e treinados sem sobrecarga por 07 dias).

GT5=TRATAMENTO 5 (10 ratos com AR induzida e treinados sem sobrecarga por 07 dias).

Os 60 ratos foram distribuídos em 15 gaiolas galvanizadas modelo GK115PP - Marca Beiramar (4/gaiola) na cor branca para otimizar a limpeza e com bebedouro e comedouro acoplados na parte superior das caixas, tipo kaefiq, produzidas em polipropileno, autoclaváveis, resistentes a ácidos (as quais foram forradas com maravalha) e 4 extratores em forma de L, com borda reforçada contendo orifícios para drenagem da água quando lavada, reforço em "X" no fundo da caixa, oferecendo maior resistência e durabilidade.

Durante a realização dos experimentos os ratos dos grupos:

GC e GT1: foram mantidos nas gaiolas durante todo o experimento.

GT2: não tiveram indução de AR e foram submetidos a exercícios de natação com sobrecarga adicional proporcional a sua massa corporal durante 30 dias.

GT3: tiveram indução de AR e foram submetidos a exercícios de natação com sobrecarga adicional proporcional a sua massa corporal durante 30 dias.

GT4: não tiveram indução de AR e foram submetidos ao treino sem sobrecarga por período de 07 dias.

GT5: tiveram indução de AR e foram submetidos a treino sem sobrecarga pelo período de 07 dias.

3.3. Protocolo do exercício físico (natação)

Neste trabalho foram utilizados dois protocolos de treino de natação com o intuito de se avaliar as modificações pós-prática de exercício físico nos indivíduos a eles submetidos. Um dos treinos ocorreu sem adição de sobrecarga, metodologia adaptada de Kuphal et al. (2007) e o outro com sobrecarga, metodologia adaptada de Gobatto et. al. (2001).

Antes do início dos treinos de natação, os animais passaram por adaptação ao meio líquido durante o período de 5 dias consecutivos e ininterruptos, que consistiu de:

a) Dia **1**: ratos de todos os grupos, exceto do (**GC**) e do (**GT1**) foram introduzidos na água rasa (medindo 25cm) profundidade por 5 minutos,

b) Dia **2**: os mesmos ratos foram novamente colocados em água rasa, porém, agora durante 10 minutos,

c) Dia **3**: os ratos foram colocados durante 5 minutos em água profunda (50 cm de profundidade), e foram separados um do outro por tubos cilíndricos de PVC, medindo 60 cm de comprimento por 10 cm de diâmetro.

d) Dia **4**: os ratos foram colocados em água profunda por 10 minutos também individualizados com tubos.

e) Dia **5**: os ratos foram colocados em água profunda por 5 minutos, individualizados pelos tubos e ainda com sobrecarga de 3% da massa corporal (MC) em media 13 gramas, atados ao dorso de cada animal.

Os treinos foram realizados nas dependências do Laboratório de Biodinâmica do Departamento de Educação Física do IB da UNESP de Rio Claro (SP), fazendo uso de caixa de polipropileno (115x75x60cm), com superfície lisa, o que impossibilitou o animal de atingir seu fundo, permanecendo nadando ou caminhando, submerso ou saltando. Esta recebeu aproximadamente 350 litros de água morna (31ºC± 2 ºC) devidamente monitorada com termômetro digital com medidor externo da marca G – Tech com precisão de 0,2 ºC aprovado pela Agencia Nacional de Vigilância Sanitária (Anvisa) e pelo Instituto Nacional de Metrologia Qualidade e Tecnologia (INMETRO).

Nesse ambiente foram introduzidos 5 tubos de PVC em formato cilíndrico (medindo 60cm altura x 10cm diâmetro), onde os animais foram individualizados. Depois de retirados, foram alocados em outra caixa para desacelerar o metabolismo ("volta à calma"), este procedimento foi realizado durante 7 minutos. Posteriormente os animais foram retirados do tanque e individual e manualmente secos com toalhas (100% algodão). Na sequência, foram devolvidos às caixas onde permaneceram até a próxima seção do experimento.

3.4. Protocolo de treinamento sem utilização de sobrecarga (Adaptado de Kuphal; Fibuch e Taylor, 2007)

Esse protocolo de treinamento geralmente é aplicado num período de 7 dias consecutivos, em água profunda (50 cm). No presente trabalho foram submetidos a este teste apenas os ratos dos grupos (**GT4**) e (**GT5**).

Dia **1**: os indivíduos foram submetidos a duas sessões de natação com duração de 30 segundos cada e com repouso de 120 segundos.

Dia **2**: foram realizadas duas sessões com duração de 2 minutos e repouso de 120 segundos.

Dia **3**: foram realizadas 3 sessões com duração de 10 minutos e repouso de 5 minutos.

Dia **4**: foram realizadas duas sessões de 15 minutos e repouso de 5 minutos.

Nos três dias restantes, as sessões tiveram duração de 30 minutos contínuos (sem repouso).

Para melhor visualização dos bioensaios os mesmos estão resumidos na **Tabela 1**.

Tabela 1: Treino de natação sem sobrecarga

Dia	Treino (minutos)	Repouso (minutos)	Número de sessões	Tempo total de atividade (minutos)
1º	0,5	2	2	1
2º	2	2	2	4
3º	10	5	3	30
4º	15	5	2	30
5º	30	-	1	30
6º	30	-	1	30
7º	30	-	1	30

("-" = não houve tempo de repouso)

Decorridas 24 horas após o último dia de treino, os animais foram radiografados e posteriormente foram sedados com gás CO_2 para que fossem realizadas as medidas da espessura articular, procedimentos estes que estiveram sob a responsabilidade da medica-veterinária Letícia Maria Graballos Ferraz Hebling (CRMV- SP 5.412), Polivet Clinica Veterinária, localizada em Rio Claro, SP a qual atua também como colaboradora do grupo de pesquisa **BCSTM,** UNESP de Rio

Claro, SP. A espessura articular foi tomada com paquímetro analógico da marca Mitutoyo modelo nº. 2046F com precisão de 0,01mm.

Após os procedimentos anteriormente descritos todos os ratos deste grupo foram eutanasiados também pela médica veterinária Leticia Maria Graballos Ferraz Hebling (CRMV- SP 5.412), em sala de sacrifício do biotério do Departamento de Educação Física do IB da UNESP de Rio Claro (SP) fazendo uso de 0,31 mL de Ketamina - ($C_{13}H_{16}NClO$) associada com 0,45 mL de Xilasina – ($C_{12}H_{16}N_2S$)/rato.

Na sequência da eutanásia procedeu-se a dissecção dos ratos e isolamento da perna posterior direita para a retirada das articulações dos joelhos que, foram imediatamente fixadas em formol 10%, para depois serem encaminhadas para descalcificação em ácido nítrico 5% onde permaneceram por 22 dias. Logo após esta etapa o material foi submetido aos procedimentos de desidratação em série crescente de álcoois (50% a 100%) para posteriormente serem incluídos em moldes plásticos contendo historesina Leica. Foram realizadas secções histológicas longitudinais de 3 micrômetros de espessura em micrótomo RM2255 Leica® alocado no Laboratório de Histologia do Departamento de Biologia da UNESP de Rio Claro, SP.

O descarte dos animais deu-se conforme normas estabelecidas pelo Comitê de Ética em Uso Animal (CEUA), bem como pelo Conselho Nacional de Controle de Experimentação Animal (CONCEA).

.

3.5. Protocolo de treinamento com sobrecarga (Adaptado de Gobatto, 2001)

Esse protocolo está fundamentado na Carga Crítica (Ccrit) dos animais, sendo o termo Ccrit definido como a mais elevada intensidade do exercício que pode

ser mantida durante um período considerável em condições de relativo equilíbrio metabólico, expressas em valores estáveis de consumo de oxigênio e de lactatemia. O treino deve ser individualizado, portanto, direcionado para as dimensões corporais de cada indivíduo. Esse protocolo foi baseado na intensidade (sobrecarga) do treino e no volume (tempo) do mesmo, sendo estes parâmetros inversamente proporcionais.

Para a obtenção da Ccrit, no presente trabalho os 20 ratos dos grupos (**GT2**) e (**GT3**) foram submetidos a um protocolo preditivo dividido em 4 fases, com cargas continuas de 9%, 13%, 11% e 15% da massa corporal, realizadas em dias consecutivos e distribuídas de forma alternada para não comprometer o desempenho dos animais. Os ratos nadaram até a exaustão, quando foram obtidos os tempos limites de exercício, em segundos (Tlim), (MARANGON et al., 2002). O parâmetro de exaustão adotado foi o de não manutenção dos padrões de movimento de natação por um período de até 5 segundos, com o animal não mais retornando com sucesso a superfície da água. Ao acontecer isso, os animais foram resgatados manualmente e colocados em outra caixa vazia sem água com as mesmas medidas da anterior e em condições ideais para o repouso; com pouca luminosidade, ambiente seco e acomodado em superfície revestida de tecido de algodão que absorvia mais rápido a água do corpo dos mesmos.

Como já mencionado, esses testes aconteceram em caixa de polipropileno (115x75x60cm) com água mantida a 31±1ºC.

No dia subsequente à obtenção das Ccrit, deu-se início aos treinos com os animais por um período de 18 dias, durante 4 semanas.

- Semana **1**: os grupos exercitados foram submetidos à **5** sessões de **60** minutos de natação com **85%** da Ccrit relativo ao seu peso.

- Semana **2**: os grupos treinados foram submetidos à **5** sessões de natação com duração de **50** minutos com **90%** da Ccrit especifica para a massa corporal de cada rato.

- Semana **3**: **5** sessões diárias com duração de **40** minutos cada com **95%** da Ccrit.

- Semana **4**: última semana de treino, onde foram realizadas **3** sessões diárias de **30** minutos com **100%** da Ccrit.

Ao final desta quarta semana foi realizado um re-teste da obtenção da Ccrit com o intuito de comparar os resultados da Ccrit inicial e final, respeitando as sobrecargas e o tempo necessário para sua realização, sendo 2 (dois) dias consecutivos com duas manhãs e duas tardes com percentuais de sobrecargas alternados conforme no inicio, para não interferir nos resultados.

Os parâmetros do treino estão resumidos na **Tabela 2**.

Tabela 2: Treino de natação com sobrecarga (Adaptado de GOBATTO et al., 2001).

Período	Tempo de treino (minutos)	Sobrecarga (Ccrit)	Número de sessões
1ª. semana	60	85%	5
2ª. semana	50	90%	5
3ª. semana	40	95%	5
4ª. semana	30	100%	3

Decorridas 120 horas (5 dias) do último dia de treino, os joelhos os animais foram radiografados e a medida da espessura articular foi tomada com paquímetro.

Posteriormente os ratos foram eutanasiados por aprofundamento anestésico (0,31 mL de Ketamina - ($C_{13}H_{16}NClO$) associada com 0,45 mL de Xilasina ($C_{12}H_{16}N_2S$)/rato) para a coleta das articulações dos joelhos, as quais foram encaminhadas para processamento segundo as diferentes técnicas propostas.

3.6. Exame radiográfico

Um animal vivo de cada grupo de estudo foi submetido aleatoriamente a exames radiográficos, os quais ocorreram nos tempos:

T9 (9 dias após a indução da AR) indivíduos de todos os grupos; **GC, GT1, GT2, GT3, GT4 e GT5**;

T18 (18 dias após a indução de AR e finalização do treinamento sem sobrecarga) somente os indivíduos dos grupos **GT4 e GT5**;

T43 (43 dias após a indução de AR e finalização do treinamento com sobrecarga) indivíduos dos grupos **GT2 e GT3**.

A intensidade da radiação foi de 40KV/100mA (voltagem do tubo/corrente do tubo) e os procedimentos foram realizados nas dependências da Clínica Veterinária Polivet, localizada em Rio Claro, SP.

Os médicos veterinários realizaram as projeções radiográficas e Thales Bregadioli (CRMV – SP 34.655) cirurgião ortopedista, liberou o laudo.

3.7. Microscopia eletrônica de varredura (MEV)

Essa técnica foi aplicada para a análise ultra-estruturais das articulações dos indivíduos de todos os grupos de estudo.

No Laboratório de Microscopia Eletrônica do Departamento de Biologia do Instituto de Biociências da UNESP de Rio Claro (SP) as articulações foram fixadas em solução de Karnovsky (KARNOVSKY et al.,1965), descalcificadas e desidratadas em série crescente de álcool etílico (70%-100%, com duração de 15 minutos cada banho), seguidas de dois banhos em acetona PA (para análise), também durante 15 minutos cada. Após dessecação do material em ponto crítico, estes foram colados com fita adesiva dupla face em suportes de alumínio para metalização com ouro em "sputtering" Balzers modelo SCD 050. Logo após, as amostras foram examinadas e fotografadas em Microscópio Eletrônico de Varredura Hitachi TM 3000.

3.8. Histologia

No Laboratório de Histologia do Departamento de Biologia do Instituto de Biociências da UNESP de Rio Claro (SP) as articulações dos indivíduos de todos os grupos de estudo foram fixadas em formalina neutra tamponada 10% (pH 7- 7.4) e acetona, na proporção de 9:1, durante 48 horas, a 4^{o}C.

Após a fixação, as articulações foram descalcificadas em EDTA durante 22 dias, desidratadas em concentrações crescentes de álcool (70%, 80%, 90% e 95%) com banhos de 30 minutos cada, transferidas para resina de embebição, incluídas, e seccionadas com 3 μm de espessura. A embebição e a inclusão deram-se em resina Leica. As secções foram recolhidas em lâminas de vidro previamente limpas. As lâminas foram então coradas pela hematoxilina-eosina (HE).

- Técnica da Coloração pela Hematoxilina de Harris e Eosina Aquosa (HE) (JUNQUEIRA; JUNQUEIRA, 1983)

As secções histológicas depois de obtidas foram reidratadas em água destilada por 1 minuto, coradas por 10 minutos, em hematoxilina e lavadas em água. Na sequencia, foram coradas pela eosina por 10 minutos, novamente lavadas e as lâminas foram secas e montadas em Bálsamo do Canadá para observação ao microscópio de luz de campo claro Leica DM4000 para registro fotográfico.

3.9. Morfologia

Foi confeccionado um esquema geral ilustrativo a partir de secções histológicas medianas para mostrar a organização da articulação do joelho, identificando os ossos que fazem parte dela, bem como as regiões da articulação e zonas da cartilagem.

RESULTADOS

4. RESULTADOS

Os resultados deste estudo estão sendo aqui apresentados sob a forma de artigos científicos, já publicado (Capítulo 1) e submetido (Capítulo 2) em periódicos internacionais.

4.1.CAPÍTULO 1

SANTOS, J.P.; Arnosti, A; Camargo-Mathias, M.I. **“**Critical load evaluation in male adult Wistar rats with zymosan-induced arthritis”**.** *International Journal of Sports Science.* 2016, 6(6): 237-242 DOI: 10.5923/j.sports.20160606.06 (publicado)

RESUMO

O presente trabalho teve como objetivo verificar o efeito da artrite induzida experimentalmente na carga crítica (Ccrit) de ratos submetidos ao exercício de natação. Para tanto, foram utilizados 10 ratos Wistar machos adultos, os quais foram distribuídos em dois grupos (**G1** e **G2**), cada um contendo cinco animais. O grupo (**G1**) foi composto por ratos saudáveis (sem artrite) e o (**G2**) com artrite, induzida por injeção de solução de Zymosan, polissacarídeo derivado da parede celular do fungo *Saccharomyces cerevisae*, composto conhecido por induzir a inflamação quando injetado na articulação de ratos, camundongos e coelhos provocando a proliferação subaguda e persistente da sinóvia, bem como a desorganização da cartilagem, induzindo a expressão de macrófagos e provocando resposta inflamatória prolongada. Os animais de ambos os grupos foram submetidos à atividade de natação com cargas críticas, obtidas por meio de teste físico em meio líquido com sobrecargas proporcionais as suas massas corporais, durante dois dias consecutivos. Após, a região articular do joelho foi submetida à análise radiográfica. Somando-se a isso, realizou-se também a análise estatística dos valores das cargas críticas obtidas. Os resultados da análise radiográfica mostraram a ocorrência de leve alteração na articulação dos joelhos dos animais do grupo (**G2**), possivelmente decorrente da instalação do processo inflamatório e, a análise estatística, revelou que a média dos valores das cargas críticas do grupo (**G2**) foi significativamente inferior (27,385 ±0,0065) aquela do (**G1**) (35,735 ±0,007), com p< 0,001. Os dados aqui obtidos deixaram evidente que a inflamação instalada na

região articular do joelho dos ratos do grupo (**G2**) comprometeu o desempenho do exercício de natação dos mesmos e, por sua vez, a obtenção da carga crítica. Além disso, os resultados demonstraram também que nos ratos com a articulação comprometida, foi exigido maior esforço físico.

Palavras-chave: artrite, carga crítica, ratos Wistar, inflamação, natação, joelho

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Critical Load Evaluation in Male Adult Wistar Rats with Zymosan-Induced Arthritis

Juan Parente Santos, André Arnosti, Maria Izabel Camargo-Mathias*

Universidade Estadual Paulista, Júlio de Mesquita Filho, UNESP, Rio Claro, S.P., Brazil

Abstract This study aimed to verify the effects of experimentally induced arthritis on the critical load of rats submitted to swimming exercise. For this, ten male Wistar rats were divided into two groups: G1, formed by healthy rats, and G2, constituted by rats with Zymosan-induced arthritis. Zymosan is known for inducing inflammation when injected in the knee joint of rats, mice and rabbits, causing subacute proliferative arthritis characterized by persistent synovitis and the disorganization of the cartilage by inducing macrophage expression, causing prolonged inflammatory responses. Both groups were submitted to swimming exercise for two consecutive days, with critical loads obtained through physical test in water with overload proportional to the animals' body mass. The posterior joint region of the animals was radiographically analyzed and the statistical analysis of the critical loads values was performed. The radiography showed the occurrence of slight joint alterations in the animals from group G2, possibly due to the onset of an inflammatory process, and the critical load mean value obtained for the group G2 was significantly lower (27.385 ± 0.0065) than that of G1 (35.735 ± 0.007), with $p < 0.001$. The data obtained in this study demonstrated that the inflammation induced in the posterior joint region of the rats from group G2 affected the performance of swimming exercise, and, consequently, the critical load value. In addition, the results showed that the joint impairment required a greater physical effort from the animals tested to perform the exercise.

Keywords Physical exercise, Swimming, Effort intensity, Anaerobic threshold, Knee joint, Inflammation, Radiographic examination

1. Introduction

Studies on exercise physiology have used animal models, especially regarding the practice of invasive manipulations. The use of mammals in laboratory research simulates physical stress conditions observed in human beings, aiming to find the most suitable follow-up procedures to observe systemic exercise alterations. [1-3] Swimming is one of the most commonly used evaluation methods, once it is an activity associated with the ergonomic cycle. Rats are the most used biological models due to their reduced size, easy handling and good response to exercise. In addition, the physiological response of these animals is similar to that of human beings [4, 5]. Moreover, the choice for this experimental model is justified by the possibility to measure injuries (in this case, the joint cartilage) and analyze the systemic biological effects that involve different therapeutic methods [6, 7].

According to the literature, the effort intensity in rats can be determined through the quantification of the blood lactate or by obtaining the critical load [4].

* Corresponding author:
micm@rc.unesp.br (Maria Izabel Camargo-Mathias)
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The critical load (CL), or critical power (CP), corresponds to the highest intensity at which the exercise can be performed without exhaustion; therefore, it is related with aerobic capacity. Thus, the critical load model has the advantage of determining, by mathematical method, both the aerobic capacity (CL) and anaerobic power stock (ASC = anaerobic swimming capacity) of the test organism [8, 9].

The critical load parameter is defined as the highest exercise intensity that can be maintained for a considerable period, where conditions of relative metabolic balance are expressed in stable values of oxygen consumption and lactatemia. Thus, it is regarded as a parameter indicating aerobic performance capacity; i.e., the boundary between the domains of heavy and severe intensity [10-12].

The noninvasive protocol used to evaluate the critical power in Wistar rats was established by [13]. The animals were submitted to four tests with loads (L) ranging from 9% to 15% of their body mass on consecutive days. The time to exhaustion (tlim) was determined for each intensity and applied to the hyperbolic equation, linearly adjusted by the function: Load= CL + ASC.1/tlim.

According to literature, the experimentally induced arthritis is similar to the naturally occurring in human beings. Several substances can be used to induce arthritis, such as adjuvants, streptococcal cell wall fragments, type II collagen,

retroviruses, lactobacilli, mycoplasma, mycobacteria [14-16] and Zymosan (Zy) [17-19]. Zymosan is a polysaccharide derived from the cell wall of *Saccharomyces cerevisae* (baker's yeast), and is known for inducing inflammation when injected in the knee joint of rats, mice and rabbits, causing subacute proliferative arthritis characterized by persistent synovitis and cartilage degradation as well, reproducing most of the symptoms observed in human beings. In addition, studies using Zymosan showed that periarticular tissues and regions suffer alterations similar to those caused by autoimmune diseases [20, 21].

Considering this information, the present study had the objective to compare the critical load values imposed to healthy male adult Wistar rats and those with Zymosan-induced arthritis to verify the influence of the disease on the swimming exercise critical load.

2. Material

In this experiment, ten male adult Wistar rats (150 days oldweighing approximately 500g were used. The rats were kept in collective polypropylene cages in the facilities of the Biodynamics Laboratory of the Physical Education Department from UNESP campus Rio Claro (SP) at controlled temperature (22°C±2°C), 12h photoperiod, receiving water and food *ad libitum*. The animals were divided into two groups: a) **G1:** five healthy individuals, and b) **G2:** five individuals with induced arthritis. Both groups were submitted to swimming exercise, critical load evaluation and radiographic examination to detect the level of inflammation.

This experiment was approved by the Ethical Committee on Animal Use (ECAU), UNESP/Rio Claro, SP, protocol number 005957.

3. Methods

3.1. Inoculation

The inoculation procedure was performed in the Biodynamics Laboratory of the Physical Education Department, UNESP, campus Rio Claro, SP. Rats from group **G2** were anesthetized in time 0 (**T0**) with ketamine hydrochloride (80mg/kg MC/IP) xylazine hydrochloride (20mg/kg MC/IP) and inoculated once with an intra-articular injection with 0.1 mL/rat of Zymosan - Sigma (*Saccharomyces cerevisae)* solution containing 1mg of Zymosan/0.5 mL of saline solution 0.9%.

The disease onset occurred on the 3rd day and the physical activity intervention started on the 15th day after inoculation.

The anesthetizing and inoculation procedures were supervised by the veterinary physician Letícia Maria Graballos Ferraz Hebling, CRMV – SP 5.412, collaborator to the research group led by Prof. Dr. Maria Izabel Camargo Mathias, responsible for the development of this study.

3.2. Radiographic Examination

The radiographic examination was performed in the veterinary clinic Polivet located in Rio Claro (SP), under the supervision of the licensed veterinary Letícia Maria Graballos Ferraz Hebling CRMV/SP 5.412. Simple, latero-lateral and anteroposterior radiographic images were produced at the intensity of 40KV/100mA.

The animals from groups **G1** and **G2** were radiographed in time **T9** (9 days after the inoculation) to verify the presence and level of inflammation caused by the arthritis induced on the rats belonging to group **G2**.

3.3. Determination of Critical Load (CL) [22]

The whole training was performed in the Biodynamics Laboratory of the Physical Education Department, UNESP campus Rio Claro in times **T11** and **T12** (11th and 12th day after inoculation). A polypropylene box 60cm x 75cm x 115cm) was filled with approximately 350 L of water at 31°C±2°C. A PVC tube measuring 60 cm x 10 cm was attached to the box, where the rats were placed one by one to swim. With this methodology, each animal was analyzed individually, allowing an accurate observation to obtain the times to exhaustion.

After each swimming bout, the animals were dried with a cotton microfiber towel and returned to the cages.

3.4. Adaptation to Water

Prior to the experiment, the animals were adapted to water for five consecutive days, The purpose of the adaptation was to reduce stress without promoting exercise training adaptations. The following procedure was adopted: a) on the first day the animals were placed in shallow water for 5 minutes; b) on the second day, the rats remained in the water for 10 minutes; c) on the third day the animals were placed in deep water, individualized in tubes, remaining for 5 minutes; d) on the fourth day the rats remained in deep water, in tubes, for 10 minutes; e) on the last day the animals were placed in deep water, in tubes, for 5 minutes with an overload corresponding to 3% of their body weight (BW).

3.5. Overload Training

After the adaptation, the animals were submitted to two swimming tests with loads tied to their back (lead fish sinkers), corresponding to 9%, 11%, 13% and 15% of their body weight (BW).

This test consisted in submitting the rats to exhaustion for intercalated periods with different loads. In the morning of the first day, a 9% load was used for each rat. In the afternoon of the same day, a 13% load was used. In the morning of the second day, a 11% load was used, and, in the afternoon, a 15% load. The times to exhaustion (tlim) for each rat with the respective loads were recorded and inserted in the hyperbolic equation to swimming rats, where x = 1/Tlim (Tlim= time limit to exhaustion) and y = load (% BW), which provided the critical load value per rat (CL/rat) [13] adapted by [23].

Finally, the critical load means for the groups were obtained and statistically analyzed using ANOVA test with TUKEY post-test. Differences with $p< 0.05$ were not considered significant.

4. Results

4.1. Radiographic Evaluation

The results obtained through the radiographic images showed that the articulations of the rats from group **G1** were intact and did not present radiopacity.

(Fig. 1A e B). Contrarily, the individuals from group **G2**, presented slight inflammation and a discreet increase in the periarticular radiopacity of the joint capsule, mainly in the craniodistal portion (Fig. 1C and D).

4.2. Critical load (CL) Evaluation

The evaluation of the critical load obtained from 5 individuals from group **G1** and 5 individuals from group **G2** showed that the respective means and standard deviations were significantly lower in the individuals from group **G2** (27.385±0.0065) in comparison with the ones from group **G1** (35.735±0.007) with $p<0.01$. For better visualization, the data is plotted on **Table 1**.

Table 1. Parameters used in the quantitative analysis of the rats from groups G1 (without arthritis and submitted to swimming) and G2 (with arthritis and submitted to swimming)

Groups	BW/Rat (g)	Load (% PC) (g)	Time limit (sec)	CL/rat (g)	Mean ± Standard deviation
G1	437	39.33[a]	313	37.51	**35.735 ± 0.007**
		48.07[b]	86		
		56.81[c]	73		
		65.55[d]	36		
	477	42.93[a]	157	31.58	
		52.47[b]	71		
		62.01[c]	61		
		71.55[d]	42		
	399	35.91[a]	182	27.41	
		43.89[b]	91		
		51.87[c]	77		
		59.85[d]	49		
	504	45.36[a]	208	44.83	
		55.44[b]	71		
		65.52[c]	83		
		75.60[d]	31		
	530	47.70[a]	85	37.37	
		58.30[b]	38		
		68.90[c]	59		
		79.50[d]	28		
G2	423.5	38.07[a]	126	27.83	**27.385 ± 0.0065[(*)]**
		46.53[b]	68		
		54.99[c]	83		
		63.45[d]	48		
	507	45.63[a]	122	30.72	
		55.77[b]	63		
		65.91[c]	76		
		76,05[d]	48		
	497	44.73[a]	136	29.82	
		54.67[b]	85		
		64.61[c]	84		
		74.55[d]	51		
	498.5	44.82[a]	147	30.07	
		54.73[b]	73		
		64.74[c]	90		
		74.70[d]	57		
	603	54.27[a]	85	18.51	
		66.33[b]	87		
		78.39[c]	56		
		90.45[d]	46		

BW= body weight; **%** **BW**= body weight percentage; **CL**= critical load; a= 9% BW; b= 11% BW; c= 13% BW; d= 15% BW; (*) = significantly different in comparison with group **G1**, with $p< 0.01$

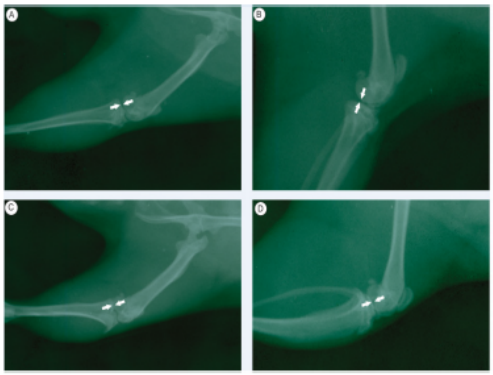

Figure 1. Radiographic images of the tibiofemoral joint of the individuals from G1 and G2 showing the preservation of the rat joints in G1 (images A and B), and radiopacity in the rats belonging to group G2 (images C and D)

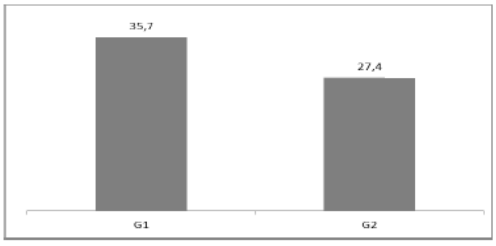

Figure 2. Graphic representation comparing G1 and G2 critical load mean values

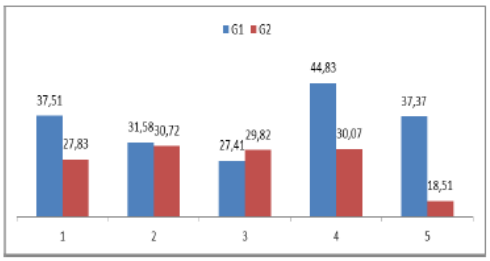

Figure 3. Graphic representation comparing the critical loads obtained for each individual from groups G1 and G2. Blue column: individuals without induced arthritis (G1). Red column: individuals with induced arthritis (2)

5. Discussion

This study analyzed male adult Wistar rats with Zymosan-induced arthritis aiming to investigate (through radiographic analysis) the onset of the inflammation in the knee joints of the individuals and the influence of such alteration on the critical load (CL); i.e., the ideal intensity to be applied on the exercise, in this case, swimming.

The results showed that the critical load values for the rats with induced arthritis (group **G2**) were significantly lower in comparison with the values obtained for the healthy animals (group **G1**). It was clearly demonstrated that the inflammation affected the exercise performance, and, consequently the critical load. In addition, the effort required from the animals with articulation impairment was higher in comparison with the individuals belonging to the control group, indicating that these individuals should be saved in terms of motor performance. These data corroborate the literature, showing that rats with induced arthritis present evident morphological alterations, such as edemas and joint amplitude limitations, which would prevent the performance of simple motor activities; e.g., locomotion on solid ground. Symptoms as acute pain, joint swelling and stiffness were also observed, causing consequent alteration in the performance of the physical activity and difficulty in locomotion. Several studies on arthritis have demonstrated that the disease affects the joint lining (inflammation of the synovial membrane), making the joints hypertrophic and causing significant cartilage damage, once the friction between the bones is considerably increased.

The radiographic data obtained here showed that the level of inflammation in the knee joint region of the rats from **G2** was relatively mild, once the amount of Zymosan solution injected was four times lower than that recommended in the literature [21]. This procedure was performed to show that an articular inflammation process, even mild, is able to affect the swimming critical load parameter. Therefore, it can be suggested that the prescription of exercise intensity and volume shall be determined on an individual basis [24].

The critical load mean value obtained for group **G1** (35.735 ± 0.007) was significantly higher than the one obtained for the group **G2** (27,385 ± 0.0065). This demonstrates that these values would be directly proportional to the inflammation level and to the osteoarticular impairment occurred in these rats, once the healthy animals reached the time to exhaustion much later than the ones from the group with induced arthritis.

Reference [25] has demonstrated that long low-intensity swimming training could bring benefits to rats with formalin-induced inflammation. Some clinical studies have suggested that physical exercise would be able to reduce the chronic pain associated with fibromyalgia, osteoporosis, inflammation of the facial/neck muscles, and cancer [26-32]. Contrarily, others reported that physical exercise would increase the chronic pain associated with fibromyalgia [33] and chronic fatigue syndrome [34], which signalizes the need of further research establishing the ideal conditions to optimize the results of the existing therapeutic methods.

Thus, the data obtained here demonstrated that the symptoms articulation pathologies, mainly in the inferior limbs (rheumatoid arthritis) could be relieved with the association of pharmacological treatment and assisted physical activity, prescribed on an individual basis, considering the most suitable type of activity, volume and intensity for each individual in order to obtain relevant and satisfactory results.

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4.2.CAPÍTULO 2

SANTOS, J.P.; Arnosti, A; Camargo-Mathias, M.I. "Comparative study of the morphophysiological behavior of the knee joint of arthritic and non-arthritic Wistar rats submitted to water exercise", submetido ao periódico *Zeitschrift für Rheumatologie .*

RESUMO

No presente trabalho avaliou-se comparativamente a instalação de danos nas cartilagens articulares do joelho de ratos expostos e não expostos ao Zymosan (*Saccharomyces cerevisae*), indutor artificial de artrite reumatóide, submetidos ou não ao exercício físico (natação). Sessenta ratos Wistar machos adultos, foram distribuídos em 6 grupos/10 animais cada (**GC -** controle, **GT1 -** artríticos e sedentários, **GT2 -** sadios submetidos ao exercício físico por 30 dias com carga, **GT3 -** artríticos submetidos a exercício físico por 30 dias com carga, **GT4 -** sadios submetidos a exercício físico por 7 dias sem carga e **GT5 -** artríticos submetidos a exercício físico por 7 dias sem carga). Para avaliação dos resultados foram utilizadas as técnicas de MEV, Raios-X e histologia de rotina. Os resultados mostraram que os indivíduos do grupo (**GT5**) apresentaram recuperação na articulação, visto que nela não foi mais observada a presença de fissuras, fendas e erosões de modo geral; aqueles dos grupos (**GT2**) e (**GT4**) não apresentaram alterações quando comparados aos do grupo controle (**GC**) e, aqueles dos grupos (**GT1**) e (**GT3**) apresentaram danos nas articulações representados pelo agravamento da inflamação na articulação, com consequente significativa redução do espaço inter-articular. Esses resultados indicaram que indivíduos portadores de AR quando da prática de exercícios físicos devem observar os mesmos sejam suaves e sem sobrecarga, controlados sistematicamente no seu volume e na intensidade. Além disso, pode-se confirmar no presente estudo que a associação de diversas ferramentas para análise e diagnóstico da doença AR, tais como MEV, Raios-X e histologia, fornece com maior precisão indicativos de qual tratamento deve ser implantado para que os resultados sejam mais eficientes e, tragam assim benefícios que melhorem a qualidade de vida dos portadores desta doença.

Palavras-chave: artrite reumatoide (AR); Zymosan; natação; ratos Wistar; articulação, morfologia

Zeitschrift für Rheumatologie

"Comparative study of the morphophysiological behavior of the knee joint of arthritic and non-arthritic Wistar rats submitted to water exercise"

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Corresponding Author:	Maria Izabel Camargo-Mathias, Ph.D Universidade Estadual Paulista Julio de Mesquita Filho BRAZIL
Corresponding Author's Institution:	Universidade Estadual Paulista Julio de Mesquita Filho
Corresponding Author Secondary Information:	
Corresponding Author's Secondary Institution:	
Corresponding Author E-Mail:	micm@rc.unesp.br
Order of Authors:	Juan Parente Santos, M.D. André Arnosti, Ph.D Maria Izabel Camargo-Mathias, Ph.D
Order of Authors Secondary Information:	
First Author:	Juan Parente Santos, M.D.
First Author Secondary Information:	
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Abstract:	The present study carried out a comparative analysis of articular damage in the knee joint cartilage of rats exposed or not exposed to Zymosan (Saccharomyces cerevisae), artificial inducer of rheumatoid arthritis, and submitted or not submitted to physical exercise (swimming). Sixty adult male Wistar rats were divided into 6 groups/10

	animals each (CG-control, TG1- sedentary and arthritic animals, TG2- healthy animals submitted to physical exercise with overload for 30 days, TG3- arthritic animals submitted to physical exercise with overload for 30 days, TG4- healthy animals submitted to physical exercise without overload for 7 days and TG5- arthritic animals submitted to physical exercise without overload for 7 days). The results were evaluated using SEM, X-ray and histological techniques. The animals from group TG5 showed signs of joint recovery, once fissures, splinters and erosions in general were no longer observed; those from groups TG2 and TG4 did not display alterations when compared with the animals belonging to the control group (CG) and those from groups TG1 and TG3 presented damage in the articulations, with intensification of the articular inflammation and consequent reduction of the intra-articular space. The results indicate that individuals with RA should perform physical activities at a mild intensity, and without overload. In addition, the present study confirmed that the association of tools for diagnosis and analysis of RA, such as SEM, X-rays and histology provides useful data for the prescription of the most appropriate treatment to improve the patients' quality of life.

Comparative study of the morphophysiological behavior of the knee joint of arthritic and non-arthritic Wistar rats submitted to water exercise

ABSTRACT

The present study carried out a comparative analysis of articular damage in the knee joint cartilage of rats exposed or not exposed to Zymosan (*Saccharomyces cerevisae*), artificial inducer of rheumatoid arthritis, and submitted or not submitted to physical exercise (swimming). Sixty adult male Wistar rats were divided into 6 groups/10 animals each (**CG**-control, **TG1**- sedentary and arthritic animals, **TG2**- healthy animals submitted to physical exercise with overload for 30 days, **TG3**- arthritic animals submitted to physical exercise with overload for 30 days, **TG4**- healthy animals submitted to physical exercise without overload for 7 days and **TG5**- arthritic animals submitted to physical exercise without overload for 7 days). The results were evaluated using SEM, X-ray and histological techniques. The animals from group **TG5** showed signs of joint recovery, once fissures, splinters and erosions in general were no longer observed; those from groups **TG2** and **TG4** did not display alterations when compared with the animals belonging to the control group (**CG**) and those from groups **TG1** and **TG3** presented damage in the articulations, with intensification of the articular inflammation and consequent reduction of the intra-articular space. The results indicate that individuals with RA should perform physical activities at a mild intensity, and without overload. In addition, the present study confirmed that the association of tools for diagnosis and analysis of RA, such as SEM, X-rays and histology provides useful data for the prescription of the most appropriate treatment to improve the patients' quality of life.

Key words: rheumatoid arthritis (RA); Zymosan; swimming; Wistar rats; knee joint, morphology

1.INTRODUCTION

Rheumatoid Arthritis (RA) is estimated to affect more than 250 million people worldwide, about 1% of the global population, and 25% of individuals over 60 years old. Additionally, RA is associated with a heavy burden on society in terms of disability and health and economic costs [1, 2, 3]. According to [3], the incidence of rheumatoid arthritis (RA) is expected to increase over the next 10 years due to the increasing proportion of elderly people, and the female population over 55 years old are predicted to be the most affected [4]. Studies on RA have reported that the disease can be inherited, and some risk factors are associated with its evolution, such as aging, body mass index (overweight) and gender (direct agents). Lack or excess in exercising along with a poor diet significantly contribute to the degeneration of articular cartilages in general [5].

Due to the multifactorial nature of arthritis, the physiopathology and the evolution of the disease can vary [6]. With specific regard to rheumatoid arthritis, the most common symptoms are: pain, synovial capsule inflammation and articular rigidity, with consequent functional loss. On the other hand, the evolution of RA can be asymptomatic, only detected through radiographic analysis, which is able to demonstrate joint space narrowing, synovial thickening and the presence of osteophytes [7].

Zymosan is a polysaccharide derived from the fungus *Sacharomices cerevisae*, with immunomodulating properties and widely used in *in vivo* experiments aimed to study the behavior of white blood cells in inflammatory processes [8]. In the present study, the animals tested had the intra-articular region inoculated with Zymosan, which caused glycosaminoglycan loss in the extracellular matrix, with consequent RA onset [9-11]

Studies on RA have used several techniques and tools, and histology is among them. Histological analyses demonstrate the characteristics of a cartilage that has been morphologically modified, defining the aspects of the cells and of the extracellular matrix as well. Scanning Electron microscopy is another important technique, allowing the observation of alterations on the surface of the biological models as well as of the different levels of damages [12].

Thus, the present study had the objective to carry out a comparative ultramorphophysiological analysis of damages in the articular cartilages of rats exposed and not exposed to Zymosan (artificial RA inducer), and those of healthy animals. We also compared the alterations occurred in the animals submitted to physical exercise (swimming).

2.MATERIAL AND METHODS

Sixty 150-day old Wistar rats weighing approximately 450g were used in this experiment. The animals were provided by the Animal Facilities of UNESP Botucatu, SP, Brazil.

The rats were kept in collective polypropylene cages (40x34x16cm), in the facilities of the Biodynamics Laboratory of the Physical Education Department from UNESP campus Rio Claro (SP), at controlled temperature (22°C±2°C), 12h photoperiod (light from 6 am to 6 pm), and received water and food *ad libitum.* The animals were fed with commercial food **Presence** (INVIVO NUTRIÇÃO E SAÚDE ANIMAL LTDA.*)* and the sawdust bedding was changed on a daily basis.

The animals were marked with indelible non-toxic marker pen on the dorsal part of their bodies (01 to 60).

2.1.INDUCTION OF THE INFLAMMATORY PROCESS

For RA induction, the rats were inoculated with an intra-articular injection of Zymosan (*Saccharomyces cerevisae)* solution (SIGMA-ALDRICH Brazil LTDA), reference Z4250, 250 mg, batch number BCBL6140V.

2.2.STUDY GROUPS

The animals were divided into six study groups (10 rats/group):

CG = CONTROL (10 sedentary rats, not submitted to induced arthritis)

TG1 = TREATMENT 1(10 sedentary rats with induced arthritis)

TG2 =TREATMENT2 (10 rats without arthritis and trained with overload for 30 days).

TG3 = TREATMENT 3 (10 rats with induced arthritis and trained with overload for 30 days).

TG4 = TREATMENT 4 (10 rats without arthritis and trained with overload for 7 days).

TG5 = TREATMENT 5 (10 rats with induced arthritis and trained with overload for 7 days).

The rats used in the experiment were handled as follows:

CG and TG1: were kept in the cages throughout the experiment.

TG2: not submitted to induced arthritis, submitted to swimming exercise, with overload, proportional to their body weight, for 30 days.

TG3: submitted to induced arthritis, submitted to swimming exercise with overload, proportional to their body weight, for 30 days.

TG4: not submitted to induced arthritis, submitted to swimming exercise, without overload, for 07 days.

TG5: submitted to induced arthritis, submitted to swimming exercise without overload for 07 days.

2.3.PHYSICAL EXERCISE PROTOCOLS (SWIMMING)

Two swimming protocols were used, with [13], and without overload [14], aiming to evaluate the post-exercise modifications caused to the animals.

Prior to the experiment, the animals were adapted to water for five consecutive days:

a) Day **1**: the rats, except the ones belonging to groups **CG** and **TG1**, were placed in shallow water (25cm) for 5 minutes,

b) Day **2**: the same rats were placed in shallow water for 10 minutes,

c) Day **3**: the animals were placed in deep water (50cm) for 5 minutes and individualized in PVC cylindrical tubes (60cm length/10cm diameter).

d) Day **4**: the rats, individualized in tubes, remained in deep water for 10 minutes.

e) Day **5**: still individualized in tubes, the rats were placed in deep water for 5 minutes with an overload corresponding to 3% of their body weight (BW), 13 grams on average.

The swimming protocols were performed in the Biodynamics Laboratory of the Physical Education Department from UNESP campus Rio Claro (SP), using a 350-liter polypropylene tank (115x75x60cm) maintained at a temperature of 31ºC ±2ºC. The slippery surface of the tank prevented the animals from reaching the bottom, so that they kept swimming or moving immersed the water. .

Five cylindrical PVC tubes (60x10cm) were introduced in the tank and the animals were individualized. After each training session, the rats were placed in another empty tank of the same material and size. After remaining in the tank for 07 minutes to allow the metabolism to decelerate, the rats were dried manually and individually with 100% cotton towels.

2.4.TRAINING PROTOCOL WITHOUT OVERLOAD (Adapted from [14])

This test included only the rats from groups **TG4** and **TG5,** once the objective was to compare different overload intensities of the same physical exercise (swimming).

Day **1**: two 30-second swimming sessions were performed, with 120-second rest.

Day **2**: two 2-minute swimming sessions with 120-second rest.

Day **3**: three 10-minute swimming sessions with 5-minute rest.

Day **4**: two 15-minute swimming sessions with 5-minute rest.

On the three remaining days, the sessions were continuous and lasted 30 minutes, without rest.

For better visualization, the bioassay results were summarized in **Table 1**.

Twenty-four hours after the last training day, the animals were radiographed and sedated with CO_2 to have the articular thickness measured, under the responsibility of the veterinarian Letícia Maria Graballos Ferraz Hebling (CRMV- SP 5.412) from Polivet Veterinary Clinic, located in Rio Claro, SP. An analogic Mitutoyo caliper model 2046F with accuracy of 0.01mm was used.

Posteriorly, the rats from this group were euthanized by the same veterinarian in a room of the Physical Education Department of the Bioscience Institute, UNESP Rio Claro (SP) with 0.31 mL of Ketamine ($C_{13}H_{16}NClO$) and 0.45 mL of Xylazine ($C_{12}H_{16}N_2S$)/rat. The rats were dissected and the right posterior leg was isolated for the removal of the knee joints, which were immediately fixed in formaldehyde 10%, and decalcified in nitric acid 5% (22 days). The samples were dehydrated in crescent ethanol series (50% to 100%) and included in Leica historesin for the application of histological techniques. The material was sectioned at 3µm thickness using a Leica microtome RM2255 Leica® of the Histology Laboratory of the Biology Department of UNESP, Rio Claro, SP.

The animals used in this experiment were disposed of according to the norms stablished by the Ethics Committee on Animal Use (ECAU) and the Brazilian National Council for the Control of Animal Experimentation (CONCEA). This experiment was approved by the Ethics Committee on Animal Use (CEUA), UNESP/Rio Claro, SP, protocol number 005957, decision number 05/2015.

2.5.TRAINING PROTOCOL WITH OVERLOAD (Adaped from [13])

The animals belonging to groups **TG2** and **TG3** were submitted to a predictive protocol comprised of 4 phases, with randomly distributed overloads of 9%,11%,13% and 15% of the body weight, performed on consecutive days. The rats swam until exhaustion, and the exercise time limits were obtained, expressed in seconds (Tlim), [15]. The exhaustion parameter adopted was the incapability of the rats to maintain the standard swimming movements for 5 seconds, i.e., when the animal was not able to reach the water surface. When this happened, the rats were manually rescued and placed in an empty box, the same size as the one containing water, with ideal conditions to rest: low light, dry environment and cotton fabric bedding to absorb the water from their body.

One day following the determination of the critical loads, the training sessions started, consisting in a period of 18 days, for 4 weeks.

- Week **1**: five 60-minute swimming sessions, with 85% of the critical load.

- Week **2**: five 50-minute swimming sessions, with 90% of the critical load.

- Week **3**: five 40-minute swimming sessions per day, with 95% of the critical load.

- Week **4**: last training week, with three 30-minute swimming sessions per day with 100% of the critical load.

After the fourth week, a re-test was performed to obtain a critical load value to be compared with the initial one, respecting the overloads and the necessary performance time; i.e., two consecutive days: two mornings and two afternoons, with different overload percentages. The training parameters are summarized in **Table 2**.

The rats were euthanized with ketamine hydrochloride 0.9/ Xylazine hydrochloride 0.3, 120 hours following the last training day, for the removal of the knee joints.

2.6.Scanning Electron Microscopy (SEM)

After the dissection of the animals, the joints were removed and fixed in Karnovsky's solution [16], decalcified and dehydrated in crescent ethanol series (70%-100%, 15 minutes each bath), and washed twice in acetone P.A. (15 minutes each bath). After critical point drying, the material was placed in aluminum stands for gold sputtering using Balzers SCD 050. The samples were analyzed and documented under Scanning Electron Microscope Hitachi TM 3000.

2.7.Radiographic images (XR)

The radiographic examination was performed in the veterinary clinic Polivet located in Rio Claro (SP), under the supervision of the licensed veterinary Letícia Maria Graballos Ferraz Hebling CRMV/SP 5.412. Simple, laterolateral and anteroposterior radiographic images were produced, at the intensity of 40KV/100mA.

An animal from each group (**CG** – **TG5)** was radiographed in time **T9** (9 days after inoculation (**TG1**, **TG3** and **TG5**) to verify the presence and level of inflammation caused by induced arthritis and in time **T45** (45 days after inoculation) at the same time and under the same material conditions.

The radiographic technique was used to evaluate radiopacity, radiolucency and thinning of the joint structure and measure the intra-articular space before and after the experiment.

2.8.Histology

The articulations of all the animals tested were fixed in buffered formalin 10% (pH 7- 7.4) and acetone at the proportion 9:1, for 48 hours at 4ºC. Then, the samples were decalcified in EDTA for 22 days, dehydrated in crescent ethanol series (70%, 80%, 90% and 95%) 30 minutes each bath, embedded in Leica resin and sectioned at 3 μm thickness. The material was mounted on slides and prepared for hematoxylin-eosin staining.

2.9.Hematoxylin and eosin staining technique ([12])

The histological sections were rehydrated in distilled water for 1 minute, stained with hematoxylin for 10 minutes and rinsed in water. Posteriorly, the samples were stained with eosin for 10 minutes, rinsed again, dried, and mounted in Canada Balsam to be analyzed under bright field light microscope Leica DM4000 for photo documentation.

3.RESULTS

Following the order of appearance, the results are listed and summarized in the tables and plates below. (**Table 3**)

Figure 1

Scanning Electron Microscopy (SEM)

3.1.SEM RESULTS

Control Group (CG)

The results of the SEM techniques applied in this study showed that, in the animals belonging to the control group (CG), the facet joint cartilage and tibia were intact, with homogeneous aspect (Fig. 2H). The synovial membrane was preserved, showing no morphological alterations (Fig. 2 N).

Sedentary arthritic group (TG1)

The facet joint cartilage displayed morphological alterations and signs of degenerative processes, such as irregular surface, splinters and fissures in the articular surface, and substantial loss in the collagen layer (Fig. 2 I, O). The synovial membrane was shrunk.

Healthy group submitted to extenuating exercise (TG2)

The obtained for the animals belonging to this group were similar to those found for the control group (**CG**). The bone structures of the femur and tibia were uniform, and the articular cartilage surface was smooth and intact. (Fig. 2 D, J). The synovial membrane did not show any alterations in comparison with the control group (**CG**) (Fig. 2 D).

Arthritic group submitted to extenuating exercise (TG3)

The animals belonging to this group showed morphological alterations, such as erosion (Fig. 2 K) and evident fissures and depressions in the articular cartilage facet (Fig. 2 Q). The synovial membrane was shrunk, apparently atrophied, and thickened in the region of the joint capsule, indicating a possible inflammatory process.

Healthy group submitted to moderate exercise (TG4)

The results obtained for this group were similar to those found for the control group (**CG).** The bone structures of the femur and tibia were uniform and the articular cartilage surface had a polished and homogeneous aspect (Fig. 2 L). The synovial membrane did not present any alterations when compared to the control group (GC) (Fig. 2 F).

Arthritic group submitted to moderate exercise (TG5)

The animals belonging to **TG5** did not show morphological alterations that could affect the regular functioning of the condyles, indicating that there was no RA evolution (Fig. 2 M, S). The synovial membrane was practically intact when compared to the control group.

Figure 2:

Radiographic images- X-ray

3.2.X-ray RESULTS

RADIOGRAPHIC IMAGES (X- RAY)

For the radiographic analysis, an animal from each group was randomly chosen and had the right knee joint radiographed in two different moments; before exercising (9 days following inoculation) and after exercising (45 days following inoculation). Anteroposterior (AP) and laterolateral (LL) radiographs were taken. For the measurement of the intra-articular space, the distance between the distal femoral condyle and the proximal tibial condyle in the medial portion were calculated. The values are shown in table 4.

PRE EXERCISE

The animal belonging to the control group (**Fig. 2AB)** presented healthy joints, i.e., no signs of RA. The intra-articular space measured 0.59 mm.

The rat from group **TG1**, which comprised of arthritic and sedentary rats (**Fig. 2EF)** presented a significant increase in the intra-articular radiopacity (➔), suggesting inflammatory process onset (RA). The intra-articular space measured 0.29mm.

The articulation of the rat belonging to group **TG2**, healthy and trained for 30 days with overload (**Fig. 2IJ),** presented no alterations or signs of RA. The intra-articular space measured 0.70mm.

The animal from group **TG3**, arthritic rats trained for 30 with overload displayed an increase in intra-articular radiopacity (➔), which suggested the onset of an inflammatory process (RA). The intra-articular space could not be observed (**Fig. 2MN)**.

The rat belonging to group **TG4**, healthy and trained for 7 days without overload (**Fig. 2QR)** presented intact articulation, and no signs of RA. The intra -joint space measured 0.34mm.

The rat from group **TG5**, arthritic and trained for 7 days without overload (**Fig. 2UV)** presented an increase in the intra-articular radiopacity (➔), suggesting the onset of an inflammatory process (RA). The intra--articular space was not observed.

POST EXERCISE

The rat belonging to the control group (**Fig. 2CD)** presented a decrease in radiolucency (>) and an evolution in the femoral cortical and subchondral radiopacity (➔). No signs of RA were observed. The inter-articular space measured 0.35mm.

The animal from group **TG1**, arthritic and sedentary (**Fig. 2GH),** presented evident decrease in radiolucency (>) with an increase in femoral and tibial radiopacity (➔). The intra-articular space was not observed.

The rat belonging to group **TG2**, healthy and trained for 30 days with overload (**Fig. 2KL),** displayed a decrease in radiolucency (>), increase in the femoral radiopacity (➔), no signs of RA or medullary femoral thinning (*). The intra-cellular space measured 0.48mm.

The animal from group **TG3**, arthritic and trained for 30 days with overload (**Fig. 2OP)** showed loss of radiolucency (>) decrease in radiopacity (➔), and medullary canal thinning (*). The intra-articular space was not observed.

The rat belonging to group **TG4**, healthy and trained for 7 days without overload (**Fig. 2ST),** showed no alterations. The intra-articular space measured 0.30mm.

No signs of RA onset were observed in the subjects belonging to the group **TG5**, comprised of arthritic rats trained for 7 days without overload (**Fig. 2WX)**. There was an increase in radiopacity (➔) and decrease in radiolucency (>), as well as an increase in the intra-articular space (0.40mm).

Figure 3:

Histology – HE

3.3.HE RESULTS

Control Group (CG)

The results obtained for the animals belonging to the control group (**CG)** showed no morphological alterations in the joint tissues. The femoral and tibial epicondyle were preserved (Fig. 2 B-D). The cells and the synovial membrane displayed a regular organization pattern (Fig. 2 D). Chondrocytes were observed in the articular cartilages, single or organized into isogenic groups (Fig. 2 C, D). Adipocytes and synoviocytes were regularly distributed in the synovial membrane (Fig. 2 E).

Sedentary Arthritic Group (TG1)

The animals from group **TG1** started to show signs of synovial thickening, with the presence of fibers and formation of pannus rheumatoid (Fig. 2 F-H). Morphological alterations, such as cell disorganization and proliferation were evident. Several synoviocytes were enlarged and hypertrophic (Fig. 2 G). Lymphocytes and macrophages were detected (Fig. 2 G, I) characterizing generalized inflammation, except for the cartilage region (Fig. 2 I).

Healthy group submitted to extenuating exercise (TG2)

In the rats belonging to treatment group 2 (**TG2**), the femoral and tibial epicondyles presented intact and preserved tissues (Fig. 2 J, K). The articular cartilage surface and the synovial membrane displayed small irregularities, mainly in the periphery (Fig. 2 L). However, the chondrocytes and isogenous groups were aligned (Fig. 2 K). Adipocytes were observed in the synovial membrane, in addition to discreet cell proliferation. The synoviocytes were organized (Fig. 2 M).

Arthritic group submitted to extenuating exercise (TG3)

The animals from treatment group 3 (**TG3**) presented an apparent decrease in the joint cavity in comparison with the ones from the previous groups (Fig. 2 N). The rheumatoid *pannus* invades the intra -articular space (Fig. 2 O). The synovial membrane is thicker in comparison with the control group, and Langhans giant cells were observed (Fig. 2 P, Q).

Healthy group submitted to moderate exercise (TG4)

The articular cavity, the epicondyle and the synovial membrane of the animals from treatment group 4 (**TG4**) were preserved, with intact tissues (Fig. 2 R-T). Chondrocytes and synoviocytes were round-shaped and well defined (Fig. 2 U).

Arthritic group submitted to moderate exercise (TG5)

The joint cavity of the rats from treatment group 5 (**TG5**) presented the expected morphological features, with no signs of inflammation (Fig. 2 V). The apical region of the synovial membrane was turned to the articular center, well defined and without the presence of pannus rheumatoid (Fig. 2 W-X). The synoviocytes were round-shaped, and their morphology suggested tissue integrity with consequent recovery (Fig. 2 Y).

4.DISCUSSION

Several studies have been developed aiming to reduce or even eliminate rheumatic symptoms, and numerous have recommended physical exercises and the patient's self-management, non-pharmacological alternatives to minimize or retard the effects of rheumatoid arthritis (RA) [17, 18]. Water exercises have been considered a facilitating strategy to control the disorder, due to the reduced impact of the activity and its capability to increase the therapeutic intensity, consequently bringing several benefits to the patient [19, 20]. There are few reports in the literature regarding the positive results of swimming; however, discreet improvements in the functional capacity of elderly patients showing that the rheumatic diseases can be stabilized have been reported [21-23].

In this sense, oriented and monitored water exercise can be highly beneficial for RA patients. Thus, the present study aimed to analyze the effects of water exercise (swimming) by comparing possible morphological alterations in the articular cartilage tissue using male Wistar rats as biological model. The animals used in this experiment had different profiles, as follows: a) sedentary, with or without RA, b) arthritic submitted to different exercise intensities (with and without overload) and volumes (7 and 30 days) and c) healthy and physically active. The exercising period took place always after 7 pm, according to the protocol stablished by [24]. To obtain the arthritic profile, the animals were exposed to Zymosan – AZy (*Sacharomices cerevisae*), a substance that stimulate the onset of inflammatory processes. The intensity of the exercises ranged from moderate to high, according to the classification by [25]: **low**

(above basal levels), **moderate** (therapeutic rehabilitation), **high** (acceptable effort levels) and **elite** (athletic level for performances of competitions).

The measurement of the tibial cartilage thickness was one of the parameters analyzed in this study. The results showed that the only group that presented equal or superior means in comparison with the control group (290,00 μm) was **TG2** (active and healthy rats, trained for 30 days with overload), probably due to the need to find a compensatory mechanism to prepare the tissue for additional load after a period of intense physical activity. According to [26-28], the relationship between physical activity and a larger volume of the articular cartilage would be the result of a complex mechanism of mechano-transduction, in which the chondrocytes would respond to the body weight load with an increase in the synthesis of proteoglycans, causing the cartilage to swell.

Regarding the superficial aspect of the knee cartilage analyzed through SEM, erosions, fissures and depressions were observed both in the animals belonging to **TG1** (sedentary and arthritic) and in those from **TG3** (active, trained for 30 days with overload and arthritic). According to some authors, as [29, 30], the presence of cartilages with smooth, polished and whitish surfaces indicate the natural and healthy state of the tissue; however, [31], who analyzed human articulations using SEM, healthy cartilages can also have an irregular aspect, displaying undulations and depressions. One of the alterations observed in the present study was the presence of fissures, which probably progressed to erosions and affected deeper regions of the articulation, in some cases, the subchondral portion of the bone. This might have happened due to the combination of several factors, including the pathology onset, or the addition of extra load, which would decrease hydration by disassembling the proteoglycans, with consequent loss of surface integrity and impairment of the matrix rigidity.

The measurements of the spaces between the femur and tibia (joint cavity) were taken. The healthy joints measured 0.35mm on average, while in the altered joints the spaces measured less than 0.35mm or were not detected in the analysis. Only the animals belonging to groups **TG3** (active and arthritic, trained for 30 days with overload) and **TG4** (active and healthy, trained for 7 days without overload displayed alterations in the joint space. In **TG3**, the alterations were probably potentiated by the addition of overload, once the animals were arthritic. In some rats, the joint surfaces

were in direct contact, causing pain, decreasing movement amplitude and resulting in the impairment of the joint function. As for the animals form **TG4,** the physical exercise did not modify the joint morphology. Radiography is widely used to provide information on skeleton disorders, once it is inexpensive, non-invasive and easy to be performed [32]. Histological analysis was performed to detect alterations in the joints of the Wistar rats with different profiles submitted to water exercise (swimming). The animals belonging to **TG5**, (arthritic, trained for 7 days without overload) displayed evident chondrocyte alignment, suggesting the occurrence of cell migration to preserve the cartilage. The rats from **TG1** (sedentary and arthritic) and **TG3** (active and arthritic, trained for 30 days with overload) showed chondrocyte hyperplasia and empty spaces in the extracellular matrix, suggesting the occurrence of cell death, probably caused by the RA onset, which triggered inflammatory processes as well. Similar results were found by [33], confirming that moderate training, without overload and during a short period would promote recovery of stability of rheumatic disorders, validating water exercise as a promising alternative to control RA.

The histological analysis showed that, in the animals from group **TG2** (healthy and active, trained for 30 days with overload) and **TG3** (arthritic and active, trained for 30 days with overload), structural degeneration was stimulated, probably due to the knee joint locomotion demand. These findings corroborate [34, 35]. The higher effort demand resulted in the onset of inflammatory processes, which in turn activated the cascade of inflammatory mediators, disseminating the inflammation to adjacent tissues [36-38].

The histological analysis demonstrated that the animals from groups **TG1** (sedentary and arthritic) and **TG3** (active and arthritic, trained for 30 days with overload) presented hyperplasia in the chondrocytes and in the synoviocytes as well, which characterizes the onset of inflammation (synovitis). The emergence of a rheumatoid pannus, characterized by a significant number of small blood vessels, structural proteins, proteoglycans and inflammatory cells, contributed to the erosion of the articular tissue located between the synovial membrane and the cartilage. According to [39, 40], who developed studies using dogs, the synovial membrane would play a fundamental role in RA evolution, once the disorder is characterized by an inflammation of the joint capsule and migrates to the synovial membrane, where the release of proteolytic enzymes into the joint space degenerate the cartilage. These data

corroborate our findings, confirming that swimming would not be effective as a non-pharmacological method to treat RA [39, 40].

Therefore, the results presented herein indicate that oriented and planned swimming training (with moderate intensity, performed periodically, having exclusively the resistance of the water, i.e., without extra load), would be beneficial for the arthritic knee joints of male Wistar rats, being able to inhibit the inflammation evolution in the structure as a whole. These findings are encouraging, once water exercises could become an interesting strategy to minimize RA effects; however, the correct prescription and the elaboration of a patient-specific training routine are of the utmost importance. This study also confirms the importance of the association of different tools, such as histology, SEM, and radiography to elucidate the processes related with joint pathologies.

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LEGENDS:

Figure 1.

A: Schematic representation of the synovial joint of a Wistar rat

B-**S**: Ultramicrographs of the joint and articular cartilage joint surface of Wistar rats, with or without induced arthritis and submitted or not submitted to swimming exercise. **B**, **C**, **D**, **E**, **F**, **G**: femoral condyle, tibial plateau and synovial membrane, **H**, **I**, **J**, **K**, **L**, **M**: details of the cartilage surface, **N**, **O**, **P**, **Q**, **R**, **S**: articular cartilage and subchondral bone.

Joint: **CG** (Fig. **B**, **N**): femoral condyle, tibial plateau and synovial membrane, preserved and intact **TG1** (Fig. **C**, **O**): femoral condyle, tibial plateau and synovial membrane with signs of inflammation and edema.**TG2** (Fig. **D**, **P**): femoral condyle, tibial plateau and synovial membrane, preserved and intact.**TG 3** (Fig. **E**, **Q**): femoral

condyle, tibial plateau and synovial membrane, with joint capsule edema and cartilage depression.**TG 4** (Fig. **F**, **R**): femoral condyle, tibial plateau and synovial membrane, preserved and intact.**TG 5** (Fig. **G**, **S**): femoral condyle, tibial plateau and synovial membrane, intact.

Articular Cartilage Surface:
CG (Fig. **H**); smooth and intact.
TG 1 (Fig. **I**): with splinters and fissures
TG 2 (Fig. **J**): smooth and intact.
TG 3 (Fig. **K**): with erosion, depression and fissures
TG 4 (Fig. **L**): smooth and intact.
TG 5 (Fig. **M**): smooth and intact.

cv= articular cavity; **f**= femur; **t**= tibia; **s**= synovial membrane; **ca**= cartilage; **cp**= joint capsule; **cf**= cartilage facet; **sp**= splinters; **fi**= fissure; **dp**= depression; **er**= erosion; **sb**= subchondral bone.
Scale bars:
B-G: 2mm; **H, I**: 300μm; **J, K, M**: 200μm; **L**: 100 μm; **N, O, Q**: 1mm; **P, R, S**: 2mm.

Figure 2:

X-ray images of the tibial-femoral articulation of Wistar rats before (9 days following Zymosan inoculation and before swimming training) and after (45 days following inoculation and submitted to swimming training); **A**-**D**= control group (**CG**); **E**-**H**= sedentary arthritic group (**TG1**); **I**-**L**= healthy group trained with overload for 30 days (**TG2**); **M**-**P**= arthritic group trained with overload for 30 days (**TG3**); **Q**-**T**=healthy group trained without overload for 7 days (**TG4**); **U**-**X**= arthritic group trained without overload for 7 days (**TG5**).

Anteroposterior view (**AP**); Laterolateral view (**LL**).

Figure 3:A:

Schematic representation of the synovial knee joint of a Wistar rat.**B**-**Y**: Histological sections of the femur distal articulation and proximal tibia of Wistar rats submitted and not submitted to induced RA and water exercise, stained with HE (hematoxylin and eosin). **B**–**E**: Control (**CG**); **F**-**I**: Arthritic sedentary (**TG1**); **J**-**M**: Healthy and trained for 30 days with overload (**TG2**); **N**-**Q**: Arthritic and trained for 30 days with overload (**TG3**); **R**-**U**: Healthy and trained for 7 days without overload (**TG4**); **V**-**Y**: Arthritic and trained for 7 days without overload (**TG5**).

ac= articular cavity; **f**= femur; **t**= tibia; **s**= synovial membrane; **sy**= synoviocytes; **c**= chondrocyte; **ad**= adipocyte; **m**= mastocyte; **hp**= hyperplasia; **ir**= irregularity; **rp**= rheumatoid *pannus*; **ng**= neoangiogenesis; **co**= collagen; **ig**= isogenous group; **Lg**= Langhans giant cells.
Scale bars: **B, F, J, N**= 500μm; **C, D, R, V**= 200μm; **E, H, I, L, M, O, P, Q, S, T, W, X**= 100μm; **G, K**= 50 μm; **U, Y**= 20μm.

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Table 1

Table 1: Swimming training without overload

Day	Training (minutes)	Rest (minutes)	Number of sessions	Total activity time (minutes)
1st	0.5	2	2	1
2nd	2	2	2	4
3rd	10	5	3	30
4th	15	5	2	30
5th	30	0	1	30
6th	30	0	1	30
7th	30	0	1	30

Table 2

Table 2: Swimming training with overload (Adapted from [13])

Period	Training length/minutes	Overload (CL)	Number of sessions
1st week	60	85%	5
2nd week	50	90%	5
3rd week	40	95%	5
4th week	30	100%	3

Table 3

Table 3: Summary of the results obtained through radiography, scanning electron microscopy and histological techniques to detect the alterations in the knee joint of X rats submitted to the different bioassays

	GC Control	TG 1 AR/SED	TG 2 TR 30	TG 3 AR/TR 30	TG 4 TR 7	TG 5 AR/TR 7
Radiopacity						
Increase	X	X	X		#	X
Decrease				X	#	
Radiolucency						
Increase					#	
Decrease	X	X	X	X	#	X
Surface						
Intact	X		X		X	X
Erosion		X		X		
Fissure		X		X		
Depression		X		X		
Thickness						
≥290µm	X		X			
≤290µm		X		X	X	X
Space						
≥0,35mm	X	X	X			X
≤0,35mm				X	X	
Synovial membrane						
Intact	X		X		X	X
Inflamed		X		X		

Table 4

Table 4: Summary of the means (mm) obtained by the measurement of the right knee intra-articular space of the animals in each group

Group	Pre Exercise (mm)	Post Exercise (mm)
CG	0.59mm	0,35
TG1	0.29mm	absent
TG2	0.70mm	0.48
TG3	absent	absent
TG4	0.34mm	0,30
TG5	absent	0.40

Figure 1

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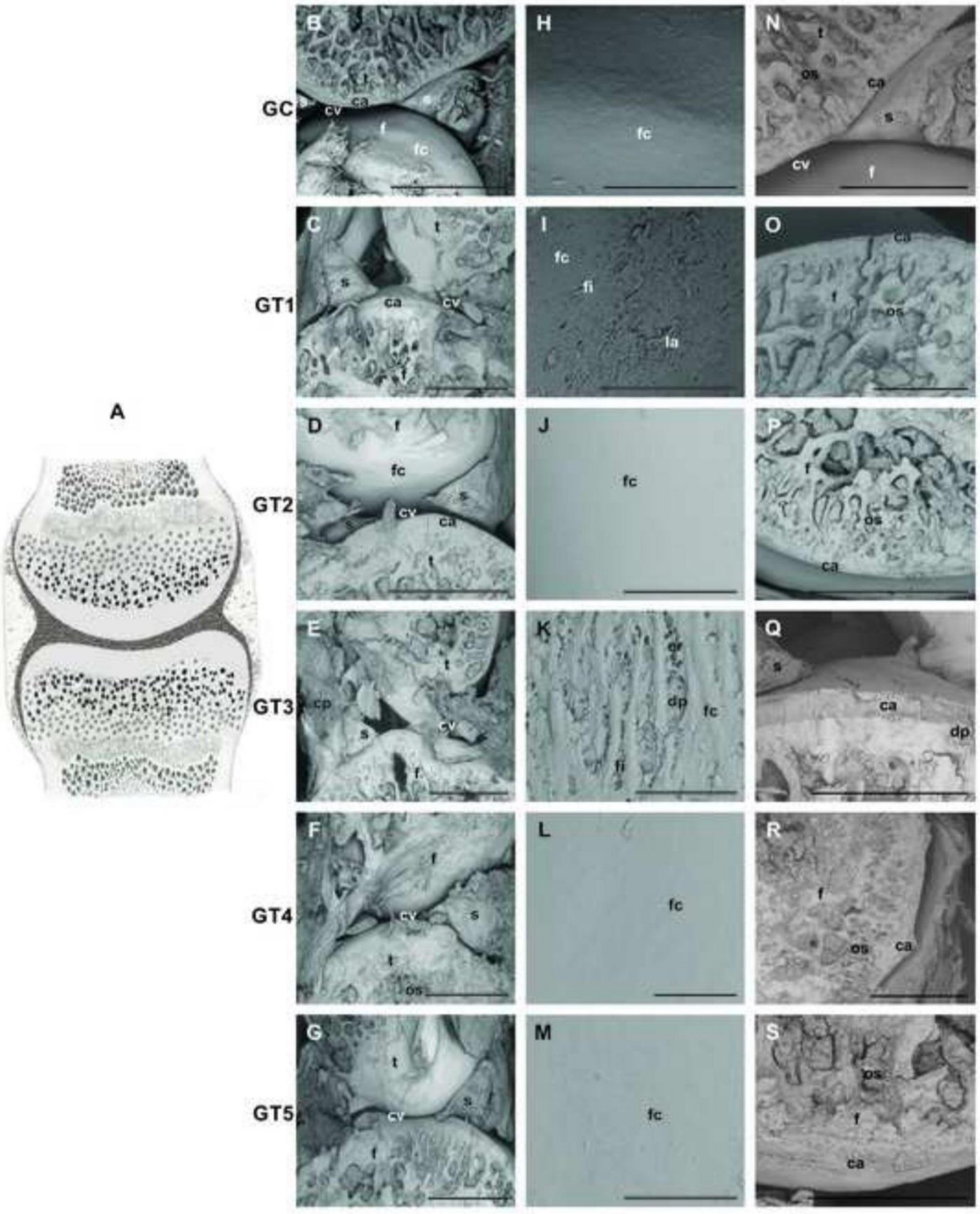

Figure 2

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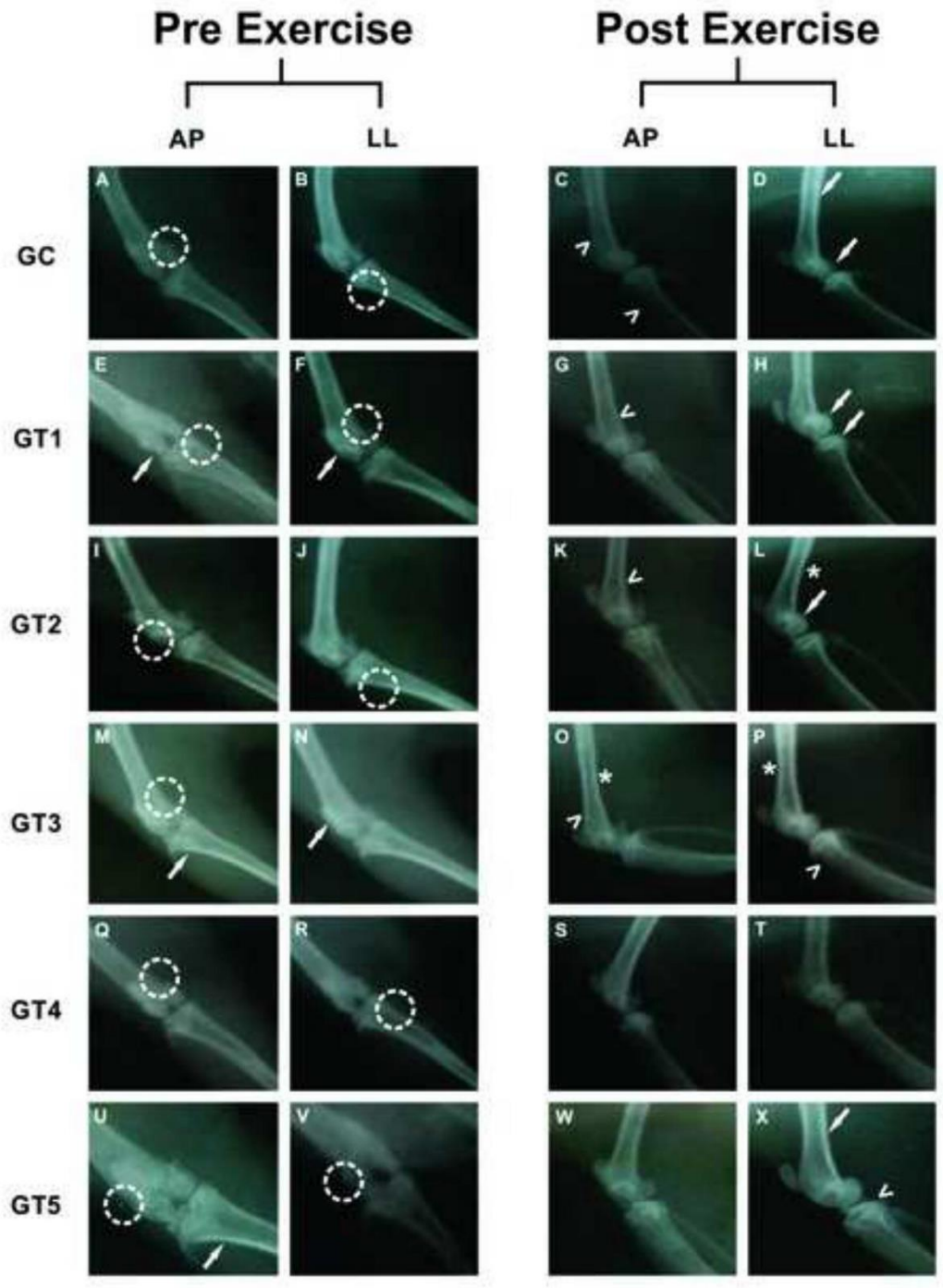

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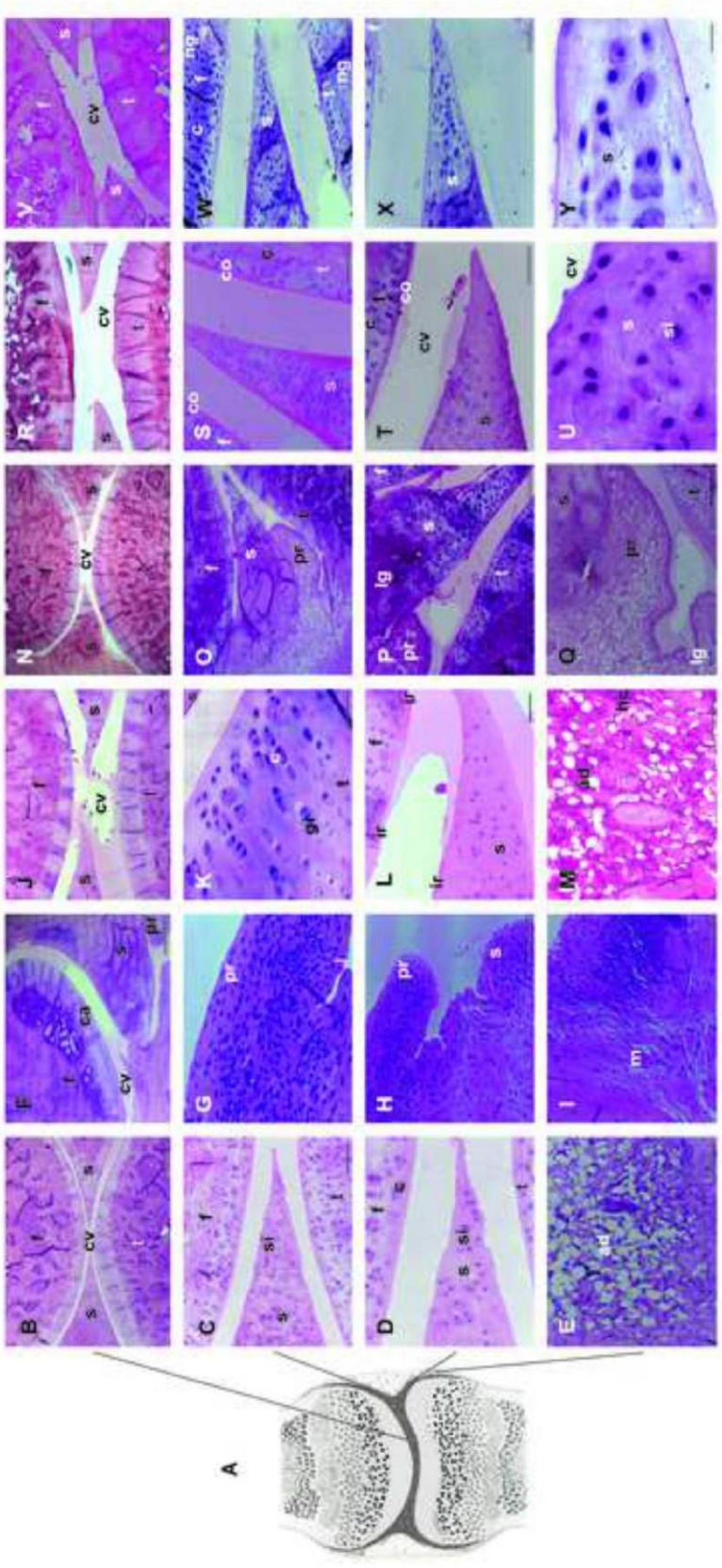

Figure 3

CONSIDERAÇÕES FINAIS / CONCLUSÕES

5.CONSIDERAÇOES FINAIS / CONCLUSÕES

O desenvolvimento do presente trabalho permitiu concluir que:

1- Ratos induzidos com AR e submetidos à prática de exercício físico de natação pelo período de 30 dias (**GT3)** e com diferentes cargas críticas (9%, 13%, 11% e 15%) tiveram os espaços inter-articulares do joelho reduzidos, o que sugeriu que exercícios físicos praticados de forma intensa e com excesso de sobrecarga ao invés de trazer benefícios, trazem prejuízos às articulações, causando danos morfológicos às mesmas e consequentemente fisiológicos para o indivíduo portador da doença.

2- Ratos induzidos com AR (**GT5)** submetidos ao exercício físico de natação por 7 dias e sem sobrecarga adicional, apresentaram significativa melhora na estrutura articular do joelho como um todo, uma vez que, fendas, lascas, fissuras, depressões e erosões que antes eram observadas, não o foram após a realização do exercício, indicando que praticar exercício na água (natação) quando de forma amena, branda e suave sem incremento de sobrecarga e por um curto período de tempo (7 dias) pode ser benéfica para as articulações de ratos portadores de AR.

3- Ratos com AR (**GT1** e **GT3**), sedentários e submetidos ao exercício físico de natação por um período de 30 dias com sobrecarga (respectivamente), apresentaram alterações significativas nas articulações do joelho, ou seja, neles foram observados *pannus* reumatóide, desorganização e proliferação celulares, células gigantes de Langhans, o que não ocorreu naqueles que foram induzidos com AR (**GT5**) e submetidos ao exercício físico por um período de 7 dias sem sobrecarga, nos quais, não houve evolução da AR.

4- A combinação de diferentes ferramentas de análise: parâmetro da carga crítica, análises estatísticas, microscopia eletrônica de varredura (MEV), imagens radiográficas (Raios-X) e histologia auxiliam no diagnóstico e, consequentemente, na adequação da intervenção terapêutica, quando da indicação de qual e que tipo de exercício físico seria mais seguro, mais eficaz e menos invasivo para melhorar a qualidade de vida de um indivíduo portador de AR.

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6. REFERÊNCIAS

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ANEXO

7.ANEXO

Figura 1

A: Representação esquemática da articulação sinovial de joelho de rato Wistar. **B**-**S**: Ultramicrografias da articulação e da superfície da cartilagem articular do joelho de ratos Wistar, com ou sem AR induzida e submetidos ou não ao exercício físico de natação. **B**,**C**,**D**,**E**,**F**,**G**: côndilo femoral, platô tibial e membrana sinovial, **H**,**I**,**J**,**K**,**L**,**M**: detalhes da superfície da cartilagem, **N**,**O**,**P**,**Q**,**R**,**S**: cartilagem articular e osso subcondral.

Articulação:
GC (Fig. **B**,**N**): côndilo femoral, platô tibial e membrana sinovial em condições preservadas e íntegras.
GT1 (Fig. **C**,**O**): côndilo femoral, platô tibial e membrana sinovial com sinais inflamatórios e edema articular.
GT2 (Fig. **D**,**P**): côndilo femoral, platô tibial e membrana sinovial em condições preservadas e íntegras.
GT3 (Fig. **E**,**Q**): côndilo femoral, platô tibial e membrana sinovial com edema na capsula articular e depressão na cartilagem.
GT4 (Fig. **F**,**R**): côndilo femoral, platô tibial e membrana sinovial em condições preservadas e íntegras.
GT5 (Fig. **G**,**S**): côndilo femoral, platô tibial e membrana sinovial em condições íntegras.

Superfície da cartilagem articular:
GC (Fig. **H**); lisa e íntegra.
GT1 (Fig. **I**): com lascas e fissuras.
GT2 (Fig. **J**): lisa e íntegra.
GT3 (Fig. **K**): com erosão, depressão e fissuras.
GT4 (Fig. **L**): lisa e íntegra.
GT5 (Fig. **M**): lisa e íntegra.

cv= cavidade articular; **f**= fêmur; **t**= tíbia; **s**= membrana sinovial; **ca**= cartilagem; **cp**= capsula articular; **fc**= face da cartilagem; **la**= lascas; **fi**= fissura; **dp**= depressão; **er**= erosão; **os**= osso subcondral.

Barras:
B-G: 2mm;
H, I: 300µm;
J, K, M: 200µm;
L: 100 µm;
N, O, Q: 1mm;
P, R, S: 2mm.

Figura 2

Imagens de Raios-X obtidas da articulação tíbio-femoral de ratos Wistar antes (9 dias após a inoculação do Zymosan e antes do treinamento físico de natação) e após (45 dias pós inoculação com Zymosan e submetido ao treinamento físico de natação); **A-D**= grupo controle (**GC**); **E-H**= grupo artrítico e sedentário (**GT1**); **I-L**= grupo sadio com treino com sobrecarga de 30 dias (**GT2**); **M-P**= grupo artrítico com treino com sobrecarga de 30 dias (**GT3**); **Q-T**=grupo sadio com treino sem sobrecarga de 7 dias (**GT4**); **U-X**= grupo artrítico com treino sem sobrecarga de 7 dias (**GT5**).

Perfil ântero-posterior (**AP**); Perfil látero-lateral (**LL**).

Figura 3

A: Representação esquemática da articulação sinovial de joelho de rato Wistar.

B-Y: Secções histológicas da articulação distal do fêmur e proximal da tíbia de ratos Wistar com e sem indução de AR e submetido ou não à prática de exercício físico na água, coradas pela técnica HE (hematoxilina e eosina).

B–E: Controle (**GC**);

F-I: Artrítico sedentário (**GT1**);

J-M: Sadio e treinado por 30 dias com sobrecarga (**GT2**);

N-Q: Artrítico e treinado por 30 dias com sobrecarga (**GT3**);

R-U: Sadio e treinado por 7 dias sem sobrecarga (**GT4**);

V-Y: Artrítico e treinado por 7 dias sem sobrecarga (**GT5**).

cv= cavidade articular; **f**= fêmur; **t**= tíbia; **s**= membrana sinovial; **si**= sinoviócito; **c**= condrócito; **ad**= adipócito; **m**= mastócito; **hp**= hiperplasia; **ir**= irregularidade; **pr**= *pannus* reumatóide; **ng**= neoangiogenese; **co**= colágeno; **gi**= grupo isógeno; **Lg**= células gigantes de Langhans.

Barras de escala:

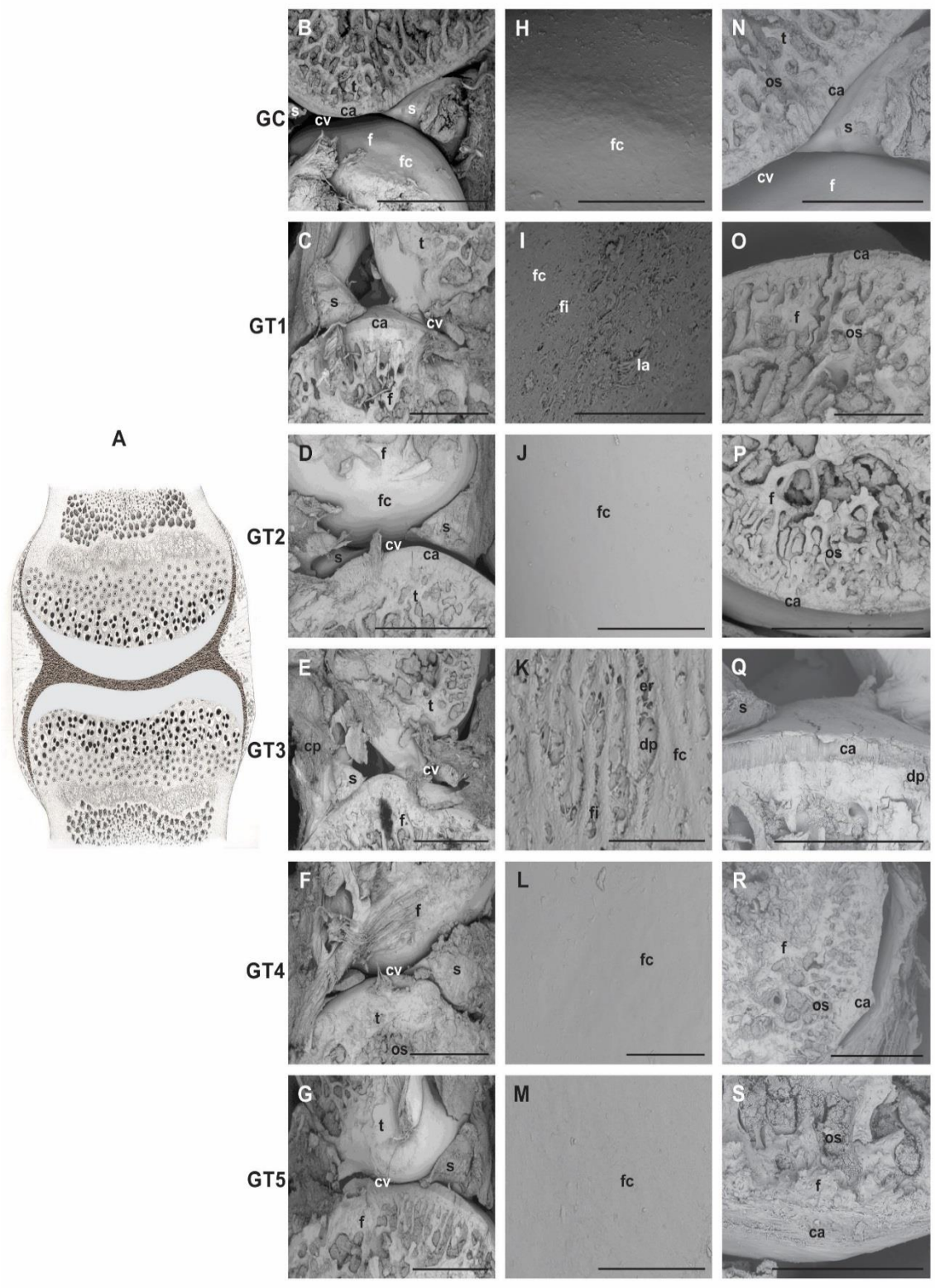
B,F,J,N= 500µm ;

C,D,R,V= 200µm;

E,H,I,L,M,O,P,Q,S,T,W,X= 100µm;

G,K= 50 µm;

U,Y= 20µm.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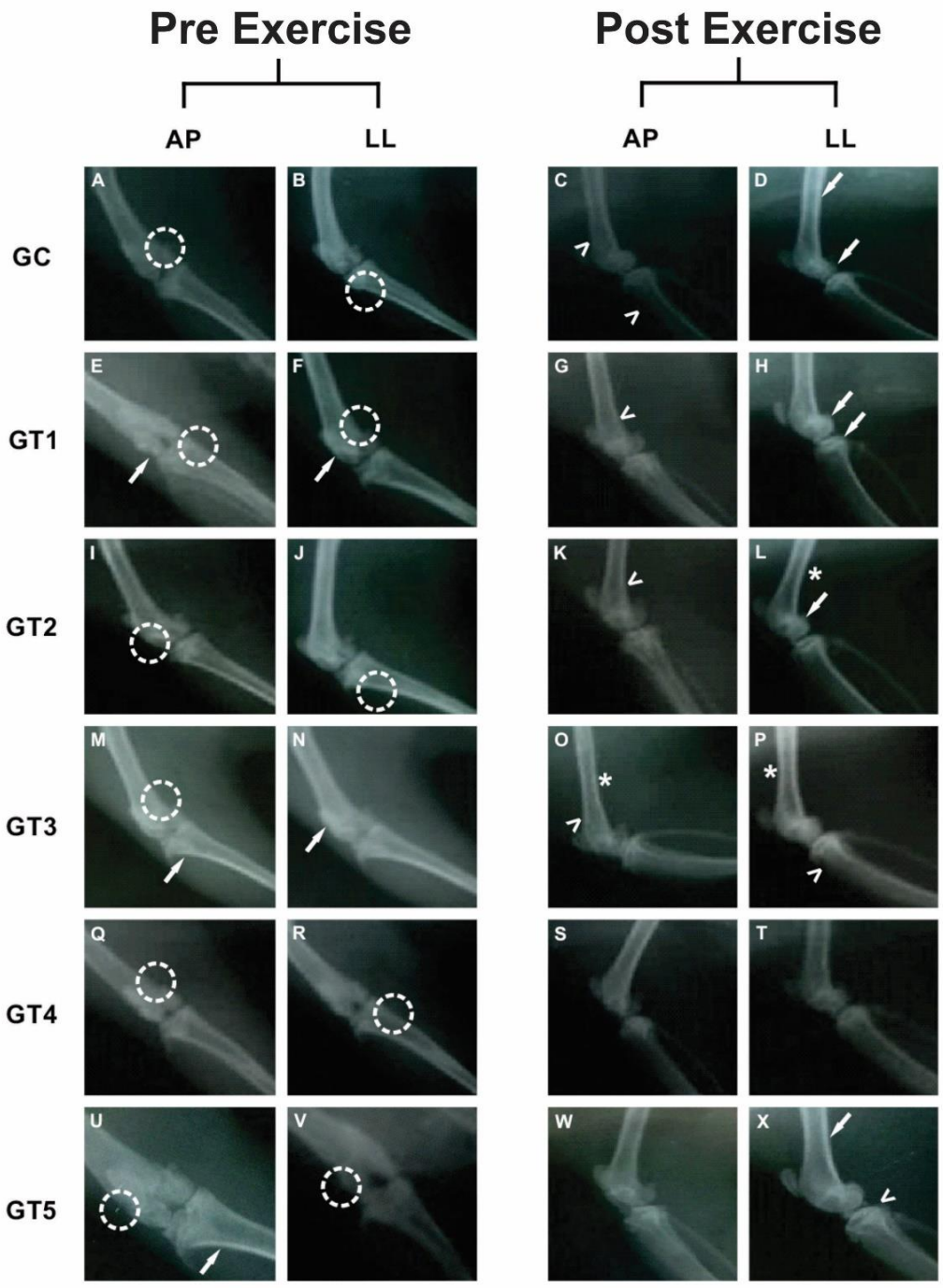
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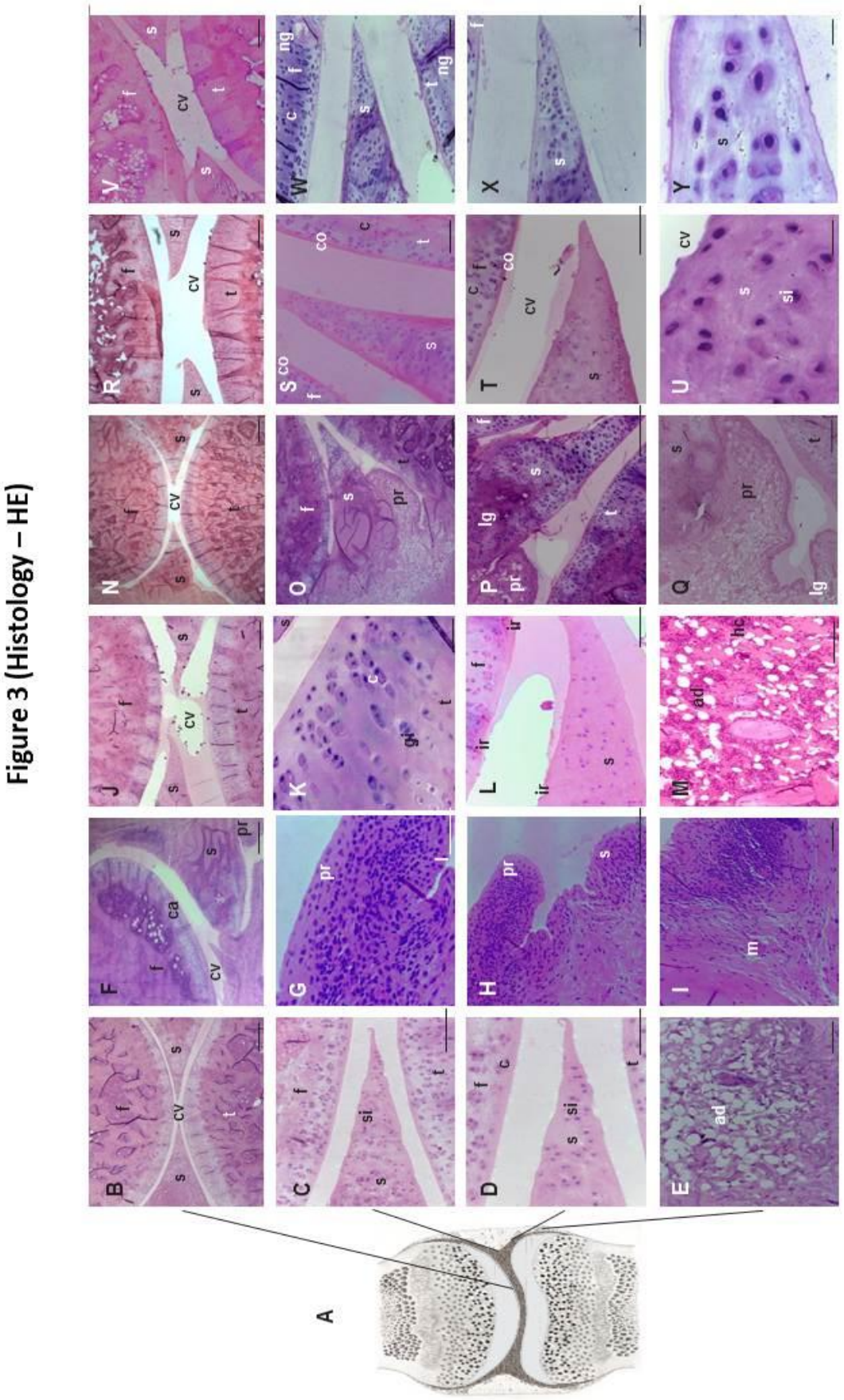

Figure 3 (Histology – HE)