CRISTIANO PEDROZO VIEIRA

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Campinas 19 de fevereiro de 2015.

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Orientador: Prof. Dr. Edson Rosa Pimentel

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ABSTRACT

Therapeutic nutrition is the administration of some nutrients, in higher doses than those recommended for the daily food needs that can prevent dysfunctions and act as pharmacological agents. Glycine has large beneficial effects in inflammatory and tumor processes. Green tea made from leaves and buds of the *Camellia sinensis* plant, is the second most consumed beverage in the world. The economic and social interest has gained space in the market and currently its consumption is part of the daily routine of many people who use this drink as a therapeutic purpose. Green tea has antimutagenic, antidiabetic, anti-inflammatory, antioxidant, antimicrobial and hypocholesterolemic properties. Tendinitis is recognized as a clinical problem that motivates the scientific community to investigate treatments that help in restoring the functional properties of tendons. The present study investigated the effect of green tea and/or diet rich in glycine after 7 and 21 days of tendinitis collagenase-induced. Biochemical, molecular, morphological and biomechanical tests were developed. Furthermore, tenocytes in culture were treated with glycine after inflammation induced by TNF-α. Our tests *in vivo* showed high concentrations of hydroxyproline and glycosaminoglycans in glycine and green tea group in 21 days of treatment. In biomechanical assay, green tea and glycine diet groups in 21 days showed a high biomechanical loads bore before rupture. In addition, better organization of collagen fibers was observed in green tea group in 7 days**.** Biochemical and molecular analyzes of myotendinous junction showed that the inflammation installed in osteotendinious region can cause significant change in that region. Remarkable changes were noted in metalloproteinases (MMP) such as MMP-2, MMP-8 and MMP-9 in animals with tendinitis treated with or without glycine and green tea. In the *in vitro* study, tenocytes from Achilles tendon were treated with TNF-α, or not following treatment with glycine in the culture medium. Before and 24 hours after inflammation was added glycine. Tenocytes inflamed and treated with glycine showed expression of collagen type I close to the treated groups with glycine previously and after the inflammation when compared to the control group. All treated groups showed less glycine MMP-2 expression. The activity of MMP-9 was high only in the group treated with glycine for 48 hours. In the cell migration assay results in 24 hours of treatment were similar to the control group. In general, both glycine and green tea influenced the synthesis of the tendon components, improve the organization of the collagenous fibers, increase the load resistance of the inflamed tendon and consequently accelerate the remodeling process after inducing

tendinitis. In addition, the treatment with glycine in tenocytes culture showed efficient reorganization of the extracellular matrix, confirming the results found *in vivo*.

Keywords: Extracellular matrix, inflammation, tendinopathy, tumor necrosis factor-alfa, collagen.

RESUMO

Nutrição terapêutica é a administração de alguns nutrientes, em doses maiores que as necessidades alimentares diárias que podem prevenir deficiências orgânicas e atuar como agentes farmacológicos. A glicina apresenta amplos efeitos benéficos em processos inflamatórios e tumorais. O Chá verde feito de folhas e brotos da planta *Camellia sinensis*, é a segunda bebida mais consumida em todo mundo. O interesse econômico e social tem ganhado espaço no mercado e atualmente seu consumo faz parte da rotina diária de muitas pessoas que utilizam essa bebida como uma finalidade terapêutica. O Chá verde possui propriedades antimutagênicas, antidiabéticos, antiinflamatórias, antioxidante, antimicrobial e hipocolesterolêmica. A tendinite é reconhecidamente um problema clínico que motiva a comunidade científica a buscar tratamentos que auxiliem no restabelecimento das propriedades funcionais dos tendões. O presente estudo investigou o efeito do chá verde e ou da ração rica em glicina após 7 e 21 dias da indução da tendinite com colagenase. Ensaios bioquímicos, moleculares, morfológicos e biomecânicos foram desenvolvidos. Além disso, tenócitos em cultura foram tratados com glicina após inflamação induzida por TNF-α. Nossos ensaios *in vivo* mostraram altas concentrações de hidroxiprolina e glicosaminoglicanos no grupo glicina e chá em 21 dias de tratamento. Nos ensaios biomecânicos os grupos chá verde e dieta de glicina em 21 dias suportaram maiores cargas biomecânicas antes da ruptura. Além disso, uma melhor organização das fibras de colágeno foi observada no grupo chá verde em 7 dias. Análises bioquímicas e moleculares da junção miotendínosa mostraram que a inflamação instalada na região osteotendinea pode provocar alterações significativas nesse local. Marcantes alterações foram notadas nas metaloproteínases (MMP) tais como MMP-2, MMP-8 e MMP-9 em animais com tendinite tratados ou não com chá verde e glicina. No estudo *in vitro*, tenócitos extraídos a partir de tendão de Aquiles foram tratados com TNF-α, seguindo ou não de tratamento com glicina em meio de cultura. Antes e após 24 horas da inflamação foi adicionado glicina. Tenócitos inflamados e tratados com glicina mostraram expressão de colágeno tipo I próxima aos grupos tratados com glicina previamente e depois da inflamação quando comparado ao grupo controle. Todos os grupos tratados com glicina mostraram menor expressão de MMP-2. A atividade da MMP-9 foi alta apenas no grupo tratado com glicina em 48 horas. A concentração de ácido urônico foi menor no grupo tratado com glicina 24 horas após a inflamação. No ensaio de migração celular, resultados em 24 horas de tratamento foram similares ao grupo controle. Em geral, tanto a glicina quanto o chá verde influenciam na síntese dos componentes do tendão, melhoram a

organizaçao das fibras colagênicas, aumentam a resistência a cargas do tendão inflamado e consequentemente aceleram o processo de remodelamento após indução da tendinite. Além disso, o tratamento com glicina em cultura de tenócitos mostrou uma reorganização eficiente da matriz extracelular, corroborando com os resultados encontrados *in vivo*.

Palavras-chave: matriz extracelular, inflamação, tendinopatia, fator de necrose tumoral alfa, colágeno.

SUMÁRIO

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“Os imprevistos da vida são como uma nuvem: as vezes branca, limpa.
As vezes negra, carregada.
Mas atrás dela sempre haverá um céu azul”
(Jade Ohara Mesquita).

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1. INTRODUÇÃO

Os tendões e ligamentos são geralmente acometidos por diferentes lesões incluindo processos inflamatórios, podendo estes desencadearem rupturas parciais ou totais no tecido. O tratamento do processo inflamatório agudo em tendão é amplamente estudado em várias pesquisas. Trabalhadores e atletas são frequentemente afastados das suas atividades por apresentarem tendões com inflamação (JÄRVINEM *et al*., 2005, RUSCHEL *el al.,* 2009, DARIO *et al.,* 2010). A inflamação é o primeiro processo a ser detectado após uma lesão. Contudo, quando a inflamação não é controlada, processos como tendinite e tendinose podem ser evidenciados.

1.1 CARACTERÍSTICAS BIOQUÍMICAS E ESTRUTURAIS DO TENDÃO

Os tendões possuem variações em relação à organização, distribuição e quantidade de seus componentes, dependendo dos tipos de forças mecânicas que estejam atuando sobre ele, como já observado em tendões de bovinos (VOGEL *et al.,* 1986; EVANKO & VOGEL 1990), coelhos (MERRILEES & FLINT, 1980), anfíbios (CARVALHO & VIDAL, 1994; CARVALHO & FELISBINO, 1999), ratos (COVIZI *et al.,* 2001), porcos (FEITOSA *et al.,* 2002 a,b; FEITOSA *et al*, 2006) e frangos (BENEVIDES *et al.,* 2004). De acordo com a organização, quantidade e propriedades dessas macromoléculas na MEC é possível obter uma diversidade de formas adaptadas às necessidades de cada tipo de tendão (CHIQUET, 1999).

Os tendões são tecidos conjuntivos densos, bem organizados e fibrosos, que geralmente transmitem a força gerada no músculo ao osso, tornando possível o movimento articular. O tendão calcanear transmite ao osso calcâneo as forças de tensão geradas pela contração do músculo tríceps sural, promovendo o movimento da articulação talocrural.

1.1.1 COMPONENTES FIBRILARES DO TENDÃO

A matriz extracelular (MEC) do tendão é formada por fibras de colágenos (65-80%), predominantemente colágeno tipo I (cerca de 95%) (BENJAMIN *et al.*, 2008) e podem ser encontrados o colágeno tipo II em região de compressão dos tendões, o tipo III, que está relacionado ao controle do diâmetro fibrilar e compõe fibrilas heterotípicas com os colágenos tipos I e V, o tipo VI que é encontrado na parte mediana e na entese do tendão calcanear, além dos tipos XII e XIV que participam na regulação do crescimento e associação fibrilar (YOUNG *et al.,* 2000, AHTIKOSKI *et al*., 2003).

O colágeno fibrilar possui uma estrutura longa, rígida e estável com conformação em fita-tripla helicoidal, composta de três cadeias polipeptídicas em que consiste de uma sequência de aminoácido repetida de glicina, Gly-X-Y, onde X e Y podem ser quaisquer aminoácidos. Cerca de um terço das posições X são ocupadas por prolina e um número semelhante de posições Y são 4-hidroxiprolina, resultante de modificações pós-traducionais de prolina. A prolina, hidroxiprolina e glicina encontradas na molécula de colágeno são fundamentais na estabilização da tripla hélice de colágeno (PIEZ & REDDI, 1984). Resíduos de lisina encontrados na molécula de colágeno passam por um processo de hidroxilação através da enzima Lisil-hidroxilase (CARVALHO & PIMENTEL, 2007) e posterior formação do grupo aldeído pela ação da Lisil-oxidase, favorecendo a formação de ligações cruzadas intra e intermoleculares, importantes para o aumento da capacidade das fibrilas de colágeno de resistir às forcas de tensão. Quanto maior o número de ligações cruzadas, maior será a resistência à força tensora (JAMES *et al,* 2008).

As moléculas de colágeno arranjam-se formando fibrilas, que por sua vez formarão as fibras que constituem os feixes que formam os tendões. Esse arranjo estrutural das fibrilas e associação com outros elementos da matriz são responsáveis pelas propriedades biomecânicas como flexibilidade, resistência e até mesmo certa elasticidade dos tendões (BENJAMIN *et al.,* 2008). As unidades estruturais das fibras colagênicas são ligadas dentro de feixes pelo endotendão (Figura 1), que é formado por tecido conjuntivo frouxo e tem papel fundamental por possuir redes de transmissão vascular, linfático e neural (JAMES & WANG 2006, JAMES

et al., 2008). Os feixes de fibras formam fascículos, esses são rodeados pelo epitendão, que também possui a função de nutrir o tecido (WANG, 2006).

Os feixes de fibras de colágeno do tendão possuem um padrão ondulado chamado “crimp”, este é facilmente detectado em microscopia de polarização como regiões transversais claras e escuras. O “crimp” fibrilar atua na absorção de energia durante o estresse e a deformação inicial pelos quais os feixes de colágeno são submetidos quando o tendão é tensionado (FRANCHI et al., 2007).

Outro componente fibrilar encontrado na MEC, é uma pequena quantidade de fibras elásticas (~2%) (JOZSA & KANNUS 1997, AQUINO *et al.,* 2005) que se dispõem ao longo de algumas fibras de colágeno, contribuindo para a distensão inicial dos tendões quando submetidos às cargas unidirecionais durante as atividades desportivas ou diárias (AQUINO *et al.,* 2005).

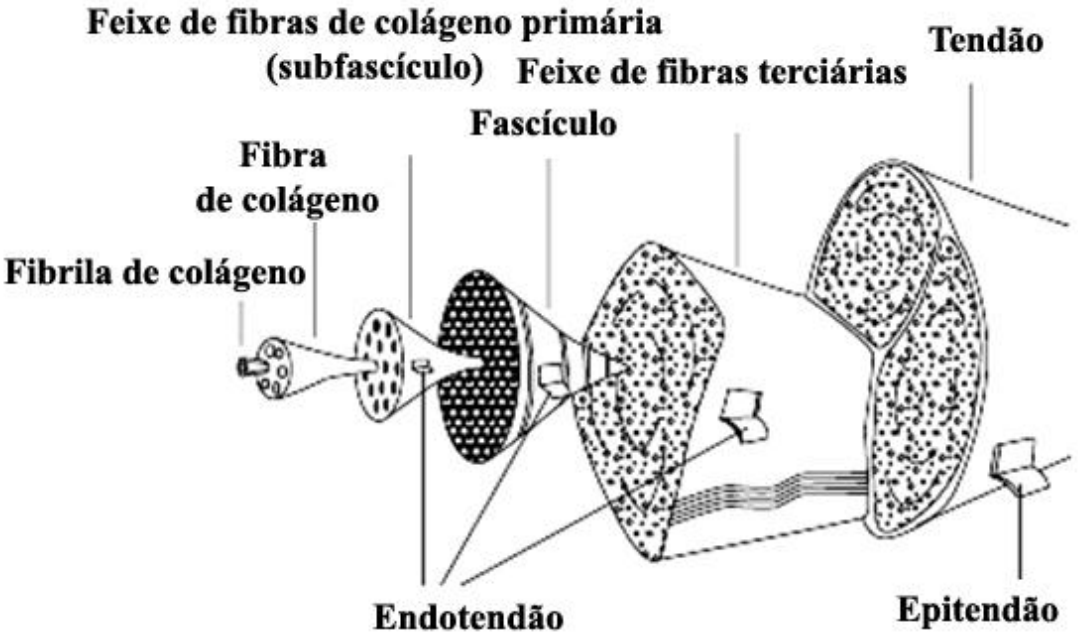

Figura 1: Organização hierárquica da estrutura do tendão (modificado de KANNUS, 2000)

1.1.2 COMPONENTES NÃO FIBRILARES DO TENDÃO

A MEC do tendão também é constituída por componentes não-fibrilares como os glicosaminoglicanos (GAGs), proteoglicanos (PGs), glicoproteínas não-colagênicas e tenócitos (JAMES *et al.,* 2008).

Os GAGs são cadeias polissacarídicas não ramificadas, compostas de unidades repetidas de dissacarídeo. Os PGs, especialmente, os de alto peso molecular encontrados em regiões em que o tendão passa junto a uma protuberância óssea ou contorna uma articulação, são responsáveis pela resistência à compressão no tecido (ALIMOHAMAD *et al.,* 2005) e consistem de um esqueleto de proteína central e cadeias de GAG ligadas covalentemente. Os PGs, devido à grande quantidade de cargas negativas, formam géis hidratados que são importantes na manutenção do espaçamento entre as fibras de colágeno, facilitando o deslizamento entre as mesmas e conferindo ao tecido a propriedade de viscoelasticidade (YANG *et al.,* 2008). Os PGs de baixo peso molecular, como o fibromodulim e o decorim, estão presentes na matriz interfibrilar mantendo e regulando o diâmetro das fibrilas de colágeno, (WATANABE *et al.,* 2005) além de estarem envolvidos na modulação da MEC e no comportamento celular. Os GAGs também associam-se às proteínas fibrosas da matriz, como o colágeno, gerando estruturas supramoleculares (VIDAL & MELLO, 1984).

Proteínas não colagênicas correspondem a 0,5% do peso úmido do tendão. (FRANK *et al.,* 1987), as quais atuam entre outras funções na interação célula-matriz permitindo uma comunicação entre ambas, importante para vários processos fisiológicos. A tenascina-C contribui na estabilidade mecânica da matriz através da interação entre as fibrilas de colágeno. Fibronectina e trombospondina tem sua síntese aumentada na cicatrização. Ambas participam de processos de reparo de tendões (WANG, 2006; JAMES *et al.,* 2008).

A água representa aproximadamente 55% do peso do tendão, importante para a redução da fricção, facilitando o deslizamento das fibrilas em respostas a cargas mecânicas (JAMES *et al.,* 2008).

Entre os feixes de colágeno são encontradas células como os tenócitos (células tipo fibroblasto) que possuem papel importante na produção dos elementos da MEC, auxiliando no estágio proliferativo e no remodelamento quando o tecido sofre injúrias (O'BRIEN, 1997; WANG, 2006; BENJAMIN *et al.,* 2008). A quantidade de células presentes nos tendões e a espessura dos feixes de fibras de colágeno variam de acordo com a idade, sendo que tendões de animais adultos têm feixes de colágeno mais compactos e menor número de células, enquanto que animais mais jovens apresentam grande celularidade e feixes de fibras mais finos (VIDAL & CARVALHO, 1990). Tenócitos e os componentes da MEC formam uma interação célula-matriz que permite que as células detectem e respondam a estímulos mecânicos. Em geral, fibroblastos através de receptores de superfície celular interagem moléculas da MEC com seu citoesqueleto, assim permitindo que diferentes vias de sinalização possam ser desencadeadas, quando necessárias (CHIQUET 2004, FRANCHI *et al.,* 2007).

Os tendões possuem uma vascularização relativamente limitada. A área ocupada por vasos sanguíneos representa 1-2% de toda a MEC, provenientes principalmente do epitendão (KJAER, 2004). Em regiões onde os tendões recobrem superfícies ósseas há uma diminuição ainda maior do suprimento sanguíneo, e essa característica pode ter uma relação direta com as forças de compressão aplicadas nestas regiões (BENJAMIN *et al.,* 2008).

A integridade da matriz envolve a síntese e degradação dos componentes da MEC (GONZÁLEZ *et al.,* 2002). As enzimas proteolíticas essenciais para a degradação e remodelamento tecidual são endopeptidases dependentes de cálcio (Ca^{++}) ou zinco (Zn^{++}) chamadas metaloproteinases (MMPs). A família das MMPs compreende pelo menos 23 membros que são reguladas ao nível de transcrição gênica e ativação enzimática por inibidores teciduais de metaloproteinase (TIMP). Em condições normais, as MMPs estão presentes em níveis baixos, geralmente em forma latente, e são ativadas para manter o remodelamento fisiológico do tecido. Esses elementos são os grandes responsáveis pela homeostasia do tecido após uma condição patológica ou remodelamento de matriz (PARKS *et al.,* 2004; CLUTTERBUCK, 2010). As principais MMPs estudadas em tendões são MMP-2 e MMP-9, ambas envolvidas em processos de degradação e consequente reorganização do tecido. MMP-9 é principalmente encontrada em processos inflamatórios, sendo secretada principalmente por células inflamatórias (CLUTTERBUCK, 2010; VIEIRA et al., 2012).

1.2 TENDINOPATIAS

A tendinopatia é caracterizada por dor e degeneração do tendão, sendo associada com o uso repetitivo e de sobrecarga. A condição não é apenas restrita à atletas, 25 a 30% das pessoas afetadas não são atletas, e essa condição pode favorecer a perda de um número significativo de dias de trabalho e ter um enorme impacto financeiro para a sociedade, adicionando substancialmente aos trabalhadores os custos de compensação. Na prática esportiva, 50 % das lesões encontradas são injúrias tendíneas, com aumento de sua incidência (JÄRVINEM *et al.,* 2005; RUSCHEL *et al.,* 2009; DARIO *et al*., 2010; FUNG *et al.,* 2010). A prevalência de tendinopatia no tendão de Aquiles é cerca de 11% em corredores, 8% em dançarinos, e menos de 2% em jogadores de tênis (MAFFULLI *et al.,* 2005).

A Tendinite é uma condição no qual o tendão agredido produz uma resposta inflamatória. No caso de animais, a afecção com a maior incidência na carreira do cavalo atleta é a tendinite, estimada em 11% a 46% (DAHGREN, 2007). Essa lesão causada por movimentos repetitivos pode exceder a capacidade de reparação do tendão, este poderá romper-se, ocasionando uma inflamação. Sobrecarga mecânica, hipertemia local, isquemia ou hipoxemia são fatores apontados como as causas mais comuns das lesões tendíneas (McLAUCHLAN e HANDOLL, 2003). Os principais sintomas da tendinite são a dor, sensação de queimação e edema (MAFFULLI *et al.,* 2005; MARCOS *et al*., 2012).

Em todos os casos de lesões tendíneas, a inflamação é o início do processo de reparação tecidual. Embora, dependendo de sua gravidade essa pode desencadear a ruptura parcial ou total do tendão (SHARMA e MAFFULLI, 2006). A ruptura tendínea é uma grande preocupação para cirurgiões, pois a sutura é uma das intervenções que altera a organização dos feixes de colágeno. O tendão quando sofre ruptura, não retorna à sua condição normal (JOZSA e KANNUS, 1997; JÄRVINEM *et al.,* 2005).

O reparo do tendão lesado é semelhante ao reparo de outras lesões de tecidos conjuntivos, em que ocorrem um processo ordenado de estágios múltiplos, como proliferação e migração de muitos tipos celulares. Uma cascata de acontecimentos contribuem para que isso se torne possível, tais como a liberação de quimioatraentes e infiltração de células como neutrófilos e macrófagos, ativação de enzimas específicas na degradação e regeneração da matriz, ocasionada pelas metaloproteínases (MMP) e seus inibidores (TIMP), fatores de crescimento tais como

TGF-β envolvido com a produção de uma nova MEC pelos fibroblastos e que será o ponto chave para a recuperação do tendão (MARSOLAIS *et al.,* 2007; ENOCH e LEAPER, 2007). O acúmulo de células inflamatórias baseia-se no recrutamento de novas células provenientes do sistema circulatório ou na mitose de células inflamatórias residentes MARSOLAIS *et al.,* 2007; SHÖNBEIN-SCHMID, 2006). Os neutrófilos são geralmente as primeiras células a aparecer nos locais da inflamação, e são os responsáveis por liberar uma variedade de agentes destrutivos, tais como radicais livres, proteases e citocinas que atraem macrófagos. Sabe-se que os fibroblastos entram em apoptose quando o tendão está sob processo inflamatório. Indicando que a apoptose desempenha um papel na degeneração do tendão (HOSAKA *et al.,* 2005; EGERBACHER *et al.,* 2008). Além de fagocitar neutrófilos e demais células apoptóticas, macrófagos podem liberar diferentes fatores de crescimento que induz a síntese de matriz extracelular e inibe sua degradação. Muitas citocinas são liberadas durante o processo inflamatório. Entre elas, IL-1β que auxilia a desencadear alguns sintomas da inflamação, como a febre, e é capaz de induzir a produção de algumas citocinas, tais como IL-2, IL-6 e IL-8, além de regular a expressão da MMP-9, enzima característica de processos inflamatórios (YOO *et al*., 2002; ALTEN *et al.,* 2008).

Colágeno e proteoglicanos são secretados e quaisquer rupturas no tecido são eventualmente reparadas por fibras de colágeno, principalmente do tipo III, sendo posteriormente substituídas por colágeno do tipo I (SHARMA e MAFFULLI, 2006; ENOCH e LEAPER, 2007). O tendão apresenta a resistência e a firmeza, pelas ligações cruzadas que ocorrem entre as moléculas de colágeno, nesse processo de recuperação do tecido, as fibras danificadas são reparadas e podem ser sintetizadas novas fibras para reaver a força e rigidez características do tendão (KANNUS, 2000).

A tendinose é uma forma crônica de tendinopatia e é conhecida por induzir uma matriz colágena danificada e desorganizada (JÄRVINEM *et al.,* 2005). O uso excessivo e ou casos de esforços de algumas articulações promovem a ocorrência de microrrupturas na estrutura do tendão, mais especificamente no local onde se insere ao osso. Posteriormente, ocorre a deposição de matriz e células nas lesões, diminuindo a locomoção do tendão e facilitando sua ruptura. Neste caso, pode ter existido um processo de tendinite, mas a patologia vai além, uma vez que a cicatrização e o processo de reparo nessa área foi defeituosa e deficiente. Embora,

ocorra similaridades dos dois processos, a tendinite é caracterizada pelo ínicio da inflamação nos tendões podendo evoluir ou não para um quadro mais severo como a tendinose.

1.3 ALTERNATIVAS PARA TENDINITE

Atualmente, o tratamento da tendinite consiste basicamente de alguns procedimentos. A primeira medida que deve ser tomada para tratar a tendinite é interromper qualquer movimento no local onde ela estiver situada, isto é, imobilizar a articulação. Entre os medicamentos recomendados está o paracetamol, uso de antiinflamatório não-esteroidal e corticosteroides (em casos mais graves). Fisioterapia e eletroacupuntura são outras opções para o tratamento da tendinite. Tratamento cirúrgico é necessário em casos mais graves (BRETT *et al*., 2008; WEINFELD *et al.,* 2014)

O uso de terapia alternativas para diferentes enfermidades tem sido atribuídas como um fator positivo nos dias de hoje. O uso de plantas medicinais como o chá verde e/ou de suplemento de aminoácidos, como a glicina tem sido complementos saudáveis para muitos tipos de patologias (CHATTOPADHYAY *et al.,* 2004; AMRA PERVA-UZUNALIC *et al.,* 2006; FIGUEIREDO *et al.,* 2009; CARMANS *et al.,* 2010; STOFFELS *et al.,* 2011). Visando um modelo de tratamento alternativo e eficiente, o uso de ambos elementos atribuídos (separadamente ou em conjunto) em animais com tendinite, pode ser um modelo eficiente e sem efeitos colaterais ao tratamento dessa condição.

1.4 GLICINA

A glicina tem papel crucial na nutrição e metabolismo. Na composição da estrutura de enzimas, a glicina provem flexibilidade para seus sítios ativos. Além disso, há multiplas vias da utilização da glicina para gerar glutationa, creatinina, purinas (RNA e DNA), grupo heme (hemoglobinas) e serina. A glicina é o aminoácido mais encontrado no ácido biliar de mamíferos, desempenhando um papel importante na digestão e absorção de lipídios e vitaminas liposolúveis. Também, a glicina é um neutrotransmissor do sistema nervoso central, possuindo

comportamento regulador, na ingestão de alimentos e na homeostase do corpo (WANG *et al.,* 2013)

A glicina é um aminoácido com estrutura molecular simples e, apresenta amplos efeitos biológicos, como moduladora na cascata inflamatória sistêmica e melhoria na microcirculação. Além de auxiliar na inibição de moléculas pro-inflamatórias tais como TNF-α e IL-1β (FIGUEIREDO *et al.,* 2009). Em adição, a glicina possui propriedades terapêuticas em muitos modelos de processos inflamatórios (HARTOG *et al*., 2007; WEELER *et al.,* 2009; CARMANS *et al.,* 2010; STOFFELS *et al.,* 2011), na prevenção e tratamento para cânceres (WEELER *et al.,* 2009) e efeitos benéficos contra a toxicidade do fígado (LI *et al.,* 2001; MIKALAUSKAS *et al.,* 2011).

A glicina é um aninoácido não essencial e pode ser encontrada no leite, queijos, iogurte, ovos, peixes e carnes. No colágeno, a glicina está presente em cada terceira posição da montagem da triplice hélice da proteína. A glicina compõe em torno de 35% da composição da molécula de colágeno (ALBERTS *et al.,* 2010). A capacidade metabólica da biossíntese de glicina não satisfaz a necessidade da síntese de colágeno, e um suplemento para garantir o mebolismo saudável é necessário (MELÉNDEZ-HEVIA *et al.,* 2009). Uma dieta rica em glicina pode auxiliar a formação de novas fibras colágenas, quando este passa por um processo como a tendinite, permitindo possivelmente uma recuperação mais rápida e de uma forma mais eficaz.

1.5 CHÁ VERDE (*Camellia sinensis*)

Camellia sinensis (L.) Kuntze é uma árvore de pequeno porte, de origem asiática, pertencente à família Theaceae (DUARTE e MENARIM, 2006). Folhas recém-coletadas e imediatamente estabilizadas e sem fermentação caracterizam o chá verde. Quando submetidas à fermentaçào, rápida ou prolongada, constituem o tipo oolong e o chá preto, respectivamente (KUHN e WINTSON 2000; CABREBRA et al., 2006).

O interesse econômico e social do chá verde tem ganhado espaço no mercado e atualmente seu consumo faz parte da rotina diária de muitas pessoas que utilizam essa bebida com finalidade terapêutica. Embora muitos benefícios para saúde tivessem sido atribuídos ao consumo de chá verde desde o início de sua história, a investigação científica sobre esta bebida

e seus componentes está em curso há menos de três décadas (CABRERA *et al.,* 2006). As propriedades farmacológicas do chá verde são atribuídas pelo seu alto conteúdo de polifenóis (catequinas e ácido gálico), principalmente epigalocatequina-3-galato (EGCG), onde sabe-se que maior parte do efeito do chá é atribuído a esse polifenol, além de conter carotenóides, acido ascórbico, minerais (Cr, Mn, Se ou Zn), e componentes fotoquímicos (CHATTOPADHYAY *et al.,* 2004; EFTHIMIOU e KUKAR, 2006).

A composição química do extrato da planta de chá verde (*Camellia sinensis*) envolve: proteínas (15-20%), enzimas representam a maior fração das proteínas; aminoácidos (1-4% do peso seco), carboidratos (7,5% do peso seco), lipídios, vitaminas B, C e E, cafeína, minerais e oligoelementos (5% do peso seco) tais como Ca, Mg, Cr, Mn, Fe, Cu, Zn, Mo, Se, In, P, Co, Sr, Ni, K, F e Al (CABRERA *et al.,* 2006).

A concentração de catequinas, flavonóides e proantocianidinas encontrada no chá verde é determinada por diferentes métodos de extração (Tabela 1). As principais catequinas encontradas são: EGCG (epigalocatequina-3-galato), EGC (epigalocatequina), ECG (epicatequina-3-galato), EC (epicatequina) (AMRA PERVA-UZUNALIC *et al.,* 2006; CHATTOPADHYAY *et al.,* 2004). O extrato aquoso dos polifenóis do chá verde possui propriedades antimutagênicas, antidiabéticos, antiinflamatórias, antimicrobial e hipocolesterolêmica (CABRERA *et al.,* 2006; CLARK 2007; SHARANGI 2009; BASU *et al.,* 2010; EL-MOWAWFY *et al.,* 2010).

Solvente	**Temperatura (°C)**	**Eficiência de extração (%)**		**Conteúdo (g/kg do extrato seco)**		
Água		**Cafeína**	**Catequinas**	**Catequinas**	**Flavonóis**	**Protoantocianinas**
	70	56.0	65.9	430	17.4	16.2
	80	81.5	84.4	448	9.1	16.9
	85	63.8	61.3	385	17.0	16.8
	95	89.1	57.2	258	13.4	13.7
	100	57.0	37.3	237	11.4	12.0

Tabela 1: Extração dos componentes do chá verde em água em diferentes temperaturas (AMRA PERVA-UZUNALÍC *et al.,* 2006).

Estudos revelaram que o EGCG inibe o fator de transcrição nuclear kappa-B (NF-kB) e IL-1β, resultando numa redução de óxido nítrico e prostaglandina E2, assim como, tem uma papel importante sobre a quinase c-Jun-N-terminal, que é primariamente responsável por doenças inflamatórias e degenerativas (AUGUST *et al.,* 1999; PENG *et al.,* 2006). EGCG também promove a inibição da expressão e atividade de MMP-1 e MMP-13 *in vivo* (EFTHIMIOU e KUKAR, 2006), embora o chá verde ainda não tenha sido relacionado com seu efeito anti-inflamatório sobre a tendinite.

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OBJETIVOS

Gerais: Avaliar os efeitos do chá verde e/ou da dieta rica em glicina na tendinite do tendão calcanear de ratos e verificar o efeito da glicina como tratamento para a inflamação induzida por TNF-α em cultura de tenócitos.

Específicos

1. Quantificar proteínas não-colagênicas, GAGs sulfatados e hidroxiprolina em tendões dos animais que receberam os dois tipos de tratamento.
2. Analisar a organização das fibras de colágeno dos grupos experimentais em 7 e 21 dias de tratamento.
3. Identificar a presença e a atividade enzimática das MMP-2 e MMP-9, dos grupos experimentais.
4. Identificar através de western blotting a presença dos colágenos tipo I.
5. Analisar as propriedades biomecânicas do tendão dos grupos experimentais.
6. Avaliar o aspecto estrutural tais como contagem celular e medida de epitendão dos grupos experimentais.
7. Detectar a expressão de colágeno 1, MMP-2, TIMP1, TIMP2, MMP-3 dos tenócitos com inflamação induzida e tratados com glicina através de PCR.
8. Observar atráves do ensaio de migração e proliferação celular os grupos experimentais envolvendo tenócitos.
9. Quantificar dissacarídeos condroitim sulfato 2S, 4S e 6S dos grupos experimentais dos tenócitos.

CAPÍTULO 1

Glycine improves biochemical and biomechanical properties following inflammation of the Achilles tendon

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ABSTRACT

Tendinopathy of the Achilles tendon is a clinical problem that motivates the scientific community to search for treatments that assist in restoring its functional properties. Glycine has broad biological effects, acting as a modulator of the inflammatory cascade, and is the predominant amino acid in collagen. A 5% glycine diet provided beneficial effects against toxicity and inflammation since glycine may restructure the collagen molecules faster due to its broad anti-inflammatory effects. The purpose was analyze the effects of a 5% glycine diet in rats as a treatment for the inflammatory process. The experimental groups were as follows: C (control group), G1 and G3 (inflammatory group), and G2 and G4 (glycine + inflammatory group). G1 and G2 were euthanized 8 days following injury, and G3 and G4 were euthanized 22 days following injury. The concentrations of hydroxyproline, non-collagenous proteins, and glycosaminoglycans, as well as the activity of MMP-2 and -9 were analyzed. Biomechanical and morphological tests were employed. Higher concentrations of hydroxyproline and glycosaminoglycans were found in G4 and an increased activity of MMP-2 was found in G2. Higher birefringence was noted in group G2. The biomechanical results indicated that the tendon was more resistant to loading to rupture upon treatment with a glycine diet in group G4. Glycine induced the synthesis of important components of the tendon. A rapid remodeling was noted when compared with the inflamed-only groups. These data suggest that glycine may be a beneficial supplement for individuals with inflammation of the Achilles tendon.

Keywords: Tendinopathy; Achilles tendon; glycine; inflammatory process; extracellular matrix;

INTRODUCTION

Tendon injuries often occur in tendons that are exposed to high mechanical forces and that undergo more extensive matrix remodeling, such as the Achilles, supraspinatus and patellar tendons (Benjamin et al., 2008; Wang et al., 2012). The biomechanical properties of tendons depend on the structural arrangement of the collagen fibrils, fiber diameter, and the molecular aggregation of the collagen. The fibrils aggregate into collagen fibers that are responsible for the resistance of the tendon, and the efficiency of this mechanism depends on the parallel arrangement of these fibers in the direction of the tension forces (James et al., 2008; Wang et al., 2012; Aro et al., 2012). However, biomechanical properties of the tendons are often compromised and the tendon may undergo significant changes following injuries (Maganaris et al., 2004).

The incidence of Achilles tendon lesions is common, indeed increasing in sports, comprising 30 to 50% of the total number of injuries (Järvinem et al., 2005; Ruschel et al., 2009; Dario et al., 2010; Fung et al., 2010). Each year in the United States, there are about 16.4 million cases of tendon and ligament injuries, and about 100,000 cases that involve the Achilles tendon (Järvinem et al., 2005). Antibiotics and various medications are frequently used to treated these injuries (Kader et al., 2002; de Olivieira et al., 2013). Tendon injuries are difficult to repair surgically and are typically slow to heal. Furthermore, is known that after the tendon ruptures, it does not return to its pre-injury condition (Jozsa and Kannus, 1997).

The extracellular matrix (ECM) components of the tendons undergo structural and biochemical changes during injuries caused by the local inflammation (Vieira et al., 2012; Vieira et al., 2013) and with partial ruptures (Almeida et al., 2012; Guerra et al., 2013). In the inflammatory process, macrophages, monocytes and neutrophils are primarily responsible for the action of enzymes and subsequent degradation of ECM components. Various types of collagenases are involved in inflammation in addition to the matrix metalloproteinases (MMP) that include MMP-2 and MMP-9. A balance between MMPs and their inhibitors is important to maintain and prevent excessive tissue degradation (Gill and Parks, 2008; Clutterbuck et al., 2010). Furthermore, the molecular

mechanisms of tendinopathy are still unclear, and therefore, the results of current treatments are largely empirical and often less effective for these types of injuries (Wang et al., 2006).

Glycine is an amino acid with a simple molecular structure, and it displays important biological activities by acting as a modulator of the systemic inflammatory cascade, improving the microcirculation and assisting in the inhibition of TNF-α and IL-1β (Hartog et al., 2007; Figueiredo et al., 2009). Many studies have proposed glycine as a useful treatment for many types of inflammatory processes (Hartog et al., 2007; Weeler et al., 2009; Carmans et al., 2010; Stoffels et al., 2011). In addition, glycine is used in the prevention and treatment of cancers such as melanomas (Weeler et al., 2009). A 5% glycine diet has provided beneficial effects against liver toxicity and inflammation in rats. (Li et al., 2001; Mikalauskas et al., 2011). The metabolic capacity for glycine biosynthesis does not satisfy the need for collagen synthesis, thus dietary sources are important for normal metabolism (Meléndez-Hevia et al., 2009).

The hypothesis that was tested in this study was whether a 5% glycine diet could accelerate recovery of the biochemical and biomechanical properties of tendons following inflammation. The collagenase-induced inflammation model in rats was employed to test this hypothesis. The main objective of this study was to evaluate glycine supplementation as a viable alternative or supplementary treatment for Achilles tendon inflammation.

MATERIAL AND METHODS

EXPERIMENTAL DESIGN

Animal care was in accordance with the European Convention for the Protection of Vertebrate Animals used for Experimental and Other Scientific Purposes, and it was consistent with the ethical principles of animal experimentation adopted by the Brazilian College of Animal Experimentation. The protocol was approved by the Ethics Committee on Animal Experiments of the State University of Campinas (UNICAMP), SP, Brazil, and filed under no. 2307-1.

In this study, young adult (60 days) male Wistar rats, weighing on average 300 grams, were obtained from the Multidisciplinary Center for Biological Investigation (CEMIB) of the State University of Campinas. The rats were housed two per cage under a 12-h light/dark cycle at 23°C with free access to standard rat chow and water prior to the treatment with glycine and the control diet (without glycine). The animals were divided into five groups: C, control group; G1 and G3, inflamed group; and G2 and G4, inflamed and supplied with a glycine diet. Groups G1 and G2 were euthanized on day 8 after induction of inflammation, and groups G3 and G4 were euthanized on day 22 after induction of inflammation.

To induce inflammation, the animals were anesthetized with isoflurane (Forame) and given an intratendinous injection in the right Achilles tendon of 10µl of collagenase (10 mg/ml; Sigma, C6885) dissolved in sterile buffered saline (PBS) (Lake et al., 2008). After induction of tendinitis, the animals in the treatment groups (G2 and G4) received a diet containing 5% glycine. The animals in the induced tendinitis groups G1 and G3 and the control group (C) control received the control diet without glycine. The treatments lasted 7 days for groups G1 and G2 and 21 days for groups G3 and G4. The entire right tendon was taken after euthanasia. Five animals in each group were used for biochemical studies and the other five were used for morphological analysis. Six animals per group were used in biomechanical analyses. The left tendon was not used.

A diet containing 5% glycine (AJINOMOTO, BRAZIL) was provided by the Institute of Biology, Animal Physiology, State University of Campinas, following the guidelines of AIN-93M (Reeves et al., 1993). The diet was administered to the animals and the consumption was recorded daily.

EXTRACTION PROCEDURES

The Achilles tendon was removed and treated with 50 volumes of 4 M guanidine hydrochloride (GuHCl) containing 20 mM EDTA and 1 mM PMSF in 50 mM sodium acetate buffer, pH 5.8, for 24 h at 4°C with stirring (Heinergard and Sommarin, 1987). Then, the material was centrifuged (13,000×g, 25 min, 4°C), and the supernatant was used for the quantification of non-collagenous protein and glycosaminoglycans (GAGs).

QUANTIFICATION OF PROTEINS AND SULFATED GAGs

The extracts collected from the experimental groups were used to quantify the relative amounts of protein according to the method of Bradford (1976). Bovine serum albumin was employed as a standard. The quantification of GAGs was performed using the dimethylmethylene blue method (Farndale et al., 1986) with chondroitin sulfate as the standard. The absorbance was measured at 595 nm for proteins and at 540 nm for GAGs using an ASYS Expert Plus Microplate Reader (Biochrom, Holliston, MA, USA).

HYDROXYPROLINE QUANTIFICATION

Fragments of the tendon were hydrolyzed in 6 N HCl (1 mL/10 mg of tissue) for 4 h at 130°C. Then, the lysate was treated with 1.41 % chloramine-T solution and 15 % p-dimethylaminobenzaldehyde, as described by Stegemann and Stalder (1967). After incubation for 15 min at 60 °C, the hydroxyproline solution was cooled, and the absorbance was measured at 550 nm using an Ultrospec 2100 Pro spectrophotometer (Amersham Biosciences, England).

ZYMOGRAPHY FOR MMP-2 AND 9

The tendons were treated according to the method described by Marqueti (2006). For protein extraction, the fragments of the tendon were immersed in a solution of 50 mM Tris-HCl (Synth) pH 7.4, 0.2 M NaCl, 10 mM CaCl2 (Ecibra, São Paulo, SP, Brazil), and 0.1% Triton X-100 (Nuclear) with 1% of a protease inhibitor cocktail (Sigma) at 4°C for 2 h. After the first extraction, the samples were incubated with 1/3 of the volume of the solution described above at 60°C for 5 min. Then, 20 μg of the protein extract was loaded onto the gel. The protein samples were electrophoresed at 4°C on a 10% polyacrylamide gel containing 0.1% gelatin, and after completion of electrophoresis, the gel was washed with 2.5% Triton X-100 (Nuclear) and incubated for 21 hr in a solution of 50 mM Tris-HCl (pH 7.4), 0.1 M NaCl, and 0.03% sodium azide (Sigma) at 37 °C. The gel was stained with Coomassie brilliant blue R-250 for 1 hr. After staining, the gels were washed with a solution containing 50% methanol and 10% acetic acid to observe negative bands of proteins corresponding to enzymatic activity. As

a positive control, 20 mM EDTA was used in the incubation buffer. EDTA inhibits the activity of gelatinase and confirms the identification of MMPs in the gels. The bands in the negative image were quantified by densitometry using Scion Image software Alpha 4.0.3.2 (Scion Corporation, Frederick, MD, USA).

LIGHT MICROSCOPY ANALYSIS AND MORPHOMETRY

The tendons were fixed in a solution containing 4% formaldehyde in Millonig buffer (0.13 M sodium phosphate, NaOH 0.1 M, pH 7.4) for 24 hours at room temperature. The tendons were then washed for 6 hours in tap water, dehydrated with a sequence of increased ethanol concentrations, diaphonized with xylene and embedded in paraffin (Histosec, Merck), according to Neto et al. (2003). Longitudinal serial sections of 7 μm in thickness were prepared for microscopic analysis. To visualize the overall structure of the tissue, some sections were stained with toluidine blue (TB) (0.025%) in McIlvaine buffer (0.03 M citric acid, 0.04 M sodium phosphate dibasic, pH 4.0).

Digital images were observed with a camera coupled to a light microscope (Olympus BX 60) and then were used to perform all morphometric analyses using Image-Pro Plus 6.0 software. Ten random images (200x magnification) were used to measure the thickness of the epitenon and the same number of images was used (400x magnification) to count the cells. Ten images were used per animal (n=5).

BIREFRINGENCE: IMAGE ANALYSIS AND MEASUREMENTS

Birefringence properties were studied using an Olympus BX51-P BX2 polarizing microscope with an image analyzer (Image-Pro Plus 6.3, Media Cybernetics, Inc.-Silver Spring, MD, USA). Since birefringence appears visually as brilliance, this phenomenon was measured with the image analyzer and expressed as gray average (GA) values in pixels, after its calibration (8 bits=1 pixel). The major tendon axis was positioned at 45° to the crossed analyzer and polarizer during the measurements. Considering that collagen

bundles exhibit two kinds of birefringence: intrinsic birefringence (Bi) and form or textural birefringence (Bf) (Vidal and Volpe 2005; Vidal and Mello 2010), the total birefringence (sum of Bi and Bf) was used in this study. The measurements of the transected region of the tendons in each experimental group were made after immersing the sections in water.

BIOMECHANICAL TESTS

The biomechanical tests were performed using a TAXT2 texturometer (Stable Micro Systems, from the Department of Engineering, Faculty of Food Engineering, UNICAMP), according to Almeida et al., (2009). We used six tendons in each experimental group for this analysis. The tendons were maintained in saline solution until the time of testing. Prior to testing, the lengths, widths and thicknesses of the tendons were measured with a caliper, and the latter two parameters were determined at the midpoint of the tendon; the cross-sectional area was calculated from these measurements. The adapters that were encased in the machine secured the tendons by their ends.

During the test, the tendons were subjected to a gradually increasing load at a constant displacement rate of 1 mm/s, using a load of 5 N until the tendons ruptured. The maximum stress was calculated from these data. The stress (MPa) was calculated using the ratio between the load (N) and the cross-sectional area (mm^2). The maximum displacement was determined as the load that the tendon could withstand prior to rupture.

STATISTICAL ANALYSIS

The data were presented as the mean±SD of the results obtained from five animals per group. To compare the data, statistical analysis was performed using one-way analysis of variance (ANOVA) with the Tukey post hoc test. The Mann–Whitney test was used only for analysis of the birefringence measurements. A value of $P<0.05$ was considered statistically significant, and the statistical program GraphPad PrismVR, version 3.0, was used for all analyses.

RESULTS

Biochemical analysis via quantification of hydroxyproline and an indirect quantification of collagen showed a greater concentration of hydroxyproline in G4 ($p<0.05$) in comparison to the other inflamed and treated groups. In addition, the amount of hydroxyproline in G4 was similar to that found in control group, C (Figure 1). Apparently, the action of glycine on the hydroxyproline concentration occurred within 7 to 21 days, as there were no changes in the first 7 days of treatment.

In the quantification of total sulfated GAGs (Figure 2), a higher concentration was observed in G2 ($p<0.01$) and G4 compared to G1 and G3. In G2 and G4, the concentrations of GAGs were similar to that found in group C.

Moreover, quantification of the non-collagenous proteins showed a greater concentration in G2 when compared to G3 and G4 ($p<0.001$). G4 showed a lower concentration of proteins compared to all other groups (Figure 3).

Regarding the activity of metalloproteinases in the zymography for MMP-2 (Table 1), a greater concentration of pro-MMP-2 was observed in the control group (C) compared with the other groups. However, the active isoform of MMP-2 was greater G2 relative to the other groups; however, this result was not significantly different among the groups. Densitometry of the gel zymography bands showed no significant difference in the activity of MMP-9 among the groups. As expected, the C group did not show the presence of MMP-9 (Figure 4).

The biomechanical characteristics of the Achilles tendon presented interesting results in the animals that received glycine as a treatment for inflammation (Table 2). In the maximum load analysis graph, G4 withstood a larger load (N) than the other groups that were inflamed ($p<0.05$), and this value remained close to that observed in the control group. Considering the maximum stress (MPa), no differences were found among the groups; however, the values of this parameter had a tendency to increase in G4 compared to G3. Regarding the displacement and cross-sectional area, there were no significant differences among the groups.

The birefringence images showed a high birefringence of collagen molecules in G2 when compared to G1 (Figure 5 and 6). There were no differences among the groups in 21 days. The epitenon was thicker in G2 (Figure 7) when compared to the others groups ($p<0.05$). However, there were no differences among the groups, in relation to the number of cells (Table 3).

DISCUSSION

The inflammatory process applied to the Achilles tendon was remediated via its biochemical and biomechanical properties after treatment with a 5% glycine diet. In this study, biochemical components of the ECM that are involved directly in the remodeling process after the induction of a lesion were analyzed. In addition remarkable changes were observed in the endurance of the tendon after submitting it to a biomechanical test.

Glycine is predominant (approximately 35%) in the collagen molecule, and some studies have utilized it for therapeutic purposes for inflammatory processes and for tumor and hepatotoxic conditions (Yamashina et al., 2007; Hartog et al., 2007; Stoffels et al., 2011; Mikalauskas et al., 2011). The results from the present study on the benefits of a glycine-rich diet on the biochemistry, structural and biomechanics of the inflamed Achilles tendon also suggest potential new therapeutic approaches for this and related injuries.

The collagen molecule includes hydroxyproline, proline and glycine, which assist in the stabilization of the triple helix structure of collagen (Piez and Reddi, 1984). The glycine-rich diet provided to the animals with inflamed tendons resulting in levels of hydroxyproline that reached the values observed in the control, despite the degradation and disorganization of collagen fibers associated with the inflammatory process (Clutterbuck et al., 2010; Guerra et al., 2013). These observations suggest that the higher levels of hydroxyproline may help maintain the integrity of collagen fibers, perhaps making them more resistant to degradation.

Concentrations of GAGs are decreased in inflamed tendons (Vieira et al., 2012). In the present study, GAGs concentrations were observed to return to normal levels in

tendons of animals treated with a diet rich in glycine, from 8 to 22 days following the induction of inflammation. GAGs are part of the proteoglycans molecules, which are integral to the regulation of many cellular events, including cell proliferation, migration and adhesion (Jozsa and Kannus, 1997; Banos et al., 2008). The high concentration of GAGs may promote a more rapid remodeling of the extracellular matrix after inflammation. It is also known that the small proteoglycan, decorin, is involved directly with the fibrillogenesis of collagen molecules and is responsible for controlling the diameters of these fibers (James et al., 2008; Banos et al., 2008).

During the inflammatory process, signaling molecules and proteins of the ECM are cleaved (Vieira et al., 2012). Regarding the increase of non-collagenous proteins in G2, the data suggests that glycine may promote or facilitate protein synthesis during inflammation.

Previous studies have shown that glycine contributes to the inhibition of TNF-α and IL-1β (Hartog et al., 2007; Figueiredo et al., 2009). These inflammatory molecules are involved in the induction of apoptosis (Kim et al., 2010) and the activation of metalloproteinases involved in the excessive degradation of matrix components (Tsuzaki et al., 2003). MMP-2 is the enzyme responsible for extracellular matrix remodeling, and it shows high activity during pathological processes such as injury and partial rupture of the tendon (Almeida et al., 2012; Guerra et al., 2013). A greater trend towards increased MMP-2 activity was observed in 7 days in the present study. It is known that this enzyme is required to restore the tissue after injury, and it has beneficial effects in the remodeling process (Clutterbuck et al., 2010). The trend toward increasing activity may promote tissue remodeling in the ECM and thus more effectively restoring tendon biomechanical properties.

Cells and their associated enzymes recruited during the initiation of inflammation can induce disorganization of the collagen bundles and promote degradation of ECM components, which may lead to severe chronic pathologic processes. This process may result in a reduction in the tensile strength of the tendon and predisposes the tendon to rupture (Sharma and Maffulli, 2006; Den Hartog, 2009; Silva et al., 2011). The

biomechanical analyses showed that G4 sustained a higher biomechanical load compared to the other inflamed groups. The profile found in G4 remained closer to the control group. The tendons from this group more resistant to rupture after treatment with glycine compared to G1, G2 and G3. These data were corroborated by the high concentrations of glycosaminoglycans and hydroxyproline found in G4. In case of tendinopathy, the weakening of the tendon causes morbidity and disability among athletes and people in general (Tallon et al., 2001). The biomechanical characteristics of the Achilles tendon are primarily responsible for the ability to withstand large muscular forces with minimum deformation (Maganaris et al., 2002; Casalechi et al., 2012). The addition of dietary glycine in the present study was associated with a higher biomechanical resistance in the Achilles tendon after the induction of inflammation. It is known that glycine is present in the triple helix of the collagen molecule in large quantity and the addition of greater dietary glycine may have promoted collagen synthesis.

High birefringence noted in G2 showed that glycine supplementation resulted in better organization of collagen fibers after induced inflammation compared with the non-supplemented groups. G3 and G4 showed similar measurements in the birefringence images. However, the biomechanics properties noted in G4 were significantly better compared to the inflamed group in the same period. The biomechanical properties of tendons change during physical activity, stretching and pathological processes (Wang et al., 2012; Marqueti et al., 2006). The improvement or maintenance of the biomechanical properties of tendons after a pathological event, such as inflammation, suggests that glycine supplementation improved or maintained effective connective tissue remodeling processes.

The epitenon was thicker in G2. This result suggests a greater cellular infiltration in this region. During inflammation, the tissue becomes weaker due to the cascade of events arising from the injury, such as release of cytokines that are involved in the infiltration of macrophages and neutrophils from the blood stream, as well as the imbalance between MMPs and their inhibitors (TIMPs). The MMP-2 and -9 can be secreted by neutrophils (Clutterbuck et al., 2010; Casalechi et al., 2012). During the

recovery time, the tendon is more susceptible to further injuries. Glycine apparently functions on the active isoform of MMP-2 in the first 7 days of treatment, and no further effect was observed after 21 days. This enzyme may be involved for the rapid remodeling of collagen fibers and arrangement of proteoglycans, as well as for improving the functionality of the tendon to withstand higher loads in 21 days, when compared to animals that had no inflammation in the tendon.

This study demonstrated effects of a glycine-rich diet on inflammation of the Achilles tendon. The glycine diet stimulated the synthesis of hydroxyproline, glycosaminoglycans, non-collagenous proteins and appeared to maintain or improve the organization of collagen molecules. The biomechanical results indicated that the tendon was more resistant to mechanical loading upon treatment with a glycine diet. Glycine also induced a rapid remodeling of tissue when compared with the groups without treatment. The data from this study suggest that dietary glycine supplementation may be a useful therapeutic adjunct for individuals with inflammatory injuries of tendons, such as Achilles tendon injuries, and perhaps other types of connective tissue injuries and inflammatory events.

ACKNOWLEDGMENTS

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Figures

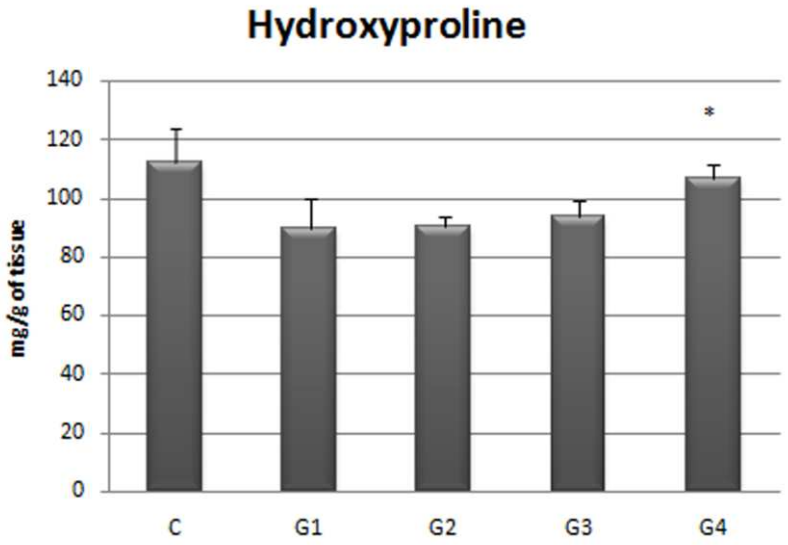

Figure 1: Concentration of hydroxyproline (mg/g dry tissue) in different groups. C: control group; G1: inflamed group in 7 days; G2: inflamed and supplied with a glycine diet in 7 days; G3: inflamed group in 21 days; G4: inflamed and supplied with a glycine diet in 21 days. G4 showed values similar to those of C and higher than the other groups ($p<0.05$*).

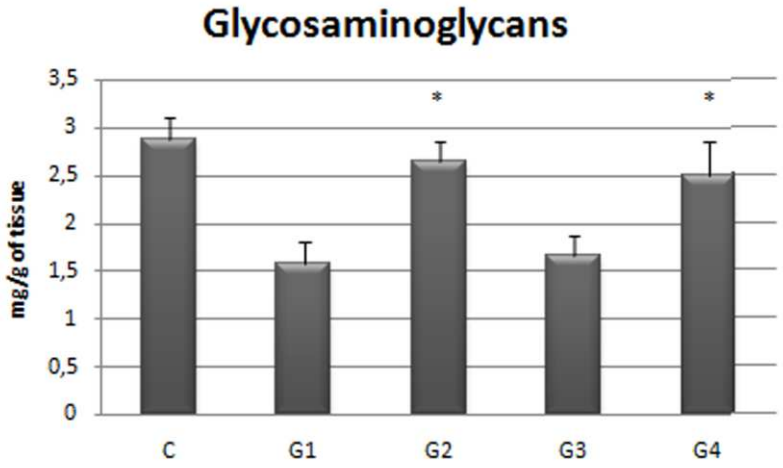

Figure 2: Concentration of sulfated glycosaminoglycans (mg/g wet tissue). C: control group; G1: inflamed group in 7 days; G2: inflamed and supplied with a glycine diet in 7 days; G3: inflamed group in 21 days; G4: inflamed and supplied with a glycine diet in 21 days. Note the higher concentration in G2 and G4 compared to G1 and G3 ($p<0.05$*). Both treated groups presented values similars to those of C.

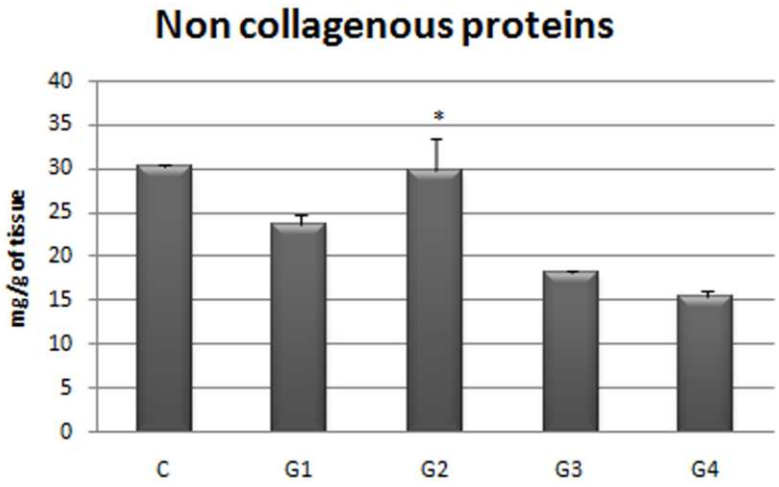

Figure 3: Concentration of non collagenous proteins. C: control group; G1: inflamed group in 7 days; G2: inflamed and supplied with a glycine diet in 7 days; G3: inflamed group in 21 days; G4: inflamed and supplied with a glycine diet in 21 days. Observe a higher concentration of proteins in G2 when compared to G1 ($p<0.01$*). There were not differences between G3 and G4. Note the concentration in G2 was similar the found in C.

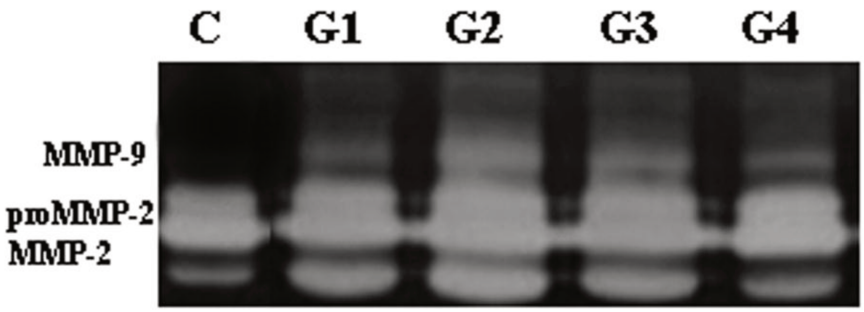

Figure 4: Zymography for MMP-2 and 9. The activity of MMP-2 showed a trend of increase in G2. However, these results were not significant. C: control group; G1: inflamed group in 7 days; G2: inflamed and supplied with a glycine diet in 7 days; G3: inflamed group in 21 days; G4: inflamed and supplied with a glycine diet in 21 days.

Table 1. Densitometry of the bands corresponding to zymography of the MMP-2 and MMP-9 isoforms.

Groups	pro-MMP-2 (72 and 68 kDa)	MMP-2 active (62 kDa)	MMP-9 active (82 kDa)
C	160.9 ± 30.20*	28.76 ± 9.2	0
G1	84.6± 12.0	40.43 ± 14.3	29.51 ± 9.1
G2	66.76 ± 21.04	62.40 ± 4.1	49.91 ± 12.9
G3	94.71 ± 8.9	31.11 ± 7.2	27.70 ± 8.7
G4	75.87 ± 9.6	36.12 ± 6.9	54.27 ± 23.6

(*) $p<0.05$; the activity of pro-MMP-2 was elevated in the C group; a trend increase was observed for the active MMP-2 in G2 when compared to the other groups, but the difference was not significant; It was not observed differences among the groups in relation to MMP-9. C: control group; G1: inflamed group in 7 days; G2: inflamed and supplied with a glycine diet in 7 days; G3: inflamed group in 21 days; G4: inflamed and supplied with a glycine diet in 21 days.

Table 2. Biomechanical properties of the experimental groups.

Groups	Maximum load (N)	Maximum displacement (mm)	Maximum stress (MPa)	Cross-sectional area (mm^2)
C	53.2 ± 7.0	1.40± 0.5	57.3 ±6.3	1.01 ±0.4
G1	33.2± 4.7	1.10 ± 0.2	52.1 ± 9.4	0.66 ±0.1
G2	33.0 ± 7.1	1.20 ± 0.1	51.0 ± 19.0	0.76 ±0.1
G3	35.2 ± 6.5	1.08 ± 0.4	39.6 ±12.2	0.90 ±0.2
G4	42.5 ± 4.3*	1.25 ± 0.4	54.6 ± 11.9	0.72 ±0.1

The maximum load were higher in G4 (*$p<0.05$). These values were similar to those of C. The maximum stress were smaller in inflamed tendons (G3) but this result was not differences significant. The cross-sectional area and displacement were similar in all groups. C: control group; G1: inflamed group in 7 days; G2: inflamed and supplied with a glycine diet in 7 days; G3: inflamed group in 21 days; G4: inflamed and supplied with a glycine diet in 21 days.

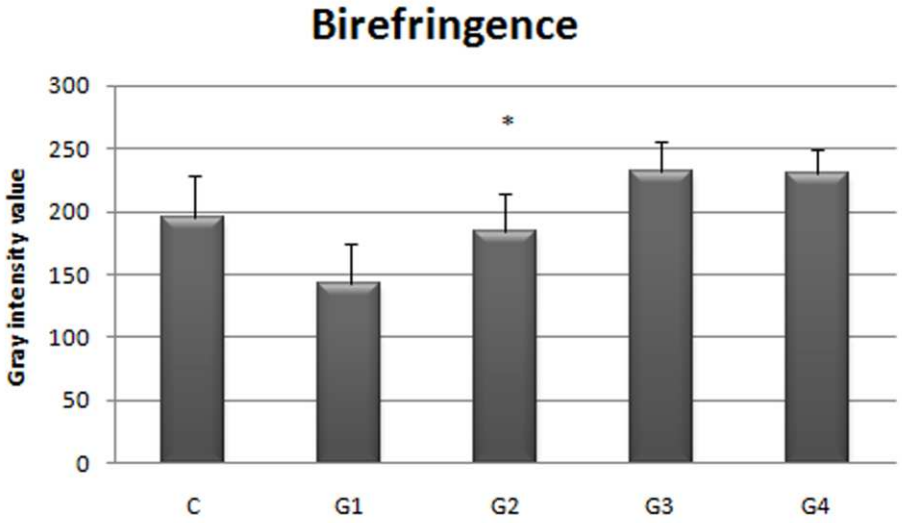

Figure 5: Birefringence measurements of different groups of tendon. C: control group; G1: inflamed group in 7 days; G2: inflamed and supplied with a glycine diet in 7 days; G3: inflamed group in 21 days; G4: inflamed and supplied with a glycine diet in 21 days. The measurements of G2 was higher than G1 (*). G3 and G4 showed similar measurements.

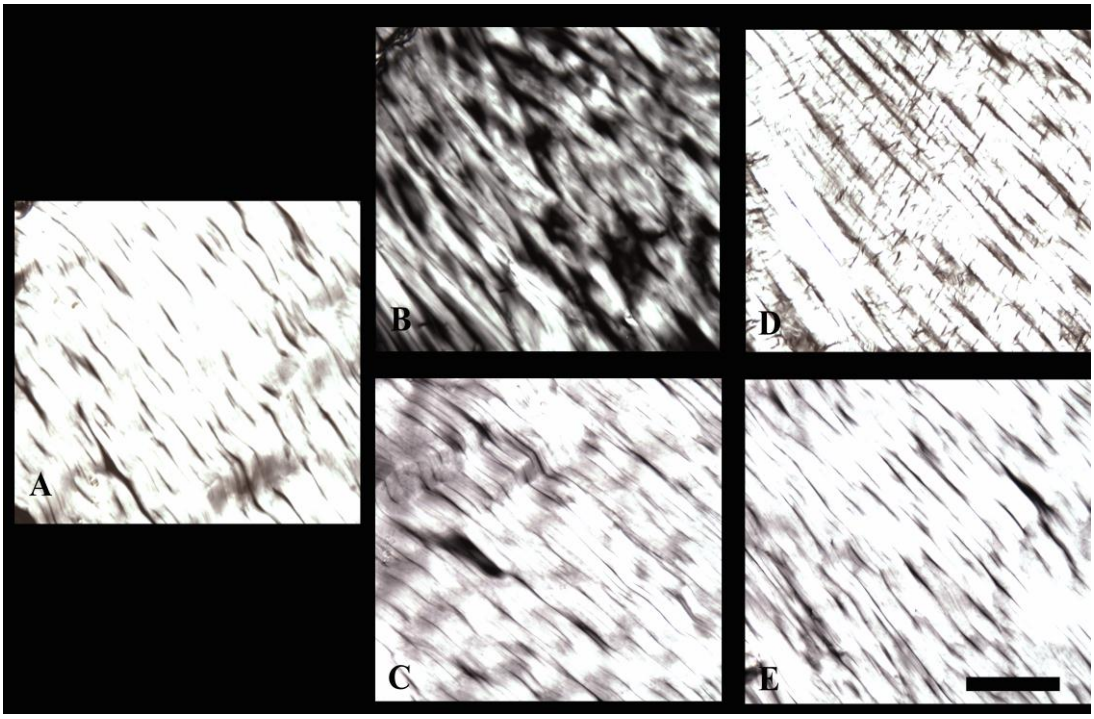

Figure 6: Birefringences of longitudinal sections of tendons from the different groups, observed by polarization microscopy. The largest axis of the tendon is positioned at 45° in relation to the crossed polarizers. A: C group; B: G1; C: G3; D: G2; E:G4. C: control group; G1: inflamed group in 7 days; G2: inflamed and supplied with a glycine diet in 7 days; G3: inflamed group in 21 days; G4: inflamed and supplied with a glycine diet in 21 days. It is possible to note the high birefringence of collagen molecules in G2 when compared to G1. In G3 and G4 the organization of collagen fibers was the same. Bar: 20µm.

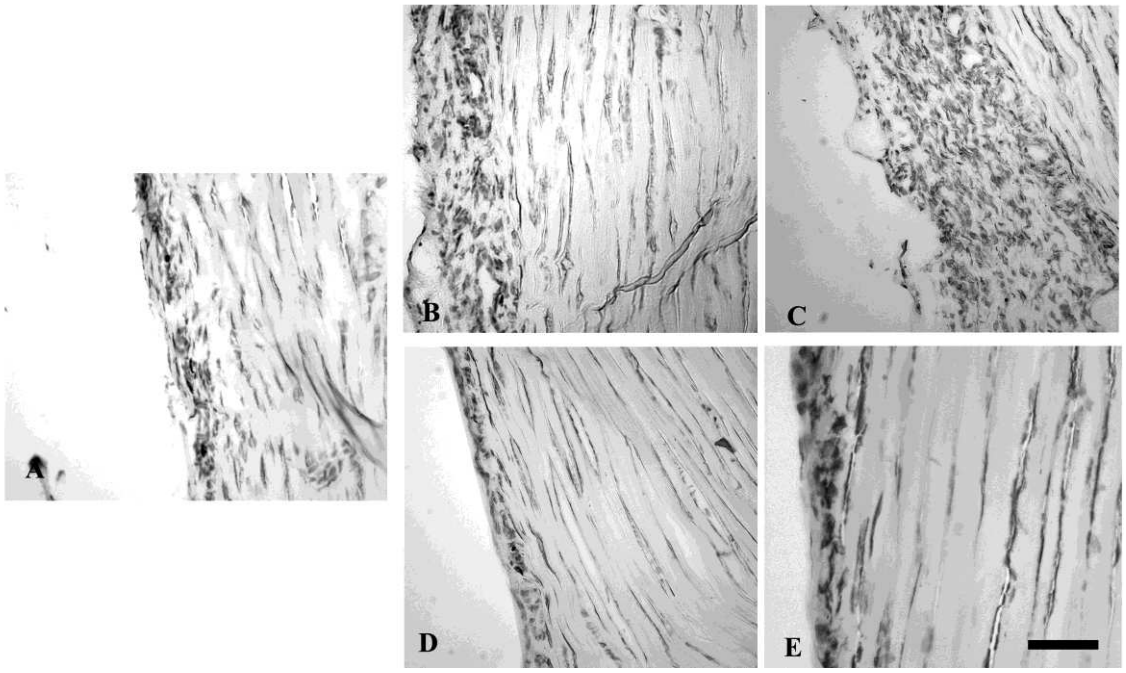

Figure 7: Histological sections stained with toluidine blue. A: C group; B: G1; C: G2; D: G3; E: G4. Note the epitenon thicker in G2 compared to the other groups. Bar: 40µm.

Morphometry of tendon sections stained with Toluidine Blue represented by the mean and standard deviation.

Parameters	**C**	**G1**	**G2**	**G3**	**G4**
Cells/$\mu m^2(10^{-4})$	8.0±0.78	6.0±0.8	7.0±0.8	6.0±0.8	5.0±0.8
Epitenon/ µm	93.2±25.2	233.7±45.0	268.7±44.0*	111.7±23.2	138.0±22.2

There was no differences observed in the amount of cells of tendon. In relation to epitenon, it was noted thicker in G2 compared to the others experimental groups. C: control group; G1: inflamed group in 7 days; G2: inflamed and supplied with a glycine diet in 7 days; G3: inflamed group in 21 days; G4: inflamed and supplied with a glycine diet in 21 days.

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CAPÍTULO 2

Green tea and glycine aid in the recovery of tendinitis of the Achilles tendon of rats

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ABSTRACT

Purpose: Green tea is widely used due to its anti-inflammatory properties. Previous studies have shown beneficial effects of a glycine diet on the remodeling process in inflamed tendons. Tendinitis is commonly observed in athletes and is of concern to surgeons due to the slowness of the recovery process. Our hypothesis is that green tea + a glycine diet may improve tendinitis. **Aim of the Study:** To analyze the effect of green tea and/or glycine in the diet on tendinitis. **Materials and Methods:** Wistar rats were divided into seven groups (G): control group (C); G1 and G4, tendinitis; G2 and G5, tendinitis supplied with green tea; and G3 and G6, tendinitis supplied with green tea and a glycine diet for 7 or 21 days, respectively. We performed zymography for metalloproteinase, biochemical, morphological and biomechanics tests. **Results:** G2, G3 and G5 showed high levels of hydroxyproline in relation to G1, while G4 showed high levels of glycosaminoglycans. High activity of metalloproteinase-2 was detected in G3. The organization of collagen bundles was high in G2 and G3. G5 showed similar birefringence measurements compared with C. G5 withstood a larger load compared with G4. **Conclusions:** The presence of metalloproteinase-2 indicates that a tissue is undergoing a remodeling process. High birefringence suggests a better organization of collagen bundles. After 21 days, G5 sustained a high load before rupture, unlike G4. The results suggest that green tea + a glycine diet has beneficial effects that aid in the recovery process of the tendon after tendinitis.

Keywords: Achilles tendon, tendinitis, extracellular matrix, biomechanics, metalloproteínase

INTRODUCTION

Tendinopathy is characterized by pain and degeneration of the tendon and is associated with repetitive and overloading use of the tendon. Tendon injuries are frequently observed in sports, representing 30-50% of the total of lesions. In the past few years, an increase in the incidence of tendon injuries has been observed [1,2,3]. In the United States, musculoskeletal disorders are very common. It is estimated that approximately 315 million medical visits occur annually because of this type of injury [4]. This condition is not only restricted to athletes: 25-30% of the people affected by tendon injuries are not athletes and can lose a significant number of working days. The prevalence of tendinopathy of the Achilles tendon is approximately 11% in runners, 8% in dancers and less than 2% in tennis players [5,6].

The tendon is a highly organized extracellular matrix (ECM) comprised of collagen fibers, with a predominance of type 1 collagen, proteoglycans, glycosaminoglycans (GAGs), non-collagenous proteins (NCP), metalloproteinases (MMP) and tenocytes [7]. Under inflammatory processes, collagen fibers become disorganized [8]; the tenocyte population is altered, likely due to apoptosis [9]; inflammatory cells are directed to the location of the injury; and GAGs and proteins undergo degradation through the activation of enzymes such as MMPs [10]. Notable, during tendon healing, the tendons are more susceptible to ruptures, and their biomechanics are compromised due to the disorganization of the collagen fibers [11].

Many researchers are currently searching for reasonable alternatives to treat tendon pathologies. Green tea (GT) (*Camellia sinensis*) has been widely used for therapeutic purposes due to its beneficial properties, including antimutagenic, antidiabetic, anti-inflammatory and antioxidant effects [12,13,14,15]. The anti-inflammatory effect of GT can be explained by its ability to inhibit nuclear factor-kappa B (NF-kB), interleukina-1 beta (IL-1β) transcription, tumor necrosis factor alpha (TNF-α) synthesis and kinases [4,16,17]. All of these molecules can induce the activation of other transcription factors as well as inhibiting MMP such as MMP-1 and MMP-13, which show high levels under chronic inflammation [18].

Moreover, the inclusion of 5% glycine in the diet has been shown to have an important effect on the treatment of inflammatory processes [19,20] and tumors [21]. Recently, treatment with glycine in the diet was found to lead to improvements in biomechanic properties, the organization of collagen bundles and the synthesis of ECM molecules in tendinitis induced in rats [22]. Glycine is the most prevalent amino acid in the triple helix of collagen and can facilitate the remodeling process. In addition, glycine is the amino acid that is most commonly found in the bile acid of animals and plays an important role in the digestion and absorption of lipids and fat-soluble vitamins [23].

Collagenase is known to exhibit proinflammatory and degenerative properties and has been employed to induce acute tendinitis in animal models [24,25]. Despite the well-defined pharmacological properties of GT and its widespread consumption around the world and low cost, there are no studies addressing its effect on tendinitis of the Achilles tendon. Due to these characteristics, we believe that treatment with GT and glycine together in the diet may improve the structure and biochemistry of the Achilles tendon in the presence of tendinitis.

MATERIALS AND METHODS

Experimental Groups

Animal care was in accordance with the European Convection for the Protection of Vertebrate Animals used for Experimental and Other Scientific Purposes and it's consistent with the ethical principles of animal experimentation adopted by the Brazilian College of Animal Experimentation. The protocol was approved by the Ethics Committee on Animal Experiments of the State University of Campinas (UNICAMP), SP, Brazil, and filed under no. 2307-1.

In this study we used male Wistar rats, young adults (60 days), weighing on average 300 g from the Multidisciplinary Center for Biological Investigation of the State University of Campinas. The rats were housed two per cage in a 12-h light/dark cycle at 23 °C, with free access to standard rat chow and water.

The animals were divided into seven groups: C, control group; G1, tendinitis in 7 days; G2, tendinitis supplied with green tea; G3, tendinitis supplied with green tea and glycine diet in 7 days; G4, tendinitis in 21 days group; G5, tendinitis supplied with green tea in 21 days; G6, tendinitis supplied with green tea and glycine diet in 21 days.

The animals were first anesthetized with isoflurane (Forame) and given an intratendineous injection of 10 μL of collagenase (10 mg/mL; Sigma, C6885) dissolved in sterile buffered saline (PBS) [25] in the Achilles tendon of the right leg.

Immediately, after the induction of tendinitis, the animals in the treated groups received GT orally and a glycine diet for 7 or 21 days.

Preparation of the Green tea and Glycine diet

The plant *Camellia sinensis* was obtained commercially from Yamamoroyama, SP. The leaves of *Camellia sinensis* were used after extraction according to Amra Perva-Uzunalic and collaborators [26]. The leaves were weighed, and distilled water was added, followed by incubation in a water bath at 80°C. The extraction mixture was stirred constantly. After 20 minutes of extraction, the extraction mixture was cooled and filtered, and the leaves were removed. The solution was left at room temperature after administration to the animals. This extraction showed an efficiency of 84.4 % for catechins and 81.5% for caffeine. At a ratio 100 mL:1 g the contents of catechins in the GT extract solution (mg/100 mL) were 100 mg of epigallocatechin gallate (EGCG), 45 mg of epigallo catechin (EGC), 15 mg of epicatechin gallate (ECG) and 2 mg of epicatechin [26].

The concentration of the GT was established in our laboratory following biochemical tests of the blood serum of animals. A dose of 700 mg/kg/day was given orally via gavage to rats with inflamed tendons. We analyzed the levels of albumin, creatinine, serum proteins, urea, triglycerides, aspartate aminotransferase (AST) and alanine aminotransferase (ALT) to verify whether there was any toxicity in relation to the concentration of green tea administered. In all analyses, the animals that did not receive the treatment with green tea were compared with the animals that received green tea via gavage. The reference value of

each of the elements analyzed was respected in the rats. Differences from the reference values for these compounds in the blood serum indicate toxicity. We did not observe significant differences in the values obtained from these analyses between the groups. Hence, we confirmed there was no toxicity in relation to the concentration of GT given to the animals.

In addition, the animals were weighed weekly to determine whether there were any alterations in relation to the weight of the rats after/during the treatment with GT. There were no significant differences in relation to the animals' weight after/during treatment with a glycine diet and GT.

The diet was administered to the animals, and the consumption was recorded daily. A diet containing 5% glycine (AJINOMOTO, BRAZIL) was provided by the Institute of Biology, Animal Physiology, State University of Campinas, following the guidelines of AIN-93M [28].

Extraction of Extracellular Matrix

The Achilles tendon was removed and treated with 50 volumes of 4 M guanidine hydrochloride (GuHCl) containing 20 mM EDTA and 1 mM PMSF in 50 mM sodium acetate buffer pH 5.8 for 24 h at 4 °C with stirring [29]. Afterward, the material was centrifuged (13,000×g, 25 min, 4 °C), and the supernatant was used for quantification of NCP and GAGs. Five animals were used per group in these analyses.

Quantification of Proteins and Sulfated Glycosaminoglycans

Samples (5 µL) of the GuHCl extracts collected from the experimental groups were used to quantify the relative amounts of NCP according to the method of Bradford [30]. A bovine serum albumin (BSA) solution containing from 1 to 10 µg of protein was employed to obtain standard curves. The BSA solutions and samples were mixed with 200 µL of protein reagent (0.01% Coomassie Brilliant Blue G-250, 4.7% ethanol and 8.5% phosphoric acid). The quantification of GAGs was carried out using the dimethylmethylene blue method [31], with chondroitin sulfate as a standard. The

absorbance was measured at 595 nm for proteins and at 540 nm for GAGs using an Asys Expert Plus Microplate Reader (Biochrom, Holliston, MA, USA).

Quantification of Hydroxyproline (HOPro)

Fragments of the tendon were hydrolyzed in 6 N HCl (1 mL/10 mg tissue) for 4 h at 130 °C. Then, the lysate was treated with 1.41 % chloramine-T solution and 15 % p-dimethylaminobenzaldehyde, as described by Stegeman and Stalder [32]. After incubation for 15 min at 60 °C, the hydroxyproline solution was cooled, and the absorbance was measured at 550 nm in an Ultrospec 2100 Pro (Amersham Biosciences, England) spectrophotometer.

Zymography for MMP-2 and 9

The tendons from the experimental groups (n=5) were treated according to the method described by Marqueti [33]. For protein extraction, the fragments of the tendon were immersed in a solution of 50 mM Tris-HCl (Synth) pH 7.4, 0.2 M NaCl, 10 mM CaCl2 (Ecibra, São Paulo, SP, Brazil), and 0.1% Triton X-100 (Nuclear) with 1% of a protease inhibitor cocktail (Sigma) at 4 °C for 2 h. After the first extraction, the samples were incubated and added 1/3 the volume of the solution described above, at 60 °C for 5 min. Then, 20 μg of the protein extract was loaded on the gel. The protein samples were electrophoresed at 4 °C on a 10% polyacrylamide gel containing 0.1% gelatin, and after completion of electrophoresis, the gel was washed with 2.5% Triton X-100 (Nuclear) and incubated for 21 h in a solution of 50mM Tris-HCl (pH 7.4), 0.1M NaCl, and 0.03% sodium azide (Sigma) at 37 °C. The gel was stained with Coomassie brilliant blue R-250 for 1 h. After staining, the gels were washed with a solution containing 50% methanol and 10% acetic acid to observe negative bands of proteins corresponding to enzymatic activity. As a positive control, 20 mM EDTA was used in the incubation buffer. EDTA inhibits the activity of gelatinase and confirms the identification of MMPs in the gels. The bands in the negative image were quantified by densitometry using Scion Image software Alpha 4.0.3.2 (Scion Corporation, Frederick, MD, USA).

Birefringence: Image Analysis and Measurements

The tendons from experimental groups (n=5) were fixed using a 4% formaldehyde solution in Millonig's buffer (0.13 M sodium phosphate, 0.1 M NaOH pH 7.4) for 24 h at 4º C and washed in water, ethanol dehydrated, diaphanized with xylene and paraffin-embedded. Longitudinal sections of 8 µm were analyzed. The measurements of the tendons (5 regions of each tendon) in each experimental group were made after immersing the sections in water. Birefringence properties were studied using an Olympus BX51-P BX2 polarizing microscope and an image analyzer (Image-Pro Plus 6.3, Media Cybernetics, Inc.—Silver Spring, MD, USA).

Since birefringence appears visually as brilliance, this phenomenon was measured with the image analyzer and expressed as gray average (GA) values in pixels, after its calibration (8 bits = 1 pixel). The major tendon axis was positioned at 45° to the crossed analyzer and polarizer during the measurements. Considering that collagen bundles exhibit two kinds of birefringences: intrinsic birefringence (Bi) and form or textural birefringence (Bf) [34,35] total birefringence (sum of Bi and Bf) was used in this study.

Morphometry Analysis Through Light Microscopy

To visualize the overall structure of the tissue, sections were stained with hematoxylin and eosin. Digital images were observed with a camera coupled to a light microscope (Olympus BX 60) and were then used to perform all morphometrical analyses using the software Image-Pro Plus 6.0. Ten random images (200x magnification) were used to measure the thickness of the epitenon, and the same number of images was used (400x magnification) to count cells. Ten images were employed per animal (n=5).

Biomechanical Tests

The biomechanical tests were performed in texturometer, TAXT2 model, Stable Micro Systems brand, of the Department of Engineering, Faculty of Food Engineering, UNICAMP, according to Almeida and colleagues [36]. Six tendons of each experimental group were used. The tendons were kept in saline solution until the time of testing. Prior

to testing, the length, width and thickness of the tendons were measured with a caliper, and the last two were determined at the midpoint of the distal tendon of each, and the cross-sectional area was calculated from these measurements. The adapters that were encased in the machine arrested the tendons for their ends.

During the test, the tendons were subjected to a gradually increasing load at a constant displacement rate of 1 mm / s, using a load of 5 N until the tendon was ruptured. From this data was calculated the maximum stress. The stress (MPa) was calculated by the ratio between load (N) and the cross-sectional area (mm^2). The maximum displacement was determined so far that the tendon can withstand before breaking.

Statistical Analysis

Data were presented as mean ± SD of results obtained from five/six animals per group. To compare the data, statistical analysis was performed using one-way analysis of variance (ANOVA) with the Tukey post-hoc test. The Mann–Whitney test was used only for analysis of the birefringence measurements. A value of $p<0.05$ was considered statistically significant, and the statistical program GraphPad Prism®, version 3.0 was used for all analyses.

RESULTS

The biochemical, morphological and biomechanical analyses revealed striking alterations in the groups treated with GT and GT + glycine diet. The body weight of the animals before and after the treatments was verified. There were no differences between the treated groups compared with the control and inflamed groups. Additionally, we did not note any toxicity in relation to the concentration of GT used (data not shown).

Biochemical results

The results of HOPro quantification (Fig. 1A) showed higher values in both G2 and G3 compared with G1 ($p<0.05$). In G5, a higher concentration of HOPro was observed compared with G4 ($p<0.01$). Differences in the HOPro concentration are equivalent to the variation in the number of collagen molecules found in the ECM after injury.

Regarding the total dosage of sulfated GAGs (Fig. 1B), it was observed that group G6 exhibited a higher concentration of this component in comparison with G4 ($p<0.05$). Therefore, considering the GAGs concentration in group C, group G6 was the only group that showed similar levels of GAGs compared with group C.

G3 exhibited a lower level of NCP in relation to C, G1 and G2 ($p<0.05$). G6 showed a lower level of NCP in comparison with G5 ($p<0.001$) (Fig. 1C).

Zymography was performed to analyze gelatinases (MMP-2 and MMP-9). Group C displayed a higher level of proMMP-2 in comparison with the other groups. The active isoform MMP-2 presented more activity in G3 compared with G1 ($p<0.05$). There were no differences between the groups after 21 days of treatment in relation to active MMP-2. MMP-9 was not detected in group C (result already expected) (Fig. 2).

Morphological results

Birefringence determines the organization of collagen fibers in the extracellular matrix. Under polarization microscopy, every tendon in every group exhibited strong brightness due to the high organization of the collagen bundles, though there were differences found when the different groups were compared (Fig. 3). The birefringence measurements were expressed as the average gray level (Fig. 4). A higher birefringence was found in G2 and G3 in comparison with G1 ($p<0.05$). G3, G5 and G6 presented similar measurements to group C. In G5 and G6, there were no differences in the obtained measurements. However, G4 showed high birefringence compared with G5 and G6 ($p<0.05$).

Morphological analysis revealed that G1 presented a lower number of cells. G2 exhibited a greater number of cells and thicker epitenon compared with 7-day groups. G3

presented a thinner epitenon compared with G1 (Fig. 5). The value was close to that found in C. After 21 days, there were no significant differences between the groups (Table 1).

Biomechanical results

The biomechanical properties of the Achilles tendon underwent alterations after lesion. All of the groups exhibited a lower load in relation to group C, as expected. However, group G5 endured a greater load (N) in comparison with G4 ($p<0.05$). The values obtained for group G4 were closer to those of group C. G6 displayed high displacement (mm) compared with G4. The displacement results for C and G6 were similar. Additionally, it was observed that the cross-sectional area was greater in group G3 compared with G1 ($p<0,05$) (Table 2).

DISCUSSION

Tendinopathy is very complicated to treat, mainly due to the poor vascularity and cellularity of the tendon [37]. GT and glycine have been widely used due to their beneficial effects on inflammatory processes [4,16,17,19,20]. Here, we analyzed the effect of GT and GT + a glycine diet on collagenase-induced tendinitis of the Achilles tendon of rats. Remarkable changes in the biochemical, structural and biomechanical properties of the Achilles tendon were noted after both treatments after 7 or 21 days.

The higher concentration of some ECM components observed after treatment with GT and GT+a glycine diet was considered a striking change, as inflammation normally triggers significant degradation of ECM molecules (10,37). Alterations in the collagen concentration, estimated based on the hydroxyproline dosage, were detected. Hydroxyproline is the characteristic amino acid in the triple helix structure of collagen and plays a role in the stabilization of this molecule (38). G2, G3 and G5 exhibited higher hydroxyproline levels compared with the inflamed groups, G1 and G4. Collagen fibers are the main components of the tendons. During tendinitis, the collagen bundles are affected, causing alterations in the biomechanical properties of the tissue that may lead to tendon rupture (39). When the integrity of collagen fibers is preserved, as demonstrated after the 7- and 21-day treatments, injuries resulting from tendinitis can be avoided.

GAGs are attached to a protein core to form proteoglycans, which play an important role in cell migration and differentiation, in addition to their regulatory role in collagen fibrillogenesis [41]. A significant increase was observed in G6 in comparison with the other inflamed groups, suggesting that GT + a glycine diet had a positive effect on the production of new proteoglycans, most likely on the small proteoglycan decorin, which helps to maintain and regulate the diameter of collagen bundles and is essential for repair processes [42,43,44].

Group C presented higher levels of the proMMP-2 isoform. The proMMP-2 isoform is expected to be present at high levels in normal tissue. Its activation involves disruption of the Cys73–Zn2+ bond, which results in the active isoform. MMPs are generally activated by other proteinases. Activation of MMPs is commonly observed in pathological cases (44–47). High activity of the active MMP-2 isoform was found in G3 compared with G1. Gelatinases are mainly involved in the process of ECM remodeling and degradation(48). MMP-2 is the main enzyme in the remodeling process, and high levels of MMP-2 can indicate an active remodeling process of the ECM after injury (8). G3 displayed a lower level of NCP in relation to G1 and G2, and HOPro levels were lower in G3 compared with G2, which we believe to have been caused by MMP-2. Notably, this type of degradation is a part of process to restructure the tissue after lesions (48,49).

Regarding MMP-9, the absence of significant differences between the groups after 7 and 21 days was most likely due to high enzyme activity at the beginning of acute or chronic inflammations [38,48,51,52]. Only a low level of activity was observed at 7 days, and an even lower level was found after 21 days.

Normal tendons observed under a polarized light microscope present high brightness, arising from aligned collagen fibers, indicating that the tissue shows its best organization (34,52). Under injury, collagen fibers lose their organization and appear darker when viewed under a polarizing microscope(51). High birefringence values were found in group G2 and G3, indicating a better organization of collagen bundles at seven days after treatment. The organization of collagen bundles was poorer after 21 days in G5 and G6 compared with G4. However, the measurements of birefringence in G5 (209.9) and G6 (215.8) were closer the

value in C (195.3). This result indicates possible similarity of the organization of collagen fibers in the treated groups and control group in this period of treatment

Tendons are subjected to large mechanical loads that can often cause injury. An excessive mechanical load on the tendon triggers decreased tensile strength and collagen disorganization, in addition to inducing inflammatory mediators and cells [54,55]. These characteristics are noted in typical tendinopathies [56]. When an injury occurs, the intermolecular cross-links are compromised, leading to disorganization of collagen fibers into fibrils and decreased tensile strength, thus promoting a weaker tendon [55,57]. We observed that after 21 days of treatment with GT, the tendons sustained a high load (49 N). These values were close to those in group C (53 N). Treatment with GT led to higher resistance to the load on the tendon before rupture, as observed in group GT. We believe that the combination of the higher levels of GAGs, HOPro and NCP found in group GT may be related to enhanced stability of these fibers, allowing the tendon to bear a greater maximum load, despite the poorer organization of the collagen bundles in relation to group G4.

In a previous study, we verified that a glycine diet aids in a rapid recovery following inflammation by inducing a high organization of collagen bundles and synthesis of ECM components that are responsible for tissue repair [22]. However, in this study, positive changes were observed when GT was administered together with a glycine diet. Our group has searched for different therapies, such as the use of a low level laser [8,52], electroacupuncture [46] and plant extracts [47, 58], to improve the structure of the Achilles tendon, considering biochemical and structural elements after partial rupture. These alternatives mainly aim to accelerate tissue healing because the tendon shows low vascularity and cellularity, which directly influences the tissue remodeling and recovery time.

These data demonstrated the beneficial effects of GT (G2 and G5) and GT+a glycine diet (G3 and G6) on tendinitis of the Achilles tendon. GT and glycine are known to show inhibitory action in relation to TNFa and IL-b (4,16,17). In turn, both pro-inflammatory cytokines trigger the action of MMPs, which function in the degradation of extracellular matrix components. Therefore, improvement of the contents of elements of the ECM may be related to the lower rate of degradation. The levels of HOPro and GAG improved after 21

days of treatment to values close to those in group C. MMP-2 exhibited high activity in G3, indicating that a remodeling process was taking place. The measurements of birefringence indicated that after seven days, there was high birefringence in the treated groups compared with the inflamed group. Although, after 21 days, the inflamed group showed high birefringence, it was not sufficient to show beneficial results in relation to biomechanics properties, as observed in the GT group (G5) after 21 days.

These data obtained in this study are scientifically relevant because GT and GT + a glycine diet were shown to aid in faster remodeling of the ECM under inflammatory processes. The use of therapeutic nutrition consisting of GT and GT + glycine may constitute a healthy new alternative that is accessible, low cost, and without side effects and is effective for promoting the remodeling process during tendinitis in tendons.

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Figures

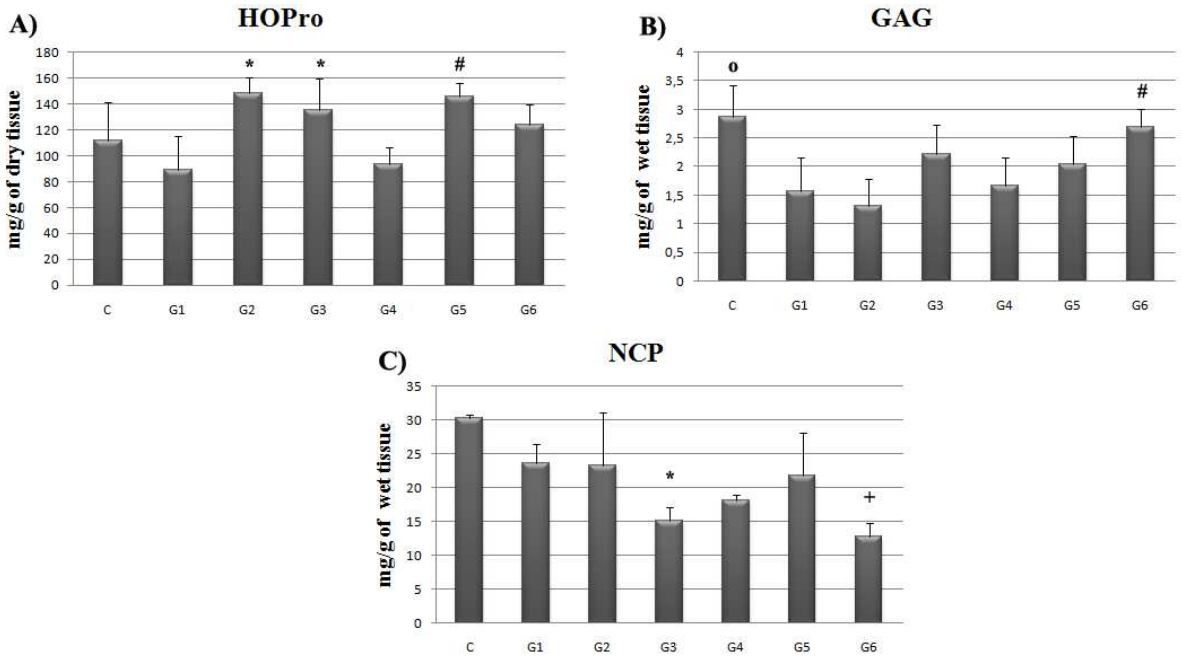

Figure 1. Quantification of extracellular matrix components. C: control group; G1: tendinitis in 7 days; G2: tendinitis supplied with green tea; G3: tendinitis supplied with green tea and glycine diet in 7 days; G4: tendinitis in 21 days group; G5: tendinitis supplied with green tea in 21 days; G6: tendinitis supplied with green tea and glycine diet in 21 days. **(A)** Concentration of hydroxyproline (HOPro): G2 and G3 showed high levels of this amino acid in relation to G1 (*$p<0.05$); additionally, G5 exhibited a high concentration of hydroxyproline compared with G4 (#$p<0.05$). **(B)** The concentration of sulfated glycosaminoglycans (GAG): G6 displayed a higher concentration than the G4, (#$p<0.05$); C showed a high concentration in relation to all other groups except for G6 (o). **(C)** The concentration of non-collagenous proteins (NCP): G3 exhibited a lower level of NCP in relation to C, G1 and G2 (*$p<0.05$); G6 showed a lower concentration in relation to G5 (+$p<0.05$).

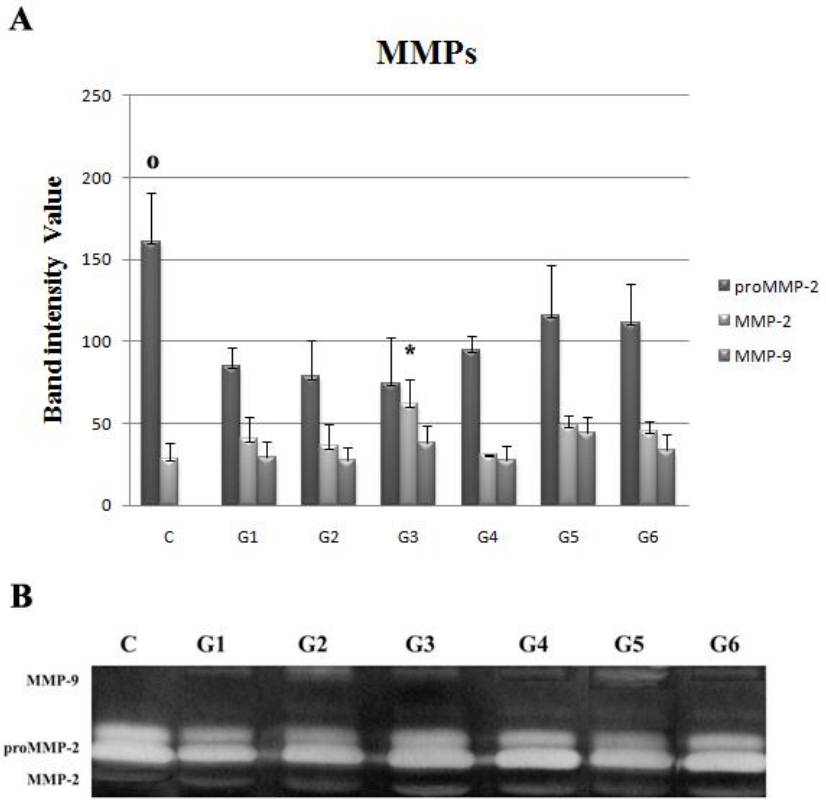

Figure 2: Densitometry of metalloproteinase bands (A) and zymography gel (B). C: control group; G1: tendinitis in 7 days; G2: tendinitis supplied with green tea; G3: tendinitis supplied with green tea and glycine diet in 7 days; G4: tendinitis in 21 days group; G5: tendinitis supplied with green tea in 21 days; G6: tendinitis supplied with green tea and glycine diet in 21 days. ProMMP-2 showed high activity in C compared with the other groups (**o** p<0,05). MMP-2 displayed a higher level in G3 compared with G1 (*p<0,05.). There were no differences between the experimental groups in relation to MMP-9. **o**Significant difference compared with all inflamed groups; * significant difference compared with G1.

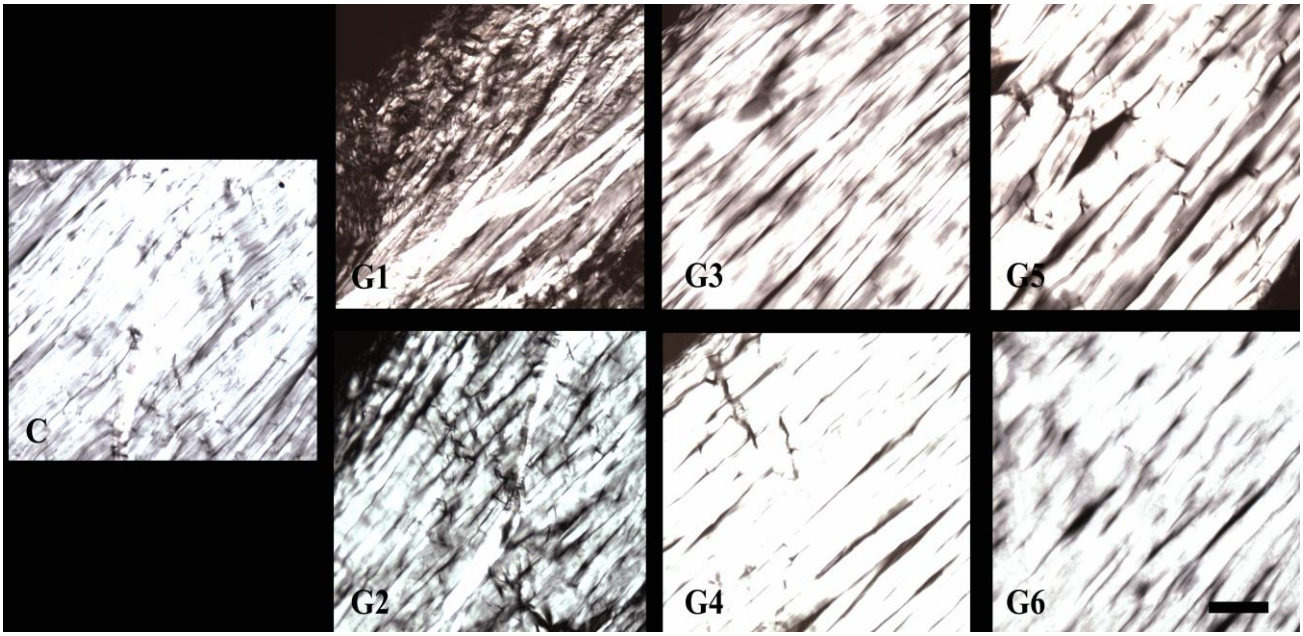

Figure 3: Birefringence viewed in longitudinal sections of tendons from the different groups, observed via polarization microscopy. The longest axis of the tendon is positioned at 45° in relation to the crossed polarizers. C: control group; G1: tendinitis in 7 days; G2: tendinitis supplied with green tea; G3: tendinitis supplied with green tea and glycine diet in 7 days; G4: tendinitis in 21 days group; G5: tendinitis supplied with green tea in 21 days; G6: tendinitis supplied with green tea and glycine diet in 21 days. Lower brightness was observed in G1 compared with the others groups. G3 showed similar characteristics to C when compared with G1. G4 exhibited high brightness compared with G5 and G6. However, G3, G5 and G6 displayed brightness similar to C.

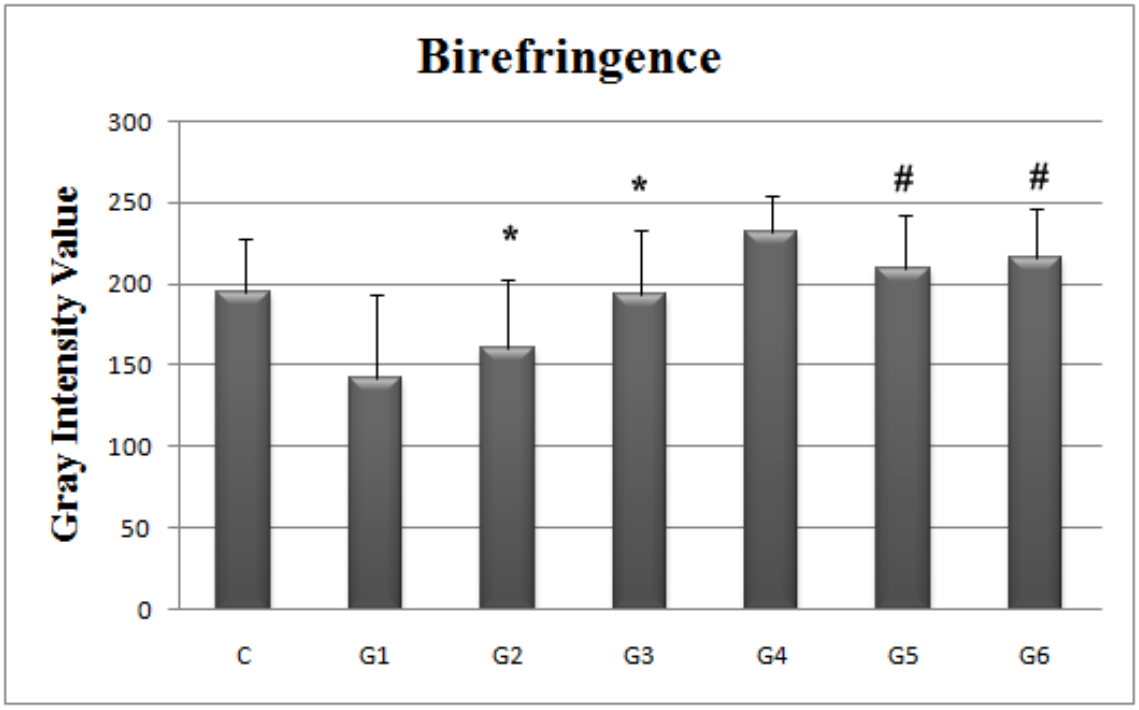

Figure 4: Birefringence measurements in different groups of tendons. C: control group; G1: tendinitis in 7 days; G2: tendinitis supplied with green tea; G3: tendinitis supplied with green tea and glycine diet in 7 days; G4: tendinitis in 21 days group; G5: tendinitis supplied with green tea in 21 days; G6: tendinitis supplied with green tea and glycine diet in 21 days. The birefringence in G2 and G3 was higher than in G1 (*$p<0.05$), and the level in G4 was higher than in C, G5 and G6 (#). However, the measurements obtained in G3, G5 and G6 were similar to those found in group C. *#$p<0.05$.

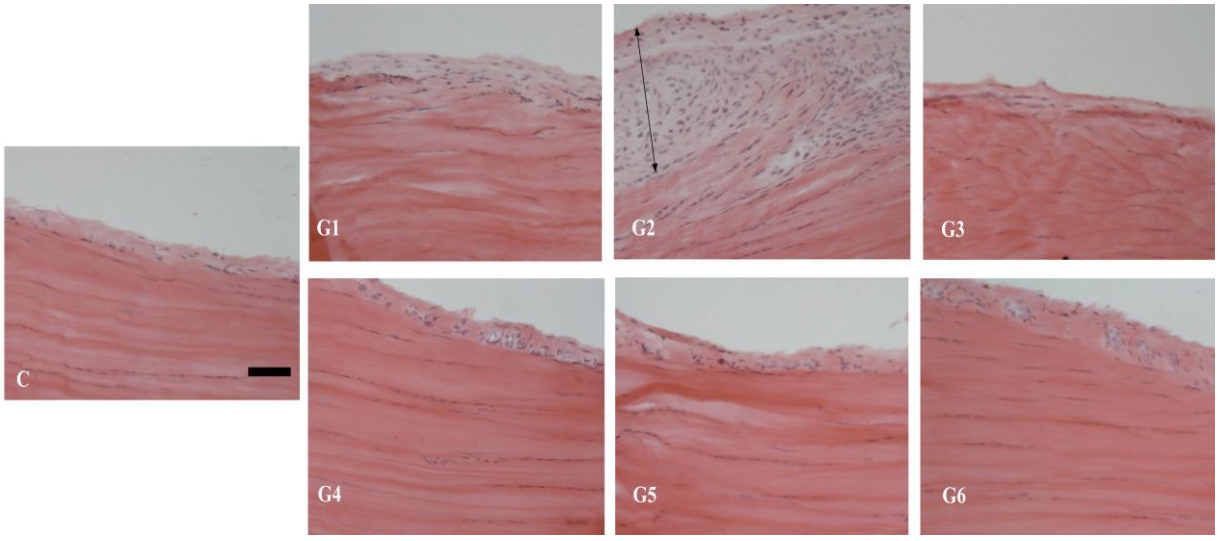

Figure 5: Histological appearance of tendon sections staining with Hematoxylin-Eosin. C: control group; G1: tendinitis in 7 days; G2: tendinitis supplied with green tea; G3: tendinitis supplied with green tea and glycine diet in 7 days; G4: tendinitis in 21 days group; G5: tendinitis supplied with green tea in 21 days; G6: tendinitis supplied with green tea and glycine diet in 21 days. It is possible to note the thicker epitenon (arrow) in G2 when compared with C, G1 and G3. There were no differences in relation to epitenon thickness among G4, G5 and G6. The amount of cells were higher in G2 in comparison with G1. There were no differences in relation to the cells in the other groups. Bars: 50 µm;

Table 1: Morphometry of tendon sections stained with hematoxylin-eosin represented by the mean and standard deviation. C: control group; G1: inflamed group at 7 days; G2: inflamed and supplied with green tea after 7 days; G3: inflamed and supplied with green tea + a glycine diet after 7 days; G4: inflamed group after 21 days; G5: inflamed and supplied with green tea after 21 days; G6: inflamed and supplied with green tea + a glycine diet after 21 days.

Parameters	**C**	**G1**	**G2**	**G3**	**G4**	**G5**	**G6**
Cells/$\mu m^2(10^{-4})$	8.0±0.78	6.0±0.8	9.0±1.0[a]	6.0±0.5	6.0±0.8	5.0±0.3	5.0±0.4
Epitenon/ µm	93.2±25	233.7±45	302.4±58[b]	89.9±30[c]	111.7±23	186.0±70	220±69

The number of cells was greater in G2 compared with G1 and G3 ([a]). In relation to the 21-day groups, there were no differences noted. The epitenon was thicker in G2 compared with the other experimental groups after 7 days of treatment ([b]). G3 showed a thinner epitenon compared with G1 ([c]).

Table 2: Biomechanical properties of the Achilles tendons.

Groups	Maximum Load (N)	Displacement (mm)	Maximum Stress (MPa)	Cross-sectional area (mm^2)
C	53±7.0	1.40±0.5#	57.3±6.3	0.86±0.2
G1	36±4.8	1.14±0.2	52.1±9.4	0.66±0.1
G2	38±9.9	1.01±0.2	40.0±13.1	0.90±0.1
G3	36±8.0	1.11±0.2	47.3±7.7	1.04±0.2*
G4	40±6.5	1.02±0.4	39.0±12.2	0.85±0.2
G5	49±3.7#	1.09±0.2	43.0±9.2	1.07±0.2
G6	44±6.4	1.37±0.2#	42±6.2	1.00±0.1

The maximum load were higher in G5 compared to G4. These values were close to those of C. G6 showed the largest cross-sectional area compared to G1. Also, G6 showed high displacement compared to G4. *statistical difference in relation to G1. # statistical difference in relation to G4 ($p<0,05$).

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CAPÍTULO 3

Effect of the tendinitis and of the treatment with glycine diet and green tea on the myotendinous junction of rats

Abstract

Objectives: Our purpose was to analyze the effects of tendinitis induced in the osteotendinous region (OR) of the Achilles tendon in the myotendinous junction (MTJ) and, to verify the treatment with green tea and glycine diet, considering their anti-inflammatory effects. Methods: We used male Wistar rats divided into five groups: C, control; G1, tendinitis; G2, tendinitis supplied with green tea; G3, tendinitis supplied with glycine diet; G4, tendinitis supplied with green tea and glycine diet. The treatment with green tea (700mg/kg/day) and 5% glycine diet last for 7 days. Non-collagenous protein (NCP) and glycosaminoglycans (GAG) were quantified. Zymography for MMPs (2, 7, 8 and 9) and western blotting for collagen I, IL-1β and MMP-8 was also performed. Results: Although the tendinitis was induced in the OR, it was enough to triggered inflammatory effects in the MTJ. The treatments regulates the activity of MMP-2, -9 and -8, and induces the synthesis of collagen type I, HOPro, GAG and NCP. Conclusions: The MTJ is the weakest region of the tendon, and could be very compromised in tendinitis. The therapies, mainly when combined, modulates the inflammatory process and induce the synthesis of elements involved to restructure the tissue after lesion.

Introduction

Tendons are anatomic structures that generally promote the insertion of the muscles into bones [1]. Basically each muscle has two tendons, one proximal and one distal. The point of attachment of tendon into the muscle is called myotendinous junction, while the junction of tendon into the bone is called osteotendineous junction [2].

The myotendinous junction (MTJ) is a specialized anatomical region of the skeletal system, where the tension generated by a muscle contraction is transmitted from the intracellular contraction of muscle proteins to extracellular proteins that constitute the collagen fibers. The MTJ is a major area for transmitting force from the skeletal muscle system and acts in joint position and stabilization. The final portion of tendons form many finger-like processes to increase the contact area of muscle and tendon, that attributes high adherence of this region [3, 4, 5]. Although various findings suggest that under loading conditions the MTJ is the weakest element in the muscle-tendon unit, making it susceptible to strain injuries. Studies regarding the pathological changes in this region and of its response to the atrophy process is very limited [5].

In collagenase induce-tendinitis is possible to note a high disorganization of collagen fibers probably by degradation of collagen molecules, its biomechanics properties are compromised, the presence of inflammatory cells, an unbalance between metalloproteinases e its inhibitors can be noted (Fig 1) [6, 7]. It is known that, if tendinitis is not controlled it can become a serious picture characterized as tendinosis.

Diet supplemented with glycine is a good alternative as treatment of many types of inflammatory process [8, 9, 10, 11]. Glycine modulates inflammatory response indicators through changes in the expression of pro-inflammatory cytokines such as IL-1, IL-6, IFN-γ, TNF-α [12]. Also, glycine minimizes injury caused by D-galactosamine [13] or cyclosporine A [14], and has been used in the prevention and treatment of model of cancer cachexia [15] and melanomas [9] as well as prevents liver damage after exposure to alcohol [16]. Glycine is the most prevalent amino acid in the triple helix of collagen. The collagen (95% of wet weight) is the main component of tendons and it is known that the glycine biosynthesis does not satisfy the need for collagen synthesis [17].

Moreover, therapeutic benefits from green tea consumption have seen in inflammatory and cardiovascular diseases as well as several types of cancer [18, 19, 20]. GT decreased production of MMP-1, MMP-2, MMP-3, IL-6, IL-1β e consequently the presence of TNF-α. [21, 22, 23]. Among the components of GT, (-)- epigallocatechin-3-gallate was effective in preventing IL-8 production in airway epithelial cells and to inhibit inducible NOS (iNOS) and COX-2 expression in cartilage [24].

In our previous study, we treated the tendinitis of Achilles tendon in rats with green tea (GT) and glycine diet (GD), and it was noted a better organization of collagen bundles following of an improved in the biomechanical properties. Besides a high amount of glycosaminoglycans and hydroxyproline content were noted [7, 25].

Of note, there is no studies about the effect of tendinitis and the post-treatments with GT and GD in the MTJ. Considering that the tendinitis effects have been analyzed in previous studies, our purpose was to analyze the effect of induced tendinitis in the osteotendinous region of the Achilles tendon in the MTJ and, to verify the post-treatment with GT and GD as a new possible alternative for the treatment of this injury.

Materials and Methods

Experimental groups

In this study, we used male Wistar rats (60 days), weighing on average 300 grams from the Multidisciplinary Center for Biological Investigation of the State University of Campinas. The rats were housed two per cage in a 12-h light/dark cycle at 23 °C, with free access to standard rat chow and water. Animal care was in accordance with the European Convection for the Protection of Vertebrate Animals used for Experimental and Other Scientific Purposes and it's consistent with the ethical principles of animal experimentation adopted by the Brazilian College of Animal Experimentation. The protocol was approved by the Ethics Committee on Animal Experiments of the State University of Campinas (UNICAMP), SP, Brazil, and filed under no. 2307-1.

The experimental groups were animals divided into five groups: **C**, control group; **TE**, tendinitis; **GT**, tendinitis supplied with green tea; **GD**, tendinitis supplied with glycine diet; **GTD**, tendinitis supplied with green tea and glycine diet. Five animals in each group were used for the analysis.

To induction of tendinitis, the animals were first anesthetized with isoflurane (Forame) and given an intratendineous injection of 10µl of collagenase (10mg/ml; Sigma, C6885) dissolved in sterile buffered saline (PBS) [26] in the osteontendinious region of Achilles tendon. After induction of tendinitis, the animals of the treated groups received green tea orally and glycine diet during 7 days.

Preparation and administration of the Green tea and Glycine diet

The leaves of *Camellia sinensis* were used and follow the proceedings of extraction according to Amra Perva-Uzunalic and collaborators [27]. The plant *Camellia sinensis* was obtained commercially by Yamamoroyama, SP. The leaves were weighed and it was added distilled water followed by water bath at 80^{o}C for 20 minutes. The animals received 700mg/kg/day given orally by gavage [25]. A diet containing 5% glycine (AJINOMOTO, BRAZIL) was provided by the Institute of Biology, Animal Physiology, State University of Campinas, following the guidelines of AIN-93M [28]. The glycine diet was administered to the animals, and the consumption was recorded daily.

The animals that received both treatment weighed weekly to detect if there were some alteration in relation to the weigh rats after/during the treatment. Our analyzes did not show significant differences in relation to animals weight.

Extraction procedures

The MTJ was separated and removed of the right tendon and pulverized in liquid nitrogen. Then, the MTJ was treated with 50 vol of 4 M guanidine hydrochloride (GuHCl) containing 20 mM EDTA and 1 mM PMSF in 50 mM sodium acetate buffer pH 5.8 [29] for 24 h at 4 °C with stirring. Afterward, the material was centrifuged (13,000×g, 25 min, 4 °C),

and the supernatant was used for non-collagenous protein and glycosaminoglycans dosages and Western blotting for IL-β and collagen type I.

To the samples of the zymography assay was used the method extraction described by Marqueti [30]. The MTJ fragments after pulverization were immersed in a solution of 50 mM Tris-HCl (Synth) pH 7.4, 0.2 M Na Cl, 10 mM Ca Cl2, and 0.1% Triton X-100 with 1% of a protease inhibitor cocktail at 4°C for 2 h. After the first extraction, the samples were incubated with 1/3 of the volume of the solution described above at 60°C for 5 min.

Quantification of proteins and sulfated glycosaminoglycans

The extracts collected of MTJ were used to quantify the relative amounts of protein according to the method of Bradford [31]. Bovine serum albumin was employed as a standard. The quantification of GAGs was performed using the dimethylmethylene blue method [32] with chondroitin sulfate as the standard. The absorbance was measured at 595 nm for proteins and at 540 nm for GAGs using an ASYS Expert Plus Microplate Reader (Biochrom, Holliston, MA, USA).

Zymography for MMPs

It was used 15 μg of the protein extract was loaded onto the gel. The protein samples were electrophoresed at 4°C on a 10% polyacrylamide gel containing 0.1% gelatin, and after completion of electrophoresis, the gel was washed with 2.5% Triton X-100 (Nuclear) and incubated for 21 hr in a solution of 50 mM Tris-HCl (pH 7.4), 0.1 M NaCl, and 0.03% sodium azide (Sigma) at 37 °C. The gel was stained with Coomassie brilliant blue R-250 for 1 hr. After staining, the gels were washed with a solution containing 50% methanol and 10% acetic acid to observe negative bands of proteins (MMPs) corresponding to enzymatic activity [30]. The protein standards assist to identify the position of enzymes in the gel. As a positive control, 20 mM EDTA was used in the incubation buffer. EDTA inhibits the activity of gelatinase and confirms the identification of MMPs in the gels. The bands in the negative image were quantified by densitometry using Scion Image software Alpha 4.0.3.2 (Scion Corporation, Frederick, MD, USA).

Quantification of Hydroxyproline

The fragments of the MTJ were pulverized in liquid nitrogen and they were immersed in acetone for 48 hr and then in chloroform: ethanol (2:1) for 48 hr. Thereafter, the fragments were hydrolyzed with 6 N HCl (1 mL/10 mg tissue) for 16 hr at 110◦C. The hydrolysate was neutralized with 6 N NaOH and treated with a 1.41% solution of chloramine-T (Sigma) and 15% p-dimethylaminobenzaldehyde as described by Stegemann and Stalder [33]. Subsequently, the samples were incubated for 15 min at 60◦C. The solution was cooled to room temperature, and the absorbance was read at 550 nm using a Diode Array spectrophotometer (Model 8452A, Hewlett Packard).

Western Blotting

For collagen I detection, were precipitated 10 µg of total protein from the guanidine extract, using a solution containing 1 M sodium acetate buffer pH 7.4 (100 mL)and 9 volumes of ethanol (1350 mL) for 24 h at 4 ◦C. After three washes (150 mL acetate buffer 1 M sodium pH 7.4 and 1350 mL of ethanol), the precipitate obtained was dried at 37 ◦C and resuspended in reducing sample buffer (0.5 M Tris–HCl pH 6.8, 26% glycerol, 20% SDS, 0.1% Bromophenol Blue). For MMP-8 and IL-1β detection, were precipitated 80 µg of total protein, the same procedure was made, but the precipitate obtained was ressuspended in reducing sample buffer containing β-mercaptoethanol 5%. Proteins MTJ subjected to electrophoresis on SDS-polyacrylamide (6%) were transferred to nitrocellulose membrane, as described by Towbin et al. [34].

After electrophoresis, the proteins were transferred to Hybond-ECL nitrocellulose membranes (Amersham, Pharmacia Biotech, Arlington Heights, USA) at 70 V for 3 h and incubated at 4°C for 10 min with the following primary antibodies: collagen type I (C2456, Sigma) diluted in basal solution with 1% BSA (1:50), MMP-8 (SAB4501895, Sigma) diluted in basal solution with 1% BSA, IL-1β (sc- 74138, Santa Cruz) diluted in basal solution with 1% BSA (1:100), and glyceraldehyde 3-phosphate dehydrogenase (GAPDH: sc-25778, Santa Cruz), used as an endogenous control, diluted in basal solution with 1% BSA (1:50). The membranes were incubated for 10 min with goat or mouse secondary HRP-conjugated antibody (A0545 and A0412, respectively, both Sigma) diluted (1:100 and

1:500) in basal solution with 1% BSA. The peroxidase activity was detected by incubation with a diaminobenzidine (DAB) chromogen for 5 min.

Statistical Analysis

Data were presented as mean ± SD of results obtained from five/six animals per group. To compare the data, statistical analysis was performed using one-way analysis of variance (ANOVA) with the Tukey post-hoc test. A value of $p<0.05$ was considered statistically significant, and the statistical program GraphPad Prism®, version 3.0 was used for all analyses.

Results

In this study, we verify two types main results: (1) the effect of collagenase induce-tendinitis on MTJ, since the inflammatory process was applied in the osteotendinious region and (2) the post-treatment with GT and GD isolated and combined. The elements involved in the remodeling process of extracellular matrix were analyzed and it was possible to note indicators of beneficial effects of GT and GD in the tendinitis of the Achilles tendon.

Non-collagenous protein (NCP) amount was decreased in TE when compared to C and GTD group ($p<0.001$). It was noted that GTD showed a high concentration of NCP in relation to TE, GT and GD ($p<0.05$), the value found was closer to C group. Also, GT group showed a lower amount of NCP in comparison with TE (Figure 2).

In relation to total sulfated GAGs, GD and GTD showed a high content in comparison with C group ($p<0.01$). Among the tendinitis groups there were not differences (Figure 3).

In the hydroxyproline dosage, an indirect method to quantify the amount of total collagen of the tissue showed high amount in G3 when compared to C, TE, GT and GTD ($p<0.05$). Among the others groups there were no differences (Figure 4).

Western blotting for collagen type I showed high amount in GTD in comparison to TE and GD ($p<0.05$) Among the others treated groups there was no statistical difference (Figure 5A-5B).

All the tendinitis groups showed high presence of IL-1β. Besides, IL-1β content was higher in GD in comparison GT (Figure 5C).

The MMP-2 has three isoforms known (Figure 6). Considering the proMMP-2 (62 kDa, latent isoform), there was an increase in TE in comparison with all the others groups. The active MMP-2 (72 kDa) showed high activity in all groups with tendinitis. In addition, GTD exhibited high activity of MMP-2 in comparison to C, TE and GD group ($p<0.05$). Regarding the intermediate isoform (68 kDa), no differences were observed among the groups (Figure 7A).

We also analyzed all the isoforms of the MMP-9 (Figure 6). The proMMP-9 (92 kDa, latent isoform) exhibited less presence in GTD when compared to TE. The value found was similar to the C group. TE showed high presence of the proMMP-9 in comparison with C. Homodimeric MMP-9 (220 kDa) showed high presence in C when compared to TE, GD and GTD. The proMMP-9-lipocalin-complex (130 kDa) was similar in all groups. Considering the active isoform of MMP-9 (83 kDa), we observed less activity of the enzyme in GTD in comparison with TE. All the tendinitis group exhibited presence of the active isoform of MMP-9 ($p<0.05$). There was no active isoform of MMP-9 in C group (Figure 7B).

In relation to the MMP-8 (75 kDa) it is possible to note high activity in all tendinitis groups in the western blotting (Figure 5D). There were no differences in the activity of MMP-8 among the TE, GT, GD and GTD (Figure 7C). However, GD and GTD showed less activity of MMP-8 when compared to TE in the zymography gel (Figure 7C) ($p<0.05$).

MMP-7 (28 kDa) showed less preence in GT in comparison to C and TE (Figure 7D).

Discussion

Under pathological process, it is known that the MTJ is the weakest component in the muscle-tendon unit [5]. Actually, little is discussed about the effects of lesions/inflammatory process in this specific region (MTJ) of the Achilles tendon. Here, we evaluated through biochemical and molecular analyses the effect of collagenase induced-tendinitis in MTJ, after the induction in OR. Also, the post treatment with GT and GD isolated and combined was analyzed.

The collagenase induce-tendinitis degrades collagen bundles allowing the decrease of this component in the tendon [26]. Collagen type I presented high amount in C compared to TE as expected. In GTD was noted an increased of the collagen type I. Apparently, the GD and GT together induce the synthesis of the main element of tendons.

In general, individuals with tendinopathy have pain and discomfort during the gait [35, 36, 37]. With appropriate treatments, the pain and discomfort can reduce, consequently, promote a better movement and induce the collagen molecules formed to trigger an alignement of the collagen fibers [36].

Apparently, GTD group enabled a faster recovery of the collagen type I after excessive degradation that occurred after inducing tendinitis. It is important to consider that all tendon is composes predominantly by collagen type I (95% of wet weight) [38, 39]. The biomechanics properties are improved when the collagen is reconstituted after a lesion [40].

Hydroxyproline is the typical amino acid found in collagen molecules [41]. Our previous study, it was verify a decrease in the amount of HOPro in the Achilles tendon with tendinitis [7, 25]. Here, it was not noted differences between C and TE group. However, GD showed a high contend of HOPro. The increase was superior when compared to the others inflamed group and C. Although, the HOPro content indicates the presence of collagen in the tissue, this does not mean the presence of intact collagen. This is can explain why exist differences between the amout of HOPro and collagen type I presence.

There were differences in the profile of the effect of tendinitis in the MTJ when compared with all the Achilles tendon. Some elements of extracellular matrix increased in MTJ, while in analyze of the all the tendon the components decreased [7, 25]. Apparently, the action of GT and GD together avoid the degradation or induce the synthesis of NCP in the tissue after induce tendinitis. The amount found in G4 was closer to those in C.

It is known the great part of GAG of the tendon is in the compression region [42]. The MTJ is the tension region and is not characterized by great amount of GAG [3, 4], due the collagen bundles aligned in one direction, being characterized by a tension region of the Achilles tendon. There were not evidenced significant differences between TE and C in relation to amount of GAGs. GAGs have important role in the collagen fibrilogenesis controlling the diameter of collagen fibers [39]. GTD group showed differences in relation to C. Here, we suggest that GT + GD together can assist a better improvement of the composition of these elements during the tendinitis. The high GAG content can be a beneficial for the recovery process of tendinitis, considering that during the tendinitis process a less GAG amount is noted in analysis of the all the tendon [7, 25].

The gelatinases are extensively studied, mainly by be active enzymes during pathological process in tendons [43, 44, 45, 46]. MMP-2 have three isoforms [47]. The latent isoforms are proMMP-2 and intermediate MMP-2. With both data is possible to determinate how many enzymes are in latent state during the lesion. The intermediate isoform was similar in all groups. However, the latent isoform showed a high presence in the TE group when compared to the others groups. In turn, the MMP-2 had high activity in GTD in comparison with the others groups. This enzyme is involved to remodeling process after lesion [48, 49] and it can suggest the faster remodeling process in animals treated with GT and GD together after tendinitis.

MMP-9 is secreted mainly by neutrophils, macrophages and others inflammatory cells [43, 49, 50]. In general, MMP-9 is found in high activity in acute and chronic inflammatory process [45, 51]. Here, we analyzed different latent isoforms of MMP-9 and its active isoform. All the isoforms of MMP-9 showed less activity in GTD in comparison with the others groups. The lower activity of MMP-9 can suggest that the tissue is on regulation, such as, the fast remodeling induced by MMP-2 could be related with the action of MMP-9 during the tendinitis. Apparently, both treatments (GT + GD together) regulate the activity of these enzymes. The glycine and green tea had properties in inhibiting TNF-α and IL-1β, respectively [8, 22, 23]. Both pro-inflammatory cytokines induce activation of gelatinases and other enzymes involved in inflammatory process [52, 53, 54]. In fact, the IL-1β showed an increase in all the tendinitis groups. GT and GTD exhibited a tendency in

reduce the values of this cytokine in comparison to tendinitis group. Otherwise, the high presence of IL-1β noted only in GD did not change the activity of MMPs.

Although, in the zymography gel contained gelatin have been used to detect the gelatinases activity (MMP-2 and -9), we were able to identify other MMPs, present in the electrophoresis gel, as MMP-8 and MMP-7 which can also degrade gelatin [49, 55]. For that, we believe the isoform of 75 kDa is the MMP-8, a collagenase with capacity to degrade collagens, glycoproteins, proteoglycans and gelatin [49]. Also, the enzyme of 28 kDa can be the MMP-7, a matrilysin with properties to degrade proteoglycans and gelatin.

We verify MMP-8 through two methods: zymography gel and western blotting. In relation to MMP-8, high activity was detected in tendinitis groups. This is already expected, in view of that during the collagenase induce-tendinitis occurs excessive degradation of collagen. The disruption of collagen molecules through of its fragments is involved in the activation of different types of collagenase, gelatinases and others enzymes [56]. GD and GTD group controlled the increase of MMP-8 aactivity noted in the tendinitis process. It is important to say that the degradation rate is as important as the synthesis of new elements and a control is very necessary in both [57]. We suggest the during the inflammatory process like tendintis occured the inhibition of MMP-7, mainly in GT group.

Conclusion

Although the tendinitis was induced through the intratendineous injection in the osteotendinous region of the Achilles tendon, it was enough to triggered inflammatory effects in the MTJ. The post-treatment with GT and GD assists the recovery process of tendinitis. As already seen in our previous study, both treatments induce the synthesis of main elements involved to restructure of tissue after lesion and the biomechanics properties of the Achilles tendon [7, 25]. Here, we noted the same beneficial profile. Mainly in the data corresponded to GT + GD treatment together. Apparently, these two elements are involved directly to control the inflammatory process in regulate the activity of MMP-2, MMP-9 and MMP-8. Also, the collagen type I, HOPro, GAG and NCP exhibited amount observed in the C, it indicates the better recovery of the main elements that composed the tendon after treatment with GT and GD. Since the MTJ is the weaskest region of the

tendons and under tendinitis this region is also affected, we showed that with the treatment with GT and GD together could be a new alternative to rapid recovery after the lesion.

Figures

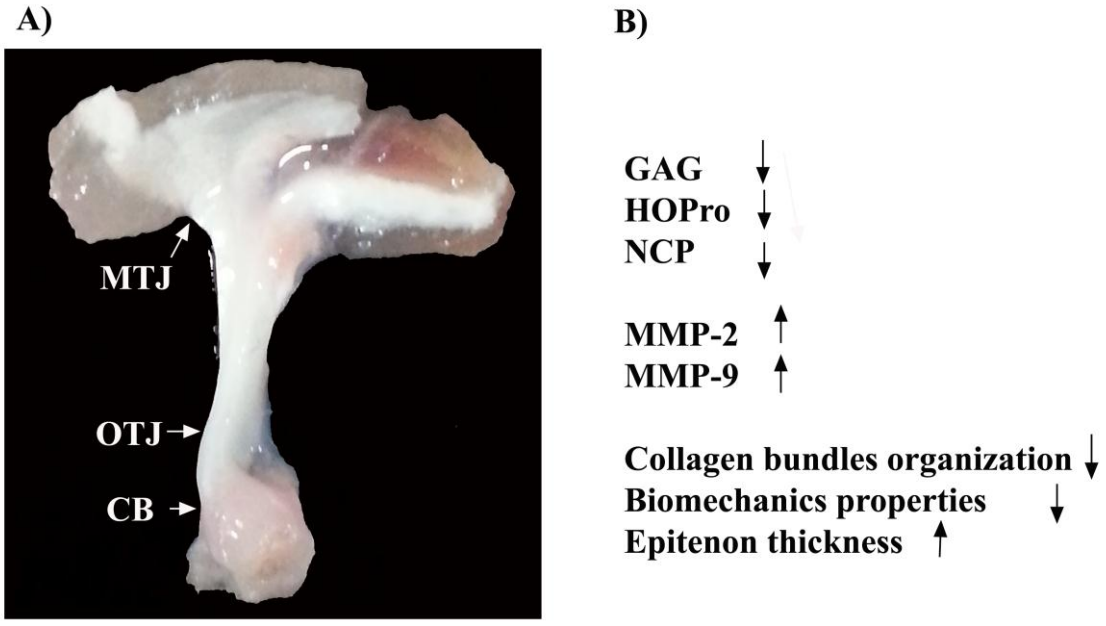

Figure 1: (A) Achilles tendon with its regions: MTJ: myotendinous junction; OTJ: osteotendinious region; CB: calcaneal bone. The induction of tendinitis was in OTJ and the effects were analyzed in MTJ. (B): In Achilles tendon under tendinitis of our previous study, we noted a decrease of GAG, HOPro and NCP content, greater activity of MMP-2 and MMP-9, low collagen bundles organization, low biomechanics properties and high epitenon thickness.

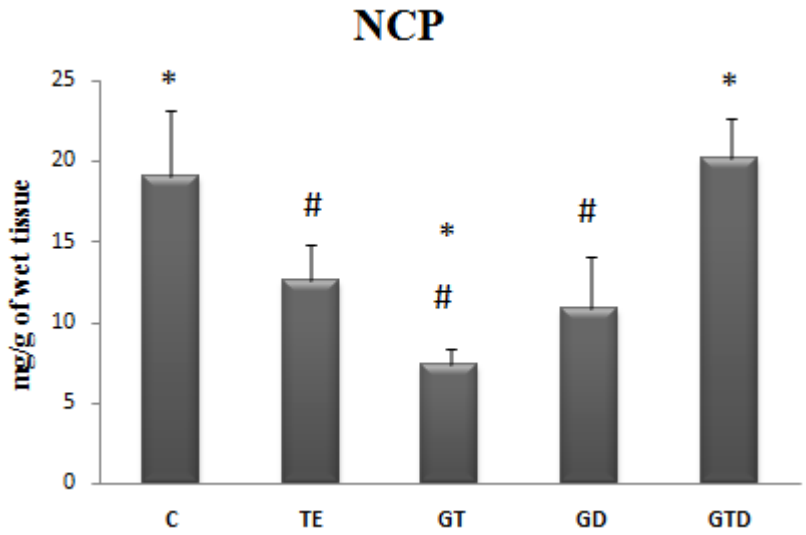

Figure 2: Quantification of Non-collagenous proteins (NCP). TE showed less amount of NCP in relation to C and GTD group ($p<0.01$*). GTD showed a high content of NCP in comparison with TE, GT and GD ($p<0.05$). This value was closer to found in C group. GT showed a decrease of NCP when compared with TE ($p<0.05$*). *significant difference compared with TE; # significant difference compared with C.

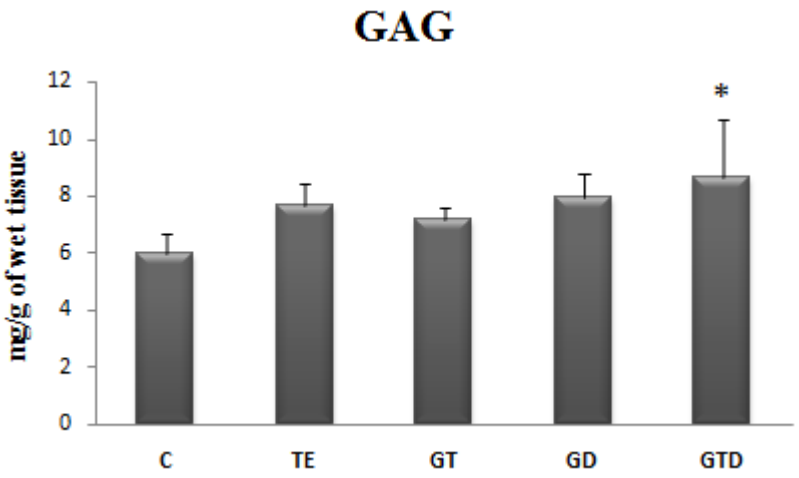

Figure 3: Quantification of sulfated glycosaminoglycans. No differences were found between C and TE. GTD showed high amount of GAG in relation to the C ($0<0.05$#). Among the tendinitis group were not noted statistical differences. # significant difference compared with the C.

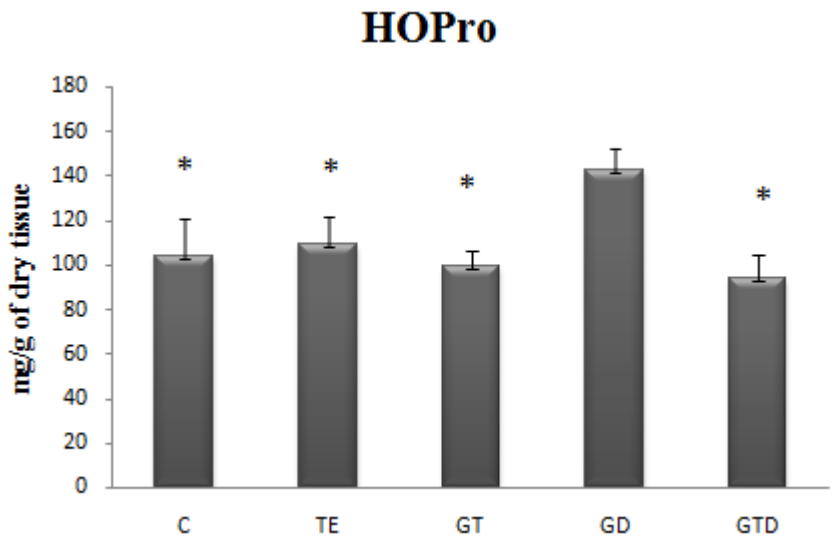

Figure 4: Quantification of Hydroxyproline. High amount of HOPro was noted in GD when compared to C, GT and GTD ($p<0.05$*). There were no difference among the others groups. *significant difference compared with GD.

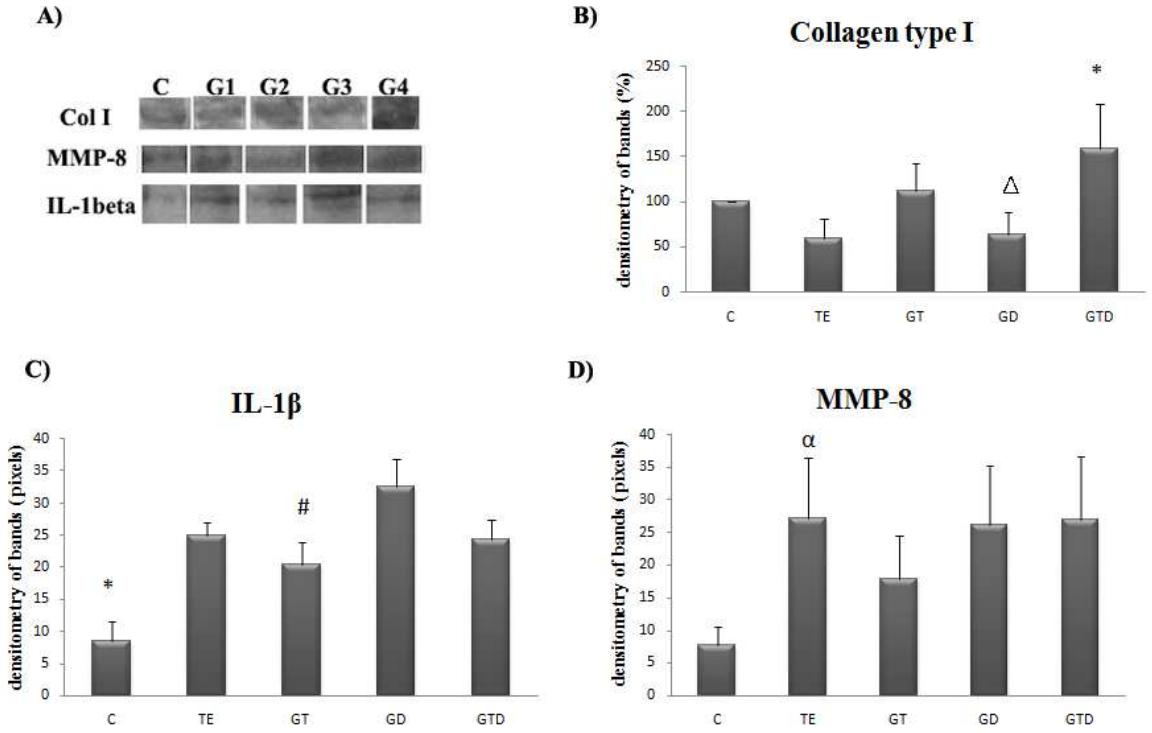

Figure 5: Densitometry of western blotting for collagen type I, MMP-8 and IL-1β. **A)** Presence of proteins in nitrocellulose membrane; **B)** Densitometry of collagen type I: A high amount of collagen type I was noted in GTD in comparison with TE ($p<0.05$*). Also, lower content of collagen type I was noted in GD in comparison with GTD ($p<0,05\Delta$). Among the others treated groups there was no statistical difference.*significant difference compared with TE; Δ significant difference in relation to GTD. **C)** Densitometry of IL-1β: All tendinitis group exhibited high presenc of IL-1β in relation to C. There were statistical differences between GD and GT. GT showed a high amount of IL-1β in comparison with GD ($p<0,05$#) *significant difference compared to all tendinitis group; #significant difference compared to GD. **D)** Densitometry of MMP-8: All tendinitis groups showed high presence of MMP-8. It were not noted differences among them. α significant difference in relation to C

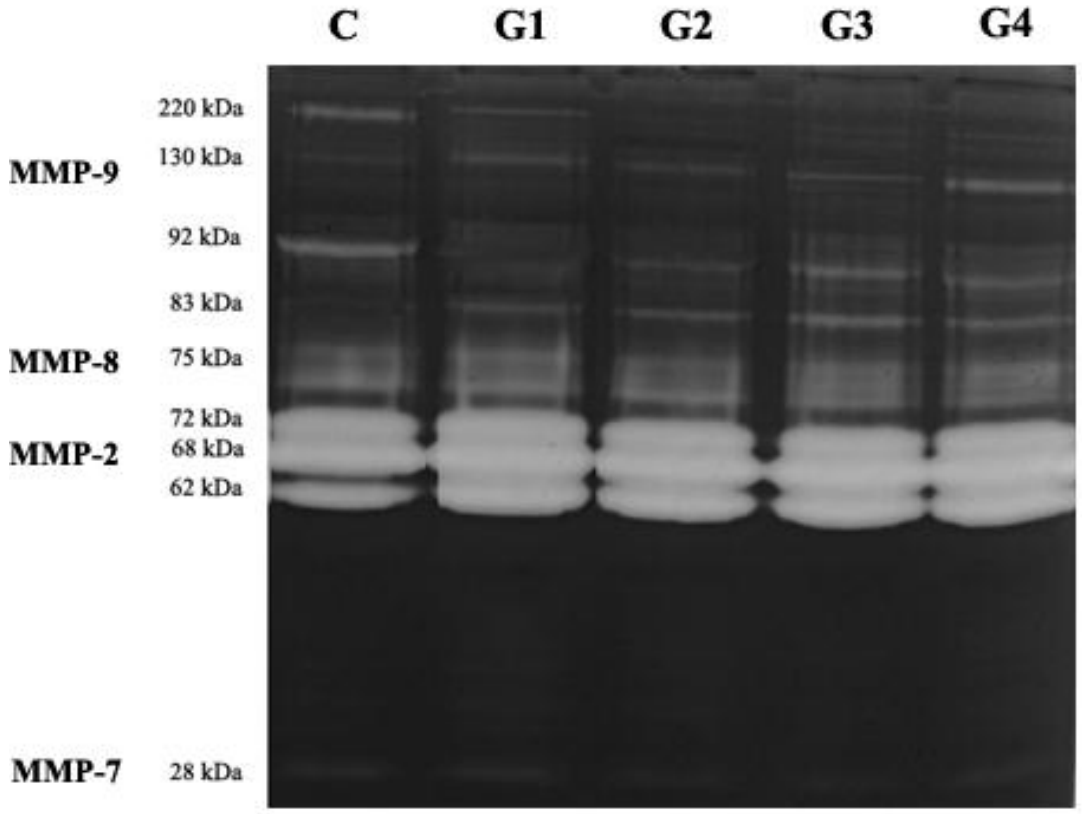

Figure 6: Zymography gel for MMPs. The profile of the isoforms of MMP-9, MMP-8, MMP-7 and MMP-2 was evidenced. The latent isoforms of MMP-9 are in 220 kDa, 130 kDa and 92kDa on the gel. The active isoform of MMP-9 is 83 kDa. The MMP-8 was noted in 75 kDa position. The proMMP-2, intermediate and active isoform are in 72, 68 and 62 kDa, receptively. The 28 kDa position is the MMP-7 isoform. The weight of the proteins followed a standard of proteins known. The analysis of weight of enzymes on the gel was important to perform the densitometry of bands in figure 7.

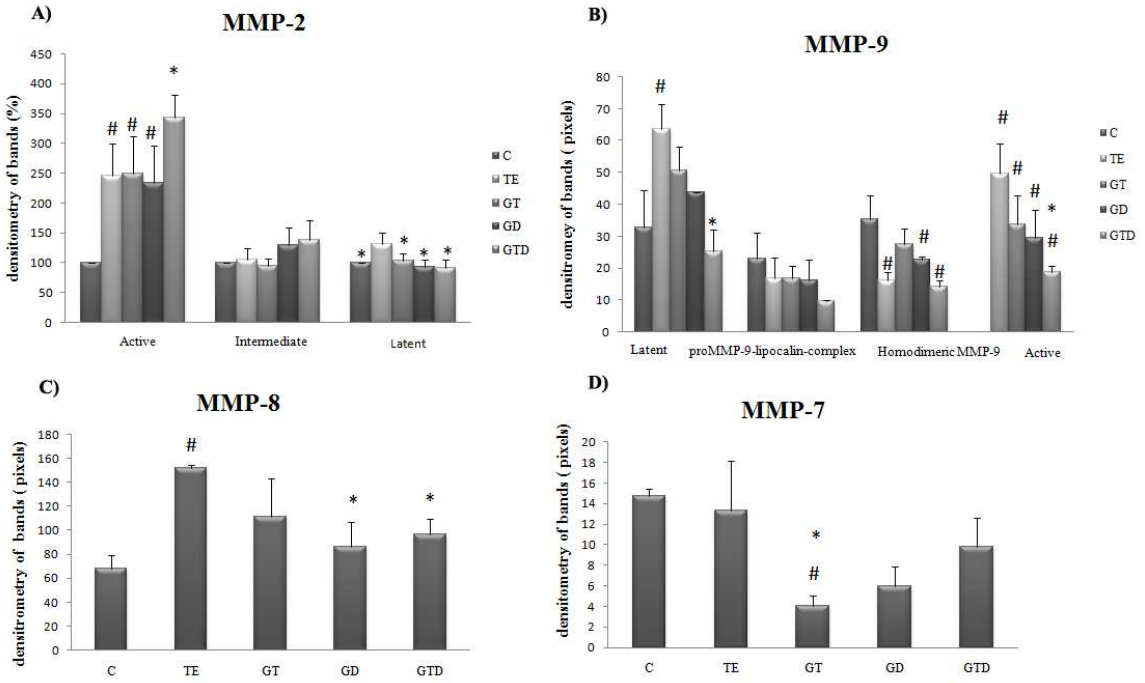

Figure 7: Densitometry of zymography gel for MMPs activity. A) Three isoform of MMP-2 were analyzed. There was an increase of proMMP-2 between TE and C, GT, GD and GTD ($p<0.05$*). The active isoform of MMP-2 showed an increase in all the tendinitis groups (TE, GT, GD and GTD) in comparison with C ($p<0.05$#). GTD showed a greater activity of MMP-2 in comparison to TE ($p<0.05$*). B) There was low presence of proMMP-9 in GTD in comparison with TE ($p<0.05$*). A high presence of the isoform was detected in TE when compared to C ($p<0.05$#). Homodimeric MMP-9 (220 kDa) showed high presence in C in comparison with TE, GD and GTD ($p<0.05$#). The active isoform of MMP-9 had a high activity in TE in relation to C ($p<0.05$). GTD exhibited less activity of the MMP-9 in comparison with TE ($p<0.05$*). The isoform of MMP-9 active was not evidenced in C. C) There were no differences in the activity of MMP-8 among the tendinitis groups. An increase of activity of MMP-8 was noted in TE in comparison with C ($p<0.05$#).vIn GD and GTD was detected a decrease of the activity of MMP-8 ($p<0.05$*). D) MMP-7 (28 kDa) showed less preence in GT in comparison to C and TE ($p<0,05$*#). *significant difference compared with TE; # significant difference compared with the C.

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CAPÍTULO 4

Glycine improves the inflammation induced by TNF-α in tenocytes culture

Abstract

Glycine has been used for therapeutic purpose in inflammatory process. *In vivo*, the glycine diet showed beneficial effects in biochemical, biomechanical and morphological properties of Achilles tendon with tendinitis. In this study, it was verified the effect of glycine in tenocytes submitted to inflammatory process in culture medium. For that, the inflammation was induced with TNF-α (10 ng/mL) with and without previous treatment the cytokine with glycine (20 mM). Expression of MMPs, TIMPs and collagen I was analyzed by PCR. Zymography assay was performed to observe the profile of activity of MMP-2 and MMP-9. Also, Uronic acid dosage, Proliferation and migration cellular were studied. Glycine was able to change the inflammatory effect of TNF-α in tenocytes. Expression of mRNA for collagen I, MMP-2 and TIMP-2 were similar to group without inflammation. The activity of MMP-2 and MMP-9 increased in 48h in comparison to 24h group treated with glycine. Uronic acid content was low in 24 h in the glycine group. The cellular migration was faster in better in the treatment of glycine in 24h in comparison to the others groups. Here, we noted the remodeling process was happening in the extracellular matrix after inducing the inflammatory process. All data together suggest the glycine can revert the inflammatory action of TNF-α in tenocytes. This study indicates the glycine as an therapeutic alternative to treat tendons with tendinitis.

Introduction

Tendinitis is an acute inflammation that alters the biochemical and morphological properties of the tendon, making it weaker and predisposed to rupture. (Järvinem et al., 2005; Lake et al., 2008, Vieira et al., 2014a). Tendinopathy cases are very common in athletes and the population in general. Nowadays, the use of different drugs are increasing considerable, as well as, the alternative therapy for the treatment of tendinopathies. The huge concern on surgeous is the low cellularity and vascularity found in tendons (James et al., 2008).

Tenocytes are fibroblast-like cells that are the main cells found in tendons. Besides, these cells has a important role in the maintenance, repair and remodeling of tendons through of synthesis of extracellular matrix (ECM) elements. TNF-α is typical proinflammatory cytokine that can influence various cellular functions, including the metabolism of the extracellular matrix (Furuyama et al., 2008). TNF-α and IL-1β stimulate tenocytes in vitro to produce further pro and anti-inflammatory cytokines (IL-1beta, TNF-α, IL-6 and IL-10) and ECM degrading enzymes such as MMPs. This suggest an autoactivation of tenocytes by exogenic cytokines (Tsuzaki et al., 2003; John et al., 2010). Several studies have demonstrated an alteration in the levels of MMPs in tendinopathy. Breakdown can be stimulated by increased MMPs activity and reduced levels of tissue inhibitor of metalloproteinases (TIMP) (Riley et al., 2008, Vieira et al., 2012; De Oliveira et al., 2013; Del Buono et al., 2013).

Glycine has been used in treatment for inflammatory process in different tissue and, beneficial effects were evidenced (Carmans et al., 2010; Stoffels et al., 2011, Vieira et al., 2014a,b). The inhibitory role of glycine in some cytokines (IL-1β and TNF-α) is a good sign to modulate inflammatory process (Hartog et al., 2007; Hasegawa et al., 2012; Blancas-Flores et al., 2012). In fact, glycine is the predominant amino acid of the triple helix of collagen molecule (Jozsa and Kannus 1997) and it has been noted that a treatment with glycine can improve the recovery process of the tissue after tendinitis. The effect of glycine in induced-tendinitis *in vivo* showed the synthesis of important elements of ECM such as collagen, non collagenous proteins, glycosaminoglycans and the activation and

inhibition of MMP-2 and MMP-9 respectively, involved directly in the remodeling process (Vieira et al., 2014a,b).

Of note, it is important to understand the behavior of tenocytes in the inflammatory process aimed a alternative therapy to the pathologies in tendons. Therefore, considered the beneficial effects of glycine in the tendinitis of Achilles tendon, we investigated its action in medium culture of tenocytes under inflammatory process induced by TNF-α.

Materials and Methods

1. Isolation of tenocytes

Achilles tendon of rats was removed and immersed in PBS + antibiotics (penicillin-streptomicin 1%). Small fragments of tendon were cut in pieces of $1mm^2$ followed by treatment with collagenase 0.2 % in PBS by 15 min at 37 °C.

The remained fragments was put in the wall of plate of 25 mL upright for 4 hr on Dulbecco's modified Eagle's medium (DMEM) with 20% fetal bovine serum (FBS). After the period of attachment of the sections on the plate, the flask was lying down. The first passage of cells was around ten days (Figure 1A). The tenocytes culture consisted of DMEM contained 10% FBS. Cultures were grown at 37 °C in a humidified atmosphere with 5% CO_2. Tenocytes in the 5^{th} passage were used. This study was approved by Ethics Committee on Animal Experiments of the State University of Campinas, and filed under no. 2307-1.

2. Treatment with TNF-α and glycine

The $1.5.10^5$ cells were cultivated in 6 wells plate. The cells were put in quiescence for 24 h before of the beginning of the treatments. During all treatments the cells were cultivated under DMEM without FBS. For the induction of the inflammatory process was used TNF-α (10ng/mL) (Zhu et al.,2011; Vignozzi et al., 2012) and to the treatment with glycine was used 20 mM (Hasegawa et al., 2012). One group received only glycine before

the TNF-α treatment, in the period of quiescence. After the first 24 h, TNF-α was added in the plate of cells. The cells received glycine after the same period. The analyses was after 24 and 48 h with glycine and only with TNF-α in the culture medium and cells.

3. Immunofluoresce labelling of tenocytes

Tenocytes were characterized by staining for vimentin (Giusti et al., 2014). Cells were washed twice with PBS and fixed with acetone for 30 min at 4 °C. The cells were blocked with PBS/BSA 1% for 2 hours at room temperature and incubated overnight at 4 °C with primary antibodies, anti-mouse vimentin (Sigma), diluted 1:200 in PBS containing 2% bovine serum albumin (BSA). The secondary antibody used was FITC (0.5 mg/mL, eBioscience, San Diego, CA/USA) diluted 1:250 and incubated for 40 minutes at RT. We labeled nuclei with DAPI (0.1 mg/mL in methanol) for 15 minutes at 37 °C. The slides were analyzed in the Fluorescence Microscope (Olympus BX60, camera QColor 3) with objectives 10 and 20 x and the images were captured using the program QCapture 4.0.

4. Proliferation assay (MTT assay)

The proliferation assay followed the MTT protocol. It was added 8.000 tenocytes in the plate of 96 wells and all experimental treatments were performed. After the confluence of cells, the culture medium was removed and added 200 µL of new culture medium. Afterwards, 50 µL of MTT (Sigma, M2128) was added to the plate and let to incubate for 4 h at 37 °C. Posteriorly, the culture medium and MTT were removed and 200 µL of DMSO were added, dissolving the MTT crystals formed. Then, glycine buffer was added and immediately the absorbance was measured at 570 nm (Edmondson et al., 1988).

5. Migration cellular assay

Tenocytes were plated on 6 well-dishes in DMEM containing 10% FBS and cultured to confluence. The treatments with TNF-α and glycine were performed. During the treatments the tenocytes were in DMEM without FBS. Afterwards, with the use of a sterile

200-µL pipette tip, the cells were stratched vertically. Cultures were photographed using Olympus BH-2 microscope and an Olympys C- 5060 camera. Pictures were taken on the time zero, 1, 2, 3, 4, 5, 6, 24 and 48 h. The area not covered by cells was measured using the ImageJ software, and the percentage of the initial wound area covered by cells over time was computed.

6. Quantification of uronic acid

The determination of uronic acid was evaluated according to Fila and collaborators (2001). 200 µL H_2SO_4 were added in 25 µL of sample, incubated in 80 °C. Afterward, the plate was let in room temperature for few minutes. The absorbance was measured at 492 nm. After the first reading, 40 µL of m-hidroxyfenil was added in the plate and after fifteen minutes, the absorbance was measured again. The difference between the first and second absorbance was the result of uronic acid concentration in the samples.

7. PCR

Total RNA was isolated using TRIsol reagent (Life Technologies) according to the manufacturer's instructions. Reverse transcription was performed from 4µL of total RNA using a high-capacity cDNA reverse transcription kit (Applied Biosystems). PCR systems used was SYBR FAST Master Mix according to the manufacturer's instructions. The PCR software used was APPLYRT, that it consists at 10 min in 25 °C followed of 2 hrs at 37 °C. The PCR was developed for 2 min at 94 °C, 30 sec at 52° C and 30 sec at 72 °C. 32 cycles were made. Afterwards, agarose gel (2%) was performed with the samples and specific standard. The β-actin was used as housekeeping. The mRNA analyzed were Col I, MMP-2, -3, TIMP-1 and -2.

8. Zimography gel for metalloproteinases

The medium cells was collected and 30 µL was used to zymography gels. The protein samples were electrophoresed at 4 °C on a 10% polyacrylamide gel containing 0.1%

gelatin, and after completion of electrophoresis, the gel was washed with 2.5% Triton X-100 and incubated for 21 h in a solution of 50mM Tris-HCl (pH 7.4), 0.1M NaCl, and 0.03% sodium azide (Sigma) at 37 °C. The gel was stained with Coomassie brilliant blue R-250 for 1 h. After staining, the gels were washed with a solution containing 50% methanol and 10% acetic acid to observe negative bands of proteins corresponding to the enzymatic activity (Marquetti et al., 2006). As a positive control, 20 mM EDTA was used in the incubation buffer. EDTA inhibits the activity of gelatinase and confirms the identification of MMPs in the gels. The bands in the negative image were quantified by densitometry using Scion Image software Alpha 4.0.3.2 (Scion Corporation, Frederick, MD, USA).

Results

Tenocytes in culture received TNF-α as inductor of inflammatory process and glycine was added in the cell culture, before and after the TNF-α be added. The effect of glycine was analyzed until the first 48 hours. Imunoassay was performed to verify if the cells extracted of tendons were tenocytes. As tenocytes are modified-fibroblasts, the marker was vimentin. The assay confirmed the type of cells expected (Figure 1B).

The analysis of the treatment with glycine showed different results considering the mRNA for collagen type I, MMP-2, MMP-3, TIMP-1 and TIMP-2 (Figure 2). The expression of collagen type I and MMP-2 was high in cells treated with TNF-α. Cells treated with glycine in advance exhibited less expression of collagen type I and MMP-2. In 24 hours of glycine in culture, it was possible to note collagen type I expression similar to the group treated with glycine beforehand, however, considering MMP-2 expression, it was observed less expression in comparison with this group and with TNF-alfa group. Tenocytes submitted in 48 h of treatment with glycine showed less expression of collagen type I, MMP-2 and MMP-3 in comparison to TNF-α group with the same period. The expression of TIMP-1 was high in tenocytes treated with glycine for 48h. The value was close to the C group. Tenocytes treated with glycine before of TNF-α showed high

expression of TIMP-2, although groups treated with glycine after of TNF-α showed less expression of TIMP-2 in 24 and 48h of treatment.

The activity of MMPs was analyzed by zymography gel (Figure 3C). Tenocytes that received glycine 24 h before of the inflammation showed lower presence of active and latent isoform of the MMP-2. After 48 h of treatment is possible to note a trend of increase of active isoform in comparison with inflamed group of this period. Pro-MMP-2 was lower in all treated groups in 24 and 48h (Figure 3A). The latent and active isoform of MMP-9 exhibited less presence in tenocytes in culture treated before and after of the inflammatory process in 24 h in comparison with the inflamed group. However, after 48 h, there was an increase of presence of MMP-9 in treated group with glycine (Figure 3B). The isoform active of this enzyme was not observed in tenocytes without inflammation.

Less content of uronic acid was noted in the group treated with glycine 24 h (Figure 4). However, in the group of 48 h of glycine treatment the amount of uronic acid was high in comparison to TNF-α in the same period.

In MTT assay for cell proliferation, we noted all groups with the same characteristics (Figure 5). However, we observed a trend in increase only the TNF-α. Considering the wound closure of tenocytes, it was noted a similar closure between control group and glycine post 24h. Both promoted a faster wound closure in relation to TNF-α group and glycine pre 24h. In 48h, all groups exhibited similar characteristics (Figure 6 and 7).

Discussion

The main difficulty in treatment of lesions in tendons is due to the poor vascularity and cellularity found in this tissue (Lesic and Bumbasirevic et al., 2004; Sharma and Maffulli 2005). Here, it was analyzed tenocytes isolated from Achilles tendon that received or not TNF-α followed of the treatment with glycine before and after the inflammation to be installed. It is important to understand the behavior of tenocytes in the inflammation aimed a therapy alternative to the pathologies in tendons.

TNF-α is proinflammatory cytokine related to death cellular as well as is responsible to trigger the degradation of extracellular matrix through of inducing activity of MMPs (Magra and Maffulli 2005; Fang et al., 2006;). In general, the baseline production of MMPs

is low, but their synthesis or activation may be induced by cytokines (interleukin -1, -4, -6 and 10, and TNF-α), growth factor and cell-cell or cell-matrix interactions (Gan et al., 2001; Hosaka et al., 2010, Clutterbuck et al., 2010; Gupta 2012). The expression of MMP-2 and MMP-3 have been changed in tenocytes in culture with TNF-α and treated with glycine. Both enzymes showed increased levels in inflamed group, however, when tenocytes were treated with glycine, an low expression was evidenced. The MMP-3 gene is already known to be related in the pain of tendinopathies (Raleigh et al., 2009). In these case, glycine induced the decrease of the expression of MMP-3. Probably, the inhibition of transcription of this gene can be a favorable effect of the glycine to diminish the degradation process and perhaps, the pain in tendinopathy process.

The turnover of the ECM is a dynamic process in which synthesis and degradation are finely balanced (Magra and Maffulli 2005). The MMPs are able to degrade the connective tissue and change the properties of ECM. Of note, its regulation is necessary to efficient remodeling. MMP-2 is a gelatinase which degrade smaller collagen fragments released, and cleave denatured collagens (Chakraborti et al., 2003; Clutterbuck et al., 2010). When glycine was added in the culture in advance of the TNF-α, we noted that the activity of MMP-2 was low. However, in 48 h of glycine treatment is possible to note a trend of increase of active isoform of MMP-2. MMP-2 and -3 mediate the tissue degradation and later remodeling during lesions of tendons (Del Buono et al., 2013).

Glycine showed inhibitory effects in the activity of isoform active of MMP-9 in the pre-treatment and 24 hours after inducing of the inflammatory process. However, this isoform exhibited high activity after 48 h of the treatment. MMP-9 is secreted by inflammatory cells and is related with acute and chronic inflammatory process. This enzyme may be induced by others MMPs and pro inflammatory cytokines (Chakraborti et al., 2004; Adair-Kirk and Senior 2008; Gill and Parks 2008). It was noteworthy glycine modulated the expression and activity of MMPs. Being these data necessary during the reorganization of tissue under pathological states.

Considering the inhibitors of MMPs (TIMPs), it was observed that the mRNA expression of TIMP-1 was high after 48h of glycine treatment. Moreover, TIMP-2 expression was low in this same group and 24 h after the glycine treatment in comparison to TNF-α group in these respective periods. The role of TIMPs is to regulate the MMPs in

high levels during pathologic process (Gill and Parks 2008; Xu and Murrell 2008). In chronic diseases such asthma, arthritis and tendinopathies, there is no control of MMPs, which can be one of reasons for the healing process is difficult (Ohbayashi 2002; Ahmed et al., 2004; Riley 2008; Oikonomidi et al., 2009; Vieira et al., 2013). Tenocytes that received glycine in culture medium before addition of TNF-α expressed high mRNA of TIMP-2. Thus, we suggest the prior treatment of glycine can be a better alternative to avoid unbalance of MMPs during inflammatory process.

Collagen type I showed a less expression in treated groups. Despite, TNF-α group have exhibited a high expression of this mRNA, the expression of collagen type I in all the treated group was closer to that found in tenocytes not submitted to inflammation. Collagen type I is the predominant protein found in ECM of tendons, approximately 65 % to 80 % of the dry mass of the tendon and represents 95 % of the total collagen in a normal tendon (Benjamin et al., 2008; James et al., 2008). In tendinopathy, the collagen content and composition have been changed. An increase of ratio of type III to type I collagen, high expression of type I collagen mRNA and percentage of denatured collagen are characteristics observed (Birch et al., 1998; Ireland et al., 2001; Xu and Murrell 2008). In fact, glycine can promote the necessary expression of the collagen type I in tenocytes submitted to TNF-α in culture. It can be a good effect of glycine considering that the alteration in the expression of this gene in tendinopathy cases.

Uronic acid dosage can indicate a indirect method to detect the amount of GAG in the tissue (Fila et al 2001). GAGs display a important role in the fibrilogenesis of collagen and are responsible to control the diameter of their fibers (Comper 1996). Tenocytes treated with TNF-α followed or not by treatment with glycine secreted a low amount of GAG. The GAG content of injured and healing tendon reflected the loss of normal tendon architecture (Fu et al., 2007; Xu and Murrell 2008; Vieira et al., 2012; Guerra et al., 2014). After 48 h, it was noted greater amount in glycine group in comparison to the TNF-α group. Our data *in vivo* showed after the treatment with glycine during 7 and 21 days, the amount of GAG increased in comparison to tendinitis group in the same period. In fact, glycine presented late effects (48 h of treatment) in relation the synthesis of GAGs by tenocytes.

The cellular migration assay exhibited favorable effects in the glycine treatment in 24 h after the addition of TNF-α in the tenocytes. The wound closure was faster in this group in

comparison to TNF-α group and the treatment of glycine treatment before of the inflammation. This last one showed the slow wound closure. It is considerable to note the differences between the treatment of glycine before and after the inflammatory process. Both showed opposite effects. During the pathological process such as rupture and tendinitis, the migration of cells is necessary to induce important elements that are able to rebuild a new ECM (Midwood et al., 2004; Sharma and Maffulli 2005). *In vivo*, the reorganization of ECM is due to division of resident tenocytes and inflammatory cells , as well as, the release of cytokines and activation of MMPs (Riley 2008; Bedi et al., 2012; Hosaka et al., 2010; Hoppe et al., 2013). Here, tenocytes responded better to the treatment with glycine in 24 h enabling an efficient migration of these cells during the inflammatory process induced by TNF-α. The cell proliferation did not show significant differences among the groups.

In summary, glycine was able to change the effect of TNF-α in culture medium of tenocytes. Expression of mRNA of MMP-2, MMP-3, TIMP-1, TIMP-2 and collagen I were altered and the most of them, the expression were similar to the group which did not receive the proinflammatory cytokine. Zymography analysis showed the activity of MMP-2 and MMP-9 elevated in 48 h in comparison to 24 h of inflammation installed. Also, uronic acid content increased after 48h of glycine treatment in the culture medium. The migration cellular was better in the treatment of glycine in 24h. To conclude, all data together suggest the positive effects of glycine in tenocytes submitted to TNF-α in culture. The treatment with glycine *in vitro* showed effects in the remodeling process of ECM, corroborating with the data found *in vivo*.

Figures

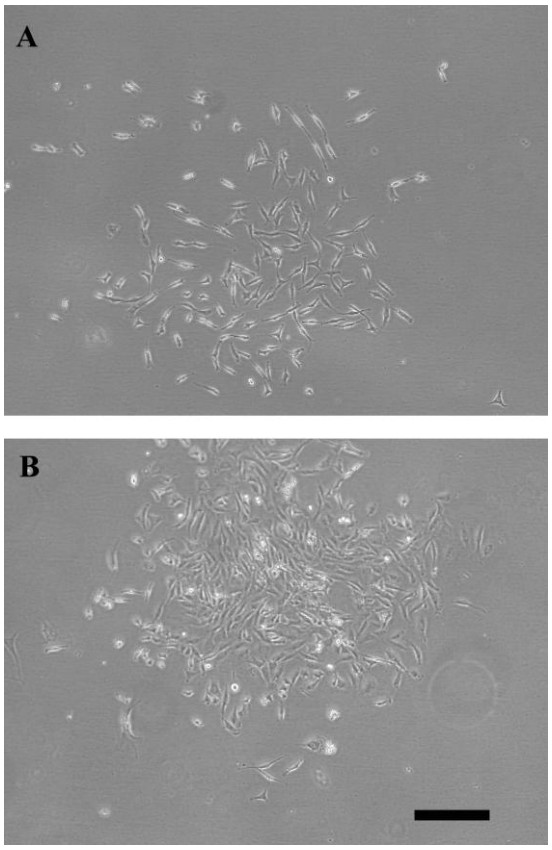

Figure 1 A: Tenocytes isolated from Achilles tendon. Images of the cells in 4 (A) and 6 days (B) after the extraction from tendon; Bar: 60µm

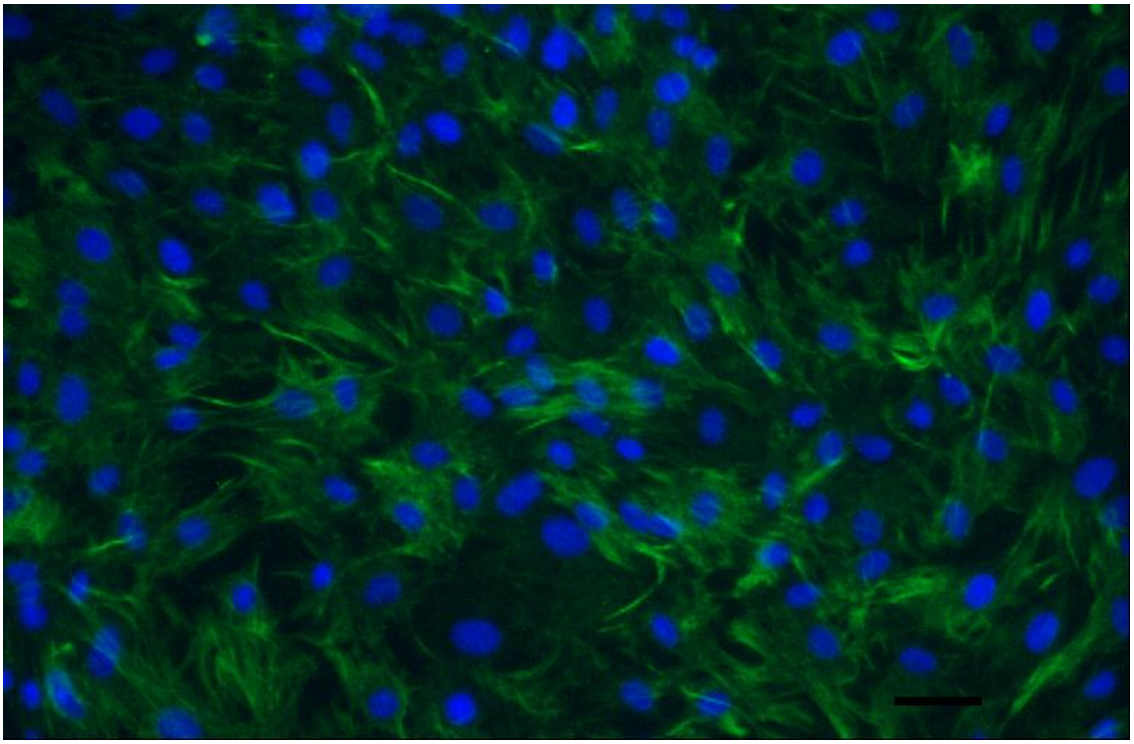

Figure 1B: Immunofluorescence of cells. Tenocytes were characterized by staining for vimentin. We labeled nuclei with DAPI and the secondary antibody was FITC. Bar: 40µm.

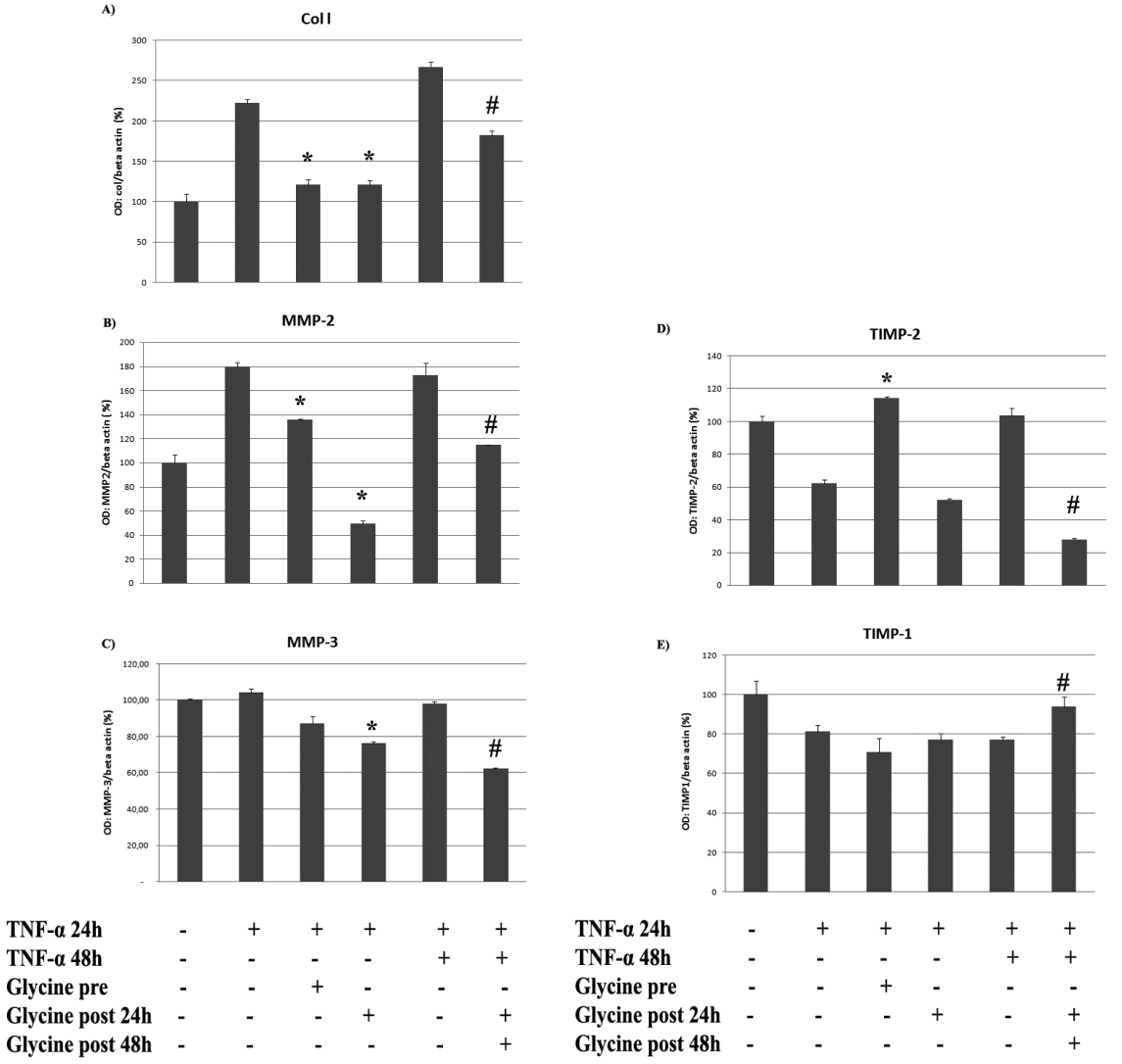

Figure 2. Type I collagen, MMP-2, MMP-3, TIMP-1 and TIMP-2 gene expression in tenocytes. **A)** Type I collagen , **(B)** MMP-2 and **C)** MMP-3 gene expression was low in relation to TNF-α groups. In A, we noted the amount of gene expression found in 24 similar to the tenocytes without inflammation. **D)** TIMP-2 gene expression was higher in tenocytes treated in advance with glycine. In tenocytes treatead in 24 and 48h, a decrease of the TIMP-2 gene expression was noted in relation to inflamed group. **E)** TIMP-1 gene expression was high in 48 h of treatment with glycine in comparison to inflamed group with the same period. Difference of the treated groups in relation to TNF-α 24h (*) or TNF-α 48 h (#).

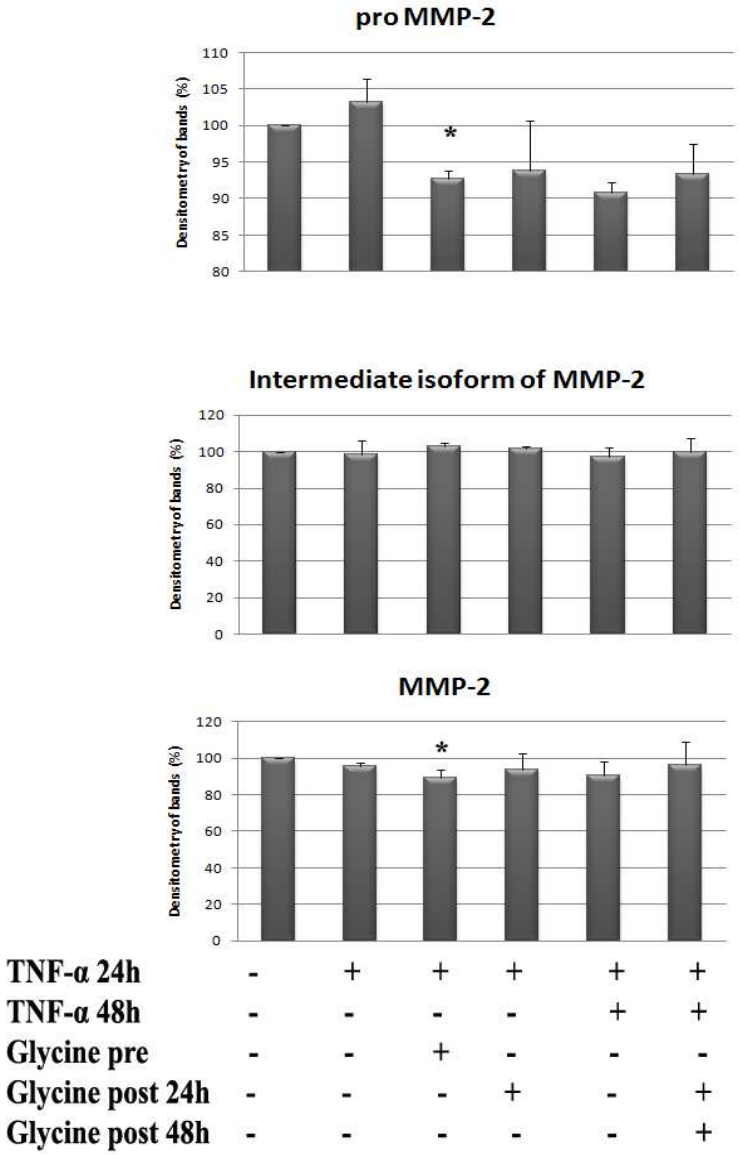

Figure 3A: Densitometry of bands of MMP-2. Pro MMP-2 showed low presence in all the treated groups with glycine and TNF-α in 48h. Active isoform of MMP-2 showed a decrease in the group with previous treatment. Intermediate isoform of MMP-2 did not show differences among the groups. Difference of the treated groups in relation to TNF-α 24h (*) or TNF-α 48 h (#).

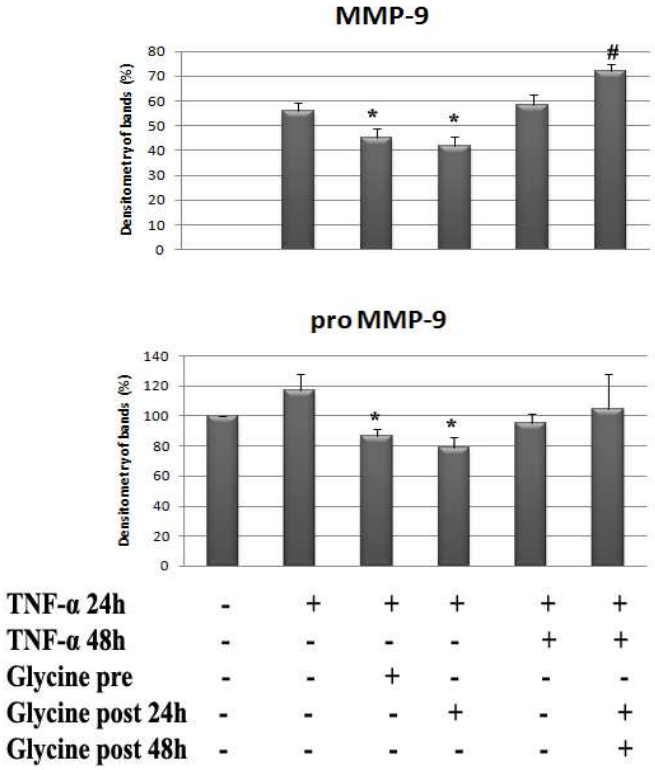

Figure 3B: MMP-9 was not detected in tenocytes without inflammation. ProMMP-9 and MMP-9 exhibited a decrease of presence in 24 h groups with prior treatment or not with glycine. In 48h, an increase of MMP-9 was observed in glycine treated group by 48h. Difference of the treated groups in relation to TNF-α 24h (*) or TNF-α 48 h (#).

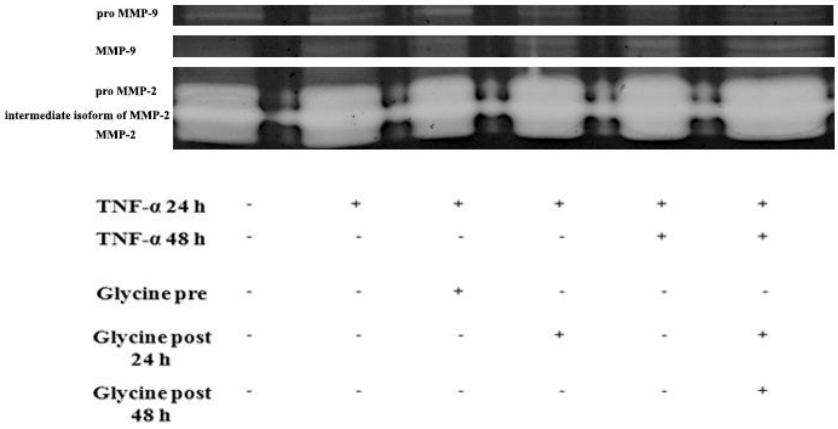

Figure 3C: Zymography gels of isoforms of MMP-2 and MMP-9.

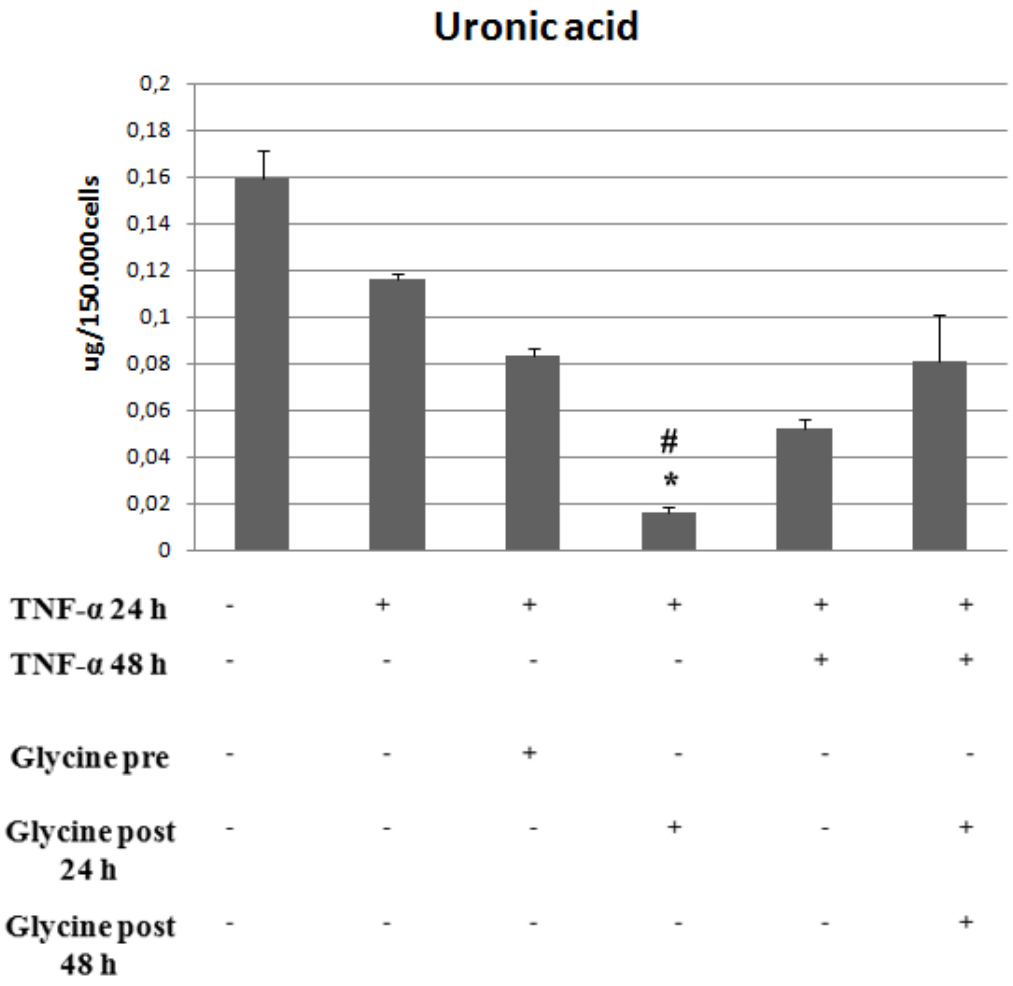

Figure 4: Uronic acid dosage. Less content of uronic acid was noted in group treated with glycine in 24h. In 48h of glycine treatment the uronic acid content was high in comparison with TNF-α in the same period. Difference of the treated groups in relation to TNF-α 24h (*) and tenocytes without inflammation (#).

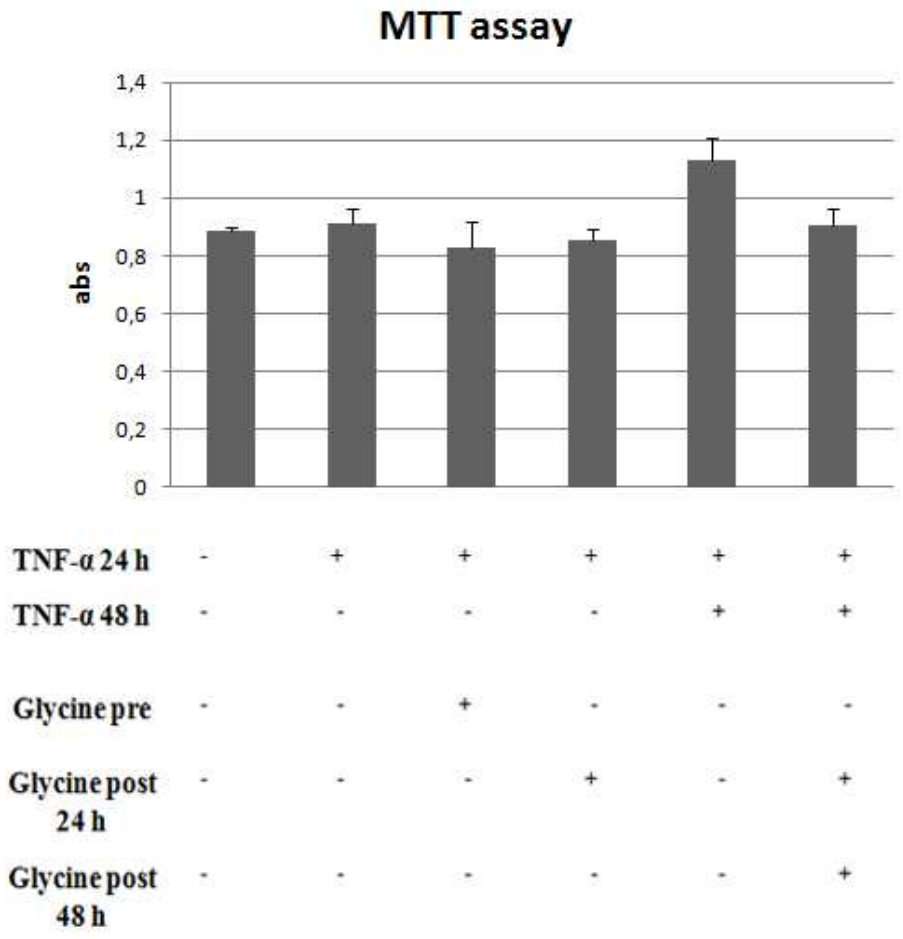

Figure 5: MTT assay. It was not observed differences among the treatments.

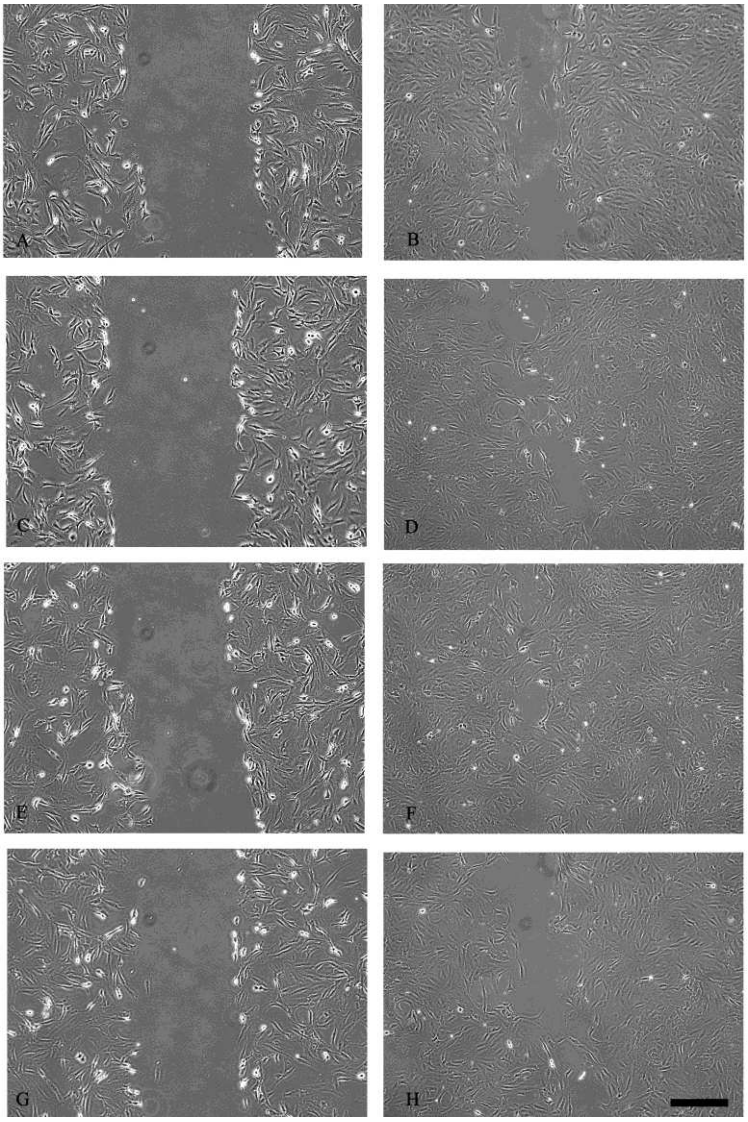

Figura 6: Cellular migration assay. A and B: groups without treatments; C and D: glycine treated before of TNF-α; E e F: TNF-α; G and H: glycine treated after TNF-α; In A, C, E and G, the imagens were taken in time zero. In B, D, F and H, the images were 24 h. Bar 60µm.

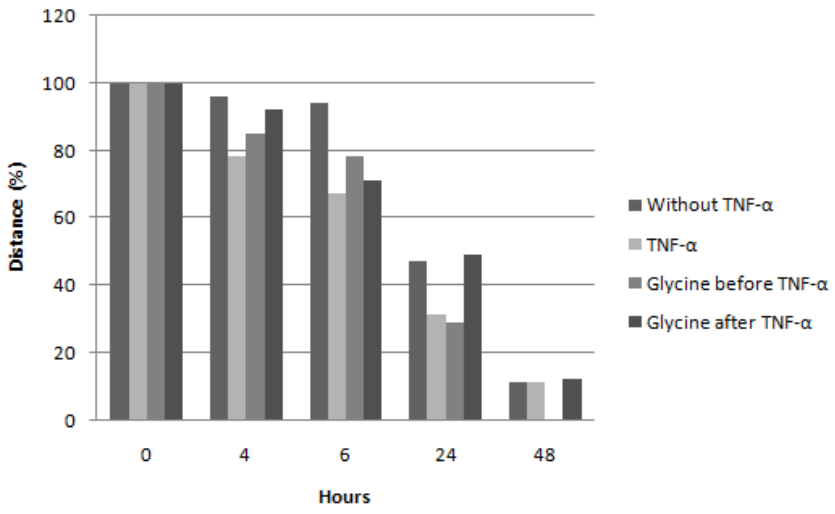

Figure 7: Graphic of cellular migration. Since of time zero the measurements were performed. The area not covered by cells were measured. The group treated with glycine before TNF-α showed a faster cell migration when compared to TNF-α group in the same period. When glycine was added after TNFα, we noted cell migration similar to tenocytes without inflammation. In 48h, all the groups were similar.

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CONSIDERAÇÕES FINAIS

Nesse estudo foi possível observar os efeitos da glicina e chá verde na tendinite do tendão de Aquiles.

No capítulo 1, observou-se alterações positivas na matriz extracelular do tendão de Aquiles com tendinite após 7 e 21 dias de tratamento com glicina. Alta concentrações de hidroxiprolina, glicosaminoglicanos e proteínas não colagênicas foram notadas em animais com tendinite tratados com glicina. Além disso, ensaios biomecânicos mostraram que tendões foram mais resistentes àruptura após 21 dias de tratamento. A Glicina induziu a síntese de elementos envolvidos no processo de remodelamento do tecido quando comparados à grupos sem o tratamento.

No capítulo 2, animais com tendinite foram tratados com chá verde e glicina. Nesse estudo, avaliamos o efeito separado do chá verde e com a dieta de glicina. Houve um o aumento do conteúdo de hidroxiprolina e aumento da atividade de MMP-2, uma melhor organização das fibras de colágeno e melhor resistência biomecânica antes da ruptura nos grupos tratados com chá verde e glicina. Nesse estudo, os resultados su geriram que ambos, chá verde e glicina, podem ser administrados juntos e com isso acelerar o processo de reparo do tendão após tendinite em ratos.

No capítulo 3, analisamos os efeitos da tendinite e do tratamento de glicina e chá verde na junção miotendínosa do tendão de Aquiles. Embora a tendinite foi induzida na região osteotendínea, essa desencadeou efeitos inflamatórios na junção miotendinosa. Ambos tratamentos regularam a atividade de MMP-2, MMP-8 e MMP-9 e induziram a síntese de colágeno tipo I, hidroxiprolina, glicosaminoglicanos e proteínas não colagências.

No capítulo 4, destacamos os efeitos da glicina em cultura de tenócitos extraídos do tendão de Aquiles. A glicina alterou o efeito inflamatório de TNF-α em cultura de tenócitos. A expressão de genes como colágeno tipo I, MMP-2, MMP-3, TIMP-1 e TIMP-2 foi alterada e na maioria deles, os resultados foram similares aos encontrados em tenócitos sem inflamação. A Atividade de MMP-2 e MMP-9 e o conteúdo de ácido urônico foram elevadas em 48 h de tratamento em comparação à 24 h. A migração celular foi melhor em 24 h no grupo tratado com glicina. O tratamento com glicina *in vitro* mostrou efeitos similares aos encontrados em nossos estudos *in vivo* e, reforçou nossa teoria que a glicina pode ser um alvo terapêutico para inflamações em tendões.

COMITÊ DE ÉTICA

DECLARAÇÃO

Declaro para os devidos fins que o conteúdo de minha dissertação de Mestrado/tese de Doutorado intitulada "Efeito da nutrição terapêutica a base de Camellia Sinensis (chá verde) e ração rica em glicina sobre a tendinite do tendão calcanear de rato".

( ) não se enquadra no § 4º do Artigo 1º da Informação CCPG 002/13, referente a bioética e biossegurança.

Tem autorização da(s) seguinte(s) Comissão(ões):

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Aluno: Cristiano Pedrozo Vieira

Orientador: Edson Rosa Pimentel

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Carimbo e assinatura

Prof. Dr. ALEXANDRE ROGERIO
Presidente da Comissão de Ética no Uso de Animais CEUA/UNICAMP

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