MARCOS DOS SANTOS DE ALMEIDA

“ANÁLISE DE DIFERENTES PROTOCOLOS DE ACUPUNTURA NOS ASPECTOS BIOQUÍMICOS, ULTRAESTRUTURAIS E ORGANIZACIONAIS DE TENDÕES DE RATOS EM PROCESSO DE CICATRIZAÇÃO”

"ANALYSIS OF DIFFERENT ACUPUNCTURE PROTOCOLS ON BIOCHEMICAL, ULTRASTRUCTURAL AND ORGANIZATIONAL ASPECTS OF RAT TENDONS IN HEALING PROCESS"

Campinas, 2015

UNIVERSIDADE ESTADUAL DE CAMPINAS

INSTITUTO DE BIOLOGIA

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"Análise de diferentes protocolos de acupuntura nos aspectos bioquímicos, ultraestruturais e organizacionais de tendões de ratos em processo de cicatrização"

"Analysis of different acupuncture protocols on biochemical, ultrastructural and organizational aspects of rat tendons in healing process"

Tese de Doutorado apresentada ao Programa de Pós-Graduação em Biologia Celular e Estrutural do Instituto de Biologia da Universidade Estadual de Campinas para obtenção do Título de Doutor em Biologia Celular e Estrutural, na área de Anatomia.

Doctorate thesis presented to the Cellular and Structural Biology Postgraduate Programme in the Institute of Biology, the State University of Campinas, required for the completion of a Doctorate degree, in the area of Anatomy.

Orientador: Prof. Dr. Edson Rosa Pimentel

Este exemplar corresponde à versão final da tese defendida pelo aluno *Marcos dos Santos de Almeida* e orientado pelo Dr. Edson Rosa Pimentel.

Assinatura do Orientador

Campinas, 2015

Ficha catalográfica
Universidade Estadual de Campinas
Biblioteca do Instituto de Biologia
Mara Janaina de Oliveira - CRB 8/6972

AL64a

Almeida, Marcos dos Santos de, 1980-
Análise de diferentes protocolos de acupuntura nos aspectos bioquímicos, ultraestruturais e organizacionais de tendões de ratos em processo de cicatrização / Marcos dos Santos de Almeida. – Campinas, SP : [s.n.], 2015.

Orientador: Edson Rosa Pimentel.
Tese (doutorado) – Universidade Estadual de Campinas, Instituto de Biologia.

1. Cicatrização de feridas. 2. Acupuntura. 3. Eletroacupuntura. 4. Eutanásia. 5. Tendões. 6. Ratos Wistar. I. Pimentel, Edson Rosa,1949-. II. Universidade Estadual de Campinas. Instituto de Biologia. III. Título.

Informações para Biblioteca Digital

Título em outro idioma: Analysis of different acupuncture protocols on biochemical, ultrastructural and organizational aspects of rats tendons in healing process
Palavras-chave em inglês:
Wound healing
Acupuncture
Electroacupuncture
Euthanasia
Tendons
Rats, Wistar
Área de concentração: Anatomia
Titulação: Doutor em Biologia Celular e Estrutural
Banca examinadora:
Edson Rosa Pimentel [Orientador]
Tatiana Carla Tomiosso
Juliana Castro Monteiro
Wagner Costa Rossi Junior
Estela Maria Correia Sant'ana
Data de defesa: 20-02-2015
Programa de Pós-Graduação: Biologia Celular e Estrutural

Campinas, 20 de fevereiro de 2015.

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1. ABSTRACT

Several studies indicate that acupuncture (AC) has both local mechanotransductor and systemic anti-inflammatory (AI) effects. These effects could potentially accelerate the healing process. Therefore, the aim of this study was to analyze the effect of different protocols of AC on the biochemical, organizational and ultrastructural aspects of rat tendons during the healing process. Male Wistar rats at 60 days of age were divided into the following groups: not tenotomized or normal group (N), tenotomized group (T), tenotomized and submitted to AC at ST-36 point (ST36), tenotomized and submitted to AC at BL-57 point (BL57), tenotomized and submitted to manual AC at ST-36 and BL-57 points (SB) and tenotomized and submitted to electroacupuncture (EA) on the two points mentioned. The animals were euthanized on 7^{th}, 14^{th} and 21^{th} days after injury. Thereafter, the Achilles tendons were collected, processed and analyzed to quantify non-collagenic proteins using the method of Bradford. For organizational analysis, it was quantified the collagen fibers birefringence by polarizing microscopy. In the ultrastructural analysis were measured the diameters of collagen fibrils and then histograms of distribution of these diameters were built as well as mass-average diameter - MAD - of collagen fibrils was calculated. The results showed that the associated use of ST-36 and BL-57 points increases both the birefringence of collagen fibers at 14^{th} and 21^{th} days and the MAD at the three periods analyzed. The reorganization of collagen fibrils also increased at 21^{th} day with the application of the two points mentioned. However, the application of EA did not increase the birefringence or the reorganization of fibrils as well as decreased the MAD of fibrils at 21^{th} after injury. Thus, we conclude that the use of manual AC at ST-36 and BL-57 points is effective in restoring the structural and ultrastructural properties of the collagen of tendons during the healing process. These results suggest strengthening the tendon structure with consequent increased resistance to re-rupture and the potential use of AC in rehabilitation protocols.

Keywords: Acupuncture, tendon healing, ultrastructure, birefringence.

1.1. RESUMO

Vários estudos indicam que a acupuntura (AC) tem efeito anti-inflamatório (AI) sistêmico e mecanotransdutor local. Estes efeitos podem potencialmente acelerar o processo de cicatrização. Portanto, o objetivo deste estudo foi analisar o efeito de diferentes protocolos de AC nas características bioquímicas, organizacionais e ultraestruturais de tendões de ratos durante o processo de cicatrização. Ratos Wistar machos com 60 dias de idade foram divididos nos seguintes grupos: grupo não tenotomizado ou normal (N), grupo tenotomizado (T), tenotomizado e submetido à AC no ponto E-36 (E36), tenotomizado e submetido à AC no B-57 (B57), tenotomizado e submetido à AC manual no ponto E-36 e B-57 (EB) e tenotomizado e submetido à eletroacupuntura (EA) nos dois pontos citados. Os animais foram submetidos à eutanásia nos dias 7, 14 e 21 após a lesão. Em seguida, os tendões calcâneos foram coletados, processados e analisados para quantificação de proteínas não colagênicas através do método de Bradford. Para a análise organizacional, foi utilizada a quantificação da birrefringência das fibras de colágeno através da microscopia de polarização. Na analise ultraestrutural foram mensurados os diâmetros das fibrilas de colágeno e em seguida foram construídos histogramas de distribuição destes diâmetros assim como foi calculado o diâmetro da massa-média - MAD - das fibrilas de colágeno. Os resultados mostraram que o uso associado dos pontos E-36 e B-57 aumenta tanto a birrefringência das fibras de colágeno nos dias 14 e 21 após a lesão quanto o MAD nos três períodos analisados. A reorganização das fibrilas de colágeno também aumentou no dia 21 após a lesão com a aplicação dos dois pontos citados. No entanto, a aplicação da EA não promoveu aumento da birrefringência ou da reorganização das fibrilas assim como diminuiu o MAD no dia 21 após a lesão. Desta forma, concluímos que o uso da AC manual nos pontos E-36 e B-57 tem efeito no restabelecimento das propriedades estruturais e ultraestruturais do colágeno de tendões durante o processo de cicatrização. Estes resultados sugerem o fortalecimento da estrutura tendínea com consequente aumento da resistência a re-ruptura e o potencial uso da AC em protocolos de reabilitação.

Palavras-chave: Acupuntura, tendão, cicatrização, ultraestrutura, birrefringência.

SUMÁRIO

AGRADECIMENTOS

Em primeiro lugar agradeço a Deus e a espiritualidade por ter me nutrido com as energias necessárias para vencer os obstáculos e me manter sempre forte no cumprimento dos meus objetivos.

Agradeço especialmente meus pais, Mauro e Regina, pelo incentivo, pelas preces e por todo suporte o qual somente um pai e uma mãe são capazes de manifestar por um filho.

À Lívia, meu eterno agradecimento por todo companheirismo, incentivo, amor e carinho que me fortaleceram e fortalecem, tornando meus dias mais suaves e cheios de vida.

Ao meu eterno amigo Gabriel, cuja amizade e prontidão em sempre ajudar, foram essenciais para que eu conseguisse iniciar, e consequentemente complementar, minha jornada acadêmica na UNICAMP até o presente momento. Obrigado meu irmão!

Ao meu orientador Prof. Edson, minha eterna admiração e gratidão! Obrigado por ter acreditado em mim e me incentivado, pela amizade, paciência e por todos ensinamentos que me iniciaram na vida acadêmica e me influenciarão por toda minha carreira.

À Profa. Laurecir Gomes (Laure), obrigado pela amizade, conselhos e ensinamentos que me fizeram crescer pessoalmente e profissionalmente.

Aos amigos do laboratório Flávia, Andrea, Cristiano e Letícia, muito obrigado pelo companheirismo e por toda convivência prazerosa desses anos todos.

À Liliam pela amizade, paciência e por ser um anjo sempre pronto a nos socorrer nos momentos em que estamos perdidos! Muito obrigado Liliam!!

Ao Francisco (Chico) pela amizade, pela paciência, pelas conversas sobre temas variados e pelos ensinamentos e todo suporte técnico para a execução dos experimentos.

Agradeço à profa. Heidi e a Karine, primeiramente pela amizade, e por me introduzir nos conhecimentos de microscopia eletrônica e pela colaboração de maneira muito importante na construção desse trabalho.

Ao Programa de Pós-graduação em Biologia Celular e Estrutural da UNICAMP por possibilitar a realização desta tese.

A CAPES e ao CNPq e pelo apoio financeiro durante o desenvolvimento deste trabalho.

2. INTRODUÇÃO

A acupuntura (AC) tem sido utilizada como parte do sistema de saúde Chinês por pelo menos 2500 anos (NIH consensus conference, 1997). Ela pode ser definida como a inserção de agulhas na pele e tecidos subjacentes em locais específicos, conhecidos como pontos de AC, para fins terapêuticos ou de prevenção (Kavoussi e Ross, 2007). Atualmente, vários pesquisadores têm se interessado pela AC com o intuito de transformar suas bases filosóficas em conhecimentos científicos. Tem sido demonstrado em estudos recentes que a AC no ponto *Zusanli* (E-36) exerce efeito anti-inflamatório (AI) pela inibição da síntese de vários mediadores inflamatórios (Wang et al, 2009; Yim et al, 2007; Lee et al, 2006; Ouyang et al, 2011; Tian et al, 2003). Outros estudos demonstram que a inserção e manipulação de uma agulha de AC resultam em uma ligação mecânica da agulha com o tecido conjuntivo. Após esse acoplamento entre agulha e tecido, um sinal mecânico é transmitido para os fibroblastos próximos ao local do estímulo, através do tensionamento das fibras de colágeno durante a manipulação da agulha, podendo determinar a síntese de várias proteínas constitucionais da matriz extracelular (MEC) por esses fibroblastos (Langevin et al, 2007; Langevin et al, 2006; Langevin et al, 2001). Essa habilidade das células em transformar estímulos mecânicos em mudanças bioquímicas é conhecida como mecanotransdução (Wang et al, 2012). Dentre as proteínas potencialmente sintetizadas pelos fibroblastos estão o colágeno e as proteínas não colagênicas (PNCs), estas últimas com papel importante na manutenção ou na cicatrização de tendões (Langevin et al, 2001; Yoon e Harper, 2005; Docheva et al, 2005). Em adição ao efeito mecanotransdutor da AC, esta técnica também age na organização ou alinhamento das fibras de colágeno nas proximidades do agulhamento (Langevin et al, 2001).

Considerando esses efeitos AI e mecânicos (mecanotransdutor e na organização das fibras de colágeno) nós hipotetizamos que a AC possa agir no processo de cicatrização do tendão calcâneo. Desta forma nós utilizamos a aplicação dos pontos E-36 e *Chengshan* (B-57). O ponto B-57 está situado na junção miotendínea do tríceps sural com o tendão calcâneo, sendo que este ponto foi utilizado com o intuito de elicitar o efeito mecânico da AC na área de lesão tendínea. Portanto, o objetivo deste estudo foi analisar o efeito da

aplicação isolada ou associada dos pontos E-36 e B-57 nas características bioquímicas, organizacionais e ultraestruturais de tendões durante o processo de cicatrização.

2.1.Acupuntura

2.1.1. Visão filosófica da acupuntura na medicina Tradicional Chinesa (MTC)

Na China, a AC tem sido utilizada para o tratamento de várias doenças por pelo menos 2500 anos. Ela é uma antiga arte de cura que sobreviveu e evoluiu na China e atualmente está prosperando nos Estados Unidos e na Europa, tanto como terapia primária quanto terapia adjuvante para uma variedade de doenças crônicas (Zijstra, et al, 2003). De acordo com a teoria da medicina oriental, a AC é definida como a inserção de agulhas na pele e tecidos subjacentes em locais específicos, conhecidos como pontos de AC, para fins curativos ou de prevenção. As agulhas podem ser estimuladas manualmente, com uma corrente elétrica de baixa tensão, a chamada eletroacupuntura (EA) ou podem ser aquecidas com incenso de artemísia (prática tradicional) ou com uma lâmpada de calor (prática moderna). De acordo com esta prática antiga, o mecanismo de ação do agulhamento terapêutico é o ajuste do fluxo de *Qi* (energia vital), o qual se acredita que circula numa rede de 12 canais de energia primários, também chamados meridianos, que conectam 360 pontos de AC (Kavoussi e Ross, 2007). Acredita-se que a estimulação das agulhas promova respostas psicofísicas profundas pela harmonização ou equilíbrio do Qi, assim como do fluxo sanguíneo através do corpo (Kim e Bae, 2010).

2.1.2. Prática e indicação atual da acupuntura

A prática clínica da AC está crescendo em popularidade sendo o tratamento alternativo e complementar mais popular em uso hoje. Nos Estados Unidos a AC é amplamente praticada, com um aumento no uso de 4,2% para 6,3% da população, o que representa 8.19 e 14.0 milhões de usuários em 2002 e 2007, respectivamente (Zhang et al, 2012).

Atualmente, a Organização Mundial de Saúde (OMS) aprova a AC para o tratamento de várias condições e o Instituto Nacional de Saúde (NIH) dos Estados Unidos emitiu uma declaração de consenso propondo a AC como uma intervenção terapêutica para

a medicina complementar (Bonafede et al, 2008; NIH consensus conference, 1997). Dentre as doenças que o NIH propôs como tratáveis com AC estão o pós-operatório adulto, náusea e vômito relacionados à quimioterapia, dor dental pós-operatória, vícios, reabilitação de acidente vascular cerebral, dor de cabeça, cólicas menstruais, epicondilite medial (cotovelo de tenista), fibromialgia, dor miofascial, osteoartrite, dor lombar, síndrome do túnel do carpo e asma.

2.1.3. Ação anti-inflamatória da acupuntura

Recentemente, tem havido um interesse crescente na pesquisa sobre AC na tentativa de reinterpretar seus conceitos tradicionais de acordo com os conceitos científicos ocidentais. Destas pesquisas uma parcela significativa se preocupa com o estudo do efeito anti-inflamatório da AC, onde modelos experimentais diversificados são empregados para demonstrar tal efeito.

Em um modelo de trauma cirúrgico em ratos, a aplicação de apenas uma sessão de EA nos pontos E-36 e *Lanwei* (extra 37) diminuiu a concentração das citocinas pró-inflamatórias interleucina-1β (IL-1β), fator de necrose tumoral alfa (TNF-α) e interleucina-6 (IL-6) tanto no plasma quanto no baço destes animais (Wang et al, 2009). No modelo em camundongos de artrite induzida por colágeno (CIA), posteriormente tratados com EA no ponto E-36, houve uma redução da concentração das citocinas TNF-α, IL-6, interferon gama (INF-γ), das imunoglobulinas G e M (IgG e IgM) e do anticorpo anti-colágeno tipo II no soro destes animais. A razão entre as células imunes atingiu valores próximos aos normais e o grau de destruição articular também foi reduzido nos animais tratados (Yim et al, 2007). Estes resultados demonstram o efeito anti-inflamatório e imunomodulador (diminuição da autoimunidade) da EA. Em um estudo de pacientes com artrite reumatóide a utilização da EA em uma combinação de vários pontos (inclusive o E-36), apresentou diminuição do nível do TNF-α tanto no sangue periférico quanto no líquido sinovial (Ouyang et al, 2011). Outro estudo, agora em modelo de colite ulcerativa, relatou que a aplicação da EA no ponto E-36 em ratos diminuiu a expressão do TNF-α tanto no tecido quanto no plasma (Tian et al, 2003). Em modelo de lesão renal aguda em ratos induzida por lipopolissacarídeo (LPS), o uso da EA no ponto E-36 e no ponto *Neiguan* (PC-6) causou uma diminuição plasmática de TNF-α, IL-1β e aumento da interleucina-10 (IL-10)

considerada anti-inflamatória (Zhang et al, 2011). Pesquisas demonstram que tanto a AC quanto a EA possuem efeitos terapêuticos, no entanto esta última técnica pode ter efeitos mais pronunciados (Ulett et al, 1998; Yim et al, 2007).

O uso da AC manual também tem sido estudado em modelo de inflamação de peritonite e induzido por carragenina. Em relação ao primeiro modelo mencionado, a estimulação manual do ponto *Sanyinjiao* (SP-6) causou o aumento da IL-10 em camundongos (Silva et al, 2011). No modelo da carragenina, utilizando tecnologia de análise por *protein array*, foi demonstrado que a AC inibe a resposta inflamatória através da diminuição significativa da IL-1β e do volume de edema na pata de ratos. Várias outras citocinas foram analisadas neste estudo, porém, não tiveram variações significativas (Chae et al, 2007).

Juntamente com o estudo citado acima, vários outros relatam o efeito AI da AC através da redução de edema ou da migração de células inflamatórias para local da lesão (Kim et al, 2008; Kim et al, 2007; Kim et al, 2006; Lee et al, 2006; Zhang et al, 2005; Zhang et al, 2004). Nestes estudos, é demonstrado que a ação AI da AC é mediada pela liberação de opióides e ativação de outras vias não opióides, liberação de catecolaminas e inibição prostaglandina E2 (PGE2). A importância da inibição da resposta inflamatória na cicatrização tendínea está relacionada ao efeito dos mediadores inflamatórios na produção de colágeno. Como mencionado, as citocinas TNF-α, IL-1β e IL-6 têm propriedades pró-inflamatórias. O TNF-α inibe a deposição de colágeno tipo I pelos tenócitos e induz a produção de mais TNF-α, IL-1β e metaloproteinase-1 (MMP-1), sendo esta ultima uma colagenase (Verrecchia e Mauviel, 2004; John et al, 2010). A PGE2, uma potente molécula pró-inflamatória, assim como o INF-γ, também diminuem a síntese de colágeno (Shen et al, 2012; Cilli et al, 2004, Riquet et al, 2000).

2.1.4. Ação mecanotransdutora da acupuntura

Além do efeito AI do ponto E-36, e sua possível ação no aumento da síntese de colágeno no processo de cicatrização tendínea, outros estudos relatam uma ação mecanotransdutora da AC com potencial para influenciar o processo citado. A mecanotransdução pode ser definida como a habilidade das células em transformar estímulos mecânicos em mudanças bioquímicas (Wang et al, 2012). Essa ação

mecanotransdutora da AC poderia influenciar a cicatrização do tendão calcâneo através do uso do ponto B-57, o qual está situado na junção miotendínea do tríceps sural com o respectivo tendão. Nossa hipótese foi que o estímulo mecânico gerado no ponto B-57 poderia ser propagado, através dos feixes de fibras de colágeno do tendão, até o sítio de lesão (porção média do tendão), estimulando os fibroblastos a atuarem na melhora da cicatrização tendínea, através de aumento da síntese de componentes da MEC.

Estudos prévios demonstram que a inserção e manipulação de uma agulha de AC resultam em uma ligação mecânica da agulha com o tecido conjuntivo, enrolamento do tecido em torno da agulha, geração de um sinal mecânico pelo tensionamento das fibras de colágeno durante a manipulação da agulha e mecanotransdução do sinal dentro das células (Langevin et al, 2007; Langevin et al, 2006; Langevin et al., 2001). Alguns estudos têm mostrado que estímulos mecânicos podem estimular a síntese de colágeno tipo I, através da via de mecanotransdução composta pelas proteínas quinases ativadas por mitógeno (MAPKs), que são as mais proeminentes quinases ativadas por estímulos mecânicos (Jeon et al, 2009; Liedert et al, 2006). As MAPKs estão envolvidas na transdução de forças mecânicas e compreendem três vias diferentes: quinase regulada por sinal extracelular 1/2 (ERK1 / 2), c-Jun N-terminal quinase (JNK) e quinase p38. Estas vias regulam a expressão gênica através da ativação de fatores de transcrição tais como a proteína ativadora-1 (AP-1) (Chen et al, 2001). Estudos recentes têm demonstrado que a aplicação de estimulação mecânica aumenta a produção de colágeno tipo I, através da ativação da via ERK-AP-1 em fibroblastos (Kook et al, 2011; Kook et al, 2009). Até mesmo estímulos mecânicos mínimos sobre o tendão, como aqueles que ocorrem em músculos paralisados por botox, são suficientes para promover a cicatrização tendínea (Andersson et al, 2012).

2.1.5. Acupuntura no tratamento de lesões tendíneas

Atualmente, alguns poucos relatos são encontrados na literatura especializada sobre o uso da AC no tratamento de lesões tendíneas. Alguns estudos clínicos têm demonstrado efeitos terapêuticos da AC nas tendinopatias, no entanto, são necessários mais estudos randomizados bem controlados para a confirmação de uma relação causal. Em geral, esses estudos preliminares demonstram que o AC melhora tanto a dor quanto a atividade funcional nos pacientes do estudo (Bi-Men et al, 2012; Papa, 2012; Lin et al, 2012;

Kleinhenz et al, 1999). Um estudo publicado por Neal e Longbottom (2012) relata que há evidencias do efeito clínico da AC nas tendinopatias, através da facilitação do fluxo sanguíneo no tendão e da atividade dos fibroblastos.

No âmbito das pesquisas laboratoriais, um estudo realizado em nosso laboratório demonstrou pela primeira vez, que a EA é capaz de aumentar a síntese e subsequente reorganização do colágeno em tendões de ratos em processo de cicatrização durante a fase proliferativa (Almeida et al, 2012). Outro estudo recentemente publicado demonstrou que a EA aumenta o número de fibroblastos e a expressão de fatores de crescimento na área de lesão no tendão (Inoue et al, 2014). Em conjunto estes dados sugerem que tanto a AC como a EA têm potencial para o tratamento de lesões tendíneas devido aos seus efeitos clínicos e laboratoriais.

2.2. Tendões

2.2.1. Conceito e aspectos gerais

Os tendões são estruturas mecanicamente responsáveis por transmitir a força gerada nos músculos ao osso, permitindo desta forma a locomoção e a estabilidade articular (Wang, 2006). O tendão calcâneo (também chamado tendão de Aquiles) é o maior e mais forte do corpo humano, sendo formado pela porção tendínea dos músculos gastrocnêmicos e sóleo (Jarvinem et al, 2005). O tendão calcâneo, assim como outros tendões, possui níveis estruturais hierárquicos de complexidade crescente. Sua formação começa com o tropocolágeno (uma cadeia polipeptídica em tripla hélice), o qual se une em fibrilas, fibras (feixes primários), fascículos (feixes secundários), feixes de fascículos (feixes terciários) formando o tendão propriamente dito (Sharma e Maffulli, 2006). Cada fibra tendínea é envolvida por uma malha reticular fina de tecido conjuntivo (endotendão). Já o tendão como um todo é revestido pelo epitendão (bainha fina de tecido conjuntivo frouxo), a qual contém vasos arteriais e venosos, linfáticos e nervos que suprem o tendão (Kastelic et al, 1978). Superficialmente, o epitendão é revestido pelo paratendão, formado de tecido conjuntivo aureolar frouxo consistindo de fibras de colágeno do tipo I e III, fibras elásticas e células sinoviais (Kvist et al, 1987). O epitendão e o paratendão compõem o chamado peritendão, o qual reduz o atrito com o tecido adjacente (Schatzker e Branemark, 1969). O

espaço entre essas duas camadas contém líquido rico em mucopolissacarídeos que fornece a lubrificação, evita a fricção e protege o tendão reduzindo o atrito.

2.2.2. Composição dos tendões

Os tendões são constituídos de colágeno, PGs, proteínas não colagênicas (PNCs), água e células.

a) Colágeno

Os tendões são ricos em colágeno, com o componente mais abundante do tendão sendo o colágeno tipo I, o qual constitui cerca de 60% da massa seca do tendão e cerca de 95% do colágeno total (Evans e Barbenel, 1975). Os restantes 5% consistem em colágenos do tipo III e V. Em tendões normais, o colágeno tipo III está localizado principalmente no endotendão e epitendão, formando fibrilas pequenas e menos organizadas (Duance et al, 1977). O colágeno tipo V é intercalado dentro da estrutura das fibrilas do colágeno tipo I e regula o crescimento da fibrila (Birk et al, 1990). Outros colágenos incluindo os tipos II, VI, IX, X e XI, estão presentes em quantidades vestigiais nos tendões (Fukuta et al, 1998). Esses colágenos são encontrados principalmente no local de inserção óssea de fibrocartilagem, onde eles reforçam a conexão através da redução da concentração de tensões na interface tecidual (Waggett et al, 1998). A unidade estrutural básica do colágeno é o tropocolágeno, que é uma proteína longa e fina produzida dentro da célula e secretada na MEC como procolágeno.

b) Proteínas não colagênicas

Além dos colágenos, os tendões também são formados pelas PNCs nas quais estão inclusas glicoproteínas tais como os proteoglicanos (PGs) e as proteínas matricelulares e as fibras elásticas (Thorpe et al, 2013; Midwood et al, 2004).

1) Proteoglicanos

Os PGs são formados por glicosaminoglicanos (GAGs) os quais são polissacarídeos sulfatados que possuem uma sequencia de repetição dissacarídica característica. Com exceção do ácido hialurônico, as cadeias de GAGs são geralmente ligadas covalentemente a

um resíduo de serina de uma proteína através de um tetrassacarídeo específico sendo o complexo resultante chamado de PG (Magnus et al, 1994). O conteúdo de PGs varia de acordo com o sitio do tendão e depende das condições de carga mecânica (região de tensão ou de compressão) do tendão (Berensson et al, 1996). Por exemplo, em regiões de compressão do tendão flexor digital profundo de bovinos, o conteúdo de PGs é de 3,5% do peso seco do tendão (Vogel e Koob, 1989). Em contraste, na região de tensão deste mesmo tendão, a quantidade de PGs representa cerca de 0,2 a 0,5% do peso seco do tendão (Koob e Vogel, 1987).

Os PGs desempenham funções diversas nos tecidos por causa da variabilidade da proteína central e dos diferentes tipos de GAGs (Iozzo e Murdoch, 1996). Decorin e fibromodulin são exemplos de pequenos PGs ricos em leucina (SLRPs) que participam da regulação da fibrilogênese (formação de fibrilas) do colágeno (Zhang et al, 2006), desempenhando uma importante função nas propriedades estruturais e funcionais dos tecidos (Vogel e Fisher, 1986). O fibromodulin liga-se ao colágeno tipo I e facilita a formação de fibrilas de colágeno maduras a espessas, modulando a força do tendão (Yoon e Harper, 2005). Já o decorin inibe a fibrilogênese do colágeno fibrilar, atuando na manutenção e regulação da estrutura fibrilar do colágeno (Yoon e Harper, 2005).

2) Proteínas matricelulares

Em adição aos PGs, nos tendões são identificadas outras glicoproteínas com importantes funções tanto na manutenção da estrutura normal quanto durante seu processo de cicatrização (Thorpe et al, 2013). Dentre elas está o tenomodulin, o qual tem funções na proliferação de tenócitos e também está envolvido no alinhamento e organização das fibrilas de colágeno (Docheva et al, 2005). Já a tenascina-C está aumentada em regiões de lesão e tem função na orientação e alinhamento da fibra de colágeno (Riley et al, 1996). A trombospondina e a fibronectina participam do processo de reparo e regeneração do tendão (Jozsa et al, 1989).). A proteína oligomérica de matriz de cartilagem (COMP), assim chamada porque foi primeiramente descoberta em cartilagem, é uma glicoproteína que interage com o colágeno e participa da fibrilogênese (Sodersten et al, 2005). Outra glicoproteína recém-encontrada na MEC dos tendões é a lubricina. Tem sido demonstrado

que a lubricina modula a resistência de deslizamento do tendão, permitindo que este deslize sobre outro tendão ou em torno das articulações (Taguchi et al, 2009).

3) Fibras elásticas

Além dos componentes citados acima, os tendões também contêm elastina, que compõe cerca de 2% do peso seco do tendão (Józsa et al, 1989). As fibras elásticas, que compreendem a elastina e as proteínas microfibrillares, podem contribuir para a recuperação da configuração ondulada das fibras colágenas após o alongamento (Butler et al, 1978).

c) Células

Embora células endoteliais, sinoviais e condrócitos estejam presentes nos tendões, os fibroblastos (tenoblastos e tenócitos) são o tipo celular dominante, os quais se alinham em fileiras entre os feixes de fibras colágenas. Tenoblastos são células tendíneas imaturas em forma de fuso, contendo abundantes organelas citoplasmáticas, refletindo sua alta atividade metabólica. Essas células se tornam alongados com o passar do tempo e passam a ser chamados de tenócitos. Juntos, tenoblastos e tenócitos correspondem de 90% a 95% dos elementos celulares do tendão (Abate et al, 2009). Os tenoblastos e tenócitos são responsáveis pela síntese de proteínas da MEC (por exemplo, colágenos, fibronectina e PGs), produzindo uma matriz de colágeno organizada e remodelando-a durante a cicatrização do tendão.

2.3. Processo de reparo do tendão

Após uma lesão, o reparo tendíneo ocorre em três fases que se sobrepõem: inflamatória (3-7 dias), proliferativa (5-21 dias) e a de remodelamento, a qual pode durar até um ano após a lesão (Abate et al, 2009).

2.3.1. Fase inflamatória

A fase inflamatória aguda dura entre 3 a 7 dias após a lesão, iniciando-se com hematoma e ativação de plaquetas (Abate et al, 2009). Na fase inflamatória inicial, que dura cerca de 24 horas, hemácias, plaquetas e células inflamatórias, particularmente neutrófilos, entram no sitio da lesão. Monócitos e macrófagos migram para o local lesado para fagocitar

materiais necrosados. Nesse meio tempo, essas células liberam substâncias vasoativas e fatores quimiotáticos, que aumentam a permeabilidade vascular, iniciam a angiogênese, estimulam a proliferação de tenócitos e recrutam mais células inflamatórias. Os tenócitos gradualmente migram para a lesão e iniciam a síntese e a deposição de colágeno tipo III. A formação de fibrilas (fibrilogênese) do colágeno também tem inicio na fase inflamatória (Sharma e Maffulli, 2006; Palma e Gigante, 2006).

2.3.2. Fase proliferativa

Após a fase inflamatória, tem início a fase proliferativa com abundante síntese e deposição de colágeno, PGs e outros componentes da MEC, os quais se apresentam ainda desorganizados. Nesta fase ocorre o pico de síntese de colágeno tipo III e a concentração de água e GAGs permanece alta com diminuição da concentração de colágeno tipo I (Sharma e Maffulli, 2006). Um tendão intacto é primariamente composto por colágeno do tipo I, correspondendo aproximadamente 95% do colágeno total, o qual confere força e elasticidade ao tecido (Wang, 2006). No pico de síntese do colágeno do tipo III, este é aleatoriamente depositado resultando em uma concentração de 20-30% em comparação a 1-3% em tendões normais. Como o colágeno do tipo III se organiza em fibrilas mais finas e menos organizadas que o colágeno tipo I, o risco de re-ruptura aumenta (Spaas et al., 2012). A fibrilogênese do colágeno gradualmente continua nesta fase com o aumento do comprimento das fibrilas, através da adição longitudinal de fibrilas (*end to end*), e aumento de sua espessura por adição lateral de fibrilas. Em seguida as fibrilas se reúnem em fibras, sendo que estas se organizam para formar o tendão como um todo (Graham et al, 2000).

2.3.3. Fase de remodelamento

A cicatrização tendínea se completa com a fase de remodelamento, a qual é caracterizada por diminuição da celularidade, do metabolismo dos tenócitos, da rede vascular e da síntese de colágeno e GAGs. Aqui, o colágeno tipo III é parcialmente substituído pelo tipo I. Durante este período, o tecido reparado muda para tecido fibroso, que novamente muda para tecido cicatricial após 10 semanas. Durante a fase de remodelamento tardia, ligações covalentes entre as fibras colágenas aumentam, o que resulta em tecido reparado com maior rigidez e força tensil. Além disso, tanto o

metabolismo dos tenócitos quanto a vascularização do tendão diminuem (Wang, 2006). A cicatrização do tendão pode ocorrer intrinsecamente, via proliferação dos tenócitos do epitendão e do endotendão, ou extrinsecamente, pela invasão de células da bainha sinovial. Cicatrização intrínseca resulta em melhora biomecânica e menos complicações. Em particular, o mecanismo normal de deslizamento dentro da bainha do tendão é preservado. Na cicatrização extrínseca, o tecido cicatricial resulta na formação de aderências que prejudicam o deslizamento. Diferentes padrões de cicatrização podem predominar em locais específicos. Por exemplo, cicatrização extrínseca tende a prevalecer nas lesões do manguito rotador (Sharma e Maffulli, 2006).

2.3.4. Fatores de crescimento

Os fatores de crescimento desempenham um importante papel, sendo as atividades de cinco destes fatores bem caracterizadas durante a cicatrização tendínea (Molloy et al, 2003). Estes são o fator de crescimento semelhante à insulina tipo 1 (IGF-1), fator de crescimento derivado de plaquetas (PDGF), fator de crescimento endotelial vascular (VEGF), fator de crescimento básico de fibroblastos (bFGF) e o fator de transformação do crescimento (TGF-β). Todos estes fatores estão expressos em altos valores e são ativos durante a cicatrização tendínea.

O IGF-1está altamente expresso durante a fase inflamatória aguda e promove a proliferação e a migração de fibroblastos e subsequente aumento da produção de colágeno e PGs (Hansen et al, 2012; Murphy e Nixon, 1997). Em tendões calcâneos de ratos, injeção intratendínea de IGF-1 acelera a recuperação funcional (Kurtz et al, 1999). PDGF é produzido rapidamente após uma lesão e estimula a produção de outros fatores de crescimento, além de promover respostas mitogênicas de maneira dose-dependente (Banes et al, 1995). VEGF estimula a proliferação endotelial, aumenta a angiogênese e também aumenta a permeabilidade capilar (Ferrara, 1999). O bFGF regula a migração e proliferação celular e também promove a angiogênese. O tratamento com este fator de crescimento aumenta a proliferação e a expressão gênica do colágeno (Chan et al, 2000; Tang et al, 2003). O TGF-β é ativo durante a fase inflamatória e proliferativa aumentando a expressão de colágeno tipo I no tecido cicatricial (Verrecchia e Mauviel, 2004).

2.3.5. Metaloproteinases

Dentre os elementos que participam do processo de reparo tendíneo, atuando ao longo das fases de cicatrização, estão enzimas como as metaloproteases (MMPs) as quais estão envolvidas no remodelamento da MEC dos tendões (Karousou et al, 2008). A MMP-2 (gelatinase) digere a gelatina a qual é uma forma desnaturada de colágeno (Snoek-Van Beurden e Von Den Hoff, 2005). É relatado na literatura que a concentração de MMP-2 está elevada até o vigésimo oitavo dia após a lesão participando tanto da degradação quanto do remodelamento do colágeno (Oshiro et al, 2003; Riley et al, 2002). Outras MMPs também participam do processo de reparo tendíneo, sendo que a expressão da MMP-9 e MMP-13 atingem o pico entre os dias 7 e 14, enquanto os níveis das MMP-3 e MMP-14, da mesma forma que a MMP-2, aumentam após a cirurgia e permanecem em níveis elevados até o dia 28 (Oshiro et al, 2003).

2.4. **Epidemiologia das lesões tendíneas**

No que diz respeito à incidência de lesões tendíneas em geral, houve um aumento substancial durante as últimas décadas. Estima-se que estas lesões somem 30% a 50% de todas as lesões relacionadas aos esportes (Järvinen et al, 2005). As lesões dos tendões são comuns e responsáveis por uma elevada proporção de encaminhamentos para reumatologistas e cirurgiões ortopédicos (Bamji et al, 1990). Os tendões mais frequentemente envolvidos são os do manguito rotador (tendão do músculo supraespinal em particular), do músculo bíceps braquial no ombro, dos músculos extensores e flexores do punho no antebraço, o tendão patelar no joelho, o tendão calcâneo na perna e o tendão do músculo tibial posterior no tornozelo e pé (Abate et al, 2009; Riley, 2008).

Com o aumento das atividades atléticas recreativas e organizadas ao longo das últimas duas décadas, a incidência de lesões do tendão calcâneo tem aumentado dramaticamente sendo que estas lesões têm sido divididas em lesões por uso excessivo (lesão crônica) e rupturas espontâneas (Järvinen et al, 2005; Järvinen et al, 2001; Kannus e Natri, 1997; Mazzone e McCue, 2002). Nas primeiras, o termo utilizado para descrever as lesões é tendinopatia e sua etiologia está intimamente relacionada ao esporte e ao exercício excessivo (Järvinen et al, 2005).

2.5.**Etiologia das lesões no tendão calcâneo**

Geralmente, as lesões do tendão calcâneo surgem de duas diferentes origens: Primeira - alguns sintomas são causados exclusivamente pela lesão ou degeneração do tendão calcâneo (sem qualquer doença sistêmica predisponente) as quais são induzidas pelo uso excessivo; segunda - por vezes, uma doença sistêmica, como a artrite reumatóide, se manifesta com sintomas no tendão calcâneo (Järvinen et al, 2005). Apenas em torno de 2% de todas as lesões e complicações no tendão calcâneo é o resultado de uma doença sistêmica. A maioria dos problemas tendíneos na população pode ser atribuída ao esporte e ao exercício excessivo (Järvinen et al, 2005; Kannus, Jozsa, 1991).

Como citado, o uso excessivo é a principal causa de tendinopatia levando a microrupturas e inflamação. A MEC do tendão normal é mantida por um processo contínuo de remodelamento, no entanto, o uso excessivo altera esse processo iniciando a tendinopatia. Outros fatores, chamados de fatores intrínsecos e extrínsecos podem atuar de forma isolada ou em combinação no desenvolvimento da tendinopatia (Kannus e Natri, 1997). Alguns dos fatores intrínsecos relacionados à tendinopatia do tendão calcâneo no esporte são: fatores gerais (sexo, idade, sobrepeso, biótipo, grupo sanguíneo, doenças predispondes e suprimento sanguíneo) e fatores anatômicos nos membros inferiores (mau alinhamento, discrepância no comprimento dos membros inferiores, fraqueza ou desequilíbrio muscular, diminuição da flexibilidade e frouxidão articular). Dentre alguns fatores extrínsecos podemos citar: drogas (uso de corticosteróides, antibióticos, esteróides anabolizantes) fatores que levam a sobrecarga sobre os membros inferiores (calçados, tipo de movimento e superfície de treinamento), erros de treinamento (alta intensidade, fadiga e erro na execução da técnica) e condições ambientais (temperatura, altitude e equipamento inadequado) (Järvinen et al, 2005). Em caso de trauma agudo, predominam os fatores extrínsecos, enquanto que lesões de uso excessivo geralmente são multifatoriais. Nos distúrbios crônicos do tendão, uma interação entre estes dois tipos de fatores é comum (Kannus e Natri, 1997). Sendo assim, pode-se dizer que a etiologia básica da tendinopatia do tendão calcâneo é multifatorial, uma vez que vários fatores extrínsecos e intrínsecos foram identificados predispondo a essas lesões (Kannus e Natri, 1997; Järvinen et al, 2001).

3. OBJETIVOS

3.1.Objetivo geral: Avaliar as propriedades bioquímicas, organizacionais dos feixes de fibras de colágeno bem como as características ultraestruturais do tecido cicatricial de tendões calcaneares de ratos submetidos à transecção parcial e tratados com diferentes protocolos de AC. Esta avaliação se dará nas fases inflamatória, proliferativa e de remodelamento da cicatrização tendínea.

3.2.Objetivo específico: Investigar a ação isolada e associada dos pontos E-36 e B-57 e quando associada com e sem estimulação elétrica, ao longo das fases da cicatrização tendínea. Para isto, avaliamos as seguintes características teciduais tendíneas:

a) A concentração de PNCs no tecido cicatricial;
b) A orientação das moléculas de colágeno dispostas nas fibras e feixes de fibras constituintes dos tendões;
c) A espessura das fibrilas de colágeno assim como a organização da distribuição de seus diâmetros.

4. JUSTIFICATIVA

De acordo com a literatura especializada sabe-se que a AC no ponto E-36 promove efeito anti-inflamatório sistêmico. Também é relatado que a presença de fatores inflamatórios pode inibir a síntese de colágeno, formando um tecido cicatricial de baixa qualidade biomecânica. Ainda, sabe-se que o estímulo mecânico da AC em si (como no ponto B-57) causa a ativação das vias de mecanotransdução em tecidos adjacentes ao sítio deste estímulo, podendo levar a síntese de componentes da MEC. Também, a literatura apresenta que a aplicação de estímulos mecânicos, muitas vezes mínimos, melhora a cicatrização tendínea. No entanto, após lesão, o tecido cicatricial do tendão nunca atinge as propriedades bioquímicas e biomecânicas de um tendão não lesado. Devido ao fato da AC possuir efeito anti-inflamatório sistêmico e mecanotransdutor local, nós hipotetizamos que a AC possa ser um tratamento efetivo nas lesões tendíneas, atuando na melhora das propriedades citadas. Sendo assim, a elucidação dos efeitos da AC e da especificidade de ação dos pontos utilizados, podem levar a elaboração de protocolos que ampliarão as alternativas de tratamento para as tendinopatias, assim como contribuir para o conhecimento dos mecanismos de ação da AC no tratamento de lesões de tecidos moles em geral.

5. MATERIAL E MÉTODOS

5.1. **Animais e Grupos Experimentais**

Ratos wistar machos com 60 dias de idade foram obtidos do Centro Multidisciplinar para Investigação Biológica (CEMIB) da Universidade Estadual de Campinas (UNICAMP). Os animais tiveram livre acesso à comida e água sendo separados nos seguintes grupos: grupo não tenotomizado ou normal (N), grupo tenotomizado (T), tenotomizado e submetido à AC no ponto E-36 (E36), tenotomizado e submetido à AC no B-57 (B57), tenotomizado e submetido à AC no ponto E-36 e no B-57 (EB) e tenotomizado e submetido à eletroacupuntura (EA) nos dois pontos citados (EA). Todos os procedimentos experimentais foram submetidos ao Comitê de Ética Institucional para uso animal para aprovação (protocolo: 2525-1).

5.2. Cirurgia

Os animais foram submetidos ao modelo de tenotomia parcial do tendão calcâneo direito (figura 1), o qual tem sido utilizado em nosso grupo de pesquisa (Almeida et al, 2012; Aro et al, 2012; Guerra et al, 2012; Tomiosso et al, 2009). Cada animal foi previamente pesado e anestesiado com injeção intraperitoneal de ketamina e xilazina (10%) a uma dose de 92 e 12 mg/kg, respectivamente. A pele sobre o tendão calcâneo foi seccionada e o tendão liberado do tecido conjuntivo adjacente por essa incisão. O tendão foi parcialmente seccionado transversalmente a meia distância entre a junção miotendínea e a inserção no osso calcâneo. A região seccionada não foi suturada. Por último, a incisão da pele foi suturada. Todos os procedimentos cirúrgicos foram realizados sob condições assépticas.

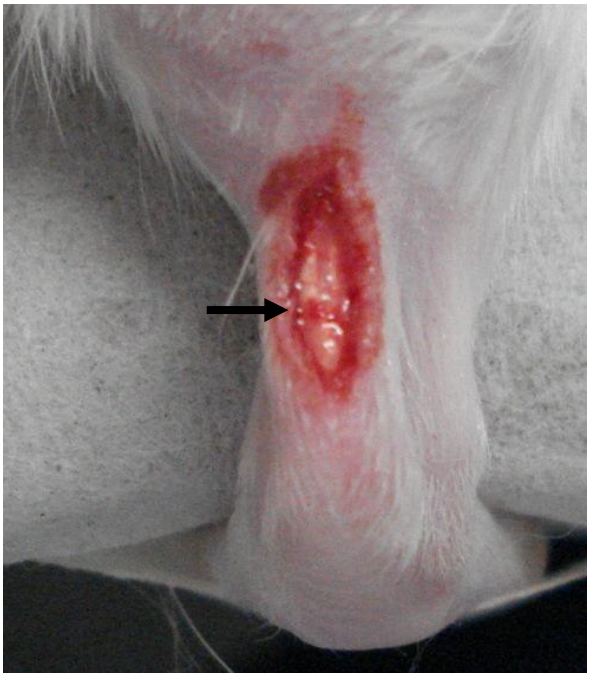

Figura 1: Imagem do tendão calcâneo após tenotomia parcial (seta). A lesão foi realizada transversalmente a meia distância entre a junção miotendínea e a inserção no osso calcâneo.

5.3. Tratamento

Previamente a todas as sessões de AC, os animais foram imobilizados em um contensor para ratos (figura 2 - C). Foram utilizadas agulhas esterilizadas, de aço inoxidável medindo 0,25 mm x 25 mm as quais foram inseridas unilateralmente, no lado da lesão. A profundidade de inserção foi de 6 mm em ambos os ponto. O ponto B-57 foi localizado a meia distância, na linha traçada entre o ponto B-40 (situado no centro da fossa poplítea) e o ponto B-60 (situado entre o maléolo lateral e o tendão calcâneo) (figura 2 – A e C). O ponto E-36 foi localizado 5 mm lateral e abaixo do tubérculo anterior da tíbia (figura 2 - A) (Almeida et al, 2012). Após a inserção das agulhas, as mesmas foram estimuladas manualmente com movimentos de rotação horária e anti-horária com um total de 16 rotações para cada lado (Langevin et al, 2007). Nos animais do grupo EA, foi realizada eletroestimulação com um equipamento que fornece onda contínua farádica bipolar assimétrica (figura 2) (Accurate Pulse 585PRO Microprocessado - Lautz, Rio Claro, SP, Brasil). A frequência de onda foi de 2 Hertz, devido ao seu efeito anti-inflamatório, composicional e organizacional sobre o colágeno (Almeida et al, 2012; Yim

et al, 2007). A intensidade de estimulação foi o suficiente para que houvesse contração muscular moderada por um período de 20 minutos. Após os procedimentos, os animais foram colocados nas gaiolas e posteriores aplicações foram realizadas em um total de 9 sessões em dias alternados (3 vezes por semana). Após os tratamentos, os ratos foram sacrificados com anestésico e as amostras foram coletadas e analisadas nos dias 7, 14 e 21 após a lesão. Os ratos sacrificados nos dias 7, 14 e 21 receberam 3, 6 e 9 aplicações, respectivamente.

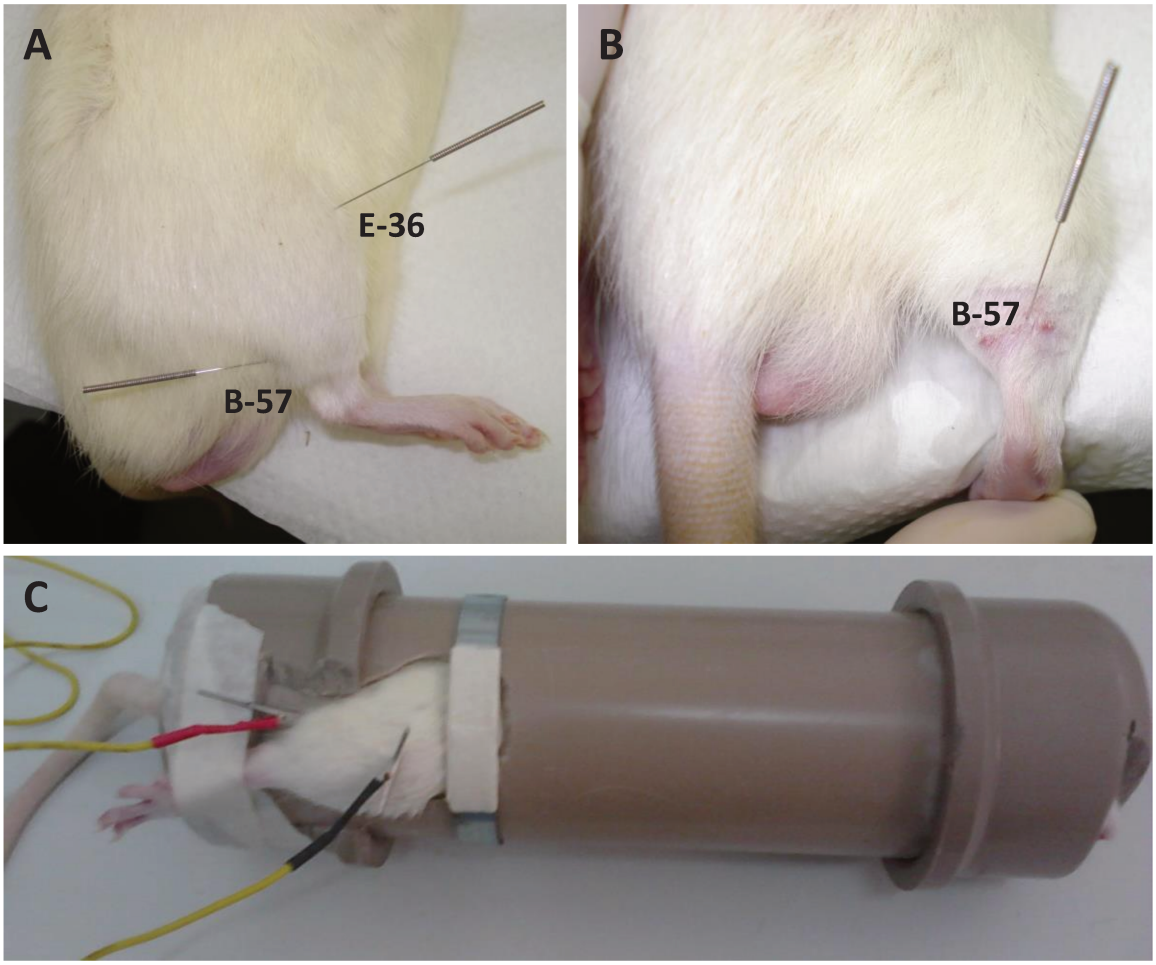

Figura 2: Imagens dos pontos de acupuntura utilizados e a forma de contenção dos animais durante o procedimento. Em A, vista lateral dos pontos E-36 e B-57. Em B, vista posterior do ponto B-57. Em C, animal dentro do contensor realizando a eletroacupuntura nos pontos E-36 (eletrodo preto) e B-57 (eletrodo vermelho). O contensor foi utilizado tanto para a eletroacupuntura quanto para a acupuntura manual.

5.4. Extração dos componentes da matriz extracelular

Em todas as experiências, apenas as regiões de tensão dos tendões foram analisadas. Nos ratos tenotomizados, as análises foram realizadas usando a região de tecido cicatricial localizado na região de tensão. Depois de uma rápida lavagem em PBS (5 mM de tampão fosfato, 0,15 M de NaCl, e 50mM de EDTA), os tendões foram picados, pesados e extraídos em 50 volumes de cloreto de guanidina a 4 M contendo
0,05 M de EDTA e 1 mM em fenilmetano sulfonil florido em tampão acetato a 0,05 M em pH 5,8 (Heinegard e Sommarin, 1987). A extração foi durante 24 horas a 4ºC em agitação constante. O material foi então centrifugado a 27.000G durante 60 minutos a 4ºC. O sobrenadante foi utilizado para a dosagem de PCNs.

5.5. Quantificação de proteínas não colagênicas

Amostras dos extratos de cloreto de guanidina obtidas a partir das amostras experimentais foram usadas para quantificar as PNCs de acordo com o método de Bradford (1976). Albumina de soro bovino foi usada como um padrão. A absorbância foi medida a 595 nm. As amostras foram lidas por um leitor de microplacas Asys Expert Plus (Biochrom, Holliston, MA, EUA).

5.6.Microscopia Eletrônica de Transmissão (MET)

Os tendões foram removidos e fixados durante 24 horas em tampão Millonig contendo glutaraldeído (5%) e ácido tânico (0,25%). A região da transecção foi isolada utilizando um microscópio de dissecação. Uma fixação secundária foi realizada em tampão Millonig contendo 0,5% de tetróxido de ósmio durante uma hora. As amostras foram lavadas em água destilada e desidratadas em acetona. Os tendões foram incluídos em epon e cortados (70-80 nm) transversalmente ao eixo longitudinal usando navalha de diamante. Os cortes foram corados com citrato de chumbo e acetato de uranila, observados no microscópio eletrônico de transmissão (LEO 906 - Zeiss) e as imagens (60.000 x ampliação) das fibrilas de colágeno dos tendões foram obtidas no total de 5 campos por amostra. Áreas contendo fibras elásticas, componentes celulares, corante residual e outros artefatos foram evitadas.

5.7. Mensuração do diâmetro das fibrilas de colágeno

O diâmetro das fibrilas foi medido utilizando o programa Image-Pro Plus 6.3 (Media Cybernetics, Inc., Silver Spring, MD, EUA). A região de interesse foi fixada para cada campo e as fibrilas contidas nestes campos foram marcadas e os seus diâmetros medidos. Utilizando estas medições, foram preparados histogramas de distribuição do diâmetro das fibrilas de colágeno, bem como o diâmetro de massa-média (MAD), que foi calculado utilizando a seguinte fórmula: $MAD = (\Sigma [ni \times di^3]) / (\Sigma [ni \times di^2])$, em que ni representa o número de fibrilas com um diâmetro específico e di é o diâmetro específico (Edwards et al, 2005; Flint et al, 1984).

5.8. Microscopia de polarização

Os tendões foram imersos em solução fixadora composta de formaldeído 4% e tampão Millonig [fosfato de sódio 0,13 M e NaOH 0,1 M (pH 7,4)] durante 24 horas a temperatura ambiente. As amostras foram lavadas com o mesmo tampão, desidratadas com etanol, diafanizadas com xilol e embebidas em parafina. Cortes seriados longitudinais (7 μm) foram então obtidos. Após a desparafinização, os cortes foram imersos em água para a medição da birrefringência das fibras de colágeno. A birrefringência foi obtida com um microscópio de polarização Olympus BX53, e as imagens foram capturadas e analisadas com o programa Image-Pro Plus 6.3 (Media Cybernetics, Inc., Silver Spring, MD, EUA). Os valores de brilho, devido à birrefringência, foram expressos como uma média de cinza em pixels, a qual traduz a organização molecular das fibras de colágeno (Vidal, 2010). Todas as medições foram realizadas com os tendões orientados a 45º em relação ao plano da luz polarizada (Vidal e Mello, 2010).

5.9. Análise estatística

Para os dados bioquímicos e para as comparações dos valores em pixels da birrefringência, foi utilizada a análise de variância (ANOVA one-way) seguida pelo teste de Tukey entre duas amostras. P ara comparar o MAD das fibrilas de colágeno foi utilizado o teste de Kruskal-Wallis com o teste de múltiplas comparações de Dunn. O teste de Kolmogorov-Smirnov para duas amostras foi usado para comparar a frequência de

distribuição dos diâmetros das fibrilas de colágeno entre os grupos. Os resultados foram apresentados como média ± desvio padrão e foram considerados estatisticamente diferentes tendo um valor de $P < 0,05$. As análises foram realizadas no Minitab 16 Statistical Software ® (State College, Pensilvânia, EUA).

6. RESULTADOS

Neste estudo, os resultados foram organizados em três manuscritos sendo que o primeiro a ser apresentado é uma revisão da literatura intitulada: "A hypothesis for the anti-inflammatory and mechanotransduction molecular mechanisms underlying acupuncture tendon healing". Os fluxogramas dos dois manuscritos restantes podem ser observados abaixo (figuras 3 e 4):

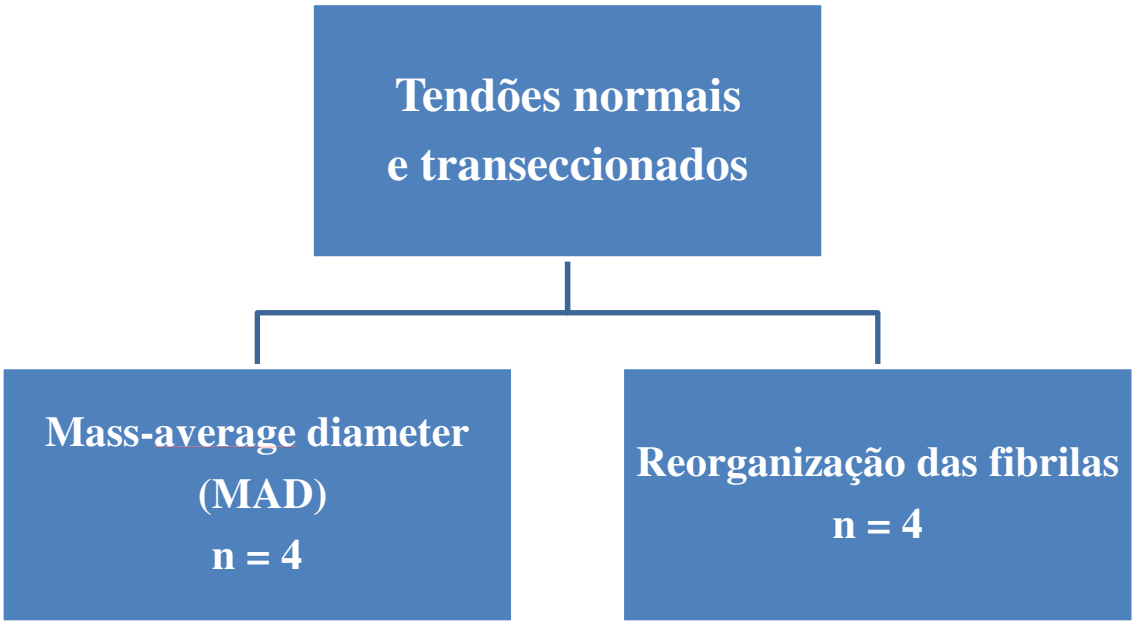

Figura 3: Fluxograma do desenho experimental utilizado no manuscrito 2 (Acupuncture Increases the diameter and reorganization of collagen fibrils during rat tendon healing). Os tendões foram analisados 7, 14 e 21 dias após a lesão. Os tendões lesados foram colocados em dois grupos: não tratados e tratados com diferentes protocolos de acupuntura.

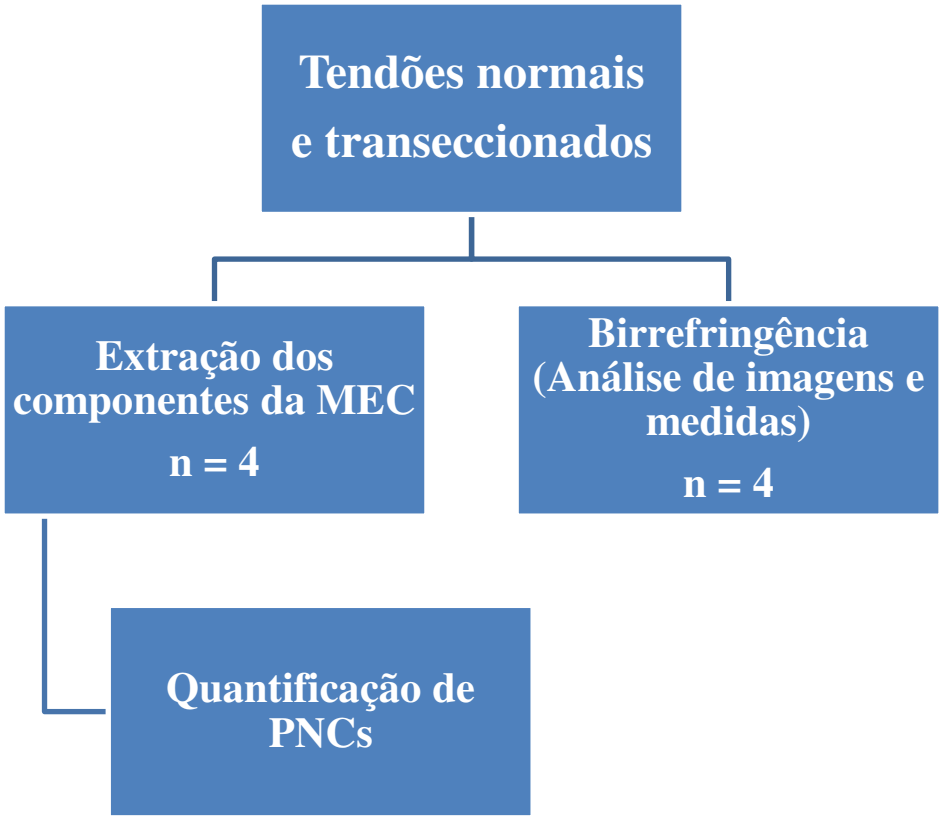

Figura 4: Fluxograma do desenho experimental utilizado no manuscrito 3 (Birefringence of collagen fibers in rat calcaneal tendons treated with acupuncture during the phases of healing). Os tendões foram analisados 7, 14 e 21 dias após a lesão. Os tendões lesados foram colocados em dois grupos: não tratados e tratados com diferentes protocolos de acupuntura.

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8. MANUSCRITO 1

Publicado na

ACUPUNCTURE IN MEDICINE

A HYPOTHESIS FOR THE ANTI-INFLAMMATORY AND MECHANOTRANSDUCTION MOLECULAR MECHANISMS UNDERLYING ACUPUNCTURE TENDON HEALING

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ABSTRACT

Previous study has demonstrated that acupuncture (AC) increases the synthesis and reorganization of collagen molecules in rat tendons after injury. Clinical studies have shown that AC improves pain and functional activity in patients with tendinopathy. However, the molecular mechanisms underlying these effects are unknown. Recent studies have demonstrated that AC can modulate both anti-inflammatory (AI) and mechanotransduction (MT) molecular pathways. Moreover, the modulation of these pathways can increase type I collagen synthesis, which is the main factor that influences tendon biomechanical properties. Our hypothesis is that AC increases synthesis and subsequent reorganization of type I collagen during tendon healing by concomitant modulation of the Toll-like receptor-Nuclear Factor-κB (TLR-NF-κB) AI pathway, the mitogen-activated protein kinases (MAPKs) pathway and the Rho/Rac-F-actin MT pathway. Increased collagen synthesis and reorganization requires that at least one acupoint is anatomically connected with the site of the injury because of the local tenoblast MT mechanism. Confirmation of this hypothesis will not only increase the knowledge of AC modulation of the previously mentioned molecular pathways, but such confirmation may also help establish the relationships between the different types of AC needle stimulation and influence of AC stimuli on pathway activity levels. In addition, the downstream therapeutic effects of AC therapy may be established. This hypothesis can be verified in a rat tendon-healing model, and subsequent clinical protocols for tendon healing can be

developed and evaluated as standalone therapies or as a component of a combination therapy.

INTRODUCTION

In China, acupuncture (AC) has been used to treat several diseases for at least 2000 years [1]. AC is an ancient healing art that has survived and evolved in China and is currently flourishing in the United States and Europe as both a primary and an adjunctive therapy for a variety of chronic conditions [2].

According to the ancient theory of oriental medicine, AC is defined as the insertion and manipulation of thin needles into the skin and subjacent tissues at specific sites, known as AC points, for preventative and curative proposes. The needles can be stimulated manually or with a low-voltage electrical current (electroacupuncture – EA) and heating them with mugwort incense (traditional practice) or with a heat lamp (modern practice). According to this ancient practice, the mechanistic system of therapeutic needling is adjustment of the *Qi* (vital energy) flow that is believed to circulate in a network of 12 primary channels, also called meridians, which connect 360 principle AC points [3]. Stimulation of the needles is believed to elicit profound psychophysical responses by harmonizing or balancing the *Qi* energy, as well as blood flow throughout the body [4].

The clinical practice of AC is growing in popularity worldwide and is the most popular complementary and alternative treatment in use today [5]. The World Health Organization (WHO) has recommended AC as a treatment for over 40 diseases [5]. In the United States, AC therapeutic intervention is widely practiced, as shown by an increase in usage from 4.2% to 6.3% of the population, representing 8.19 million and 14.01 million users in 2002 and 2007, respectively [6]. The United States National Institutes of Health (NIH) has listed several diseases that can be treated with AC, including adult postoperative and chemotherapy related nausea/vomiting, postoperative dental pain, addiction, stroke rehabilitation, headache, menstrual cramps, tennis elbow, fibromyalgia, myofascial pain, osteoarthritis, low back pain, carpal tunnel syndrome, and asthma [7].

Some clinical studies have demonstrated therapeutic effects of AC on tendinopathy, but well-controlled randomized studies are necessary for confirmation of a causal relationship. In general, these preliminary studies have shown that AC improves both pain

and functional activity in the study patients [8-11]. One study conducted in our laboratory showed that EA at the *Zusanli* (ST36) and *Chengshan* (BL57) AC points increases the concentration and organization of collagen during the proliferative phase of Achilles tendon healing in rats [12]. This study is unique because it shows that AC has the potential to improve biochemical and morphological characteristics of tendons during healing. However, the molecular pathways underlying these effects are unknown. Accumulating evidence indicates that EA or AC at the ST36 point stimulates anti-inflammatory (AI) properties [13-16]. Several studies have shown that pro-inflammatory molecules are involved in reduction of collagen synthesis [17-20]. Previous studies observed that the insertion and manipulation of AC needles activate cytoskeletal remodeling in subcutaneous connective tissue fibroblasts [21,22]. Downstream effects of cytoskeletal remodeling may include secretion and modification of extracellular matrix components [23]. Recent studies have shown that the application of mechanical stimulation increases production of type I collagen by fibroblasts [24,25]. Based on these and other studies, we discuss in this report the potential AI and mechanotransduction (MT) molecular mechanisms of AC on collagen synthesis during the tendon healing process.

HYPOTHESIS

Our hypotheses state that AC increases type I collagen synthesis and subsequent reorganization during tendon healing through co-stimulation of AC points with AI effects and AC points located in anatomical sites connected with the injury site (AC points with MT effect). Use of these AC points potentially activates AI mechanisms in inflammatory cells and MT mechanisms in tenoblasts (fibroblasts from tendons) (Fig. 1). Confirmation of this hypothesis will not only increase the knowledge of AC modulation of the previously mentioned molecular pathways, but such confirmation may also help establish the relationships between the different types of AC needle stimulation and influence of AC stimuli on pathway activity level. Therefore, the confirmation of this hypothesis may lead to novel therapeutic protocols to treat tendon injuries.

FOUNDATION OF HYPOTHESIS

Inflammatory molecules during tendon healing

After tendon injury, acute inflammation lasts for 3 to 7 days. The inflammatory process starts with hematoma formation and platelet activation followed by erythrocyte and inflammatory cell, particularly neutrophils, infiltration of the injury site. In the first 24 hours, monocytes and macrophages are the predominate cell types at the injury site and serve to phagocytosis necrotic material and attract other inflammatory cells from surrounding tissue by releasing vasoactive and chemotactic factors, such as vasodilators and pro-inflammatory molecules [26,27].

During the inflammatory phase, the cytokines TNF-α, IL-1β, IL-6 and IL-8 are known to have pro-inflammatory properties [28]. TNF-α causes tenocytes to reduce type I collagen deposition and induces production of IL-1β, IL-6, IL-8, IL-10 and prostaglandin E2 (PGE2) [19,29]. IL-1β is an important pro-inflammatory mediator that promotes prostaglandin synthesis. In damaged tissue, PGE2 functions to promote vasodilation and pain hypersensitivity [31]. PGE_2, like TNF-α and INF-γ, decreases collagen synthesis [17,18,20].

Acupuncture induces anti-inflammatory properties

As described above, during the inflammatory phase, production of pro-inflammatory molecules reduces type I collagen deposition. Healthy tendon tissue is primarily composed of type I collagen (approximately 95% of the total collagen), which provides strength and elasticity [31]. Therefore, reduction of pro-inflammatory molecule synthesis could increase synthesis of type I collagen and improve the biomechanical properties of the tendon (Fig. 1).

The ST36 is a key point on the Stomach channel with is commonly used for the treatment of gastric symptoms, such as nausea and vomiting [32]. In addition to this action of ST36, several studies have shown that AC at the ST36 exerts AI effects through inhibition of TNF-α, IL-1β, IL-6, INF-γ and PGE2 synthesis [13-16]. The cytokine IL-10

has also been implicated in AC AI effects when the *Sanyinjiao* (SP6) acupoint is used in a mouse model of peritonitis [33]. Inhibition of Toll-like receptors (TLRs) is one potential molecular mechanism responsible for the observed AC AI effects. TLRs, which are evolutionarily conserved proteins that recognize microbial molecules, initiate the innate immune response and modulate the adaptive immune system. Recent evidence suggests that in addition to TLR function as sensors of exogenous or foreign pathogen-associated molecular patterns (PAMPs), TLRs can recognize and mediate responses to endogenous stimuli [34]. Heat shock protein 60, a protein released by cells undergoing necrotic cell death, may activate innate immune cells through a TLR4-dependent mechanism [35]. Moreover, necrotic cells were recently shown to activate the nuclear factor-κB pathway (NF-κB) and inflammatory gene production in a TLR2-dependent manner [36]. In the setting of trauma, TLRs detect the release of endogenous ligands, contributing to the pro-inflammatory response to injury [37,38]. One recent study has demonstrated that TLR2, TLR4 and TLR9 play differing and selective roles in both the initial pro-inflammatory response and adaptive immune response after trauma [39].

As the downstream effectors of TLR signaling, pro-inflammatory cytokines are known to be elevated during a variety of stress responses. Specifically, the cytokines IL-1β, TNF-α and IL-6 were previously proposed to be proximal mediators during the early stages of inflammation [13]. Therefore, inhibition of the TLR-NF-κB pathway and downstream effectors may have an AI effect. Recent studies have demonstrated that AC inhibits the TLR2/4-NF-κB pathway and production of the downstream cytokines TNF-α, IL-1β, IL-6 [13,14]. This is likely one of the molecular mechanisms responsible for the AC AI effect and consequent increase in synthesis of type I collagen during the healing process.

Acupuncture and mechanotransduction

MT can be defined as the ability of cells to transform mechanical stimuli into biochemical changes [40]. Previous studies have shown that insertion and manipulation of an AC needle results in a mechanical connection of the needle to connective tissue, winding of tissue around the needle, generation of a mechanical signal by pulling of collagen fibers during needle manipulation and mechanotransduction of the signal into cells [22].

The AC mechanical signal is delivered into cells through extracellular matrix tensioning, which results in cytoskeletal remodeling and increased cell body cross-sectional area [21,22]. Cytoskeletal remodeling occurs through Rho and Rac signaling as well as actomyosin interactions [22]. Rho and Rac regulate the assembly and organization of F-actin (filamentous actin) in response to extracellular cues [41]. Filamentous actin composes part of the cytoskeleton, which has emerged as a key structural element allowing transmission of externally applied mechanical forces to the cell and conversion of these forces into biochemical responses [42]. Type I collagen secretion is one potential downstream response to the mechanical signal (Fig. 1).

Some studies have shown that mechanical stimuli can stimulate the synthesis of type I collagen through the MT pathway composed of mitogen-activated protein kinases (MAPKs), which are the most prominent kinases activated by mechanical stimuli [43,44]. The MAPKs are involved in mechanical force transduction comprising three different pathways: extracellular signal regulated-kinase 1/2 (ERK1/2), c-Jun N-terminal kinase (JNK) and p38 kinase. These pathways regulate gene expression through activation of transcription factors such as activator protein-1 (AP-1) [45]. Nowadays, the MT effects on tissue repair are best characterized in the field of physiotherapy through the application of exercises (mechanotherapy) [46]. Recent studies have shown that application of mechanical stimulation increases the production of type I collagen through activation of the ERK-AP-1 pathway in fibroblasts [24,25]. Therefore, these MT data suggest that AC mechanical stimuli have the potential to increase type I collagen synthesis through activation of MAPKs-AP-1as well as Rho/Rac-F-actin in tenoblasts close to the needle stimulation site (Fig. 1).

A previous study in our laboratory showed that EA increases the concentration and reorganization of collagen during the tendon-healing proliferative phase in rats [12]. In this previous study, we used the ST36 and BL57 AC points. Our hypothesis was that the ST36 inhibited inflammation and the BL57, which is located at the transition of the triceps surae and the Achilles tendon (an anatomical site connected with the site of the injury), activated the MT pathway in the tenoblasts. According to traditional theory, the BL57 is used as a local AC point to relax the muscles and tendons of lower leg [47]. In this model, both AC points work together to increase collagen concentration and reorganization.

DISCUSSION

As mentioned previously, the extracellular matrix of healthy tendon tissue is primarily composed of type I collagen (approximately 95% of the total collagen), which provides strength and elasticity [31]. After injury, type III collagen increases (20-30% of total collagen) compared with uninjured tendon (1-3% of total collagen). Type III collagen tends to produce smaller, less organized fibrils resulting in increased risk of tendon re-rupture [48].

Despite the process of remodeling after injury, there is consensus in the literature that the composition, structure and biomechanical properties of the scar tissue of injured tendons never return to the quality of uninjured tendons [31,48]. Therefore, the main goal of regenerative therapies is improve the quality of scar tissue and the understanding of mechanisms underlying the effects of these therapies is fundamental to develop them.

The purpose of this report is to present the possible molecular mechanisms underlying the tendon healing process. Our hypothesis is that AC modulates systemic AI and local MT molecular pathways leading to the synthesis of type I collagen. For activation of MT pathway, the selection of AC points must follow the criterion that at least one acupoint is located in anatomical connection with the site of injury. Supporting this criterion for selecting the AC points, one recent study has shown that the treatment of tendinopathy with dry needling into the pathologic tissue provide benefic effects on pain which did not return with increased physical activity [49]. Other effect of local needling is the increase of blood flow. It has been suggested that the facilitation of tendon blood flow by AC may have a role in the treatment of tendinopathy [50].

If this hypothesis is confirmed, protocols for other body site injuries, including skin, muscle, ligament, joint capsule and nerve, could potentially be developed based on the principles of systemic AI and local MT effects of AC. Collagen synthesis during the healing process leads to recovery of the biomechanical and functional properties of these structures. In the future, these protocols may be used separately or combined depending on the needs of individual patients. For example, in some patients who are intolerant to certain drug classes (e.g., AI drugs) due to gastrointestinal, liver or kidney issues, treatment with AC may prove to be a viable alternative. In addition to a collagen synthesis effect, the ST36

has an analgesic effect, which decreases the necessity of analgesic drugs during the inflammatory phase of the tissue healing [51]. Therefore, this hypothesis is important to stimulate research in the fields of tissue regeneration and AC mechanism of action.

PERSPECTIVES AND CONCLUSIONS

The hypothesis presented here suggests that AC treatment has the potential to modulate not only the AI but also the MT molecular pathways. The importance of this concept for tendon healing is the potential for increased synthesis and reorganization of type I collagen, which is the major tendon structural component. Future studies using tendon-healing models to assess the participation of molecular pathways on collagen synthesis and reorganization will enable us to understand the influence of AC therapy on tendon and other tissue healing. This line of study will potentially lead to a novel therapeutic alternative for treating soft tissue injuries.

FIGURES

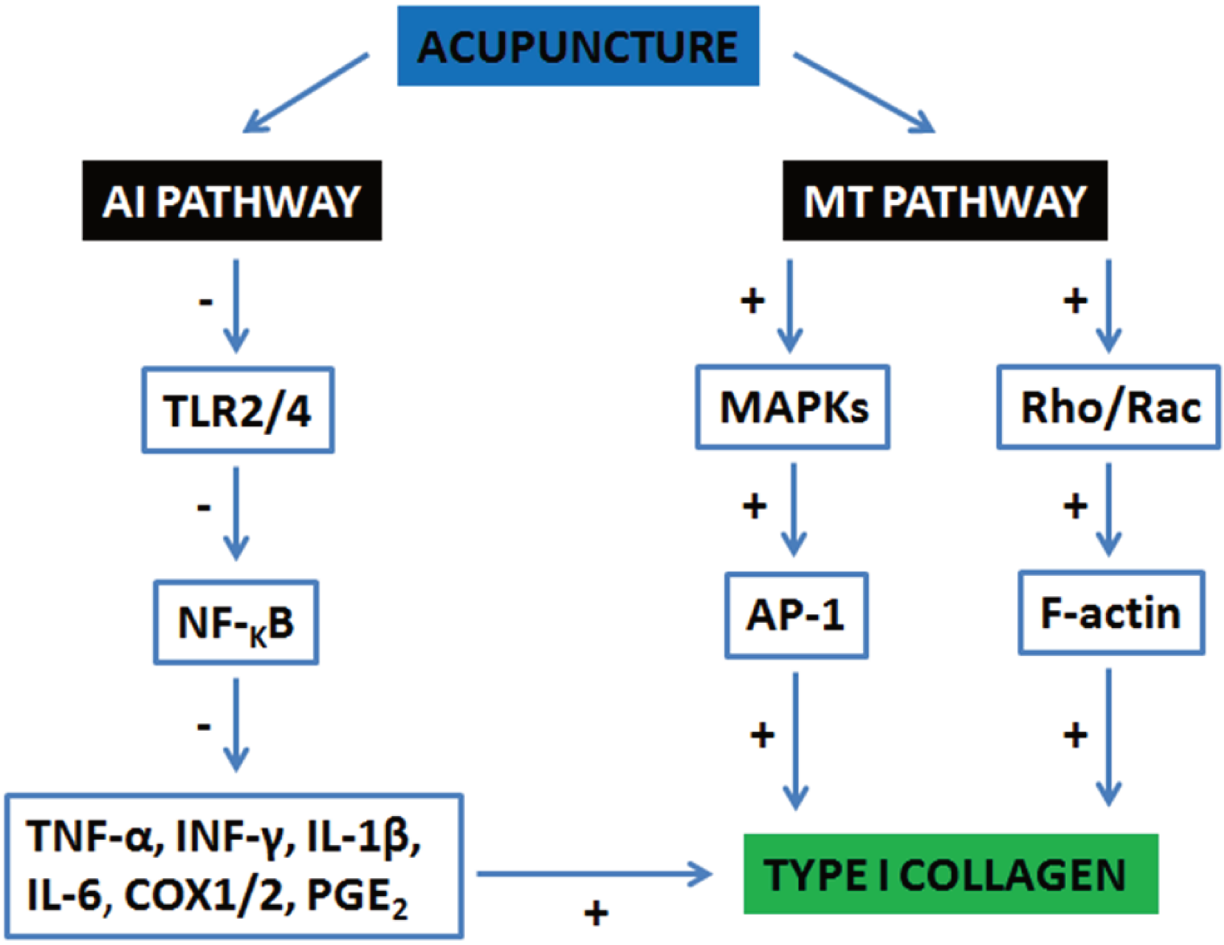

Fig. 1. The hypothesis: acupuncture increases type I collagen in the tendon during healing through inhibition (−) of the anti-inflammatory pathway in the inflammatory cells and activation (+) of mechanotransduction pathways in the tenoblasts. AP-1, activator protein-1; COX1/2, cyclo-oxygenase 1 and 2; IFNγ, interferon γ; IL-1β, interleukin 1β; IL-6, interleukin 6; MAPKs, mitogen-activated protein kinases; NF-κB, nuclear factor-κB; PGE2, prostaglandin E2; TLR2/4, toll-like receptor 2 and 4; TNFα, tumour necrosis factor α.

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9. MANUSCRITO 2

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ACUPUNCTURE IN MEDICINE

ACUPUNCTURE INCREASES THE DIAMETER AND REORGANIZATION OF COLLAGEN FIBRILS DURING RAT TENDON HEALING

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Keywords: Acupuncture, tendon healing, ultrastructure, collagen fibrils.

ABSTRACT

Our previous study showed that electroacupuncture (EA) increases the concentration and reorganisation of collagen in a rat model of tendon healing. However, the ultrastructure of collagen fibrils after acupuncture treatment is unknown.

Objectives: To assess the effect of acupuncture protocols on the ultrastructure of collagen fibrils during tendon healing.

Methods: Sixty-four rats were divided into the following groups: not tenotomised (normal group), tenotomised (teno group), tenotomised and subjected to manual acupuncture at ST36 (ST36 group), BL57 (BL57 group) and ST36+BL57 (SB group), and EA at ST36+BL57 (EA group). The mass-average diameter (MAD) and the reorganisation of collagen fibril diameters were determined during the three phases of tendon healing (at 7, 14 and 21 days).

Results: The MAD increased during the three phases of healing in the SB group. In the EA group, MAD increased initially but appeared reduced at day 21. The reorganisation of collagen fibrils was improved in EA and SB groups at days 14 and 21, respectively. EA at day 21 appeared to diminish the reorganisation.

Conclusion: These results indicate that the use of EA up to day 14 and manual acupuncture at ST36+BL57 up to day 21 improve the ultrastructure of collagen fibrils, indicating strengthening of the tendon structure. These data suggest a potential role of acupuncture in rehabilitation protocols.

INTRODUCTION

Tendons are the structures responsible for transmitting mechanical force generated in the muscles to the bones, thus enabling mobility and joint stability [1]. The incidence of tendon injuries has increased substantially over the past few decades due to increasing recreational physical activity and complications of diabetes and other metabolic diseases [2,3]. The professionally active middle-aged population is the hardest hit, leading to an economic impact due to work absenteeism [3]. In this context, the main objectives of current regenerative therapies are to improve the quality of scar tissue and accelerate healing [3,4].

Tendons consist mainly of collagen fibres composed of collagen fibrils that are damaged by injury. After injury (experimental section), tendon healing occurs in three distinct but partially overlapping phases (inflammatory, proliferative, and remodeling), during which fibroblasts synthesise collagen molecules that join to form thin fibrils (collagen fibrillogenesis) [5]. The population of these thin collagen fibrils increases after tendon injury with a consequent loss of fibril organisation [6-8]. There is a positive correlation between the diameter of collagen fibrils and biomechanical properties of tendons [9, 10]. The organisation or distribution of the fibril diameters is important for the properties of strength (thick fibrils) and elasticity (thin fibrils) of the tendons [11]. Thus the scar tissue formed by thinner and less organised fibrils, relative to that found in normal tendons, may have important consequences for patients in terms of reduced performance and the risk of re-rupture [8]. The aim of this study was to evaluate the effect of different protocols of acupuncture on collagen fibril diameter and organisation.

The clinical practice of acupuncture is growing in popularity worldwide and is the most commonly used complementary and alternative therapy today [12]. Acupuncture involves the insertion and manipulation of thin needles in the skin and subjacent tissues at specific sites, termed acupuncture points, for preventative and curative purposes. After insertion, the needles may be stimulated manually or with a low-voltage electrical current, termed electroacupuncture (EA) [13].

In a previous laboratory study, it was shown that EA at ST36 (*Zusanli*) and BL57 (*Chengshan*) increased the concentration of collagen and induced better molecular organisation of the collagen fibers in rat tendons during the proliferative phase of healing [14]. Based on these results, we hypothesised that acupuncture would increase the diameter of collagen fibrils and improve reorganisation of their diameters in rat tendons during the healing phases. To test this hypothesis, we used the Achilles tendon injury in rats as an experimental model and evaluated the effect of different acupuncture protocols on the diameter and reorganisation of the collagen fibrils during the inflammatory (7^{th} day), proliferative (14^{th} day) and remodeling (21^{th} day) phases. These protocols included the isolated and combined use of the ST36 and BL57 points with and without electrical stimulation.

METHODS

Animals

A total of 64 male Wistar rats (60 days old, weighing 250-300g) were randomly divided into six groups (n=4 per group): not tenotomised (normal group), tenotomised (teno group), tenotomised and subjected to manual acupuncture at points ST36 (ST36 group), BL57 (BL57 group) and ST36+BL57 (SB group), and electrical stimulation at ST36+BL57 (EA group). Rats were obtained from the Multidisciplinary Center for Biological Investigation (CEMIB) of the State University of Campinas (UNICAMP), São Paulo, Brazil. They were maintained with a 12-hour alternate light–dark cycle, and food and water were available *ad libitum*. The animal protocols were approved by the Ethics Committee on Animal Experiments of UNICAMP, which is in accordance with the guidelines for the care and use of laboratory animals (protocol no. 2525-1).

Achilles tendon injury model

Animals were subjected to partial injury (tenotomy) of the right Achilles tendon, which has been repeatedly used by our research group [14-17]. Each animal was pre-weighed and anesthetised with intramuscular injections of ketamine and xylazine (10%) at doses of 70 and 12 mg/kg, respectively. The skin over the Achilles tendon was incised and the tendon was released from the adjacent tissue through this incision. The tendon was then transected (~50% diameter) midway between the myotendinous junction and the insertion in the calcaneus bone. The sectioned area was not sutured. Finally, the skin incision was closed. All surgical procedures were performed under aseptic conditions.

Acupuncture treatment

Prior to all sessions, animals were immobilised in a plastic cylinder that allowed access to the right hind limb for the application of acupuncture needles. Sterilised stainless steel needles measuring 0.25mm x 25mm were placed unilaterally on the side of the lesion. The insertion depth was approximately 6mm. The BL57 point was needled midway between BL40 (*Weizhong)*, which is located in the centre of the popliteal fossa, and BL60 (*Kunlun)*, which is located between the lateral malleolus and the Achilles tendon. The ST36 point was needled 5mm lateral and inferior to the anterior tibial tubercle [14]. Needles were inserted and manually rotated in clockwise and counterclockwise directions for a total of 16 rotations each way [18]. In the EA group, electrical stimulation was given with a device providing asymmetric bipolar Faradic continuous waves (Accurate Microprocessor Pulse 585PRO, Lautz, Rio Claro, SP, Brazil). The frequency was set at 2Hz due its putative anti-inflammatory, compositional, and organizational effects [14, 19, 20]. The stimulation intensity was between 2.0 and 4.0V, so that there was moderate muscle contraction for a period of 20 minutes. After the procedures, the rats were returned to their cages pending further treatments. Acupuncture was repeated on alternate days up to a maximum of nine times (three times weekly). Rats were sacrificed by overdose of anaesthetic agents and tissue samples were collected and analysed at days 7, 14 and 21. The rats sacrificed at day 7, 14 and 21 received three, six and nine applications, respectively.

Transmission Electron Microscopy

Tendons were removed and fixed for 24 hours in Millonig buffer containing glutaraldehyde (5%) and tannic acid (0.25%). The transection region was isolated using a dissecting microscope. A secondary fixation was performed in Millonig buffer containing 0.5% osmium tetroxide for one hour. Samples were washed in distilled water and dehydrated in acetone then embedded in epon and sectioned (70-80nm) transversely to the long axis using a diamond knife. Sections were stained with lead citrate and uranyl acetate and examined under a transmission electron microscope (LEO 906, Zeiss). Images (60,000 X magnification) of the tendon collagen fibrils were obtained with five fields per sample. Areas containing elastic fibers, cellular components, residual dye and other artifacts were avoided.

Collagen fibril diameter measurement

Collagen fibril diameter was measured using Image-Pro Plus 6.3 image analyzer software (Media Cybernetics, Inc., Silver Spring, MD, USA). A region of interest of $3\mu m^2$ was fixed for each field ($15\mu m^2$ in total) and the fibrils contained in these fields were marked and their diameters measured. A total of 58,476 fibrils were measured across all samples. Using these measurements, histograms of the diameter distribution were prepared for the collagen fibrils and the mass-average diameter (MAD) was calculated using the following formula: $MAD = (\Sigma [n_i \times d_i^3])/(\Sigma [n_i \times d_i^2])$, where n_i is the number of fibrils with a specific diameter and d_i is the specific diameter [21, 22].

Statistical analysis

MAD of the collagen fibrils are shown as mean ± standard deviation and were compared using the Kruskal-Wallis test and Dunn´s multiple comparison *post hoc* test. The Kolmogorov-Smirnov test for two samples was used to compare the frequency of collagen fibril diameter distributions between the groups. The underlying distributions were considered statistically different when $P<0.05$. Statistical analyses were performed using Minitab 16 Statistical Software® (State College, Pennsylvania, USA).

RESULTS

Acupuncture increases the collagen fibril diameters

Representative electron micrographs from the different groups are shown in figure 1. The collagen fibril diameters of normal tendon are characterized by a mixture of large and thin fibrils (figure 1A). After injury, only thin fibrils were visualised in the three analysis periods in the teno group (figure 1B: day 7; figure 1C: day 14; figure 1D: day 21). Application of acupuncture at ST36 did not increase the collagen fibril diameters (figure 1E: day 7; figure 1F: day 14; figure 1G: day 21) relative to the teno group. However, when the BL57 point was stimulated and the tendons examined at days 14 and 21 after injury, large fibrils were visualised between the small ones (figure 1I and figure 1J). A similar pattern was visualised in the SB group (figure 1L: day 14; figure 1M: day 21). In the EA group at day 7, there was an increase in the fibril diameters (figure 1N) relative to all other groups on the same day (figure 1B, 1E, 1H and 1K). These large fibrils were visualised again at day 14 (figure 1O) but not at day 21 (figure 1P) in the EA group.

The MAD values of the collagen fibrils are shown in table 1. At day 7, MAD increased in SB and EA groups relative to both teno ($p<0.001$ and $p<0.05$, respectively) and ST36 ($p<0.01$ and $p<0.05$, respectively) groups. There were no significant differences between SB and EA groups. At day 14, the increase in MAD was evident in BL57, SB and EA groups relative to teno ($p<0.01$, $p<0.05$ and $p<0.001$, respectively) and ST36 ($p<0.001$, $p<0.01$ and $p<0.001$, respectively) groups. There were no significant differences in MAD between the BL57, SB, EA and normal groups. At day 21, MAD was increased in SB versus teno ($p<0.05$) and ST36 ($p<0.05$) groups. In the EA group at day 21, MAD was lower than the SB group on the same day ($p<0.05$) and the EA group on day 14 ($p<0.001$). Together the data suggest that manual acupuncture at ST36+BL57 increased collagen fibril diameters during all three phases of tendon healing, as did EA at the same points during the first two phases only (days 7 and 14) By contrast, EA at day 21 appeared to decrease the collagen fibril diameters.

Acupuncture increases reorganization of collagen fibrils

After transection, the frequency of thin fibrils increased in all groups subjected to transection, whether treated or not, at days 7, 14 and 21 after injury (data not shown). The

predominance of thin fibrils in the transected groups (disorganisation) did not match the pattern of collagen fibril diameter distribution (thick and thin fibrils) found in normal tendons (figure 2). Therefore, the pattern of collagen fibril diameters distribution was different ($p<0.05$, Kolmogorov-Smirnov test) in all groups in relation to the normal group, with the single exception of the EA group at day 14 after injury. In this group the pattern did not differ from the normal group or from the SB group at day 21 (figure 2). However, the EA group at day 14 and the SB group at day 21 both differed from the teno group at day 21 ($p<0.05$). Thus, these data suggest that EA at ST36+BL57 up to day 14 (proliferative phase) increased the reorganisation of collagen fibrils. Moreover, fibril reorganisation increased following manual acupuncture at the same points up to day 21 (remodeling phase).

DISCUSSION

Collagen fibrils are composed of individual triple helical collagen molecules arranged in a pseudo quarter-staggered array to produce fibrils with a 67nm periodic axial repeat [23]. The formation of fibrils (fibrillogenesis) occurs in three distinct steps. Firstly, small collagen fibril intermediates nucleate outside fibroblasts in distinct extracellular compartments. Secondly, these intermediates assemble lengthwise into long, thin collagen structures. Thirdly, these chains fuse laterally to form the thicker fibrils seen in fully developed tissue [24]. However, the diameter of these fibrils decreases after tendon injury [8].

Our results indicate that acupuncture at ST36+BL57 increased the collagen fibril diameters (expressed as MAD) and improved the reorganisation of collagen fibril diameters in rat tendons after injury. An increase in MAD indicates strengthening of the tendon structure, increasing its resistance to injury and/or re-rupture [25]. The MAD is defined as the diameter of a fibril that contains the average mass present in the distribution [22]. The MAD takes into account the fact that a small number of collagen fibrils with a large diameter contribute significantly to the biomechanical characteristics of the tendon [26]. A greater fibril diameter results in a higher concentration of covalent cross-links when compared to smaller fibrils because these fibrils present a low surface area to volume ratio [26]. Thus, the greater the MAD, the higher the tensile strength that the tendon can

withstand. The MAD increases from birth to maturity and it is believed that this increase occurs due to the application of mechanical stimuli to the tendon [21]. In human Achilles tendons, the MAD increases in adulthood and declines with ageing as a result of the loss of larger collagen fibrils [27].

To our knowledge, this is the first study examining the effects of acupuncture on the ultrastructural characteristics of collagen fibrils after tendon injury. In the present study, acupuncture at ST36 alone did not increase MAD during the three periods analysed. Several previous studies have demonstrated that acupuncture at ST36 has systemic anti-inflammatory effects via the inhibition of synthesis of pro-inflammatory mediators such as IL-1β, IL-6, TNF-α, IFN-γ, COX1, COX2, iNOS and PGE_2 [28-30]. TNF-α has a detrimental effect on healing due to its regulatory effects on macrophage differentiation, which subsequently increases synthesis of collagenase and reduces collagen production [31]. IFN-γ decreases collagen production and increases the time for lesion healing [32]. PGE_2 is a potent pro-inflammatory molecule and is also associated with decreased collagen production by fibroblasts [33]. Accordingly, these cytokines affect the presence and activation of fibroblasts and their role in tendon regeneration and may influence the formation and organisation of collagen fibrils in the extracellular matrix. Indeed, it has been shown that the reduction of the inflammation in the early stages of tendon regeneration promotes precocious synthesis of collagen, accelerating the repair process [34]. Despite the potential effects of ST36 acupuncture on collagen synthesis and reorganisation, needling at this point did not result in an increase in MAD relative to the teno group.

By contrast, our results showed that acupuncture at BL57 increased MAD values 14 days after injury relative to the teno and ST36 groups. At day 21, the MAD values were similar in magnitude to the normal group but were not significantly different when compared to the teno and ST36 groups. The great variability of collagen fibril diameters observed between the groups could be explained by the inter-individual variation of responses to acupuncture. In the SB and EA groups there was an increase in MAD at all three points of analysis except day 21 for EA only, relative to the teno and ST36 groups. BL57 is located in the myotendinous junction of the triceps surae, which is continuous with the Achilles tendon [35]. We hypothesise that a mechanical stimulus generated at this point through rotation of the acupuncture needle may reach the site of the tendon injury via

tension on the collagen fibers forming the conjunctive sheaths of muscle and tendon, modulating synthesis of collagen and other extracellular proteins and thereby collagen fibril diameters. This hypothesis stems from the following information. A mechanotransductor effect has been attributed to the mechanical stimuli generated by acupuncture needle manipulation [36], which can be defined as the ability of cells to transform mechanical stimuli into biochemical changes [37]. There is an intimate relationship between mechanical stimulation and the biochemical changes in tendons, and these changes appear to be adaptations to the tendons' structural properties [38]. The collagen bundles can act as cellular signaling transducers by shearing electricity, piezoelectricity and helical geometry, which could influence the tendon repair process [38]. The mechanical stimulation may lead to an increase in small leucine-rich proteoglycan (SLRP) synthesis by fibroblasts close to the injury site. SLRPs have been implicated as important regulators of collagen fibrillogenesis [39]. In resume, the choice of the ST36 and B57 points was, respectively, due their anti-inflammatory and mechanotransductor effects [40].

In contrast with the results described above, there was an apparent decrease in MAD in the EA group at day 21 relative to the SB group at day 21 and the EA group at day 14. The cumulative application of electrical stimuli may have activated enzymes such as metalloproteinases in the remodeling phase, which break down the large collagen fibrils into smaller ones [41].

Regarding the ultrastructural organisation of collagen fibrils in normal tendons, previous studies have described two discrete populations of fibrils, resulting in a bimodal diameter frequency distribution [26,42]. The bimodal distribution of the fibril diameters is important for the properties of strength (thick fibrils) and elasticity (thin fibrils) of tendons as a whole [21]. After injury, the pattern of collagen fibril diameter distribution changed from bimodal to unimodal (thin fibrils) distribution in all groups studied here, except for the EA group at day 14, which showed a recovery of large fibril populations, leading to a pattern of a collagen fibril diameters that was similar to the distribution seen in normal tendons. In the SB group at day 21, the pattern of collagen fibril diameter distribution was the same as the EA group at day 14 but different from the normal group. This improvement in the reorganisation of the distribution of collagen fibrils diameters may be due to both mechanical and electrical stimulation applied at the BL57 point leading to adaptations in

the structural properties of fibrils [38]. In line with our results, one review article has demonstrated positive evidences suggesting the acupuncture as a potential clinical treatment for tendon injuries [43]. In this review, it was shown that acupuncture may have a role in the tendinopathy through the facilitation of tendon blood flow and fibroblastic activity.

In conclusion, our data indicate that manual acupuncture at ST36+BL57 during the three phases of tendon healing as well as EA at the same points during the first two phases improves the ultrastructural properties of collagen fibrils in tendons after injury. These observations suggest strengthening of the tendon structure and resistance to re-rupture. We would suggest that electrical stimulation should be applied carefully, avoiding the remodeling phase, because of a possible detrimental effect on the MAD. Future studies need to further investigate how electrical stimulation *per se* influences the collagen fibril diameters and distribution. The influence of SLRPs in the modulation of collagen fibril diameters must be also be examined.

Competing interests: None.

FIGURES AND TABLES

Table 1. Mass-average diameter (nm) of collagen fibrils

	7 Day	14 Day	21 Day
Teno	46.6± 9.7*	56.2± 16.1*	61.6 ± 26.3*
ST36	51.0 ± 18.2*	61.7 ± 38.9*	55.5 ± 6.6*
BL57	63.3 ± 15.6*	135.8 ± 80.0# a	105.2 ± 59.0
SB	84.9 ± 17.7* # a	105.3 ± 50.5# a	107.1± 34.5# a
EA	89.0 ± 28.7* # a	134.8 ± 47.0# a	56.2 ± 10.0* c A
Normal		179.4 ± 31.3	

All data are presented as mean and standard deviation (n=4 per group). Statistical significance is indicated by $p<0.05$.
***** indicates values which differ significantly relative to the normal group (bottom row, single time-point). *Within column comparisons*: **#** = significantly different from tenotomised group; **a =** significantly different from ST36 group; **b** = significantly different from BL57 group; **c** = significantly different from SB group. *Within row comparisons:* **A =** significantly different compared with 14 day time point in EA group.

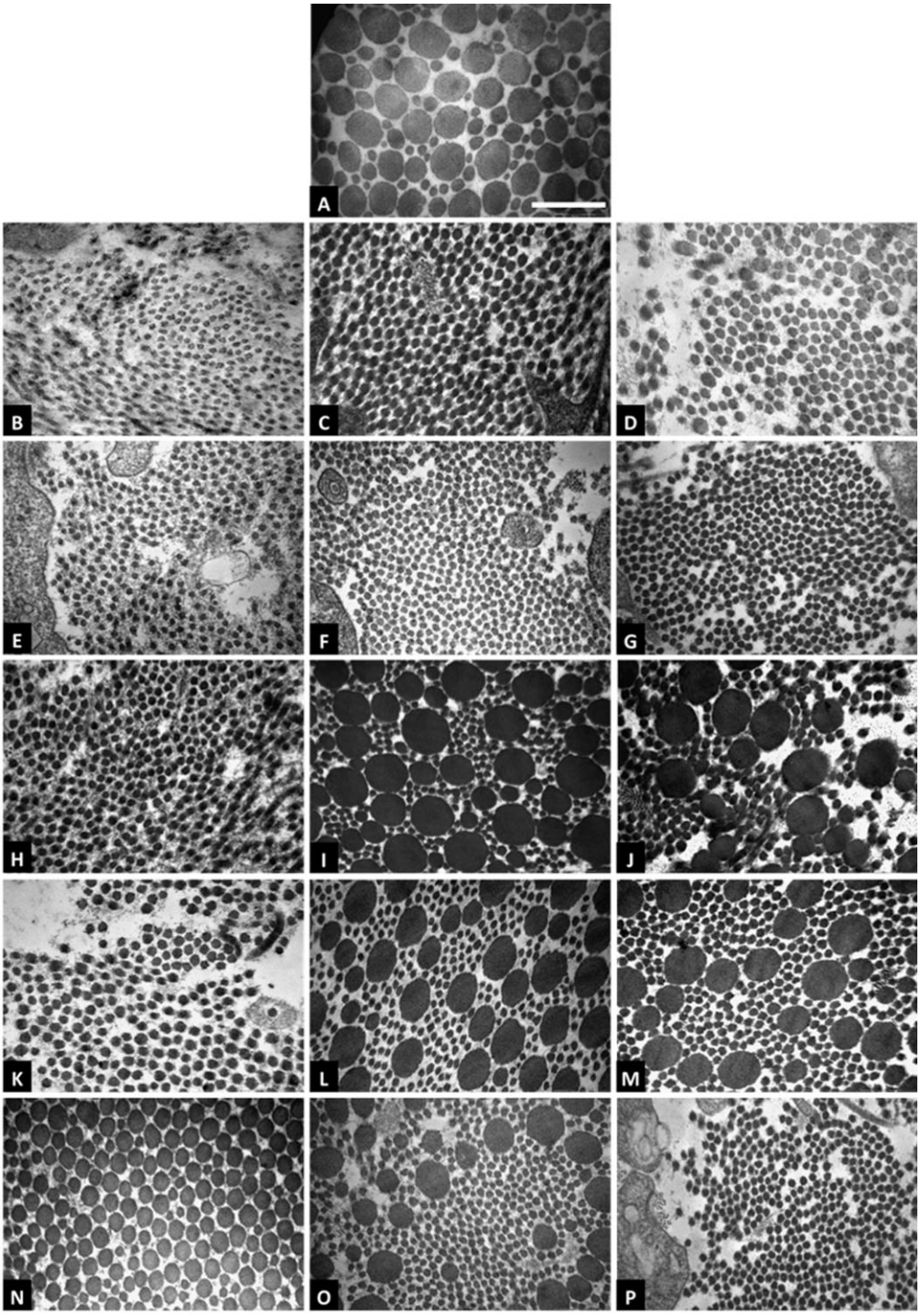
A
B
C
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Fig. 1 Electron micrographs demonstrating collagen fibril diameters in the different experimental groups (n=4 each): normal (A); tenotomised at day 7 (B), day 14 (C), and day 21 (D) after injury; manual acupuncture at ST36 at day 7 (E), day 14 (F) and day 21 (G); manual acupuncture at BL57 at day 7 (H), day 14 (I) and day 21 (J); manual acupuncture at ST36+BL57 at day 7 (K), day 14 (L) and day 21 (M); and electroacupuncture at ST36+BL57 at day 7 (N), day 14 (O) and day 21 (P). A mixture of large and thin fibrils is apparent in the normal group. After injury, the collagen fibril diameters decreased in the tenotomised and ST36 groups. Large fibrils were present in the BL57 and ST36+BL57 groups at days 14 and 21 and in the electroacupuncture group at day 14. By contrast, these large fibrils were not visualised in the electroacupuncture group at day 21. Bar = 500nm.

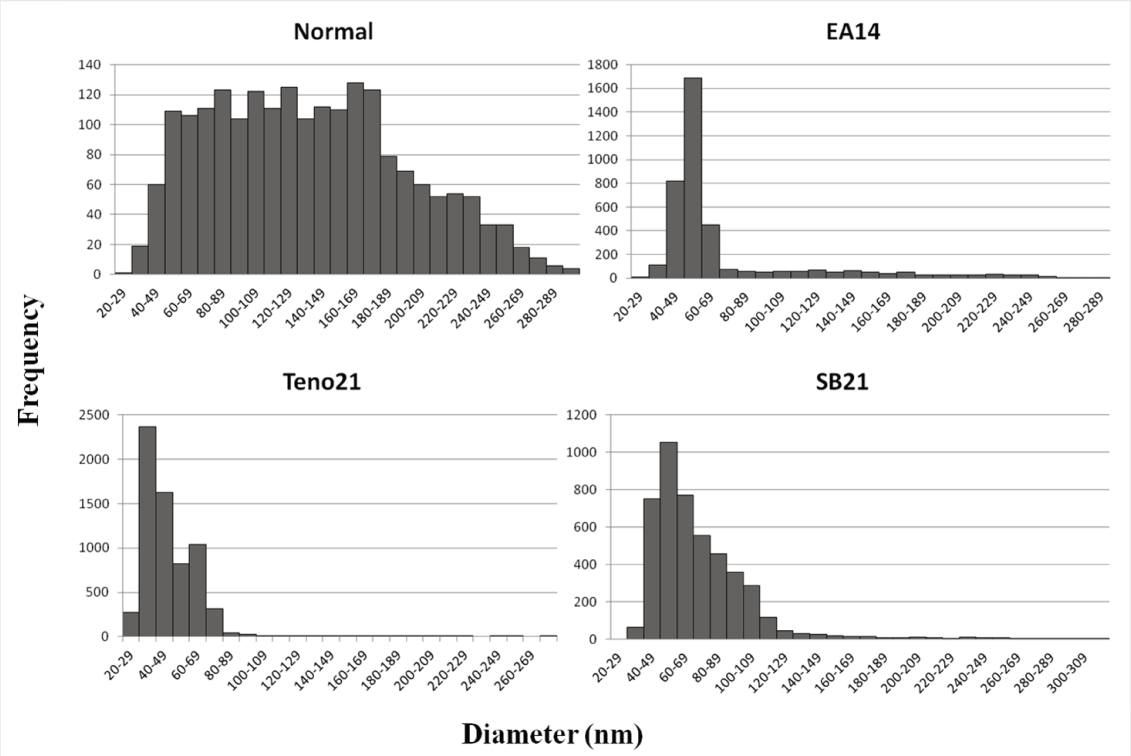

Fig. 2 The frequency distributions of collagen fibril diameters in selected experimental groups: normal, tenotomised only at day 21 (teno21), electroacupuncture at ST36+BL57 at day 14 (EA14) and manual acupuncture at ST36+BL57 at day 21 (SB21) after injury. In the injury groups there was an increase in the frequency of thin (30-69nm) collagen fibrils. The frequency of thick fibrils (100-259nm) was increased in the EA14 group and SB21 groups. The reorganisation of collagen fibril diameters was improved in the EA14 group – the values did not differ statistically from the normal (non-tenotomised) group.

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10. MANUSCRITO 3

Submetido à

COMPLEMENTARY THERAPIES IN MEDICINE

BIREFRINGENCE OF COLLAGEN FIBERS IN RAT CALCANEAL TENDONS TREATED WITH ACUPUNCTURE DURING THE PHASES OF HEALING

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ABSTRACT

Birefringence is an optical anisotropy that is investigated by polarization microscopy and has been valuable for the study of the oriented organization of collagen fibers in tendons. However, the application of this technology to evaluate the effect of different points of acupuncture during tendon healing has not yet been described.

Objectives: To evaluate the concentration of non-collagenous proteins (NCP) and birefringence in rat calcaneal tendons.

Design: Experimental controlled study.

Methods: Tendons of Wistar rats were tenotomized, treated with different acupuncture protocols and analyzed at 7^{th}, 14^{th} and 21^{th} days after injury.

Main outcome measures: Bradford method and polarization microscopy with image analysis were used to analyze the tendons through the healing process.

Results: The main result indicates that combined use of acupuncture points with anti-inflammatory and mechanotransductor effects increases the birefringence of collagen fibers in tendons at 14th and 21th days after injury.
Conclusion: This result suggests strengthening of the tendon structure with consequent resistance to re-rupture and a potential role of acupuncture in rehabilitation protocols.

Word count: 2500

Introduction

Tendon is a connective tissue, essential to the movement of limbs by transmitting the contractile (tensile) force of the muscle through the tendon to the bone.[1] This tissue is composed of an abundant extracellular matrix (ECM) that consists mainly of collagen fiber bundles, which are highly organized and arranged along the largest axis of the tendon.[2] The complex supramolecular structure of tendon collagen fibers presents optical anisotropic properties such as form and intrinsic birefringence, which have widely been assessed to study their molecular order in relation to their various physiological aspects.[3] Variation in the order of organization states has been detected and measured in collagen bundles during the processes of repair through their birefringence using polarization microscopy.[4-7] The total birefringence is determined by the addition of both birefringences (form and intrinsic), and this measurement remains the best way to study molecular order and the degree of ordered aggregation in collagen bundles.[7,8]

In addition to collagen fibers, the ECM of tendon is composed of non-collagenous proteins (NCP) such as proteoglycans, collagen oligomeric matrix protein (COMP) and thrombospondins. These proteins work increasing the formation (fibrillogenesis) and organization of collagen fibers.[9-11]

After injury, the collagen fibers organization and the NCP concentration are altered in tendon. These alterations are characterized by decrease in collagen fibers organization and increase in NCP concentration during the three phases of tendon healing: the inflammatory (7th day), proliferative (14th), and remodeling (21th) phases.[12,13] Thus, the final result of the injury is the formation of scar tissue with inferior structural properties in relation to normal tissue with risk of reinjury. In this context, the aim of therapies for

tendon heling is to improve the structural quality of the repaired tissue, accelerating the healing and avoiding the lesion reincidence.[14]

Several studies have focused on the anti-inflammatory and mechanotransductor effects of acupuncture.[15-19] These effects have the potential to improve the biochemical and structural aspects of tissues after injury, increasing the synthesis and organization of collagen.[20] Thus, we hypothesize that if acupuncture exerts the mentioned effects, the biochemical and structural components of injured tendons will improve after treatment with acupuncture. In this study, we collected data from Bradford method for NCP detection and polarization microscopy for birefringence of collagen fibers in injured tendons treated with different acupuncture protocols in the three phases of healing, aiming to address 1) the concentration of NCP; 2) the molecular organization of the collagen fibers in the scar tissue.

Experimental procedures

Animals and groups

A total of 120 male Wistar rats (60 days old, weighing 250-300g) were randomly divided into six groups (n=4 per group): not tenotomised (normal group), tenotomised (teno group), tenotomised and subjected to manual acupuncture at points ST36 (ST36 group), BL57 (BL57 group), ST36+BL57 (SB group) and electrical stimulation at ST36+BL57 (EA group). Sixty rats were used for quantification of NCP and sixty for polarization microscopy at three periods of analysis. The contralateral paws (not tenotomized) were used as normal group. The rats were obtained from the Multidisciplinary Center for Biological Investigation (CEMIB) of the State University of Campinas (UNICAMP), São Paulo, Brazil. They were maintained with a 12-hour alternate light–dark cycle, and food and water were available *ad libitum*. The animal protocols were approved by the Ethics Committee on Animal Experiments of UNICAMP, which is in accordance with the guidelines for the care and use of laboratory animals (protocol no. 2525-1).

Achilles tendon injury model

Animals were subjected to partial injury (tenotomy) of the right Achilles tendon, which has been repeatedly used by our research group.[5,12] Each animal was pre-weighed and anesthetised with intramuscular injections of ketamine and xylazine (10%) at doses of 70 and 12 mg/kg, respectively. The skin over the Achilles tendon was incised and the tendon was released from the adjacent tissue through this incision. The tendon was then transected (~50% diameter) midway between the myotendinous junction and the insertion in the calcaneus bone. The sectioned area was not sutured. Finally, the skin incision was closed. All surgical procedures were performed under aseptic conditions.

Acupuncture treatment

Prior to all sessions, animals were immobilized in a plastic cylinder that allowed access to the right hind limb for the application of acupuncture needles. Sterilized stainless steel needles measuring 0.25mm x 25mm were placed unilaterally on the side of the lesion. The insertion depth was approximately 6mm. The BL57 point was needled midway between BL40 (*Weizhong)*, which is located in the centre of the popliteal fossa, and BL60 (*Kunlun)*, which is located between the lateral malleolus and the calcaneal tendon. The ST36 point was needled 5mm lateral and inferior to the anterior tibial tubercle.[5] Needles were inserted and manually rotated in clockwise and counterclockwise directions for a total of 16 rotations each way.[19] In the EA group, electrical stimulation was given with a device providing asymmetric bipolar Faradic continuous waves (Accurate Microprocessor Pulse 585PRO, Lautz, Rio Claro, SP, Brazil). The frequency was set at 2Hz due its putative anti-inflammatory, compositional, and organizational effects.[5,17] The stimulation intensity was between 2.0 and 4.0V, so that there was moderate muscle contraction for a period of 20 minutes. After the procedures, the rats were returned to their cages pending further treatments. Acupuncture was repeated on alternate days up to a maximum of nine times (three times weekly). Rats were sacrificed by overdose of anaesthetic agents and tissue samples were collected and analysed at days 7, 14 and 21. The rats sacrificed at 7^{th}, 14^{th} and 21^{th} days after injury received three, six and nine applications, respectively.

Extraction procedures

In all experiments, only the region of scar tissue and correspond area of no tenotomized tendons were analyzed. After a quick wash with PBS (5 mM phosphate buffer,

0.15 M NaCl, and 50mM EDTA), the tendons were minced, weighed, and extracted in 50 volumes of 4 M guanidinium chloride containing 0.05 M EDTA and 1 mM phenylmethanesulfonylfluoride in 0.05 M acetate buffer at pH 5.8.[21] The extraction takes place for 24 hr at 4ºC with constant agitation. The material was then centrifuged at 27,000 X g for 60 min at 4ºC. The supernatant was used for the dosage of NCP.

Quantification of NCP

Samples of the guanidinium chloride extracts that had been collected from the experimental samples were used to quantify NCP according to the Bradford method.[22] Bovine serum albumin was used as a standard. The absorbance of the samples was measured at 595 nm in the Asys Expert Plus microplate reader (Biochrom, Holliston, MA, USA).

Polarization Microscopy

For the observation of collagen bundles by polarization microscopy, the tendon samples were immersed in a fixative solution consisting of 4% formaldehyde and Millonig's buffer [0.13 M sodium phosphate and 0.1 M NaOH (pH 7.4)] for 24 hr at room temperature. The samples were then washed with the same buffer, dehydrated with ethanol, diaphanized with xylol, and embedded in paraffin. Longitudinal serial sections (7 μm) were obtained for microscopy analysis. After deparaffinization, the sections were immersed in water for the measurement of the birefringence of the collagen fibers. The birefringence, as measured in this study, is a sum of the intrinsic (Bi) plus the form (Bf) birefringence, in which Bi is approximately 13% of the total birefringence. Thus, the Bf accounts for many of the physical properties of the tendon, including its supramolecular organization.[23,24] The birefringence properties were studied with an Olympus BX51-P BX2 polarization microscope, and the images were captured and analyzed using an Image-Pro Plus 6.3 image analyzer (Media Cybernetics Inc., Silver Spring, MD, USA). The brilliance values due to the birefringence were expressed as a gray average (GA) values in pixels, which implicates the molecular organization of the collagen fibers.[24] All measurements were performed with the tendons oriented at 45º relative to the plane of the polarized light.[3]

Statistical analysis

Data were presented as mean ± SD of results obtained from four animals per group. To compare the data, statistical analysis was performed using one-way analysis of variance with the Tukey's post hoc test. A value of $p < 0.05$ was considered statistically significant, and we used the statistical program GraphPad Prism® (Graph-Pad Software, La Jolla, CA, USA), version 3.0, for all analyses.

Results

ST36 combined with BL57 on the concentration of NCP

The concentration of NCP at 7th, 14th and 21th days after injury is shown in table 1. After injury at 7th day, the ST36 group was the only without increase of concentration of NCP in relation to normal group. However, when the ST36 was stimulated together with BL57 point, manual (SB group) and electrically (EA group), the concentration of proteins increased in relation to ST36 group.

At 14th day, there was no increase in proteins concentration in the treated groups in relation to teno group. However, comparing to normal group, the proteins increased in BL57, SB and EA groups.

At 21th day, the tenotomized groups, treated or not, did not differ in relation to each other or in relation to normal group. In teno, SB and EA groups, the proteins decrease in relation to 7th day.

These results indicate that acupuncture do not promote statistically significant increase on NCP at 7th, 14th and 21th days after injury. However, despite the NCP concentration in SB group had not reached a significant statistical increase in 7th, 14th and 21th days after injury, there was an increasing tendency in relation to teno group.

Acupuncture increases birefringence of collagen fibers

The birefringence images during the three healing phases are shown in figure 1 and its values in table 2. In terms of the birefringence brightness, the GA values obtained for the collagen fibers in the tenotomized groups were lower in relation to normal group. In

these groups, the injury decreased the organization of collagen fibers and consequently the brightness of the tissue under polarized light.

At 7th day, the application of different protocols of acupuncture did not produce a statistically significant increase of GA values compared to teno group. At 14th day, the BL57 and EB groups significantly increased the brightness and the GA values in relation to teno group. In the EA group, the GA values did not differ from teno group and were significantly low in relation to BL57 group. At 21th day, the ST36 group showed GA values significantly higher compared to teno group and ST36 groups at 7th and 14th days after injury. In the SB group, the GA values significantly increased in relation to teno group and SB group at 7th day. The EA group did not differ from teno group in terms of GA values and had values significantly lower compared to ST36 and SB groups at 21th day after injury.

Discussion

In this study, we have used polarization microscopy to analyze the collagen fibers organization through their birefringence as well as we quantified the NCP concentration in tendon after injury in the three phases of healing. It was confirmed that manual acupuncture at ST36 and BL57 points increases the molecular organization of collagen fibers in the 14th and 21th days after injury. In addition, the isolated use of BL57 at 14th day and ST36 at 21th day also increased the collagen fibers organization. However, there was not statistically significant increase in NCP concentration in all treated groups. In relation to birefringence results, possibly these results occurred due to modulation of anti-inflammatory and mechanotransduction pathways by acupuncture.

The stimulation of ST36 point increased the birefringence of collagen fibers at 21th day after injury. Several studies have shown that acupuncture at the ST36 exerts anti-inflammatory effects through inhibition of pro-inflammatory molecules synthesis.[15,16,25] These molecules affect the presence and activation of fibroblasts and their role in tendon repair, having influence in the formation and organization of collagen fibers in the extracellular matrix of tendon .[26,27] Indeed, it has been shown that the reduction of the inflammation in the early stages of tendon repair promotes precocious synthesis of

collagen, accelerating the repair process.[28] In other words, the ST36 point may have shortened the inflammatory phase, hence leading to an early remodeling phase, which are characterized by increased collagen organization.[28,29]

In relation to BL57 point (located in the transition of the triceps surae with the Achilles tendon), the scientific literature does not record study regarding to a possible anti-inflammatory effect. However, our hypothesis is that manual rotation of the needle on this point could influence the organization of collagen fibers in the scar tissue through direct mechanical stimulation on the fibroblasts in this tissue. This stimulation could reach the injury through a tensioning of the collagen fibers that form conjunctive sheaths involving the triceps surae muscle and Achilles tendon. Previous studies have shown that insertion and manipulation of an acupuncture needle results in a mechanical connection of the needle to connective tissue, winding of tissue around the needle, generation of a mechanical signal by pulling of collagen fibers during needle manipulation and delivery of this signal into the cells.[18] This signal is delivered into the cells through extracellular matrix tensioning, which results in biochemical responses.[18] The ability of cells to transform mechanical stimuli into biochemical changes is called mechanotransduction.[30] An intimate relationship has been reported between mechanical stimulation and the biochemical changes in tendons, and these changes appear to be adaptations to the structural properties of tendons .[31] In our study, there was increase of the birefringence in the BL57 group at 14th day and SB group at 14th and 21th days. Despite these birefringence results, there was not statistically significant increase in NCP concentration in these groups. However, we believe that a slight increase in NCP could have modulated the collagen fibers organization in the BL57 group at 14th day and SB group at 14th and 21th days. In other words, the mechanical stimuli on BL57 point could increase the synthesis of NCP such as proteoglycans, COMP and thrombospondins, leading to increase of birefringence. Some studies have shown that these proteins increase the fibrillogenesis and organization of collagen fibers.[9-11] Another mechanism behind the effect of BL57 point stimulation on the birefringence of tendon is the potential creation of a tension line in the tendon long axis promoting additional collagen fibers reorganization.[18]

It was shown that the combination of points (SB group) increased the collagen fibers organization at 14th and 21th after injury. This result indicates that combination of

points (anti-inflammatory and mechanotransductor effects associated) may have synergistic effects, potentiating the effect of acupuncture during the tendon healing. On other hand, when electrical stimuli was applied to the needle, the collagen fibers organization at the three phases of healing remains unchanged in relation to teno group. The cumulative application of electrical stimuli may have activated enzymes such as metalloproteinases, which acted degrading the collagen fibers and affecting the increase of its organization.[32]

Conclusion

In conclusion, this study indicates that acupuncture at ST36 and BL57 points improves the organizational properties of tendon collagen fibers during proliferative and remodeling phases of healing. In addition, this result suggests strengthening of the tendon structure with consequent resistance to re-rupture and a potential role of acupuncture in rehabilitation protocols.

FIGURES AND TABLES

Table 1. Concentration (mg/g of tissue) of NCP

	7 Day	14 Day	21 Day
Teno	51,36 ± 6,6*	41,20 ± 7,5	28,9 ± 7,8^{A}
ST36	45,86 ± 1,4	40,75 ± 8,9	31,95 ± 8,4
BL57	54,83 ± 8,6*	52,75 ± 7,8*	39,55 ± 6,1
SB	65,66 ± 4,7* ^{b}	60,89 ± 11,62*	44,86 ± 7,2^{B}
EA	66,28 ± 10,33* ^{b}	49,16 ± 2,7*	32,88 ± 5,3^{C}
Normal		28,9 ± 5,4	

All data are presented as mean and standard deviation (n=4 per group). Statistical significance is indicated by $p<0.05$.
***** indicates values which differ significantly relative to the normal group (bottom row, single time-point). *Within column comparisons*: **b =** significantly different from ST36 group; *within row comparisons:* **A =** significantly different compared with 7 day time point in teno group; **B =** significantly different compared with 7 day time point in SB group; **C =** significantly different compared with 7 day time point in EA group.

Table 2. Birefringence of tendon collagen fibers

	7	14	21
Teno	10.5 ± 0.8	11.5 ± 2.5	15.1 ± 3.4
ST36	10.5 ± 0.9	14.6 ± 1.4	21.5 ± 3.0[a, B]
BL57	13.2 ± 3.5	17.3 ± 0.7[a]	16.8 ± 3.5
SB	10.4 ± 1.1	17.6 ± 2.7[a, A]	22.6 ± 4.0[a, C]
EA	14.8 ± 1.4	12.1 ± 1.9[c]	12.7 ± 1.8[b, d]
Normal		171.7 ± 19.4	

All data are presented as mean and standard deviation (n=4 per group). Statistical significance is indicated by $p<0.05$.
Within column comparisons: **a** = significantly different from tenotomised group; **b =** significantly different from ST36 group; **c** = significantly different from BL57 group; **d** = significantly different from SB group. *Within row comparisons:* **A =** significantly different compared with 7 day time point in SB group; **B =** significantly different compared with 7 and 14 day time points in ST36 group; **C =** significantly different compared with 7 day time point in SB group.

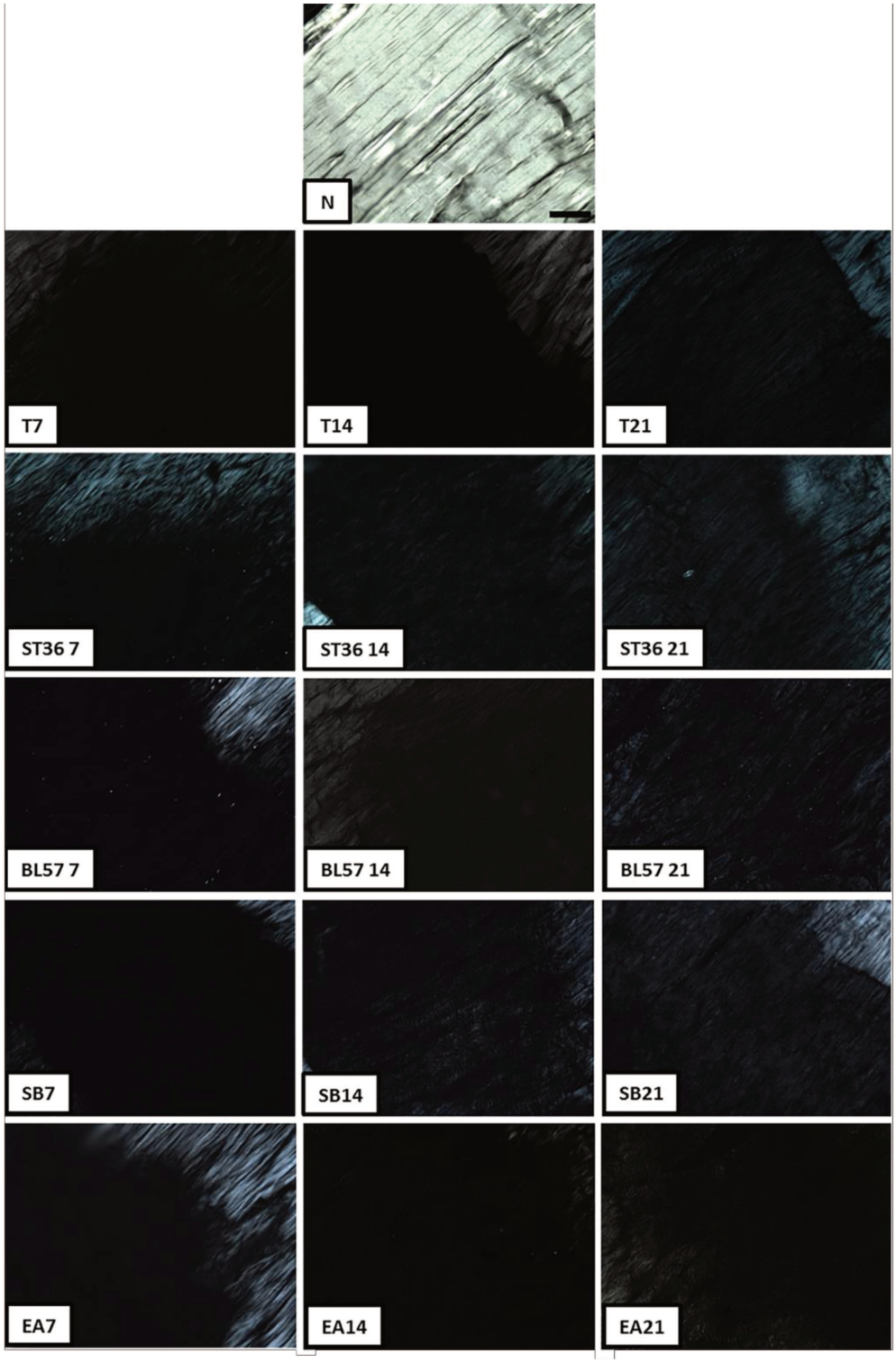
N
T7
T14
T21
ST36 7
ST36 14
ST36 21
BL57 7
BL57 14
BL57 21
SB7
SB14
SB21
EA7
EA14
EA21

Figure 1. Birefringence views of tendon collagen fibers obtained with the tendon long axis oriented at 45º relative to the plane of polarized light. In the tenotomized groups, the images were taken from injury site. The highest intensity of birefringence (brightness) is observed in the fibers of the tendons in normal group (N). At left column (7^{th} day after injury), the injury site has no brightness for every groups. At middle column (14^{th} day), the low intensity brightness appears in the ST36, BL57 and SB groups compared to the teno group (T). At right column (21^{th} day), the brightness is more intense in the groups cited above compared to T group and the same groups at 7^{th} and 14^{th} days. Observe that in the EA group the brightness was less intense in relation to ST36, BL57 and SB groups at 14^{th} and 21^{th} days.

Acknowledgements

National Council for Scientific and Technological Development (CNPq), grant number 140384/2012-0.

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11. CONCLUSÕES

11.1. Conclusão geral

A aplicação e estimulação manual dos pontos E-36 e B-57 em ratos que sofreram tenotomia parcial do tendão calcâneo resulta em aumento da birrefringência das fibras de colágeno, assim como em melhora dos aspectos ultraestruturais de suas fibrilas. Em relação à birrefringência, esse aumento é observado na fase proliferativa e de remodelamento. Já na ultraestrutura a melhora é observada nas três fases da cicatrização tendínea.

O uso da EA promoveu melhora da ultraestrutura tendínea durante a fase inflamatória e proliferativa, porém, com efeito deletério ultraestrutural na fase de remodelamento.

11.2. Conclusão específica

A estimulação manual dos pontos E-36 e B-57 em tendões lesados (transecção parcial) resulta em: 1. Aumento da organização dos feixes de fibras de colágeno no 14º e 21º dia após a lesão; 2. Aumento do diâmetro da massa média (MAD) das fibrilas de colágeno no 7º, 14º e 21º dia após a lesão; 3. Aumento da reorganização das fibrilas de colágeno no 21º dia após a lesão.

A EA nos pontos citados promove: 1. Aumento do diâmetro da massa média (MAD) das fibrilas de colágeno no 7ºe 14º dia após a lesão; 2. Diminuição do diâmetro da massa média (MAD) das fibrilas de colágeno no 21° dia após a lesão; 3. Aumento da reorganização das fibrilas de colágeno no 14º dia após a lesão.

12. ANEXO 1

DECLARAÇÃO

Declaro para os devidos fins que o conteúdo de minha dissertação de **Tese de Doutorado** intitulada **"Análise de diferentes protocolos de acupuntura nos aspectos bioquímicos, ultraestruturais e organizacionais de tendões de ratos em processo de cicatrização"**:

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

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