ANDREA APARECIDA DE ARO

"Efeito dos extratos de *Aloe vera* e *Arrabidaea chica* sobre a cicatrização do tendão calcanear de ratos após transecção parcial"

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UNIVERSIDADE ESTADUAL DE CAMPINAS
INSTITUTO DE BIOLOGIA

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Este exemplar corresponde à redação final da tese defendida pelo(a) candidato (a) *Andrea Aparecida de Aro* e aprovada pela Comissão Julgadora.

Tese apresentada ao Instituto de Biologia para obtenção do Título de Doutora em Biologia Celular e Estrutural, na área de Biologia Celular.

Orientador: Prof. Dr. Edson Rosa Pimentel

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SUMÁRIO

1. RESUMO

A utilização de extratos vegetais com atividades farmacológicas pode ser promissora no tratamento de lesões tendíneas, considerando a presença de princípios ativos que estimulam a síntese de componentes da matriz extracelular (MEC). Portanto, o presente estudo teve como objetivo investigar após 7, 14 e 21 dias da lesão, os efeitos da aplicação tópica dos extratos de *A. vera* e *A. chica* sobre tendões parcialmente transeccionados de ratos. Os grupos tratados com o extrato da *A. chica* foram denominados A7, A14 e A21 (controles S7, S14 e S21), e após tratamento com a *A. vera* foram denominados Av7, Av14 e Av21 (controles B7, B14 e B21). Foram realizadas análises bioquímicas tais como *Western blotting,* zimografia e dosagens de hidroxiprolina, de proteínas não colagênicas (PNCs) e de glicosaminoglicanos (GAGs); assim como análises estruturais, ultraestruturais e funcionais. Após aplicação do extrato da *A. chica*, a concentração de PNCs foi menor em A7 e a de hidroxiprolina foi maior em A7 e A21, em relação aos controles. Considerando a MMP-9, menor quantidade foi detectada no grupo A14 comparado ao grupo S14. As isoformas latente, intermediária e ativa da MMP-2 foram observadas em todos os grupos, porém maiores quantidades das isoformas latente e intermediária foram encontradas em A21. Os resultados de *Western blotting* mostraram menor quantidade de colágenos tipos I e III em A7 comparado ao controle. Maior quantidade de dermatan sulfato (DS) foi detectada em A14, e quantidade inferior de DS e condroitin sulfato (CS) foi observada em A21 comparada ao S21. As medidas de birrefringência detectaram maior organização das fibras de colágeno no grupo A21 em relação ao controle, e as análises ultraestruturais mostraram muitos fragmentos de colágeno na região transeccionada nos grupos S7 e A7. A análise do *CatWalk* mostrou que os animais tratados com *A. chica,* exibiram maior pressão de contato das patas durante a marcha no 7° dia.

Considerando a aplicação de *A. vera*, foi observada em SDS-PAGE banda menos intensa referente ao colágeno em Av14, confirmado por We*stern blotting*. O grupo Av21 apresentou maior concentração de PNCs comparado ao seu controle. Na dosagem de hidroxiprolina, os grupos Av7 e Av14 apresentaram maiores concentrações, ao passo que Av21 apresentou valor inferior ao controle. O grupo Av14 apresentou maior concentração de GAGs sulfatados e menor quantidade de DS em relação ao controle. Menor quantidade de MMP-9 foi encontrada em Av14, e menores quantidades das isoformas latente e intermediária da MMP-2 foram observadas em Av7 e Av14 em relação aos controles. Maior quantidade da isoforma ativa da MMP-2 foi observada em Av21 comparado a B21. As medidas de birrefringência detectaram maior organização das fibras de colágeno em Av14 em relação ao controle. Ao passo que as medidas de dicroísmo linear realizadas nos cortes corados com azul de toluidina, mostraram menor organização dos GAGs em Av14 comparado ao controle. Nossa conclusão é que a aplicação tópica dos extratos da *A. chica* e da *A. vera* é efetiva na síntese e organização de componentes da MEC durante o processo de reparo.

Palavras-chave: cicatrização tendínea, tendão, *Aloe vera*, *A. chica*, matriz extracelular.

1.1 Abstract

The use of plant extracts bearing pharmacological activities may be promising in the treatment of tendon injuries, considering the presence of active principles that stimulate the synthesis of extracellular matrix components (ECM). Therefore, this study aimed to investigate after 7, 14 and 21 days of injury, the effects of topical application of extracts of *A. vera* and *A. chica* on the healing of partially transected tendons of rats. The groups treated with the extract of *A. chica* were called A7, A14 and A21 (controls S7, S14 and S21), and after treatment with *A. vera* were called Av7, Av14 and Av21 (controls B7, B14 and B21). Biochemical analysis were performed such as Western blotting, zymography and quantification of hydroxyproline, non-collagenous proteins (NCPs) and glycosaminoglycans (GAGs); as well as structural, ultrastructural and functional analysis. After application of the extract of *A. chica*, the concentration of non-collagenous proteins (NCPs) was lower in A7 and hydroxyproline was higher in A7 and A21, compared to controls. Considering the MMP-9, lower amount was found in A14 compared to S14. The latent, intermediate and active isoforms of MMP-2 were observed in all groups, but larger quantities of latent and intermediate isoforms were found in A21. The results of Western blotting showed a lower amount of collagen types I and III compared to the control A7. Higher amount of dermatan sulphate (DS) was detected in A14 and lower amounts of DS and chondroitin sulfate (CS) were observed in A21 compared to S21. The birefringence measurements showed a higher organization of collagen fibers in the A21 group compared to control, and ultrastructural analysis showed many fragments of collagen in the transected region of groups S7 and A7. Analysis of the Catwalk showed that animals treated with *A. chica* exhibited a higher contact pressure of the legs during walking on the 7th day.

Considering the application of *A. vera*, less intense band related to collagen was observed on SDS-PAGE in Av14, confirmed by Western blotting. The group Av21had a higher concentration of NCPs compared to the control. In the dosage of hydroxyproline, Av7 and Av14 groups had higher concentrations in relation to their controls, while Av21 showed lower value than control group. The group Av14 had a higher concentration of glycosaminoglycans and lower amount of DS compared to control. Lower amount of MMP-9 was found in Av14, and lower amounts of intermediate and latent isoforms of MMP-2 were observed in Av7 and Av14 compared to controls. A higher amount of the active isoform of MMP-2 was observed in Av21 compared to B21. The birefringence measurements showed a higher organization of collagen fibers in Av14 compared to control. While linear dichroism measurements performed on sections stained with toluidine blue, showed lower organization of glycosaminoglycans in Av14 compared to control. Our conclusion is that the topical application of extracts of *A. chica* and *A. vera* is effective in the synthesis and organization of ECM components during the repair process.

Keywoeds: tendon healing, tendon, *Aloe vera*, *Arrabidaea chica*, extracellular matrix.

2. INTRODUÇÃO

Nos últimos anos, os extratos de plantas com potenciais efeitos medicinais têm sido intensamente estudados com o intuito de se obter novos princípios ativos que possam ser utilizados nos mais variados tipos de afecções. Os extratos vegetais são muito utilizados pela medicina popular e apresentam resultados positivos em muitos casos. Entretanto, nem sempre são conhecidas as mudanças que ocorrem na composição e organização do tecido afetado após o uso desses extratos. No presente estudo, investigamos separadamente o efeito da *Aloe vera* (L.) Burman f., (Liliaceae) e da *Arrabidaea chica* (Humb. & Bonpl.) B. Verlot. syn. Bignonia chica (Humb. & Bonpl), (Bignoniaceae) durante a cicatrização do tendão após transecção parcial. A *Aloe vera* tem sido amplamente utilizada em diversos tratamentos tanto pela medicina popular como pela comunidade científica, cuja principal indicação, é o tratamento de queimadura de pele com efetiva ação em processos de cicatrização. Entretanto, são poucos os estudos existentes com a *Arrabidaea chica*, cuja ação cicatrizante em pele também foi descrita recentemente. O interesse no estabelecimento de novos tratamentos para lesões tendíneas se deve ao fato da alta incidência dessas lesões na população, assim como por se tratar de um processo de reparo lento e complexo. Após rupturas, os tendões se tornam susceptíveis à recorrência de lesões devido à formação de uma cicatriz na região afetada. O presente estudo é o primeiro a analisar o efeito da *Aloe vera* e da *Arrabidaea chica* durante o processo de reparo em tendões após transecção parcial. Considerando os resultados de diversos trabalhos mostrando o efeito da *Aloe vera* na proliferação de fibroblastos, angiogênese, síntese de diversos fatores de crescimento e de importantes moléculas de matriz extracelular (MEC), tais como glicosaminoglicanos (GAGs) e colágeno, com aumento de "crosslinks" entre as moléculas de colágeno, nós hipotetizamos que a *Aloe vera* poderia estimular a síntese e organização de componentes de matriz durante o processo de cicatrização tendínea. Com relação à *Arrabidaea chica*, nós hipotetizamos um efeito benéfico na cicatrização de tendão baseados na sua ação no estímulo da proliferação de fibroblastos e na síntese de colágeno. Considerando as propriedades cicatrizantes das duas espécies, a proposta do presente estudo foi analisar os efeitos dos extratos de *A. vera* e *A. chica* separadamente durante as três fases do processo de cicatrização.

2.1 Características bioquímicas e estruturais do tendão

O tendão calcanear transmite ao osso calcâneo as forças de tensão geradas pela contração do músculo tríceps sural, promovendo o movimento da articulação tibiotársica. É formado por uma abundante matriz extracelular que consiste principalmente de colágeno tipo I (60-85%) que se organiza em fibrilas, fibras, feixes de fibras e fascículos (Kjaer, 2005), dispondo-se em arranjo helicoidal seguindo o maior eixo do tendão (O'Brien, 1997). A MEC dos tendões constituída por moléculas altamente organizadas confere as propriedades biomecânicas desse tecido (Birk et al, 1996). Além do colágeno tipo I, outros tipos de colágeno estão presentes em menores quantidades tais como os tipos II, III, IV, V e VI (ver revisão em Aro et al, 2012a).

A MEC do tendão também é constituída por componentes não-fibrilares como os proteoglicanos (PGs) (Vidal and Mello, 1984), glicoproteínas não-colagênicas (O'Brien, 1997), MMPs (Oshiro et al, 2003), células e água. Entre os PGs presentes nos tendões, o decorin e o fibromodulin são os pequenos PGs ricos em leucina mais importantes, com comprovada ação durante o processo de fibrilogênese (Derwin et al, 2001) através da manutenção e regulação do diâmetro das fibrilas de colágeno (Watanabe et al, 2005), além de estarem envolvidos na modulação da MEC e no comportamento celular (Chakravarti, 2002). A concentração desses pequenos PGs é alterada durante o processo de reparo, refletindo na reorganização tecidual após lesões tendíneas, uma vez que atuam diretamente na fibrilogênese do colágeno (Thomopoulos et al, 2002).

No tendão calcanear, as fibras de colágeno possuem grande número de ligações cruzadas, formando uma estrutura reforçada capaz de suportar altas forças de tração (Yang et al, 2008). Além das moléculas de colágeno como representantes dos componentes fibrilares da MEC, também está presente uma pequena quantidade de fibras elásticas (~2%) (Jozsa e Kannus, 1997) que se dispõem ao longo de algumas fibras de colágeno, contribuindo para a distensão inicial dos tendões quando submetidos às cargas unidirecionais durante as atividades desportivas ou diárias (Aquino et al, 2005). Vale ressaltar que o tendão calcanear apresenta duas regiões distintas quanto à sua composição e organização estrutural, devido às diferenças nas cargas biomecânicas atuantes. Essas regiões são denominadas de região proximal, em que os feixes de colágeno estão altamente

organizados apenas em uma direção considerando o maior eixo do tendão, devido à predominância de forças de tensão; e região distal, onde os feixes de colágeno estão distribuídos em várias direções de modo a oferecerem resistências às forças de compressão provenientes do osso calcâneo (Aro et al, 2008).

Os feixes de fibras de colágeno do tendão apresentam um padrão ondulado conhecido como “crimp", que pode ser observado na microscopia de polarização como áreas transversais claras e escuras (Vidal and Mello, 1984; Gathercole and Keller, 1991; Vidal, 1995; Vidal, 2003). O “crimp” característico de estruturas que apresentam colágenos fibrilares, especialmente do tipo I, presentes em tecidos conjuntivos sujeitos a altas demandas biomecânicas. O "crimp" age na absorção de energia durante o estresse e a deformação inicial pelos quais os feixes de colágeno são submetidos quando o tendão é alongado (Franchi et al, 2007; Gathercole and Keller, 1991). Pode ocorrer variação na organização do “crimp”, conforme a região do tendão, devido às diferenças nas intensidades de estresse atuantes (Vidal, 1995).

2.2 Lesões e o processo de cicatrização no tendão

2.2.1) Incidência de lesões

As lesões tendíneas apresentam alta incidência e o reparo bem sucedido ainda é um grande desafio para a ortopedia (Shearn et al, 2011). Os tendões com maior frequência de ruptura são o tendão calcanear (12-18/100 por ano) (Clayton e Court-Brown, 2008; Leppilahti et al, 1996), os tendões flexor e extensor da mão (incidência de 4,83 e 18/100 por ano, respectivamente) (Clayton e Court-Brown, 2008), e os tendões do manguito rotador (3.73/100 por ano) (Clayton e Court-Brown, 2008). O tendão calcanear humano pode resistir cargas de até 5.000 Newtons (Gerdes et al, 1992), embora uma ligeira alteração em sua composição possa ser fator determinante para a ocorrência de lesões. As lesões nesse tendão frequentemente ocorrem em uma região hipovascular, localizada entre 2-6 cm acima da entese, devido a uma rotação máxima das fibras de colágeno que prejudica o fluxo sanguíneo nessa área (Lesic and Bumbasirevic, 2004). Em geral, as lesões de tendão levam à morbidade acentuada que podem ter impacto tanto no trabalho, como nas atividades cotidianas e de recreação (James et al, 2008).

Os tendões são freqüentemente lesionados durante a prática desportiva e podem atingir atletas ocasionais, assim como atletas jovens de alta "performance" (Maffulli, 1999). A prática recreativa e até mesmo as atividades cotidianas podem ocasionar lesões tendíneas (Butler et al, 2008). A laceração ou ruptura de tendão são decorrentes principalmente de sobrecargas mecânicas súbitas (Woo et al, 2000). As diferenças nas intensidades de sobrecargas repetitivas atuantes no tendão podem causar inflamação de suas bainhas ou sua própria degeneração, ou ambas (Benazzo e Maffulli, 2000). Vale ressaltar que, os freqüentes microtraumas cumulativos provenientes de sobrecargas biomecânicas constantes causam danos ao tendão, pois não há tempo suficiente para o completo processo de reparo tecidual (Selvanetti et al, 1997). Entretanto, os microtraumas podem também resultar de estresse não uniforme dentro dos tendões, produzindo concentração anormal de cargas e forças de fricção entre as fibrilas de colágeno em determinadas regiões, causando danos às fibras (Arndt et al, 1998). Ainda com relação às cargas biomecânicas excessivas, a falta de adaptação do tendão ao estresse pode levar a liberação de citocinas pelos fibroblastos, com posterior ação na ativação de metaloproteinases (MMPs). As MMPs por sua vez, atuarão na degradação de componentes de matriz, cujo desequilíbrio pode levar ao surgimento de tendinopatias e/ou rupturas (Leadbetter, 1992; Sharma e Maffulli, 2005).

As alterações degenerativas podem levar a ruptura total dos tendões, cujas características histológicas são comuns em rupturas espontâneas (Tallon et al, 2001). Kannus e Jozsa (1991) detectaram alterações degenerativas em 865 (97%) de 891 tendões que sofreram ruptura espontânea, enquanto que essas alterações foram observadas em 149 (33%) dos 445 tendões controles (Kannus e Jozsa, 1991). A degeneração do tendão pode reduzir a resistência biomecânica do tecido, tornando-o mais susceptível a ocorrência de rupturas (Sharma e Maffulli, 2005). Outros fatores tais como idade (Caroll et al, 2008; Nakagaki et al, 2010), diabetes (de Oliveira et al, 2010; Fox et al, 2011) e hormônios (Cook et al, 2007; Soo et al, 2011), podem alterar o metabolismo do tecido conjuntivo, modificando as propriedades funcionais das fibras de colágeno. Após a ruptura, a cirurgia é encorajada nos casos de pacientes desportistas, já que o índice de re-ruptura nos pacientes tratados conservadoramente é bem maior dos que aqueles tratados cirurgicamente (Moller et al, 2001).

2.2.2) Reparo cirúrgico

O reparo cirúrgico dessas lesões tendíneas não restauram completamente sua função. A taxa de insucesso para o reparo do tendão flexor é de 4 a 13% (Zhao et al, 2007; Harris et al, 1999; Baktir et al, 1996). Para o tendão calcanear, o reparo cirúrgico têm uma taxa de re-ruptura de cerca de 2-4% (Khan et al, 2005; Strauss et al, 2007). A taxa de insucesso da cirurgia é aproximadamente o dobro quando comparada ao reparo percutâneo (4,3% e 2,1%, respectivamente). A imobilização pós-operatória seguida do uso de órtese funcional significativamente reduz a taxa de re-ruptura, quando comparada ao uso apenas de órtese (2,4% versus 12,2%, respectivamente) (Khan et al, 2005). Ainda com relação ao reparo do tendão calcanear, sua cicatrização é difícil devido à sua localização anatômica e subseqüente demanda biomecânica alta. Após o processo de reparo, dependendo da extensão da lesão, esse tendão se torna muitas vezes incapaz de suportar altas cargas mecânicas (Rees et al, 2006; Greca et al, 2005; Fredberg et al, 2008).

Resultados de reparo do manguito rotador apresentam grande variabilidade, com taxas de insucesso variando de 11 a 95% dois anos após o reparo (Galatz et al, 2004; Harryman et al, 1991; Lafosse et al, 2007). Similar ao tendão calcanear, o reparo do manguito rotador também pode requer um período de imobilização. Os pacientes tratados por lesões nesse tendão podem ser imobilizadaos durante o período de cicatrização, o que os torna mais suscetíveis a rigidez articular e atrofia muscular (Sharma e Maffulli, 2006; Thomopoulos et al, 2003).

2.2.3) Processo de cicatrização

O processo de cicatrização desse tecido é um processo lento e complexo, com a formação de uma cicatriz fibrosa que dependendo do tipo e extensão da lesão, pode causar disfunção significativa, incapacidade de movimento articular e considerável morbidade que podem durar vários meses (Almekinders and Almekinders, 1994; Favata et al, 2006). O local lesionado se torna uma região com alta reincidência de ruptura, caracterizando essas lesões ainda como um problema clínico. Após lesões, a composição e organização estrutural do tendão não são completamente restabelecidas, tornando-se um tecido biomecanicamente menos resistente (Tomiosso et al, 2009).

A cicatrização do tendão ocorre em três fases que se sobrepõem, denominadas de fase inflamatória, proliferativa e de remodelamento. Na fase inflamatória, com pico

aproximadamente no 7° dia após a lesão, ocorre a proliferação de fibroblastos, migração de células inflamatórias, eritrócitos, monócitos/macrófagos para o local lesionado, além da liberação de citocinas e fatores de crescimento (Berglund et al, 2011; Chen et al, 2004; Hoppe et al, 2012; Bedi et al, 2010). Concomitante à ocorrência da fagocitose do material necrosado, a angiogênese e síntese de colágeno tipo III encontram-se aumentadas nessa fase (Murphy et al, 1994; Oakes 2003). De acordo com dados de Tomiosso et al. (2009) e Almeida et al. (2012) obtidos em nosso laboratório, e considerando outros estudos que estão em fase de submissão (Aro et al, 2012b,c), nessa fase foram ainda observados na região lesionada, um aumento da concentração de GAGs e PNCs. Também nessa fase foi constatada disfunção durante a marcha.

Durante a fase proliferativa, a qual inicia-se após a lesão e atinge seu pico aproximadamente no 14° dia, ocorre um pico da síntese de colágeno tipo III (Oshiro et al, 2003). Nesse estágio, as fibras de colágeno tipo III formam fibras mais finas e desorganizadas na região da lesão, comparadas às fibras de colágeno tipo I (Pajala et al, 2009). Nessa fase ocorre ainda migração e rápida proliferação de fibroblastos no sítio da lesão, e síntese de outros componentes da MEC. A celularidade, o teor de água e as concentrações de GAGs, PNCs e de colágeno total se mantêm elevados durante esse estágio (Oakes, 2003; Oshiro et al, 2003; Tomiosso et al 2009). Vale ressaltar a presença de uma extensa rede de vasos sanguíneos (Fenwich et al, 2002), sendo que no final dessa fase, o reparo do tecido é altamente celular (Dahlgren et al, 2005).

Na fase de remodelamento, a qual inicia-se aproximadamente 21 dias após a lesão, ocorre a diminuição da celularidade e da síntese de colágeno tipo III (Oshiro et al, 2003), GAGs e os fibroblastos e as fibras de colágeno se tornam gradualmente alinhadas na direção do estresse biomecânico (Liu et al, 1995). Vale ressaltar que, o remodelamento da MEC inicia-se logo após a lesão, concomitante com outros eventos das fases inflamatória e proliferativa. Entretanto, o remodelamento é marcadamente aumentado após algumas semanas da lesão, com mudança de tecido celular para fibroso. Estudos realizados em nosso laboratório tem mostrado que além da região da lesão, as regiões adjacentes também passam por um processo de remodelamento, com alterações na composição e organização do colágeno e dos PGs (Aro et al, 2012b,c). Além dos colágenos tipos I e III, os tipos V e XII também aumentam durante a cicatrização do tendão (Oshiro et al, 2003). Após

aproximadamente 10 semanas, ocorre mudança gradual de tecido fibroso para tecido similar a uma cicatriz que persiste ao longo de um ano, com decréscimo da vascularização e do metabolismo dos fibroblastos do tendão (Hooley and Cohen, 1979). Entretanto, o tendão apresentará resistência biomecânica inferior comparada ao tendão normal.

Apesar dos diversos eventos celulares e bioquímicos envolvidos no remodelamento da MEC após lesão, as propriedades bioquímicas e estruturais do tendão cicatrizado não se igualam a do tendão intacto (James et al, 2008), ficando mais vulnerável a danos adicionais. Ainda não se sabe por que o tendão cicatrizado é incapaz de recuperar a quantidade normal de ligações cruzadas, embora um fator determinante seja a alteração na proporção dos diferentes tipos de colágeno, proteoglicanos ou outros fatores desconhecidos dentro do ambiente da lesão (Thornton et al, 2000).

2.2.4) O papel das metaloproteinases durante o processo de cicatrização

As metaloproteinases (MMPs) são uma família de pelo menos 23 endopeptidases zinco ou cálcio dependentes, que têm papel importante na homeostase da MEC de tendões em condições normais (Clutterbuck et al, 2010). Os níveis de MMPs são alterados durante a cicatrização, sendo essas enzimas importantes reguladoras do remodelameto da matriz nesse processo (Vu and Werb, 2000; Ireland et al, 2001). Atualmente, além do papel das MMPs na clivagem de componentes da matriz, outras funções são bem elucidadas, tais como o de regulação do crescimento celular através da clivagem da ligação entre fatores de crescimento na superfície celular e seus receptores, regulação da apoptose via liberação de fatores de morte ou de sobrevivência celular, alteração da motilidade celular através da clivagem de moléculas de adesão e modulação da bioatividade de quimiocinas (Cauwe et al, 2007). Durante a fase inflamatória do processo de reparo, regulada por citocinas e fatores de crescimento, as MMPs podem clivar esses mediadores liberando-os da superfície da célula, aumetando sua atividade. Podem ainda degradar esses mediadores, inibindo assim os sinais inflamatórios (Gill and Parks, 2008).

As MMPs estão indiretamente envolvidas no remodelamento da MEC após lesão, devido à sua capacidade de modificar o comportamento celular, e diretamente, pela degradação proteolítica de diversas moléculas, tais como colágenos, PGs e PNCs (Gill and Parks, 2008). A degradação do colágeno é iniciada na MEC principalmente por gelatinases (MMP-2 e -9) (Kjær, 2004), que rapidamente degradam colágenos desnaturados e

fragmentos de colágeno (Collier et al, 1988; Aro et al, 2012d), influenciando o *turnover* da MEC aumentado pela lesão no tendão (Madlener, 1998). As colagenases (MMP-1, -3, -8 e -13) por sua vez, degradam colágenos dos tipos I, II e III, além de outros componentes da matriz (Clutterbuck et al, 2010). A MMP-3 além de degradar vários componentes de matriz, participa ainda da ativação de outras MMPs e fatores de crescimento (Oshiro et al, 2003).

Os inibidores teciduais de MMPs (TIMPs) são inibidores endógenos responsáveis pela modulação da homeostase dinâmica da MEC durante processos de degradação e reparo (Birkedal-Hansen et al, 2008). Assim como ocorre com as MMPs, a concentração dos TIMPs também é alterada durante o processo de cicatrização, visando o controle do catabolismo após lesão (Karousou et al, 2008). Vale ressaltar que os TIMPs-1, -2 e -3 são os principais inibidores de colagenases e gelatinases (Koskinen et al, 2004).

2.2.5) O papel dos fatores de crescimento durante o processo de cicatrização

Os fatores de crescimento são definidos como moléculas sinalizadoras, envolvidas no controle do crescimento e diferenciação celular (Mehta and Mass, 2005). O interesse por essas moléculas começou há mais de 20 anos, após a descoberta de seu envolvimento na carcinogênese (McGrath, 1990). Os fatores de crescimento também tiveram seu papel comprovado no desenvolvimento fetal e na cicatrização tecidual (Conover et al, 1986). Após as lesões e em processos inflamatórios, ocorre a liberação de fatores de crescimento e citocinas provenientes de plaquetas, leucócitos polimorfonucleares, macrófagos e outras células inflamatórias (Evans, 1999). Esses fatores de crescimento induzem a neovascularização e quimiotaxia de fibroblastos e fibrócitos e estimulam a proliferação dessas células, bem como a síntese de colágeno (Abrahamsson et al, 1996; Marui et al, 1997). Sendo assim, essas moléculas desempenham um papel importante durante a cicatrização dos tendões.

Há seis fatores de crescimento, cuja atividade têm sido bem caracterizada na cicatrização do tendão: o IGF-I (fator de crescimento semelhante à insulina), PDGF (fator de crescimento derivado de plaqueta), VEGF (fator de crescimento do endotélio vascular), bFGF (fator básico de crescimento de fibroblasto), TGF-β (fator de crescimento de transformação beta) e o GDF-5 (fator-5 de crescimento e diferenciação). Após lesões do tendão, todos esses fatores são marcadamente aumentados e são ativos durante o processo

de reparo (Molloy et al, 2003). Vale ressaltar que o TGF-β é ativo nas fases inflamatória e de remodelamento do processo de reparo no tendão, com importante papel nesses estágios (Molloy et al, 2003). Em um estudo de Chhabra e colaboradores (2003), camundongos deficientes no gene para GDF-5 tiveram um processo de cicatrização deficiente, com menor organização estrutural e diminuição nas propriedades biomecânicas do tendão (Chhabra et al, 2003), evidenciando a importância desse fator durante processos de reparo tendíneo.

Os fatores de crescimento se ligam a receptores externos presentes na membrana celular, influenciando a síntese de DNA, quimiotaxia e a cascata de cicatrização tecidual (Mehta and Mass, 2005). A caracterização dessas moléculas nas lesões tendíneas estabeleceu importante conhecimento básico, a partir do qual as pesquisas relacionadas à cicatrização de tendão tem se baseado, buscando melhorar a qualidade desse processo através da aplicação exógena de fatores de crescimento. O início ou a liberação desses fatores é estimulado pelas células presentes na região da lesão e, durante a fase de remodelamento da MEC, também pela carga mecânica atuante no tendão lesionado (James et al, 2008).

2.2.6) Estratégias utilizadas na cicatrização de tendão

Diversos estudos tem demonstrado a efetividade da aplicação exógena de fatores de crescimento durante o reparo de tendão, tais como de TGF-β-1 (Kashiwagi et al, 2004), FGF-2 (Chan et al, 2000), VEGF (Zhang et al, 2003), IGF-1 (Kurtz et al, 1999) e GDF-5 (Bolt et al, 2007). A efetividade da aplicação desses fatores na região lesionada tem sido caracterizada pela aceleração do processo de cicatrização e aumento da resistência biomecânica do tendão após a lesão. Entretanto, um dos problemas relacionados à aplicação local de fatores de crescimento é a sua meia-vida curta, além da necessidade de materiais capazes de liberação lenta desses fatores (Aspenberg, 2007). Os estudos acima citados são ainda experimentais e restritos a modelos animais.

Entre as diversas estratégias desenvolvidas buscando a aceleração do processo de cicatrização, e que estão ainda em fase experimental, estão o transplante de células-tronco (CTs) através da manipulação de células do próprio paciente ou através de células provenientes de transplantes heterólogos (Witkowska-zimny e Walenko, 2011). As CTs são caracterizadas por sua capacidade de produzir auto-renovação de células progenitoras, que

podem se diferenciar em vários tipos de linhagens específicas de células. Historicamente, as CTs são subdivididas em CTs-embrionárias e CTs-pós-natal, tais como as CTs-hematopoiéticas, CTs-do sangue de cordão umbilical e CTs-somáticas adultas (Dazzi et al, 2006). Apesar da pluripotência das CTs-embrionárias, sua disponibilidade, tumorigenicidade e considerações éticas tem impedido a utilização generalizada dessas células em aplicações clínicas. Esses fatores tem ainda impulsionado a busca de CTs-mesenquimais adultas multipotentes para uso em reparo e regeneração tecidual (Tapp et al, 2009). Muitos experimentos tem revelado que as CTs-adultas podem reter o potencial de transdifernciação de um fenótipo para outro, seja *in vitro* ou *in vivo* após o transplante (Clarke et al, 2000; Ng et al, 2005). No entanto, a disponibilidade dessas células permanece um desafio para os cientistas.

A terapia com laser também tem sido estudada durante o processo de cicatrização de tendão, com alguns resultados positivos devido ao aumento da síntese de colágeno (Reddy et al, 1998) e redução do edema após cirurgia, embora sem alívio da dor ou com resultados funcionais superiores quando comparados ao controle (Ozkan et al, 2004). Vários outros estudos visando tornar o processo de cicatrização mais efetivo tem sido desenvolvidos nos últimos anos. Entretanto, algumas abordagens clínicas permanecem controversas (Okamoto et al, 2010). Em geral, os tratamentos cirúrgicos têm sido descritos com algumas vantagens, tais como menor taxa de re-ruptura (Lea and Smith, 1972; Möller et al, 2001; Carden et al, 1987), boa amplitude movimento (Gigante et al, 2008; Cetti et al, 1993), curto período de recuperação (Möller et al, 2001; Gigante et al, 2008) e sem atrofia muscular (Gigante et al, 2008; Cetti et al, 1993). Os métodos de tratamentos não cirúrgicos relatam melhor aparência estética (Lea and Smith, 1972; Gigante et al, 2008), risco de infecção reduzido (Beskin et al, 1987; Carden et al, 1987) e nenhum risco de granulomas causados pela sutura (Beskin et al, 1987; Fierro and Sallis, 1995). Apesar da existência de vários tratamentos, as lesões tendíneas são consideradas um problema socio-econômico (Dyment et al, 2012) que afetam pessoas de todas as idades. Claramente, novas estratégias precisam ser desenvolvidas.

2.3 Plantas medicinais

Os produtos naturais provenientes de plantas foram as principais fontes de grande diversidade de compostos químicos utilizados pela indústria farmacêutica no século passado. Historicamente, as empresas farmacêuticas utilizavam extratos vegetais para produzir formulações terapêuticas relativamente brutas. Entretanto, com o avanço dos antibióticos em meados do século XX, as formulações farmacêuticas de compostos bastante purificados tornaram-se mais comuns (Mishra e Tiwari, 2011). Atualmente, os medicamentos utilizados podem ser de origem sintética ou de origem vegetal, isolados diretamente ou produzidos por síntese à partir de um precursor vegetal (Newman e Cragg, 2007).

Algumas estimativas revelam que das 240 mil espécies de vegetais catalogadas em todo o mundo, apenas uma pequena porcentagem dessas espécies foram analisadas adequadamente quanto ao seu potencial medicinal para uso terapêutico. Portanto, essa flora representa um grande potencial para obtenção de novas moléculas para a descoberta de novos medicamentos (Hamburger et al, 1991; Cragg e Newman, 2005; Newman e Cragg, 2007). Assim, o uso de extratos vegetais com atividade cicatrizante pode adquirir um significado importante no auxílio aos tratamentos clínicos das lesões tendíneas, uma vez que os tratamentos convencionais ainda não são totalmente efetivos na recuperação das propriedades normais do tecido.

2.3.1) *Aloe vera*

A *Aloe vera* (L.) Burman f., (Liliaceae) é um membro entre as mais de 300 espécies pertencentes à família *Liliaceae*, freqüentemente utilizado para fins terapêuticos. É uma planta tropical crescida facilmente em clima quente e seco (Maenthaisong et al, 2007) e apresenta folhas longas e pontiagudas, constituídas de duas partes: uma casca exterior verde e uma polpa interna mais clara, que ocupa a maior parte do volume da folha. A casca exterior produz o exudato, um líquido amarelo e amargo, formado em sua maior parte por componentes fenólicos, tais como as antraquinonas (Turner et al, 2004). A polpa, também chamada de gel mucilaginoso tem sido muito utilizada para fins terapêuticos devido a sua constituição rica em polissacarídeos (Ni et al, 2004). Esses componentes são alvos de grande interesse quanto à identificação de suas atividades biológicas, sendo extraídos e

descritos especialmente para a *A. vera* (Griggs, 1996), devido ao maior número de trabalhos com essa espécie. A *A. vera* contém 75 constituintes potencialmente ativos, tais como polissacarídeos contendo glicose, galactose e xilose, taninos, esteróides, substâncias antibióticas, enzimas de diferentes tipos, vitaminas, minerais, ligninas, saponinas, ácido salicílico e aminoácidos (Atherton, 1998).

O interesse científico nas propriedades cicatrizantes da *A. vera* se deve ao fato de muitos estudos terem alcançado êxito ao constatarem sua ação efetiva no reparo principalmente de pele. Um estudo de cicatrização após queimadura de segundo grau na pele de ratos demonstrou propriedades anti-inflamatórias do gel que induziu uma cicatrização mais rápida (Somboonwong et al, 2000). Um diferente ensaio foi obtido utilizando-se camundongos tratados com uma fração de glicoproteína isolada da *A. vera*, em que também foi observada aceleração da cicatrização da pele devido à maior proliferação e migração celular para o local da lesão (Choi et al, 2001).

Outras pesquisas também apontaram para a eficácia do uso (oral e tópico) do gel da *A. vera* na cicatrização de incisões na derme (Chithra et al, 1998 a,b), devido ao aumento da síntese de componentes de MEC, tais como ácido hialurônico e dermatam sulfato, assim como devido aumento de glicohidrolases (β-glucuronidase e *N*-acetil glucosaminidase), responsáveis pelo "turnover" de GAGs (Chithra et al, 1998a). Também foi constatado o aumento do conteúdo de colágeno do tecido de granulação durante a cicatrização de pele de ratos (aplicação oral e tópica), com maior número de ligações cruzadas entre essas moléculas que contribuíram para o aumento da resistência da pele (Chithra et al, 1998b). Recente estudo realizado por Atiba e colaboradores (2011a) utilizando a administração oral do extrato da *A. vera*, mostrou aceleração do processo de cicatrização de pele após radiação, através do aumento da síntese de TGF-β1 e bFGF.

Em ratos diabéticos com lesões na pele, mais uma vez foi comprovada a ação do gel de *A. vera* (aplicação oral e tópica) sobre os componentes da MEC. Houve aceleração das fases da cicatrização como a fibroplasia, síntese de colágeno e contração do tecido, devido ao aumento de ligações cruzadas entre as moléculas de colágeno (Chithra et al, 1998c). De acordo com Atiba e colaboradores (2011b), a administração oral do extrato da *A. vera* acelerou a cicatrização da pele de ratos diabéticos através do aumento da síntese de VEGF e de TGF-β1. Ensaio *in vitro* com fibroblastos humanos demonstrou um aumento na

proliferação dessas células e nas junções do tipo gap de comunicação intercelular (GJIC), novamente sugerindo uma melhor cicatrização (Abdullah et al, 2003). A aplicação tópica da *A. vera* também foi eficaz para o tratamento de tendinite, quando associada ao uso de ultrassom (Filho et al, 2010).

Além do processo de cicatrização, pesquisas recentes têm demonstrado a bioatividade do gel de *A. vera* nos mais variados protocolos experimentais, como redução significativa dos níveis de glicose no sangue (Rajasekaran et al, 2005), e efeito protetor contra a hepatoxicidade produzida em ratos diabéticos (Can et al, 2004). No tratamento de algumas neoplasias, verificou-se que o Aloe-emodin (uma hidroxiantraquinona presente nas folhas de *A. vera)*, inibiu seletivamente o crescimento de células de tumor neuroectodermal humano em cultura de tecidos e em modelos animais (Pecere et al, 2000), além de inibir significativamente o crescimento de células do carcinoma de Merkel (MCC) em humanos (Wasserman et al, 2002). Um recente estudo mostrou que o extrato da polpa da folha da *A. vera* (injetado subcutaneamente) atuou na redução do tumor de Ehrlich, sendo este proposto também no uso profilático deste tipo de câncer (Akev et al, 2007). A atividade anti-inflamatória do extrato de *A. vera* tem sido demonstrada em modelos experimentais de úlceras gástricas (Eamlamnam et al, 2006) e em infecções estomacais causadas pela *Helicobacter pylori* (Prabjone et al, 2006). Sua ação anti-inflamatória também foi comprovada em ensaios *in vitro* devido a sua atividade antilipoxigenásica (Bezáková et al, 2005). De acordo com estudos de Zhang e Tizard (1996) e Djeraba & Quere (2000), uma fração contendo o polissacarídeo acemanana extraído do gel da *A. vera* tem efeito na ativação de macrófagos.

2.3.2) *Arrabidaea chica*

Outra planta com potencial atividade cicatrizante é a *Arrabidaea chica* (Humb. & Bonpl.) B. Verlot. syn. Bignonia chica (Humb. & Bonpl), (Bignoniaceae), a qual é constituída por 120 gêneros e 800 espécies de plantas arbustivas, arbóreas e trepadeiras, distribuídas principalmente nas regiões tropicais na América do Sul e na África (Von Poser, 2000). No Brasil, plantas dessa família ocorrem desde a região Amazônica até o Rio Grande do Sul, não possuindo habitat único, mas podendo ser encontradas nos Cerrados, na

Mata Atlântica e na Região Amazônica (Lorenzi, 1988). As folhas, quando submetidas à fermentação e manipuladas como anileira, fornecem um corante vermelho escuro ("vermelho- carajuru", "vermelho-de-chica", "vermelhão- americano"). Desde os tempos remotos, era usado pelos índios para se pintarem, assim como para tingirem os seus enfeites, utensílios, vestuário e na elaboração de tatuagens (Corrêa, 1984).

A espécie *A. chica* é conhecida popularmente como pariri (no Pará), crajiru (no Amazonas), puca-panga, coapiranga, chica ou cipó-cruz (Von Poser, 2000). A *A. chica* tem sido empregada na medicina popular como antianêmico, anti-inflamatório, anti-hemorrágico, antileucêmico e cicatrizante (Taylor, 1998). Algumas tribos indígenas da Amazônia preparam infusão das folhas da planta para tratamento não só de conjuntivites, como também para lesões dérmicas secundárias, leucorréias, cólica intestinal e inflamações de útero e ovário (Jorge et al, 2008).

O primeiro estudo fitoquímico do extrato preparado das folhas de *A. chica* realizado por Chapman et al (1927) relata o isolamento de 3-deoxiantocianidina (carajurina), Posteriormente, Scogin (1980) e Harbone & Willians (1998) propuseram que a ocorrência desse raro pigmento em Bignoniaceae era provavelmente restrita a *A. chica*. Estudos posteriores realizados por Takemura et al. (1995) e Devia et al. (2002) identificaram duas novas deoxiantocianidinas, a 6,7,4'-tri-hidroxi-5-metoxiflavona e 6,7,3',4'-tetrahidroxi-5-metoxiflavona (carajuruflavona). Foram ainda isolados fitoesteróis, flavonoides e taninos (Zorn et al, 2001; Albuquerque, 1989). O estudo da composição mineral identificou ainda a presença de cálcio, fósforo, manganês, cobre, zinco e ferro (Zorn et al, 2001; Albuquerque, 1989; Magalhães et al, 2009).

Embora existam estudos voltados para a identificação dos compostos presentes na *A. chica*, a bioatividade desses compostos tem sido pouco testada cientificamente apesar da sua ampla utilização na medicina popular. Esta planta medicinal embora não esteja ainda nos registros de fitoterápicos da Anvisa, tem grande potencial para uso médico futuro. Recentemente, um estudo enfocando a aplicação tópica do extrato dessa espécie vegetal em lesões de pele de ratos, mostrou excelentes resultados evidenciando sua ação cicatrizante devido ao aumento da síntese de colágeno e proliferação de fibroblastos (Jorge et al, 2008).

3. JUSTIFICATIVA

A cicatrização do tendão calcanear é, reconhecidamente, um problema clínico que motiva a comunidade científica a buscar tratamentos que auxiliem no restabelecimento da função do tendão após algum tipo de lesão. Ainda não se sabe por que o tendão cicatrizado é incapaz de recuperar suas propriedades biomecânicas, bioquímicas e estruturais, embora um fator determinante seja a alteração na proporção dos diferentes componentes da MEC. Portanto, o presente estudo propõe investigar o efeito cicatrizante dos extratos de *Aloe vera e Arrabidaea chica* no tendão lesionado, devido à presença de agentes químicos em sua constituição, com atividades farmacológicas que poderiam ser considerados medicamentos em potencial (Page et al, 2004). Acredita-se que em um curto espaço de tempo, várias plantas utilizadas pela medicina popular, poderão adquirir um importante significado clínico como tratamento alternativo ou auxiliar de várias patologias. Apesar de ensaios descritos anteriormente terem comprovado a efetividade da *A. vera* no estímulo da síntese e organização de componentes da MEC durante o processo de cicatrização principalmente de pele (Chithra et al, 1998a,b,c; Choi et al, 2001), não há trabalhos na literatura investigando seu efeito em um tecido conjuntivo fibroso e altamente organizado, com grande demanda biomecânica, como o tendão calcanear. Em relação à *A. chica,* além dos relatos da medicina popular, faltam estudos comprovando sua ação cicatrizante em tendões. Deve-se considerar ainda a importância do registro e da comprovação científica da atividade biológica de espécies vegetais endêmicas dos biomas brasileiros.

4. OBJETIVOS

4.1 Gerais: Investigar através de métodos bioquímicos, funcionais, estruturais e ultra-estruturais, os efeitos da aplicação tópica dos extratos de *A. vera* e *A. chica* sobre a cicatrização do tendão calcanear de ratos no 7°, 14° e 21° dias após transeccão parcial. Considerando as propriedades cicatrizantes das duas espécies, a proposta do presente

estudo foi analisar, separadamente, os efeitos dos extratos de *A. vera* e *A. chica* durante as três fases do processo de cicatrização.

4.2 Específicos:

1. Analisar a organização estrutural, através da microscopia ótica comum e de polarização, de tendões no 7°, 14° e 21° dias após a transecção parcial, em resposta ao tratamento tópico com os extratos de *A. vera* e *A. chica*.
2. Investigar através da morfometria a variação nas quantidades de fibroblastos, vasos sanguíneos e de células inflamatórias nos diferentes grupos experimentais.

3. Identificar através de ensaios imunocitoquímicos a presença dos colágenos tipos I e III no tendão de todos os grupos citados acima.

4. Quantificar proteínas, GAGs sulfatados e hidroxiprolina em tendões dos grupos tratados com

os extratos das duas espécies de plantas durante o processo de cicatrização.

5. Analisar em gel de SDS-poliacrilamida as populações de proteínas extraídas do tendão de todos os grupos experimentais.

6. Quantificar e analisar em gel de agarose a presença dos glicosaminoglicanos condroitim sulfato (CS), dermatam sulfato (DS) e heparam sulfato (HS) no tendão de todos os grupos em estudo.

7. Identificar a presença e a atividade enzimática das metaloproteinases-2 e -9 nos grupos experimentais.

8. Analisar a marcha dos animais após aplicação da *A. chica* utilizando-se o *CatWalk*.

9. Estudar a ultra-estrutura da região de transecção dos tendões após tratamento com a *A. chica*.

5. MATERIAIS E MÉTODOS

5.1 Animais: foram utilizados ratos Wistar machos, adultos jovens (68 dias), pesando em média 250 g (± 20), provenientes do Biotério Central da UNICAMP. Os animais foram

divididos em 13 grupos e mantidos em gaiolas plásticas padrão, com livre acesso à água e ração.

5.2 Grupos Experimentais: os animais foram mantidos no biotério do Departamento de Biologia Estrutural e Funcional, Instituto de Biologia – IB (UNICAMP) e manipulados de acordo com as normas da Comissão de Ética na Experimentação Animal -UNICAMP (protocolo n°1621-1). Como descrito abaixo, além dos grupos de animais que tiveram os tendões transeccionados também foram analisados tendões normais, sem lesão, como parâmetro de comparação considerando as características da MEC intacta.

5.2.1) *A. chica*: após a transecção parcial do tendão calcanear da pata direita, os animais foram divididos em **grupos salina 7 dias (S7), salina 14 dias (S14) e salina 21 dias (21):** tendões tratados com aplicação tópica de 15 µL de salina (0,85%) sobre a lesão e antes da sutura da pele, assim como nos próximos 6 dias após a transecção. Os animais foram sacrificados após 7, 14 e 21 dias. G**rupos *A. chica* 7 dias (A7)*, A. chica* 14 dias (A14) *e A. chica* 21 dias (A21):** tendões parcialmente transeccionados e tratados com aplicação tópica de 32 mg de extrato liofilizado de *A. chica* dissolvido em 15 µL de salina (0,85%). A primeira aplicação tópica também foi feita sobre a lesão e antes da sutura da pele, seguindo pelos próximo 6 dias após a transecção. Os animais foram sacrificados após 7, 14 e 21 dias da lesão.

5.2.2) *A. vera*: após a transecção parcial do tendão calcanear da pata direita, os animais foram divididos em **grupos base 7 dias (B7), base 14 dias (B14) e base 21 dias (B21):** tendões tratados com aplicação tópica de 32 mg de base (uma mistura de lanolina anidra e vaselina sólida (30:70)) sobre a lesão e antes da sutura da pele e nos próximos 6 dias após a lesão. Os animais foram sacrificados após 7, 14 e 21 dias. G**rupos *A. vera* 7 dias (Av7), *A.vera* 14 dias (Av14) *e A. vera* 21 dias (Av21):** tendões parcialmente transeccionados e tratados com aplicação tópica 32 mg da pomada de *A. vera* (contendo 16 mg de extrato liofilizado + 16 mg de base). A primeira aplicação tópica também foi feita sobre a lesão e antes da sutura da pele, seguindo pelos próximo 6 dias após a transecção. Os animais foram sacrificados após 7, 14 e 21 dias da lesão.

5.3 Obtenção dos extratos de *Arrabidaea chica e Aloe vera*

Os extratos liofilizados da *A. chica* e d*a A. vera* utilizados em nosso trabalho foram fornecidos pelo CPQBA (Centro Pluridisciplinar de Pesquisas Químicas, Biológicas e Agrícolas) – UNICAMP, onde são cultivadas (Figuras 1 a e b).

Figura 1: A) Exemplar de *Arrabidaea chica*. B) Exemplar de *Aloe vera*. Espécies cultivadas no campo experimental do CPQBA/UNICAMP.

Foram coletadas folhas de *Arrabidaea chica* (Humb. & Bonpl.) B. Verlot. (syn. *Bignonia chica* (Bonpl.)), disponível no Banco de Germoplasma, do acesso 06, do Centro de Pesquisas Químicas, Biológicas e Agrícolas da UNICAMP, localizado no município de Paulínia (22º 45' 00''Sul e 47º10'21''Oeste). O material vegetal foi moído com gelo seco em um triturador de facas, marca Stephen, modelo UM 40. As folhas moídas foram extraídas três vezes com uma mistura de etanol/ ácido cítrico 0,3%, na proporção de 1:5 (v/v). Os extratos foram filtrados, secos com Na_2SO_4 anidro, filtrados e o solvente eliminado sob vácuo, seguido de liofilização. O extrato liofilizado (32 mg) foi dissolvido em 15 µl de solução salina (0,85 %), com concentração de 2,13 g/mL para aplicação no local da lesão em cada tendão (Figura 2).

Figura 2: Fluxograma do processo de obtenção do extrato bruto etanólico da *A. chica*.

As folhas de *Aloe vera* (L.) Burman f., (Liliaceae) foram coletadas no campo experimental localizado no município de Paulínia (22º 45' 00''Sul e 47º10'21''Oeste), exsicata depositada na coleção CPMA sob a supervisão da Dr. Glyn Mara Figueira. Para obtenção do liofilizado, o exudato amarelo foi removido das folhas da *A. vera* pela gravidade. Em seguida, as folhas foram descascadas e a mucilagem homogeneizada em liquidificador. A mucilagem homogeneizada foi centrifugada a 10,000 x g por 30 minutos e a 4°C para remoção das fibras, de acordo com Chithra et al. (1998 a,b,c). O sobrenadante foi congelado e liofilizado em liofilizador Virtis modelo 8L sob vácuo, onde a água foi retirada por sublimação até secura. O tempo do processo foi em média 72 h. A base foi preparada pela mistura de lanolina anidra e vaselina sólida (30:70), seguido da incorporação do extrato da *A. vera* liofilizado (1:1) sob agitação até completa

homogeneização. Cada tendão lesionado recebeu no local da lesão a aplicação tópica de 32 mg da pomada (contendo 16 mg de extrato liofilizado + 16 mg de base) (Figura 3).

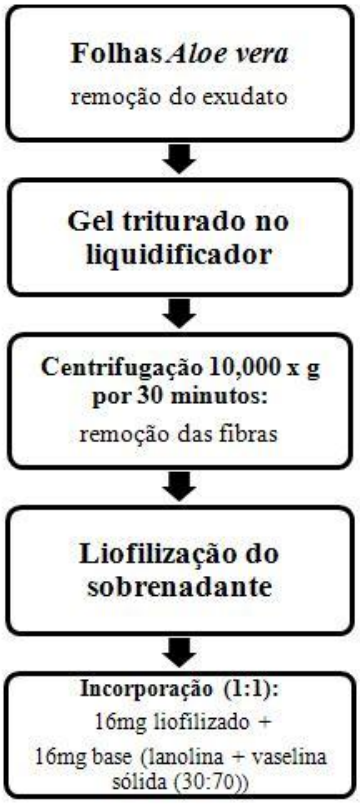

Figura 3: Fluxograma do processo de obtenção da pomada de *A. vera*.

5.4 Protocolos para a transecção parcial do tendão calcanear e aplicação tópica dos extratos das plantas *A. chica* e *A. vera*

Para os procedimentos cirúrgicos os animais foram anestesiados com injeção intra-peritoneal de Ketamina (80 mg/Kg) e Xylazina (10 mg/Kg) e as patas inferiores direitas submetidas à tricotomia e anti-sepsia com álcool iodado. Uma incisão longitudinal na pele foi feita para exposição do tendão calcanear e a transecção parcial transversal foi realizada na região proximal do tendão, sujeita predominantemente às forças de tensão, localizada a uma distância de aproximadamente 4 mm de sua inserção no osso calcâneo (Murrell et al, 1997; Tomiosso et al, 2009). Os extratos de *A. chica* e de *A. vera* (32 mg) foram aplicados no local transeccionado do tendão antes da sutura da pele. Os tendões controles receberam

apenas 15 µL de solução salina (0,85 %) no local da lesão considerando o controle negativo da *A. chica*, e 32 mg de base, no caso do controle negativo da *A. vera*, como já descrito anteriormente. Após a transecção do tendão, a pele foi suturada com fio de sutura de Nylon (Shalon 5-0) e agulha (1,5 cm). A partir do 1° dia após a cirurgia, foi feita a aplicação tópica diária dos extratos vegetais sobre a região da pele suturada, massageando suavemente até sua completa absorção, durante os 6 primeiros dias após a lesão de acordo com cada grupo formado, contados a partir do dia seguinte da lesão. Os animais foram sacrificados por aprofundamento da anestesia.

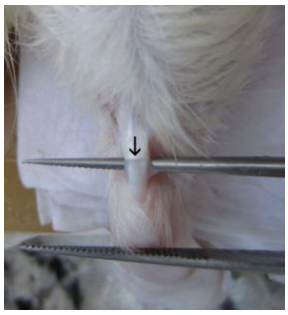

Figura 4: Imagem do tendão calcanear de rato após transecção parcial. Observe o local da transecção localizada aproximadamente à 4 mm de sua inserção no osso calcâneo.

5.5 ANÁLISES BIOQUÍMICAS:

5.5.1) Extração dos componentes MEC do tendão

Os componentes da MEC foram obtidos após extração da região de transecção do tendão calcanear com cloreto de guanidina 4 M (contendo 0,05 M EDTA e 1mM de PMSF em tampão acetato de sódio 0,05 M - pH 5,8) durante 24h e a 4º C em constante agitação, de acordo com o método de Heinergård e Sommarin (1987). Após centrifugação por 1h, a 10.000 rpm e a 4º C (centrífuga Beckman J2-21 - Rotor JA-20) o sobrenadante foi estocado em temperatura de -8°C para as diferentes análises bioquímicas.

5.5.2) Dosagens de proteínas não colagênicas

O extrato total da região de transecção do tendão foi utilizado para quantificar

proteínas pelo método de Bradford (1976), utilizando o *kit* BioRad (BioRad *protein assay*) e albumina sérica bovina (BSA) como padrão, nas concentrações 1, 2, 4, 8 16 μg/ μL . A leitura das amostras foi feita a 595 nm, em espectrofotômetro Ultrospec, modelo 2100 Pro da Amersham Biosciences.

5.5.3) Eletroforese em gel de SDS-poliacrilamida (SDS-PAGE)

As proteínas extraídas foram analisadas em gel de poliacrilamida contendo dodecil sulfato de sódio (SDS-PAGE), segundo Zingales (1984), usando um gel de gradiente de poliacrilamida (4-16%). O sistema tampão foi de acordo com Laemmili (1970), com gel de empacotamento contendo 3,5 % de acrilamida. Após a dosagem do extrato total segundo o método de Bradford (1976), 50 μg das diferentes amostras foram precipitadas em solução com tampão acetato de sódio 1M pH 7,4 (máximo de 150 μL) e 9 volumes de etanol (1350 μL), durante 24 horas à 4°C. As centrifugações foram realizadas em microcentrífuga Fischer Scientific Model 235 Va durante 5 minutos, a 8.000 rpm. Após duas lavagens (150 μL de tampão acetato de sódio 1M pH 7,4 e 1350 μL de etanol), o precipitado obtido foi seco à 37°C e ressuspendido em tampão de amostra redutor contendo β-mercaptoetanol e em tampão de amostra não redutor, contendo Tris-HCL 62,5 mM, SDS 2%, glicerol 10%, EDTA 1 mM em pH 6,8 e azul de bromofenol 0,01 %. As amostras foram incubadas por 5 minutos a 96°C. O tampão utilizado na cuba de eletroforese foi Tris 2,5 mM, glicina 190 mM e SDS 0,1% em pH 6,8. A corrente elétrica aplicada foi de 30 mA, durante aproximadamente 4 horas. Os padrões de peso molecular empregados foram: fosforilase b (94 kDa), albumina sérica (64 kDa), ovoalbumina (43 kDa), anidrase carbônica (30 kDa), inibidor de tripsina (20,1 kda), α-lactoalbumina (14,4 kDa). Para o padrão de colágeno, foi empregado o colágeno tipo I extraído de tendão de cauda de rato. Após a corrida, o gel foi fixado em uma solução fixadora (metanol 50%, ácido acético 12 %) durante 1 hora, e em seguida corado por Coomassie brilliant blue-R (CBB R-250).

5.5.4) Western blotting

50 μg de proteínas totais provenientes do extrato em guanidina foram precipitadas para detecção de colágeno tipo I e 80 μg de proteínas totais foram precipitadas para detecção de colágeno tipo III, utilizando uma solução contendo tampão acetato de sódio 1M pH 7,4 (100 ou 70 μL) e 9 volumes de etanol (1350 μL), durante 24 horas à 4°C. Após 3

lavagens (com 150 µL de tampão acetato de sódio 1M pH 7,4 e 1350 µL de etanol), o precipitado obtido foi seco à 37°C e ressuspendido em tampão de amostra redutor contendo β-mercaptoetanol 5% (0,5M Tris-HCl pH 6.8, Glicerol 26%, SDS 20%, Azul de Bromofenol 0,1%) para colágeno tipo III e em tampão de amostra não redutor para colágeno tipo I. Após a separação das proteinas em eletroforese em gel de SDS-poliacrilamida (7,5%), essas foram transferidas para uma membrana de nitrocelulose (Hybond-ECL - Amersham, Pharmacia Biotech, Arlington Heights, USA), à 70V por 3h (Montico et al, 2011; Towbin and Gordon, 1979). O bloqueio das membranas foi feito utilizando-se um reagente quimioluminescente (WBAVDCH01, Millipore) durante 20s, seguindo para lavagem com solução basal (trisma-base 0.01M, NaCl 0.15M e tween-20 0.05%) e incubação por 10 min. com os anticorpos primários para colágeno I (C2456, Sigma), colágeno III (C7805, Sigma) e GAPDH (sc-25778, Santa cruz) para controle endógeno. Todos os anticorpos foram diluídos em solução basal/BSA 1%. Após lavagem em solução basal, a membrana foi incubada durante 10 min. com os seguintes anticorpos secundários da Sigma: A0412, A8786 e A0545. Após lavagem da membrana, a atividade da peroxidase foi detectada pela incubação com DAB durante 5 min. Por último, foi realizada a densitometria de bandas de cada proteína marcada, usando-se o programa Scion Image software Alpha 4.0.3.2 (Scion Corporation,USA).

5.5.5) Quantificação de hidroxiprolina

Para estimar as alterações no conteúdo de colágeno, fragmentos da região de transecção do tendão foram picados e secos, utilizando-se banhos em acetona por 48 h (troca a cada 24h) e formol-etanol (2:1) por 48 h (troca a cada 24h). Após a desidratação, os fragmentos foram secos por 1h em estufa a 60°C, pesados e posteriormente hidrolisados em HCl 6N (1mL/10mg de tecido) por 4 horas a 130°C. O conteúdo de hidroxiprolina foi determinado segundo o método de Stegemann and Stalder (1967) e Jorge et al. (2008). A leitura da absorbância das amostras foi em 550 nm em espectrofotômetro.

5.5.6) Zimografia para detecção de MMP-2 e -9

A região de transecção dos tendões foi picada e imersa em solução contendo 50 mM Tris-HCl (pH 7.4), 0.2 M NaCl, 10 mM $CaCl_2$, 0.1% Triton e 1% de coquetel de inibidor de

protease (Sigma) para extração de proteínas (100 µL de tampão de extração para 30 µg de tecido), a 4°C durante 2h (Stegemann e Stalder, 1967). Depois dessa primeira extração, as amostras foram incubadas adicionando-se 1/3 do volume da mesma solução descrita anteriormente, a 60°C durante 5 minutos. Após quantificação de proteínas totais de acordo com o método de Bradford (1976), usando BSA (Sigma) como padrão, foi aplicado 1 µg (para análise MMP-2) e 20 µg (para análise MMP-9) de proteínas por amostra no gel. O gel de acrilamida (10%) contendo gelatina (1%) foi realizada a 4°C e, em seguida, o gel foi lavado com 2.5% Triton X-100 e encubado durante 21h, em solução de 50 mM Tris-HCl (pH 7.4) 0.1 M NaCl e 0.03% de azida sódica a 37°C. O gel foi corado com coomassie brilliant blue R-250 (Sigma) por 1h. Em seguida, o gel foi lavado com solução contendo 30% de metanol e 10% de ácido acético para observação das bandas correspondentes a atividade gelatinolítica. Adicionalmente como controle positivo, foi usado 20 mM de EDTA no tampão de incubação o qual inibiu a atividade das gelatinases, confirmando a identificação das MMPs no gel (Marqueti et al, 2006; Silva et al, 2008; Aro et al, 2012d).

5.5.7) Quantificação de GAGs e análise por eletroforese em gel de agarose

Fragmentos da região de transecção do tendão foram picados e desidratados, utilizando-se banho em acetona por 24 h. Após a desidratação, os fragmentos foram secos por 1h em estufa a 37°C, pesados e seguiram para a digestão com papaína (40 mg de papaína para cada 1g de tecido) em tampão fosfato de sódio 100 mM, pH 6,5, contendo EDTA 40 mM e β-mercaptoetanol 80 mM, por 24h à 50°C. Após a digestão, as amostras foram tratadas com TCA 90% e seguiram para a dosagem de GAGs, através do método azul de dimetilmetileno (Farndale et al, 1986), com leitura a 526 nm no espectrofotômetro Ultrospec, modelo 2100 Pro da Amersham Biosciences. Os GAGs sulfatados foram separados ataravés da eletroforese em gel de agarose (0,5%) com tampão proprileno diamino (0,05M) (Dietrich e Dietrich, 1976), a 0,1 mA durante 2h. Foi utilizado um padrão consistindo de uma mistura de heparam sulfato, dermatam sulfato e condroitim sulfato (1mg/mL de cada). O gel de agorose foi fixado com cetavlon (0,1%), seco e corado com Azul de touluidina. O perfil eletroforético dos GAGs foi visualizado após tratamento com etanol (50%) e ácido acético (1%).

5.6 ANÁLISES MORFOLÓGICAS E ULTRAESTRUTURAIS:

5.6.1) Microscopia ótica comum

Os tendões foram fixados usando formaldeído 4% em tampão Millonig (0.13 M fosfato de sódio, 0.1 M NaOH - pH 7.4), durante 24h a 4°C. Os tendões foram lavados em água, desidratados em etanol, diafanizados com xilol e embebidos em parafina. Cortes seriados longitudinais de 7 µm de espessura foram desparafinizados imediatamente antes do processo de coloração. Para observação dos proteoglicanos, os cortes foram corados com AT (0.025%) em tampão Mcllvaine (0.03M ácido cítrico, 0.04 M de fosfato de sódio dibásico - pH 4.0) (Mello e Vidal, 2003). Para análise da morfologia geral da região de transecção, os cortes foram corados com HE (Kiernan, 1981). As lâminas foram tratadas com xilol e montadas com Entellan (Merck). Para observação do tecido foi utilizado um microscópio ótico comum Olympus BX 60.

5.6.2) Microscopia de Polarização: Birrefringência - Análise de imagem e medidas

Os tendões foram fixados usando formaldeído 4% em tampão Millonig (0.13 M fosfato de sódio, 0.1 M NaOH - pH 7.4), durante 24h a 4°C. Os tendões foram lavados em água, desidratados em etanol, diafanizados com xilol e embebidos em parafina. Cortes seriados longitudinais de 7 µm de espessura foram desparafinizados e seguiram para a análise sob microscopia de polarização. Os cortes, considerando o maior eixo do tendão, foram posicionados a 45° entre o polarizador e analisador cruzados, após montagem somente em água (Mello et al, 1979; Vidal, 1965; Vidal et al, 1975; Vidal, 1980; Vidal, 1986; Vidal and Mello, 2010). Medidas de birrefringência das fibras de colágeno foram realizadas e expressas em "Gray Average (GA)" em pixels (8 bits = 1 pixel) após calibração. Média de GA e desvio-padrão foram representados em tabelas.

5.6.3) Microscopia de Polarização: Medidas de dicroísmo linear

As medidas de dicroísmo linear foram realizadas nos cortes corados com AT, após posicionamento do maior eixo do tendão perpendicular e paralelo em relação ao plano de

luz polarizada (PLP). A razão dicróica (ID=dpa/dpp) foi determinada com base na absorbância dos PGs medidas nas posições paralela (dpa) e perpendicular (dpp) em relação ao PLP (Mello e Vidal, 2003). As imagens foram capturadas utilizando o microscópio Olympus BX 53, e as medidas de dicroísmo linear foram realizadas utilizando o analisador de imagens (Image-Pro Plus 4.5.0.29, Media Cybernetics, Inc. – Silver Spring, MD, USA).

5.6.4) Microscopia eletrônica de transmissão

Apenas a região de transecção foi escolhida e fixada usando glutaraldeído (5%) e ácido tânico (0,25%) em tampão Millonig (0.13 M fosfato de sódio, 0.1 M hidróxido de sódio, 0.03 M glicose - pH 7.4) por 24h. Logo após a fixação, foram feitas 10 lavagens de 10 minutos cada sob agitação em tampão Millonig. Após as lavagens, foi feita a pós-fixação utilizando-se tetróxido de ósmio (0,5%) em tampão Millonig por 1h sob leve agitação e à temperatura ambiente. Três lavagens rápidas das amostras em água foram realizadas e em seguida, o processo de desidratação em acetona foi iniciado. Em seguida, foi feita a infiltração em resina Epon®. Cortes ultrafinso (60-80 nm) foram contrastados com acetato de uranila (3%) por 30 min. e citrato de chumbo (0.2%) em hidróxido de sódio (0.1N) por 5 min., e observados sob o microscópio eletrônico de transmissão.

5.7 ANÁLISE FUNCIONAL

5.7.1) Avaliação da intensidade máxima de contato da pata durante a marcha após transecção parcial do tendão

O sistema de Catwalk (Noldus Inc., Holanda) foi usado para analisar a recuperação da marcha dos animais após a transecção do tendão. Nesse protocolo, os ratos atravessaram uma passarela de vidro (100 cm (comprimento) x 15 cm (largura) x 0,6 cm (espessura). A aquisição de dados foi realizada com uma câmara de alta velocidade (Pulnix TM-765E CCD), e a intensidae das pegadas dos ratos foram automaticamente classificados pelo software. As pegadas foram obtidas durante os 3 dias antes da transecção parcial dos tendões, para avaliar o padrão de marcha normal dos animais, e coletadas novamente após as lesões. As medições pós-operatórias foram coletadas no 1 º, 3 º, 5 º e 7° dias após a lesão, para os grupos que foram sacrificados 7 dias após a cirurgia; no 1º, 3º, 5º, 7º, 9º, 11º,

13º e 14º dias, para os grupos que foram sacrificados 14 dias após a lesão; e finalmente, no 1º, 3º, 5º, 7º, 9º, 11º, 13º, 15º, 17º, 19º e 21º dias após a lesão, para os grupos que foram sacrificados 21 dias após a cirurgia. Os parâmetros utilizados foram "Max Contact Intensity", correspondente à pressão exercida pela pata na plataforma durante a marcha. A intensidade de ampliação variou de 0 a 255 pixels.

5.8 ANÁLISES ESTATÍSTICAS

Para as análises bioquímicas, os dados provenientes dos diferentes grupos experimentais foram analisados usando o Teste-*t* Student ($p < 0.05$), e para as análises de birrefringência e dicroísmo linear, foi utilizado o Teste de Mann-Whitney ($p < 0.05$). Para ambos os testes, foi utilizado o software GraphPad Prism® (Graph-Pad Software, La Jolla, CA, USA), version 3.0.

6. RESULTADOS

Os resultados do presente estudo foram organizados em cinco manuscritos apresentados a seguir, dos quais o primeiro consiste de uma revisão intitulada: "Biochemical and anisotropical properties of tendons". Para visualização geral do desenho experimental dos demais manuscritos, individualmente, observe os fluxogramas abaixo (Figuras 5, 6, 7 e 8).

6.1) Fluxogramas

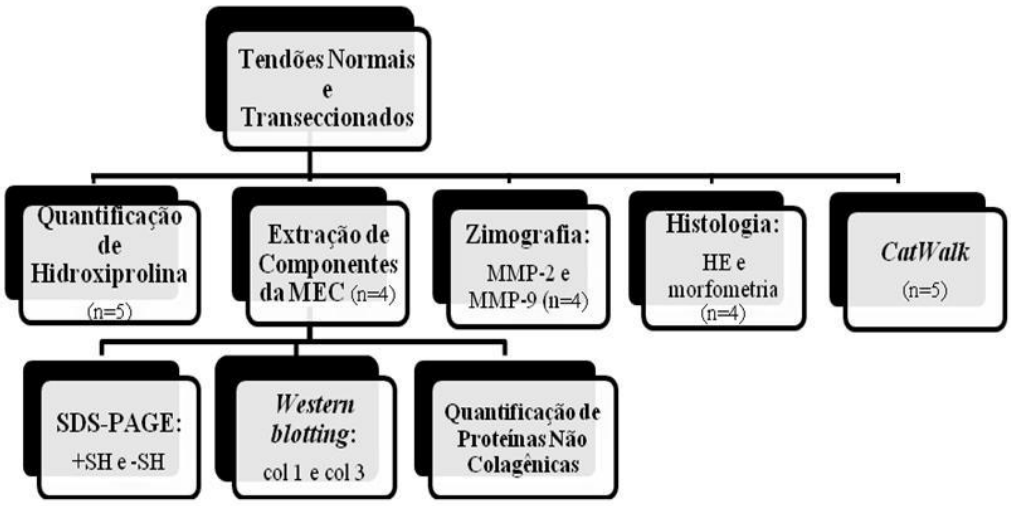

Figura 5: Fluxograma do desenho experimental utilizado no manuscrito 2, intitulado "*Arrabidaea chica* extract improves gait recovery and changes collagen content during healing of the Achilles tendon". Os tendões foram analisados após 7, 14 e 21 dias decorridos da transecção parcial. Os tendões transeccionados foram separados em dois grupos, tratados e não tratados com a *A. chica*.

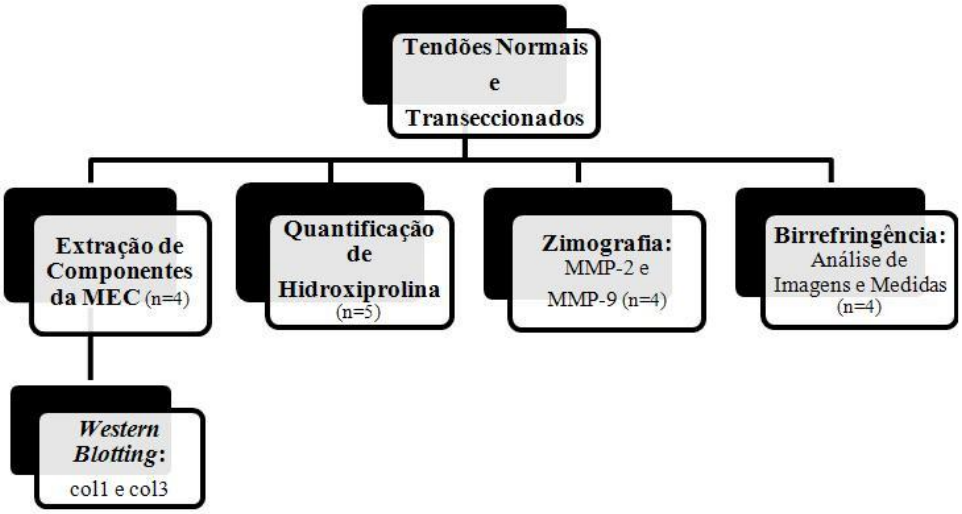

Figura 6: Fluxograma do desenho experimental utilizado no manuscrito 3, intitulado "Structural and biochemical alterations during the healing process of tendons treated with *Aloe vera*". Os tendões foram analisados após 7, 14 e 21 dias decorridos da transecção

parcial. Os tendões transeccionados foram separados em dois grupos, tratados e não tratados com a *A. vera*.

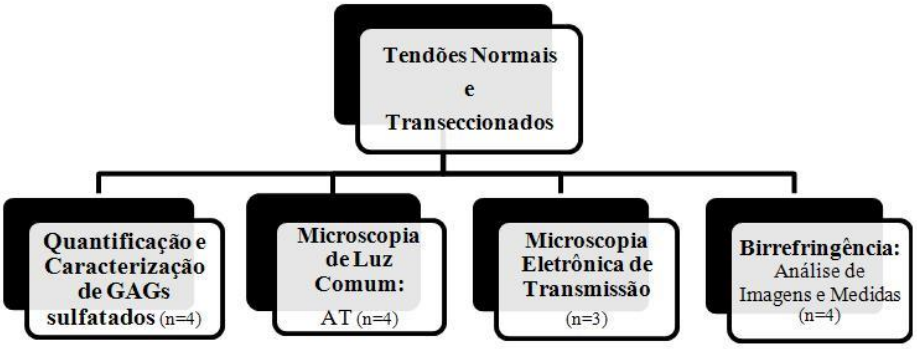

Figura 7: Fluxograma do desenho experimental utilizado no manuscrito 4, intitulado "Effect of the *Arrabidaea chica* extract on collagen fiber organization during healing of partially transected tendon". Os tendões foram analisados após 7, 14 e 21 dias decorridos da transecção parcial. Os tendões transeccionados foram separados em dois grupos, tratados e não tratados com a *A. chica*.

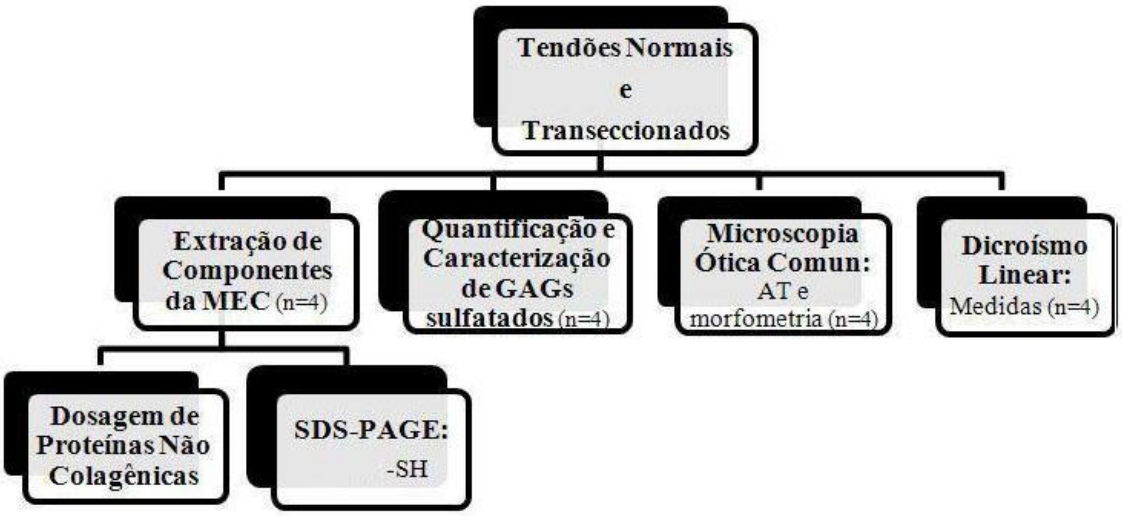

Figura 8: Fluxograma do desenho experimental utilizado no manuscrito 5, intitulado "Efeito da *Aloe vera* sobre proteínas e glicosaminoglicanos durante o processo de cicatrização em tendão de rato". Os tendões foram analisados após 7, 14 e 21 dias decorridos da transecção parcial. Os tendões transeccionados foram separados em dois grupos, tratados e não tratados com a *A. vera*.

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MANUSCRITO 1

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BIOCHEMICAL AND ANISOTROPICAL PROPERTIES OF TENDONS

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ABSTRACT

Tendons are formed by dense connective tissue composed of an abundant extracellular matrix (ECM) that is constituted mainly of collagen molecules, which are organized into fibrils, fibers, fiber bundles and fascicles helicoidally arranged along the largest axis of the tendon. The biomechanical properties of tendons are directly related to the organization of the collagen molecules that aggregate to become a super-twisted cord. In addition to collagen, the ECM of tendons is composed of non-fibrillar components, such as proteoglycans and non-collagenous glycoproteins. The capacity of tendons to resist mechanical stress is directly related to the structural organization of the ECM. Collagen is a biopolymer and presents optical anisotropies, such as birefringence and linear dichroism, that are important optical properties in the characterization of the supramolecular organization of the fibers. The objective of this study was to present a review of the composition and organization of the ECM of tendons and to highlight the importance of the anisotropic optical properties in the study of alterations in the ECM.

Key words: tendons, collagen, extracellular matrix, optical anisotropies, organization.

1. INTRODUCTION

Tendons are 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 (Birk et al., 1996). In contrast to the classical point of view that defined the ECM as an elastic inert tissue, several studies have shown that the connective tissue of the tendons is a dynamic structure that is capable of adapting to the different functional demands on the musculoskeletal system by altering the ECM's structural and biomechanical properties (Banes et al., 2009; D'Souza and Patel, 1999; Langberg et al., 1999).

Disorders and ruptures are common in the Achilles tendon although, it is one of the most resistant tendons to load in the human body (Lemm et al., 1992; DeMaio et al., 1995). During maximal eccentric plantar flexions, the Achilles tendon may experience peak stresses in excess of 70 MPa (Ker et al., 1988; Komi et al., 1992). In addition to sporting activities, the excess or absence of stress on the tendon induces alterations in its properties and permits the occurrence of lesions (Aquino et al., 2005) that may cause significant articular instability, which can lead to damage in other tissues and the development of a degenerative articular disease (Woo et al., 1999). In general, lesions of the tendon lead to an accentuated morbidity that may have an impact on work, routines and leisure activities (James et al., 2008).

Other factors such as age (Caroll et al., 2008; Dudhia et al., 2007; Esquisatto et al., 2003; Esquisatto et al., 2007; Nakagaki et al., 2007; Nakagaki et al., 2010), diabetes (Bedi et al., 2010; de Oliveira et al., 2010; de Oliveira et al., 2011; Fox et al., 2011) and hormones (Cook et al., 2007; Doessing et al., 2010; Soo et al., 2011) determine the physiological conditions that affect the connective tissues and modify the metabolism of collagen and the functional properties of its fibers (Comper, 1996). With aging and disuse, the collagen bundles in the tendons suffer alterations due to an increase in the amount of inter/intramolecular cross-linking, resulting in an increase in crystallinity and orderly aggregation of collagen fibers (Vidal and Carvalho, 1990). In addition, there are decreases in proteoglycans (PGs) and water content (Sell and Monier, 1995; Tuite et al., 1997). In general, the density and total content of collagen increase with age (Shadwick, 1990),

mainly due to a decrease in collagen turnover and a reduction in the synthesis of collagenolytic enzymes (O'Brien, 1997).

The objective of this study was present a simple and clear review of the composition and organization of the ECM of tendons and to highlight the importance of the anisotropic optical properties in the study of alterations in the ECM.

2. GROSS ANATOMY AND HISTOLOGY OF TENDONS

The main function of tendons is to transmit the contraction force produced by the muscle to the bone, thereby promoting movement and articular stability. Healthy tendons without the fascia exhibit a shiny white color and a fibroelastic texture, which shows great resistance to tension and compression forces. The structure connecting the tendon to the muscle is known as a myotendinous junction (MTJ), and the one connecting it to the bone is known as an osteotendinous junction (OTJ) (Kannus, 2000). The transmission of force of the myotendinous unit depends on the structural integrity between individual muscle fibers and the ECM (Kannus, 2000) as well as the fibrillar arrangement of the tendon that provides resistance and the absorption of energy (Alexander, 1991; Alexander and Bennet-Clark, 1997).

Tendons are surrounded by a loose connective tissue known as the paratenon, which allows for free movement of the tendon against proximal tissues and supports blood vessels that vascularize the innermost layers of the tissue, including the endotenon and epitenon. The epitenon is a fine sheath of connective tissue located underneath the paratenon (Józsa et al., 1991), which links several fascicles that comprise the tissue and contribute to the formation of its supramolecular structure. Its inner surface is continuous with the endotenon that coats and links each tertiary bundle, secondary bundle (or fascicle) and primary bundle (or subfascicle) of collagen fibers, forming large structural units of collagen fiber bundles, named fascicles. Each subfascicle is comprised of a group of collagen fibers, with each collagen fiber comprised of a collagen fibril bundle (Józsa and Kannus, 1997; Kannus, 2000). Collagen fibrils can be considered the basic unit of force transmission of the tendon (Magnusson, 2003). There are vessels, arterioles and capillaries present in the endotenon that do not penetrate the collagen bundles. These structures maintain the fibroblasts of the

tendon, which are responsible for the intense synthesis of macromolecules for the ECM (O'Brien, 1997).

The etiological factors leading to tendinous lesions can include degenerative tendinopathies (Tallon et al., 2001), steroid injection (Maffulli, 1999), and lacerations or ruptures (Woo et al., 2000) that are mainly due to sudden mechanical overcharges. Lesions in the human Achilles tendon often affect an area of the tissue located around 2-6 cm away from the insertion zone in the tendon that is hypovascular due to the maximal rotation of collagen fibers that impair the blood flow in this area (Lesic and Bumbasirevic, 2004). Currently, many studies performed have been seeking more effective treatments for injuries to the tendons, such as the employment of an allograft of amniotic epithelial cells (Muttini et al., 2010), platelet-rich plasma that contains growth factors (Bosch et al., 2010), metrenperone (Oryan et al., 2010) or mesenchymal stem cells (Schnabel et al., 2009). The aim of these new treatments was to reduce the recurrence of injuries through a remodeling of the extracellular matrix at the site of a tendon injury.

3. STRUCTURAL AND BIOCHEMICAL FEATURES

The ECM of tendons is composed of collagen and a few elastin fibrils, PGs and non-collagenous glycoproteins. Tendons are composed of 55-70% water and a substantial part of that water is associated with PGs in the ECM (Józsa and Kannus, 1997; Vogel and Meyers, 1999). The dry weight of the tendon is 60-85% collagenic proteins (Kjaer, 2004). The collagen present in the tendon is predominantly type I, and it is organized into fibrils, fibers, bundles of fibers and fascicles (Kjaer, 2004), which are organized in a helical arrangement along the largest axis of the tendon (Vidal, 1986; Liu et al., 1995; O'Brien, 1997; Vidal, 2003). **A** molecule of type I collagen is a triple helical protein 270 nm in length that assembles outside the cell, forming supra molecular structures 300 nm in diameter and several centimeters in length **(**Kadler et al., 1996). These fibers are responsible for the biomechanical properties of the tendons, such as flexibility, resistance and even some elasticity (Vogel and Koob, 1989; Józsa et al., 1991; Milz et al., 1998). Collagen types II (Benjamin et al., 1991; Rufai et al., 1992), III, IV (Hanson and Bentley, 1983; Ahtikoski et al., 2003), V and VI (Carvalho et al., 2006; Milz et al., 2002; Felisbino

and Carvalho, 1999; Józsa and Kannus, 1997; Tsuzaki et al., 1993) are also present in the tendon (For percentages see TABLE 1).

The biomechanical properties of the tendons are due to the arrangement and organization of the collagen molecules. These molecules are formed by 3 polypeptidic chains that are helically arranged with a characteristic sequence of amino acids that is essential for the stabilization of a triple helix. The main characteristic of a fibrillar collagen molecule is the long and stable structure of its helical triple helix, in which three polypeptidic chains, named α, are twisted, forming a type of super-coiled cord. Each chain contains repetitions of a characteristic sequence of amino acids, consisting of Gly-X-Y, where X and Y can be any amino acid. However, approximately one-third of the X positions are proline, and a similar number of the Y positions are hydroxyproline, which results from post-translational changes to proline. The presence of proline, hydroxyproline and glycine is essential for the stabilization of the triple helix of collagen (Piez and Reddi, 1984).

Glycine is an amino acid with the smallest radical (R), consisting only of a hydrogen. Glycine is regularly spaced every two amino acids, occupying the innermost position along the polypeptidic chain, which enables the three chains to come into contact and form the triple helix of collagen. Residues of lysine are also present in the collagen molecule. The hydroxylation of these residues by Lysyl hydroxylase (Hay, 1991) and posterior formation of the aldehyde group by Lysyl oxidase (Piez and Reddi, 1984) enable the formation of intramolecular crosslinks and the subsequent intermolecular crosslinks, increasing the capacity of collagen fibers to resist biomechanical forces. Additionally, some hydroxylysines are modified by glycosylation (Gelse, 2003). The number of crosslinks varies among the tendons depending on the biomechanical load of each tendon, as demonstrated by infrared spectroscopy analysis (Vidal and Mello, 2011).

In addition to the collagen molecules, which represent the ECM fibrillar components, there are also a small number of elastic fibers (Eyre et al., 1984; Józsa and Kannus, 1997) arranged along some collagen fibers that contribute to an initial distension of the tendons when submitted to unidirectional charges during routine or sport activities (Aquino et al., 2005). The low proportion of elastin allows for the transmission of the tension force to the bone and prevents excessive stretching of the tendon during muscle

contraction, enabling articular movement (Hall and Brody, 2001). In the elastic tendon of the chicken wing, the amount of elastic fibers is higher than in other tendons due to its biomechanical functions (Carvalho et al., 2000). This elastic tendon is responsible for maintaining the wing in the resting position (Pimentel and Carvalho, 2003) and presents collagen fibers associated with the elastic fibers as well as a variable amount of the sulfated glycosaminoglycans (GAGs), depending on the anatomical region examined in this tendon (Carvalho et al., 2000). The elastic bovine nuchal ligament also contains a large amount of elastin that can reach up 83% of its dry weight at birth and variable amounts of GAGs, such as HS and DS. When compared to the tendons, this elastic ligament contains less collagen content (Reinbotha et al., 2000).

Non-fibrillar components, such as the PGs (Vogel and Heinegård, 1985) and non-collagenous glycoproteins, also comprise elements of the tendons. The PGs consist of a central protein skeleton and at least one chain of GAGs covalently linked to the protein core (Evanko and Vogel, 1990). The PGs are molecules with a strong negative charge due to the presence of sulfated GAGs; therefore, they are highly hydrated and enveloped by a thick layer of solvation water that surrounds the molecule, causing the tissue to have a slimy characteristic (Quinn and Morel, 1997). GAGs of the proteoglycans may also associate with fiber proteins of the matrix, such as collagen, producing a supramolecular structure (Vidal and Mello, 1984).

Studies have shown that among the PGs comprising the tendon, there is the presence of particularly small PGs that are rich in leucine, of which fibromodulin and decorin (up to 1%) are the most important (Derwin et al., 2001). A non-collagenous extracellular matrix protein named cartilage oligomeric matrix protein (COMP) can also be found (Smith-Mungo and Kagan, 1997; Muller et al., 1998). COMP is a member of the thrombospondin family initially found in cartilage. This protein has been extensively studied because mutations in its gene cause some diseases, including skeletal dysplasias, pseudoachondroplasia and multiple epiphyseal dysplasia (Posey and Hecht, 2008). In tendons, COMP is located mainly at the gap region of the collagen fibrils (Fredrick et al., 2007), and the presence of fragments of this protein in the serum is related to subclinical stages of tendon injury (Smith and Heinegård, 2000). With respect to decorin, these molecules interact in an orderly manner with the collagen fibrils (Scott, 1998), acting in the

fibrillogenesis of collagen (Scott, 1995; Scott et al., 1997) and probably regulating the growth in diameter of these fibrils (Iozzo, 1996; Douglas et al., 2006). Regarding the interaction of PG molecules with collagen molecules, previous studies have already reported that the GAGs present in the PGs are arranged in relation to the collagen bundles in the tendon (Vidal and Mello, 1984) and are probably interacting electrostatically with cationic groups of the collagen molecules.

Tendons have collagen fibers that present an undulated pattern known as crimp (Vidal and Mello, 1984; Gathercole, 1991; Vidal, 1995; Vidal, 2003) that can also be detected in other tissues. The crimp is a characteristic of structures consisting of fibrillary collagens, especially type I, that are present in connective tissues submitted to tension loads; the basic undulated pattern of the fiber is altered in such a way as to adapt to the mechanical situation in progress (Gathercole, 1991). Hence, there are variations in the organization of the crimp in different regions of the same tendon (Vidal, 1995) that can be revealed by polarized light microscopy (Feitosa et al., 2002 b). The stress and deformation to which the collagen bundles are submitted require large areas of crimp, which can benefit the collagen bundles in adapting to tension (Gathercole, 1991) and shock absorption during the tendon stretching (Franchi et al., 2007).

Amongst the collagen bundles, there are fibrocytes with flat nuclei and fibroblasts with enlarged nuclei that are responsible for the intense synthesis of macromolecules of the ECM (O'Brien, 1997). These cells form approximately 90-95% of the cellular elements of the tendon, with the other 5-10% including the chondrocytes present in the compression areas and bone insertion and the vascular cells of the endotenon and paratenon (Kannus, 2000). The structural analysis has shown that fibroblasts are characterized by the presence of a well-developed endoplasmic reticulum, Golgi complex, free ribosomes and numerous peripheral vesicles (Redaelli et al., 2003) due to the high rate of synthesis of the ECM components (FIG. 1). The cellularity in tendons and the thickness of the collagen fiber bundles vary according to age, with the tendons of adult animals having more compact collagen bundles with fewer cells, while younger animals have higher cellularity and thinner fiber bundles (Vidal and Carvalho, 1990).

4. BIOMECHANICAL BEHAVIOR

The ability of tendons to resist several types of mechanical stress is directly related to the structural organization of their ECM (Esquisatto et al., 2003). The biomechanical properties of the tendons are determined by the arrangement of collagen molecules and by their association with other elements of the ECM (Benevides et al., 2004).

The compression forces and friction added to the normal tension exerted by the muscles lead to local variations in the morphology and composition of the tendons (Carvalho and Vidal, 1994; Covizi et al., 2001). Hence, the ECM adapts to different biomechanical forces and presents local differences in terms of composition and organization along the same tendon. In areas where tension is exerted in only one direction, the fibers present an orderly and unidirectional disposition (O'Brien, 1997) (FIG. 2). Several studies in our laboratory have described regional variations present along the tendons (Covizi et al., 2001; Feitosa et al., 2002 a; Esquisatto et al., 2003; Benevides et al., 2004; Feitosa et al., 2005; Feitosa et al., 2006; Esquisatto et al., 2007; Biancalana et al., 2010) in response to the different biomechanical demands imposed on the musculoskeletal system (Aro et al., 2008; Almeida et al., 2010; Nakagaki et al., 2010; Fontana et al., 2010).

Tendons that surround any articulation or run along any bone tuberosity are subjected to tension forces and especially to compression forces, causing the accumulation of PGs of a high molecular weight. A structure similar to fibrocartilage is formed in this region as an adaptative response that protects the tendons from potentially harmful pressures (Benjamim and Ralphs, 2000). These PGs retain water and offer resistance to compression forces, based upon observations in swine (Feitosa et al., 2002 a), rats (Covizi et al., 2001) and chickens (Benevides et al., 2004). In the case of the Achilles tendon, there are two fibrocartilaginous areas in its distal portion: one proximal to the bone insertion, and another due to the compression exerted by the heel bone over the tendon (FIG. 2). The molecular ordering of the PGs, especially in fibrocartilaginous regions of the tendon, contributes to the biomechanical properties of the tendon, as seen in articular cartilage (Vidal and Vilarta, 1988).

Bone compression also stimulates the production of type II collagen (Rees et al., 2000) particularly present in these fibrocartilaginous areas (Vogel and Heinegård, 1985). Elastic fibers are present along some collagen fibers in tension areas, while in compression

areas, the elastic and collagen fibers are distributed in various directions and immersed in a large amount of proteoglycans (Feitosa et al., 2002 a).

The mechanical charge leads to a physiological adaption of the tissue (Covizi et al., 2001) that responds to stress by altering its structural organization in a way to meet the new mechanical demands (Chiquet, 1999). This alteration and the maintenance of the tissue structure partially rely on mechanical stimulation (Covizi et al., 2001). Mechanical stimulation is important for the ECM homeostasis in connective tissues because it affects the expression of specific proteins of the ECM. Therefore, there must be a feedback mechanism by which cells respond to signals activated by mechanical stress through altering the expression of proteins and, consequently, remodeling the ECM to adapt to biomechanical needs. Nevertheless, the amount and composition of the extracellular matrix are controlled not only by the type and magnitude of the mechanical stress to which the tissue is submitted but also by endogenous cellular programs and growth factors (Chiquet, 1999).

The ECM of the tendon can be responsible for activities of mechanotransduction, which is an important mechanism by which mechanical stress acts on the cell and starts an intracellular signaling program that promotes growth and cellular survival (Ried et al., 1994; Frisch et al., 1996; Rouslahti, 1997; Tibbles and Woodgett, 1999; Ramirez and Rifkin, 2003). It controls the morphology and architecture of various cellular types (Chicurel et al., 1998; Tibbles and Woodgett, 1999) and influences metabolic responses (Ihlemann et al., 1999).

The feedback mechanism of the cell-ECM interaction can be explained based on the hypothesis that the molecular arrangement in the ECM is the origin of signals for the cells and the basis for special properties of different suprastructural organizations (Vidal, 1994). Hence, the collagen fibers responsible for the biomechanical properties of structures such as the tendons, ligaments, cartilage, bones and dermis, all subject to tension-distension, would work as transductors by distributing electrical signals in the cellular surface that could alter the synthesis activity of the cell. The cell would play a central role in homeostatic control of the morphology and physiology of the ECM when detecting physical or chemical alterations that occur in the extracellular environment. Depending on

these alterations, the cells could synthesize more or less of a certain component of the matrix to meet the functional demands of the tissue (Vidal, 1994) (FIG. 3).

The various types of tendon cells respond differently to mechanical changes (Hamill and Martinac, 2001). It is clear that the mechanical stimuli in the ECM of the tendon and in the skeletal muscle start an adaptation process that will make the tissue more resistant to injury and thus assure efficiency in the transmission of force by muscular contraction (Kjaer, 2004).

The interaction between the ECM and the cells induces the activation of intracellular signaling and the rearrangement of the cytoskeleton (Banes et al., 1999; Carragher et al., 1999; Chiquet, 1999; Bloch et al., 2002). The intimate molecular basis of such mechanisms is not known (Chiquet, 1999); however, the molecular order and chirality in tendons must be considered. The PGs, with their glycosaminoglycan lateral chains, can bind to growth factors and then present those factors to their respective receptors. Furthermore, the ECM can also release growth factors under mechanical stimulation (Kjaer, 2004).

Integrins seem to be important for mechanotransduction because they are the main structural molecules of the bonding complexes in the cellular membrane that link the ECM to the cytoskeleton (Burridge and Chrzanowska-Wodnicka, 1996; Chiquet et al., 1996). These molecules establish a physical continuity to which forces may be transmitted from the outside to inside of the cell and vice-versa (Goldschmidt et al., 2001). Integrins are believed to work as tension deformation sensors in the cellular surface (Ingber et al., 1994). Evidence shows that such molecules are able of converting mechanical signals into adaptative responses in the cell (Shyy and Chien, 1997; Carson and Wei, 2000).

Several studies have shown that the level of mechanical charge may also control the expression of many other components of the ECM. For example, the type XII collagen and tenascin-C present in tendons and other connective tissues such as ligaments have their expression and synthesis increased when fibroblasts are submitted to some kind of mechanical stretch in vitro. In contrast, the synthesis of those molecules is suppressed in cells that are left in a relaxation state (Chiquet, 1999; Chiquet et al., 2003).

5. OPTICAL ANISOTROPY: BIREFRINGENCE AND LINEAR DICHROISM IN THE STUDY OF THE ORGANIZATION OF THE ECM OF TENDONS.

Alterations in the organization of macromolecules of the tendon's ECM due to different mechanical stimulations can be detected by polarization microscopy. This type of optical microscopy enables the exact statistical determination of the molecular order and variations in molecular aggregation states in the tissue through the study of optical anisotropies, such as birefringence and linear dichroism. Many studies in the literature and from our group have shown the importance of optical anisotropy in the characterization of the supramolecular organization of collagen fibers not only in tendons (Vilarta and Vidal, 1989; Feitosa et al., 2002 b; Vidal, 2003; Korol et al., 2007; Aro et al., 2008; van Turnhout et al., 2009; Vidal and Melo, 2010) but also in chordae tendineae (Vidal and Melo, 2009; Jaronski and Kasprzak, 2003; Knighton and Huang, 2002), pericardium (Braga-Vilela et al., 2008), corneal stroma (Kogure et al., 2008; Aldrovani et al., 2007), skin repair (Silva et al., 2006) and cartilage (Guerra et al., 2010; Julkunen et al., 2007; Rieppo et al., 2007; Hughes et al., 2005; Alhadlaq et al., 2004).

5.1. Birefringence (B)

In addition to the study of biological structures, works relating structure to mechanical properties in polymers are gaining importance, especially in textile threads. In this field, birefringence and dichroism measurements have been prominent, together with X-ray diffraction (Vasanthan, 2005). Regarding fibers and/or collagen bundles, the systematization of studies performed by Vidal (Vidal, 1980, 1986) and dichroism measurements lead to the systematic employment of a form for birefringence (B_f) in studies of physiological variances and alterations of collagen bundles in different conditions (Whittaker et al., 1987; Vidal, 2010).

Birefringence is anisotropy allowing the difference in the refraction indexes of the object, which means propagation of light at a different speed and directions in the material birefringent. The birefringence of the collagen bundles is expressed by the difference of the two refraction indexes, according to the equation $B = n_e - n_o$, where n_e is the refraction index in the propagation direction of the extraordinary ray (in the case of collagen fibers, it is the direction parallel to the fiber axis) and n_o is the refraction index in the direction of the ordinary ray, whose direction of propagation is perpendicular to the axis of the fiber and obeys the laws of refraction (n_o = seni/senr) (Vidal, 1980, 1986, 2010).

The information regarding the birefringence of a body is acquired by measuring optical retardation: OR = $(n_e - n_o).e$, where **e** = thickness of the material (Vidal, 1986). The brightness visible in the birefringence corresponds to the OR, which varies according to the thickness of the histological sections and concentration (partial volume) of the material (FIG. 4).

The physical principles of form birefringence (B_f) are currently being applied from microgear construction to silica film production for photonic effects (Neale et al., 2005; Petrov et al., 2006). B_f depends on the geometry and dimensions of molecules, i.e., these must be compatible with the wavelength (λ) used in the analysis and thus will be nanometric. It is also necessary to have the structure's components arranged in an orderly manner.

To express the birefringence of form of birefringent bodies, Wiener (1912) proposed the following equation that is expressed by:

$$B_f = n_e^2 - n_o^2 = f_1 \,.\, f_2 \,(n_1^2 - n_2^2) \,/\, [(f_1 + 1)\, n_2^2 + f_2 \,.\, n_1^2]$$

where f_1 and f_2 are relative partial volumes of the components with refraction indexes n_1 and n_2, respectively, such that $(f_1 + f_2) = 1$; and n_1^2 and n_2^2 correspond to the dielectric constant for a given frequency, ($n_1 = \sqrt{\epsilon_1}$ and $n_2 = \sqrt{\epsilon_2}$).

The above formula is applicable to simple systems, i.e., for bodies consisting of only two components. In the case of the collagen bundles, for which the Weiner equation is not applicable (Cassim and Tobias, 1968), the approach must be based upon Vidal (1980, 1986) and consider the partial volume (concentration), aggregation state and orientation of their components. Because the collagen fibers have anisotropy of dielectric constants (given by permittivity - **ε**), it is important to underline that permittivity may be related to the refraction index as follows: $\varepsilon = n^2$, and thus, $B_f = \varepsilon_e = \varepsilon_o$. It is possible to have a quantitative relationship between the birefringence of collagen and its amount in the tissues by using the equation:

$$OR = S \,.\, e^2 \,/\, 2\, \varepsilon_o \,.\, m \,.\, \omega_0^2 = S \,.\, 2{,}510^{-15}$$

where S = number of peptidic ligations per cm^2; *e* and *m* are the charge and electron mass respectively; ε_0 is the permittivity of space; and ω_0^2 = frequency of resonance of this electron.

The images of birefringence shown below (FIG. 5, 6) illustrate molecular order and the collagen bundles morphologies and three-dimensionality because the shine observed depends on the thickness of the section and the relative position of the fibers.

5.2. Linear Dichroism (LD)

In the last few years, several studies regarding tendon repair (Mello and Vidal, 2003), aromatic molecule linking of DNA with carbon nanotubes (Rajendra et al., 2004), determination of the subunit directions of particular proteins (Bulheller et al., 2007), cerebral pathologies (Jin et al., 2003), glaucoma (Naoun et al., 2005), retina (Benoit et al., 2001), cornea of diabetic rats (Aldrovani et al., 2007) and others have used LD as a tool to analyze alterations in the organization of molecules in several experimental situations.

Linear dichroism (LD) is the selective absorption of polarized light by chromophoric groups present in certain dye molecules, when disposed parallel or perpendicular to the plane of polarized light (TABLE 2). When the polarized light interacts with the chromophoric group of the molecule, whose electrons are vibrating in a plane that coincides with the plane of the electric vector of the polarized light, maximum absorption of the polarized light occurs (Vidal, 1987).

Biological materials usually do not have chromophoric groups that allow for the detection of LD in a visible spectrum, making it necessary to use dichroic stains that chemically link to the biological material and cause an extrinsic phenomenon. Therefore, LD is an extremely accurate way to determine the molecular order because it reveals the vibrating orientation of resonant electrons in the chromophoric groups (Vidal, 1987).

According to Vidal and Mello (2005), collagen can be considered a biopolymer for the study of changes in its optical properties following the orderly linking of sulfonate acid from stains such as Ponceau SS (FIG. 7). Furthermore, according to these authors, this dye presents a well-defined geometry and topographical distribution of reactive groups ($-SO_3^-$) in naphthalene, thereby creating two anionic extremities in the molecule in addition to the

existence of two chromophoric groups (-N=N-) that align better than the large chromophoric groups present in extensive molecules of other dyes (FIG. 8), especially when linked to substrates such as collagen (Vidal et al., 1982). In conclusion, the molecules of Ponceau SS also present excellent planarity that may make them thermodynamically better in the reaction and in linking to orderly substrates such as collagen (Vidal and Mello, 2005).

5.3. Why is birefringence and/or linear dichroism used in studies on collagen?

These anisotropic properties must be used for the following reasons: 1) they provide accurate morphological information and 2) in the bidimensional surface outlined by the polarization plane of the polarizer, the observed morphology provides information about the position of fibers and their distribution and relationships in 3-D, and the detectable shine indicates the association, in degrees, of the largest axis of each fiber to the polarization planes of the polarizer. Thus, the brightness and relative distribution of fibers among each other provide three-dimensional information regarding the thickness of the material (Vidal, 2010; Vidal and Mello, 2010). Tendons, which are collagen-rich structures, are advantageous for studies under polarized light in both fresh and fixed material at different thicknesses and under different experimental conditions due to the highly dense aggregated collagen bundles (Vidal, 2010; Vidal and Mello, 2010).

6. CONCLUSION AND PERSPECTIVES

Along with other resources to investigate the effects of age, diabetes, hormones and injuries on tendons, polarization microscopy with birefringence and linear dichroism techniques is an important tool to analyze the organization of the extracellular matrix. Due to the increase in lesions in tendons mainly during sportive activities, several treatments have been used, such as cell therapy and tissue engineering techniques that show great potential for tendon repair. The application of plant extracts can also be promising for the treatment of injuries due to lesser collateral effects compared with conventional medicines. Other treatments like laser therapy and the application of growth factors have also been studied for restoring the functionality of the tendon. Aiming for a faster and more effective rehabilitation of the tendons, stretching programs combined with different types of

exercises have been frequently used. Despite the practical relevance of each kind of treatment, it is important to know the effect of each treatment on the composition and organization of the extracellular matrix.

7. ACKNOWLEDGMENTS

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FIGURES AND TABLES

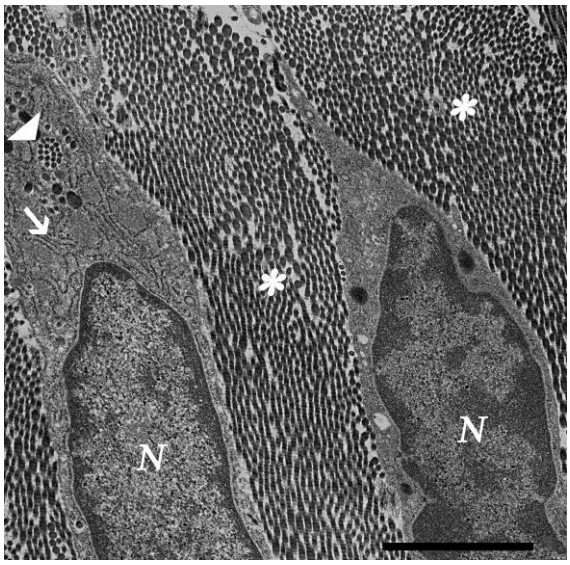

FIG. 1. Electron microscopy transmission micrographs of the tension region of rat Achilles tendon. Observe elongated fibroblasts with well-developed endoplasmic reticulum (→) and abundant sectioned collagen fibers (*). Note the presence of nucleus (N) with predominantly non-condensed chromatin and apparently secretory vesicle (▶) in the cytoplasm suggesting protein synthesis. Bar = 1 µm.

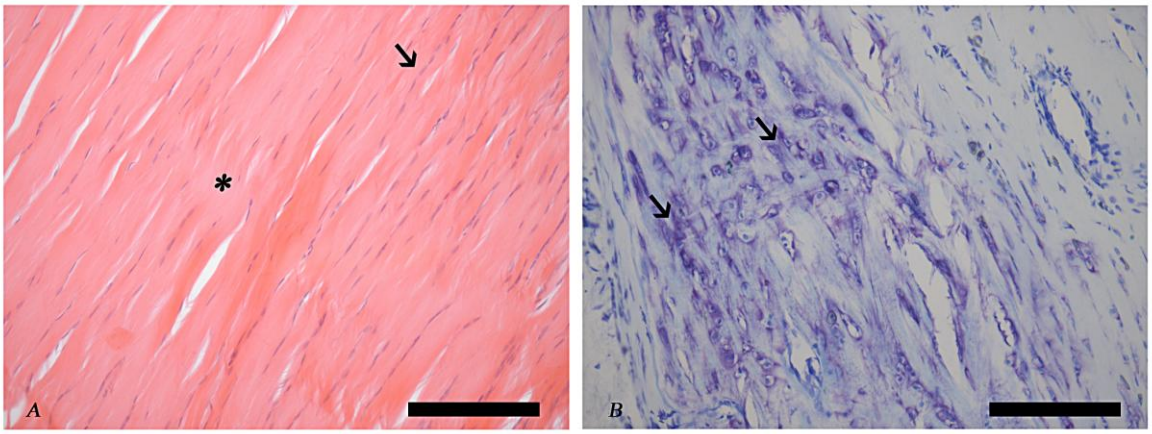

FIG. 2. Longitudinal sections of the Achilles tendon of rat. (A) Observe a typical tension region represented by numerous elongated fibroblasts willing in an abundant ECM stained with haematoxylin and eosin. This region receives exclusively tension forces. (B) Sections of compression region of the tendon stained with toluidine blue showing the metachromasy in the territorial matrix (→) and cells similar to chondrocytes, indicating high PGs amount. Bars = 80 μm.

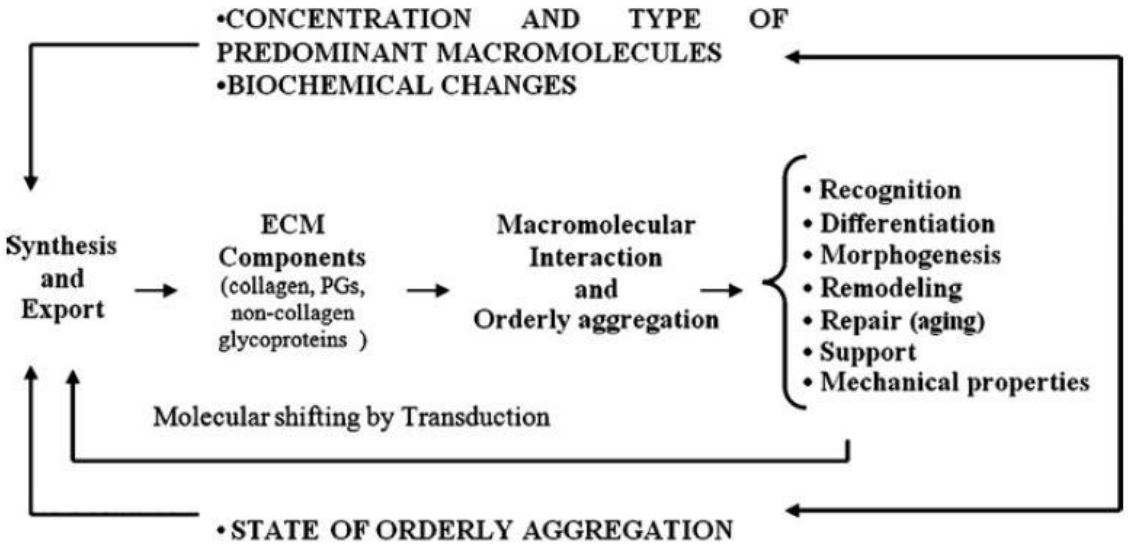

FIG. 3. Scheme representing the feedback system based on the extracellular matrix organization. The production of signals in the ECM depends on the levels of macromolecular organization and concentration of its components. These factors may influence in several metabolic responses of the tissue that result in: recognition, differentiation, morphogenesis, remodeling, repair, support and mechanical properties. These metabolic responses may also alter the distribution of signals to the cellular surface, through the transduction mechanism, interfering in the synthesis and export activities (modified from Vidal, 1994).

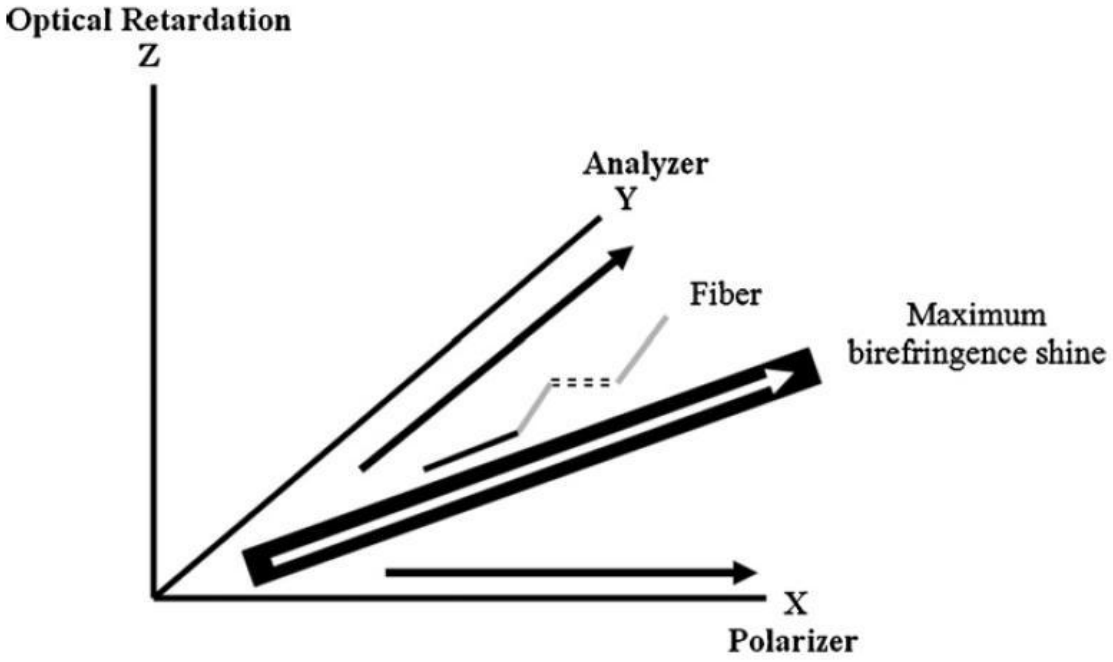

FIG. 4. Directions of the polarization planes of the polarizer (axis x), analyzer (axis y, forming a 90° angle with the axis x), and direction of propagation of the emerging light (axis z) with optical retardation (shine) caused by the collagen bundles. The differences in optical retardation (OR) along the collagen bundles supply 3-D information of the birefringence and relative direction of the fibers. Imagining any fiber, very homogeneous in which the molecules, e.g. in rods, are oriented parallel to the axis of the fiber, showing in these conditions the maximum shine in birefringence due to the position of one of the propagation axis being at 45° of the two polarizers (between the analyzer and the polarizer). The gray color in the schema represents a birefringent object with another orientation (e.g., 30°) to crossed polarizers. The dashed line represents an object in which there is a different inner fiber orientation with respect to the polarizers. The arrows parallel to the polarizers (⟶) show the extinction of birefringence indicating that the object is positioned parallel to one of the polarizers (modified from Vidal, 2010).

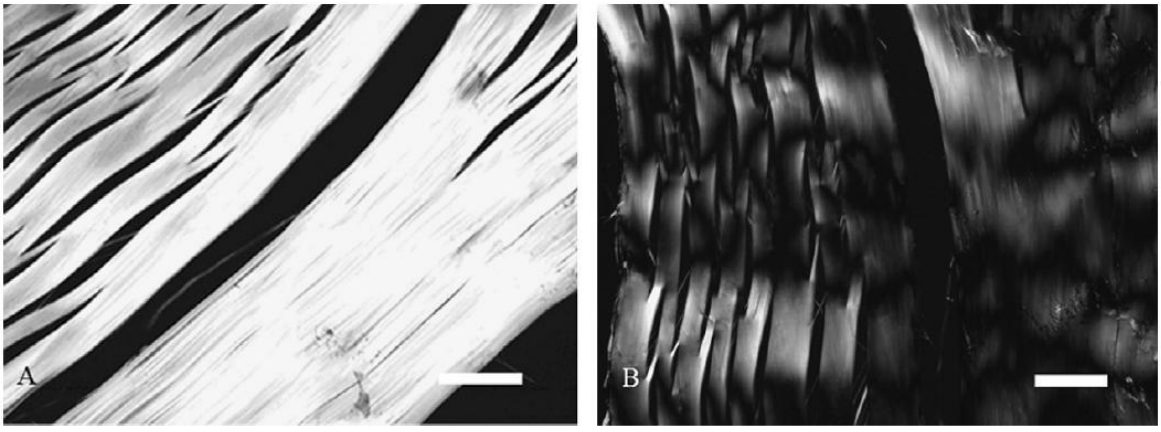

FIG. 5. Images of birefringence in sections of bovine tendons. A) Strong birefringence of the collagen fibers when the largest axis of the tendon is set at 45° of the polarizers is observed. B) The largest axis of the tendon was set parallel to the polarization plane. The dark areas indicate an area depleted of light where the fibers are placed parallel in relation to one of the polarization planes. Bar = 50 μm.

FIG. 6. Image of birefringence of the tension area typical of a bovine tendon, with its largest axis placed parallel to the polarizer. We can observe details of the organization of large areas of crimp, represented by the shine of birefringence in some segments of the tissue, and by dark areas due to the extinction of light where the fibers are placed parallel to one of the polarization planes, due to the helical arrangement of the collagen bundles along the tendon. Large dimensions of crimp areas enable collagen bundles to withstand the distension of the tendon caused by tension forces acting in this area. The crimp varies according to the animal, type and function of the tendon. Bar = 25 μm.

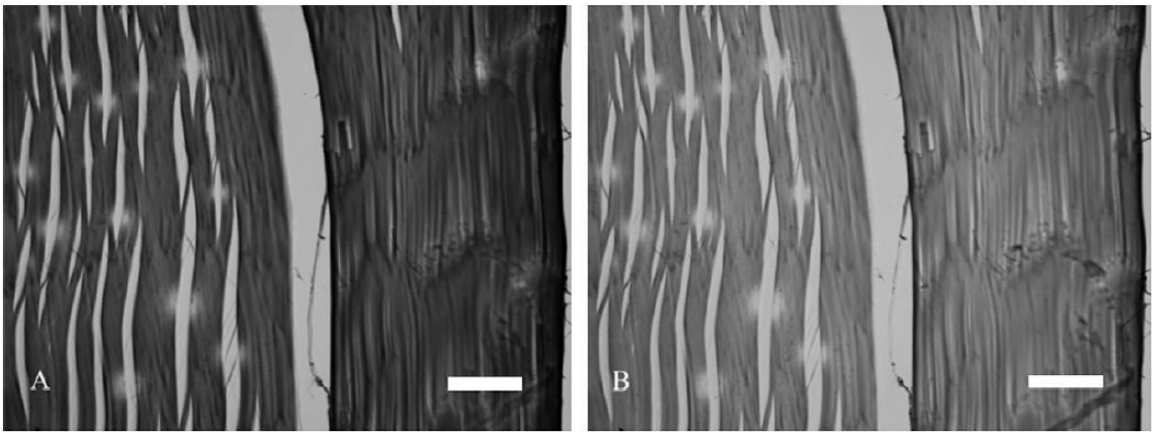

FIG. 7. Images of linear dichroism of bovine tendon sections stained with Ponceau SS. A) Stronger light absorption is observed when the largest axis of the tendon is placed parallel to the plane of polarized light (A), and weaker light absorption (B) when placed perpendicular to the polarization plane. Bar = 50 μm.

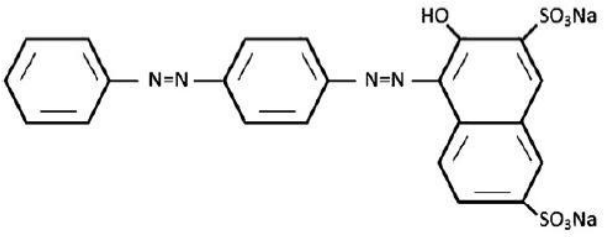

FIG. 8. Structural formula of the Ponceau SS molecule. The interaction of the stain with collagen occurs among its groups $–SO_3^-$ and the radicals $–NH_3^+$ present in the collagen molecule.

Percentage in tendons	Components
60-85%	-Total collagen (considering dry weight) (Kjaer, 2004).
~ 60%	-Collagen type I (Kjaer, 2004).
-	-Collagen type II (Benjamin et al., 1991; Rufai et al., 1992).
0-10%	-Collagen type III (Hanson and Bentley, 1983).
~ 2%	-Collagen type IV (Ahtikoski et al., 2003).
-	-Collagen types V and VI (Carvalho et al., 2006; Milz et al., 2002; Felisbino and carvalho, 1999; Józsa and Kannus, 1997; Tsuzaki et al., 1993).
up to 1%	-Proteoglycans (Vogel and Heinegård, 1985).
~2%	-Elastic fibers (considering dry weight) (Eyre et al., 1984; Józsa and Kannus, 1997).
up to 1%	-COMP (Smith-Mungo and Kagan, 1997; Muller et al., 1998).

TABLE 1. Percentage of ECM components of tendons.

Dyes	Application
Sirius red	Atherosclerosis (Purushothaman et al., 2011) Fibrosis in renal biopsies (Farris et al., 2011) Anastomotic healing (Binneboesel et al., 2010) Peripheral nervous (Kaemmer et al., 2010) Vesical prolapse lesions (Borges et al., 2007) Tendon (Feitosa et a., 2005) Collagen (Vidal et al., 1982)
Toluidine blue	Tendon (Tomiosso et al., 2009; Aro et al., 2008; Feitosa et al., 2002 b) Patellar cartilage (Appleyard et al., 1999)
Xylidine ponceau	Collagen (Vidal, 1980; Pimentel et al., 1981)

TABLE 2. Applications of dyes such as Sirius red, Toluidine blue and Xylidine ponceau in the polarization microscopy for both linear dichroism and birefringence.

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MANUSCRITO 2

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Arrabidaea chica extract improves gait recovery and changes collagen content during healing of the Achilles tendon

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ABSTRACT

Introduction: Tendon lesions are still a serious clinical problem. The leaves of the Bignoniaceae *Arrabidaea chica* (Humb. & Bonpl.) B. Verlot. (syn. *Bignonia chica* (Bonpl.)) have been used in traditional medicine and described in the literature for its healing properties. However, no study has shown the effects of *A. chica* during tendon healing. The aim of this study was to investigate the healing properties of the *A. chica* leaves extract on tendons after partial transection.
Methods: A partial transection in the tension region of the Achilles tendon of rats was performed with subsequent posterior topical application of *A. chica* extract (2.13 g/mL in 0.85% saline solution) at the site of the injury. The animals (n = 154) were separated into 7 groups: N **-** rats with tendons without transection; S7, S14 and S21 - rats with tendons treated with topical applications of saline for 7 days and sacrificed on the 7th, 14th and 21st days after surgery, respectively; A7, A14 and A21 - rats with tendons treated with topical applications of the plant extract. The transected regions of the tendons were analyzed through biochemical, morphological and functional analyses. To evaluate the type and concentration of collagen, western blotting for collagen types I and III was performed, and the hydroxyproline concentration was determined. The participation of metalloproteinases (MMP)-2 and -9 during tendon remodeling was investigated through zymography. Gait recovery was analyzed using the catwalk system. The organization of the extracellular matrix and morphometry were detected in sections stained with hematoxylin-eosin.
Results: The application of *A. chica* extract in the region of tendon injury led to an increase in the amount of hydroxyproline (mg/g tissue) on the 7th (91,5 ± 18,9) and 21st (95,8 ± 11,9) days after the tendon lesion relative to the control groups treated with saline (S7: 75,2 ± 7,2; and S21: 71,9 ± 7,9). There were decreases in collagen types I and III (as determined by densitometry) in the groups treated with the plant extract 7 days after injury (type I: 103,9 ± 15,9; type III: 206,3 ± 8,1) compared to the saline-treated groups (type I: 165,2 ± 31,1; type III: 338,6 ± 48,8). The plant extract stimulated the synthesis of MMP-2 on the 21st day after the lesion and decreased the amount of latent and active isoforms of MMP-9 on the 14th day. Analysis by the catwalk system (max contact intensity) showed that the *A.*

chica extract improved the gait of rats on the 7th day of the healing process (186,3 pixels ± 12,4) when compared to the saline group (168,5 pixels ± 10.0).
Conclusions: The use of *A. chica* extract during the healing process of the tendon leads to an increase in collagen content and improved gait recovery. Further studies will be performed to analyze the effect of this plant extract on the organization of the collagen bundles of tendons after lesions and to study its probable anti-inflammatory effect.

Keywords: Achilles tendon, collagen, *Arrabidaea chica* extract, healing, gait.

Introduction

The Bignoniaceae *Arrabidaea chica* (Humb. & Bonpl.) B. Verlot. (syn. *Bignonia chica* (Bonpl.)), popularly known as carajiru, puca panga, chica or pariri, is a liana widely distributed in the Neotropics[1,2,3] and commonly used as a medicinal plant in the Amazonian region.[4] The leaves of this plant have been used in traditional medicine and described in the literature for its healing properties[5,6,7] and for treatment of inflammation, intestinal colic, sanguine diarrhea, leucorrhoea and anemia.[4,7,8] Formerly, the red pigment from the *A. chica* leaves was traditionally used for tattos and dyeing fibers by indigenous population of the Orinoco[9] and Amazon.[10] The red colour was related to the 3-desoxyanthocyanidin named carajurin.[4,11]

The crude *A. chica* leaves extract used in the present study was standardized according to Jorge et al. (2008)[7] and included the aglycones carajurin and carajurone. These compounds are components of the anthocyanins, which are phenolic water-soluble pigments that are released after acid hydrolysis.[12] Scientific interest in these pigments has recently increased due to the effective bioactivity of anthocyanins and their aglycones[12] as anti-inflammatories,[13] antioxidants[14] and vaso-dilators,[15] among other biological properties. The studies cited before have been carried out in order to employ the *A. chica* as a phytotherapeutic agent. However, the effect of this plant in tendon remodeling during healing has not been previously reported.

For many centuries, plant extracts have been employed to treat diseases, and their active compounds possess great pharmacological potential. Some plants such as *Hippophae rhamnoides,*[16] pineapple fruit parts,[17] safflower yellow[18] and bromelain and fresh pineapple juice,[19] have been used to improve the tendon healing. However, tendon lesions are still a

serious clinical issue because the site of injury becomes a region with a high incidence of recurrent rupture due to scar formation. This problem can affect both sedentary people and athletes. This kind of injury can be acute or chronic, producing considerable morbidity, and the disability that tendon lesions cause may last for several months despite appropriate management.[20] Tendon healing is a slow and complex process due to the high level of organization of the components present in its extracellular matrix (ECM). Tendons are resistant structures with the primary function of transmitting the forces that arise from the muscles to the bones, thereby promoting articular movement. The abundant ECM present in tendons is a kind of dense and organized connective tissue in which collagen is the most abundant protein, forming collagenic fibers. Proteoglycans (PGs), elastic fibers, non-collagenous proteins (NCPs), water and cells are also important constituents of this tissue.

All members of the collagen family have a characteristic right-handed triple helix composed of three α-chains[21,22] that can include three identical chains (homotrimers) or two or three different chains (heterotrimers). In tendons, type I collagen is predominant and provides tensile stiffness to the tissue. Type III collagen is widely distributed amongst collagen I bundles, being more flexible than the type I collagen molecule, which suggests that mixing collagen types can alter the elastic modulus of collagen fibrils.[23] Collagens type I and III are fibril-forming collagens with the ability to assemble into highly orientated supramolecular aggregates[24] in tendons; their arrangement has an important role during the wound healing process in this tissue.

Tendon healing occurs in three phases called the inflammatory, proliferative and remodeling phases, which are dynamic and overlapping. In the inflammatory phase, erythrocytes and inflammatory cells such as neutrophils migrate to the site of injury, as do tenocytes, which are responsible for type-III collagen synthesis.[25] Vasoactive and chemotactic factors are then released, inducing increased vascular permeability, angiogenesis, tenocyte proliferation, and the recruitment of more inflammatory cells.[26] In the proliferative phase, water content and glycosaminoglycan concentrations are high, and a peak of type-III collagen synthesis occurs and lasts for a few weeks.[25] The remodeling phase starts after approximately six weeks, with a decrease in cellularity and collagen and glycosaminoglycan synthesis.[27] The remodeling phase can be divided into the consolidation stage (6-10 weeks), with high tenocyte metabolism[28] and a high proportion of type-I

collagen,[29] and the maturation stage (after 10 weeks),[30] with gradual alteration of the tendon; the lesion changes from a fibrous tissue to scar-like tendon tissue over the course of one year.[28,31]

Anabolic mechanisms have an important role in the formation of the matrix during the tendon repair process. Some molecules that participate in the mediation of this process are collagen, laminin, hyaluronic acid and fibronectin, a protein that establishes a communication between the matrix and cells through integrins.[32] Catabolic processes also play an important role during the healing of tendons by activating enzymes that induce matrix rearrangement.[32] In this regard, metalloproteinases (MMPs) play an important role in the remodeling of the ECM during wound healing. MMPs are endopeptidases that utilize Zn^{2+} or Ca^{2+} ions in their active site.[33] MMP-2 and MMP-9 belong to the gelatinase group and primarily digest gelatin, the denatured form of collagen.[34] These MMPs have an important role in the healing process, especially during the inflammatory and remodeling phases.[35]

Some aspects of tendon repair are still unclear, such as the process of collagen and elastic fiber fibrillogenesis, which promotes the reconstitution of the tendon.[36] Therefore, new strategies should be developed and tested to improve tendon remodeling during the healing process. Considering its use in traditional medicine and the effects of *A. chica* with improvement in skin repair,[7] our aim was to evaluate the effects of this plant on the healing of a fibrous and highly organized tissue such as the Achilles tendon. We hypothesize that the effect of collagen synthesis stimulated by the use of the *A. chica* verified in the study of Jorge et al. (2008)[7], could improve the tendon healing. Thus, we analyzed the biochemical, morphological and functional effects of *A. chica* extract on the three phases of the wound healing after tendon transection.

Materials and methods

Plant material and extraction

The lyophilized extract of the *A. chica* plant utilized in our study was provided by CPQBA (Multidisciplinary Center for Chemical, Biological and Agricultural Researches) – UNICAMP, after standardization of the aglycones according to Jorge et al. (2008)[7]. Leaves of *A. chica* (Humb & Bonpl) Verlot available in the Germplasm Bank (from acess 06) were

collected from the Center for Chemical, Biological and Agricultural Researches of UNICAMP, located in the city of Paulínia (22º45'00" South and 47º10'21" West). The plant material was ground with dry ice in a grinder of knives, Stephen brand, model UM 40. The ground leaves were extracted three times with a mixture of 1:5 (v/v) ethanol/0.3% citric acid. The extracts were filtered, dried with anhydrous Na_2SO_4 and filtered, and the solvent was removed in a vacuum, followed by lyophilization. The lyophilized extract was dissolved in saline solution (0.85%) at a concentration of 2.13 g/ml for application to the lesion site on each tendon, as described below.

Protocols for the partial transection of the calcaneal tendon and topical application of the *A. chica* extract

For surgical procedures, the animals were anesthetized with intraperitoneal injection of Ketamine (80 mg/kg) and Xylazine (10 mg/kg), and the lower right paws underwent trichotomy and antisepsis with iodine alcohol. A longitudinal incision was made in the skin to expose the Achilles tendon, and the transverse partial transection was performed in the tension region of the tendon that is located at an approximate distance of 4 mm from the tendon's insertion into the calcaneus bone[37] (Fig. 1.). We dissolved 32 mg of the *A. chica* in 15 µL of 0.85% saline solution and applied the solution at the site of the tendon transection prior to the suture of the skin. After application of the extract, the skin was sutured with nylon suture (Shalon 5-0) and needle (1.5 cm). From day 1 after surgery, we performed a daily topical application of the plant extract in the region of the sutured skin, gently massaging for its absorption. Due to the high penetrating power of the *A. chica* extract, this procedure was repeated until the 7th day after the surgery for a total of 7 topical applications. This same procedure was utilized for the transected tendons of controls that received applications of 15 µL of 0.85% saline solution. For the immobilization of the animals during the daily topical applications, a retainer rat was used (Request Privilege of Utility Model in Brazil, titled: Retainer of rats for laser beam and biocompounds application, deposited in National Institute of Industrial Property – INPI, on 04.16.10, under n° MU9000622-4, authored by researchers Andrea Aparecida de Aro and Edson Rosa Pimentel, from Institute of Biology, UNICAMP).

Experimental groups

Sixty eight-day-old male Wistar rats (n = 154, weighing 250 ± 20 g), with free access to food and water, were used in the present study. The animals were separated into 7 experimental groups: the normal group (N) – rats with tendons without transection and sacrificed at age eighty-two-days-old; the S7, S14 and S21 groups - rats with tendons treated with topical applications of saline for 7 days and sacrificed on the 7th, 14th and 21st days after surgery, respectively; and the A7, A14 and A21 groups - rats with tendons treated with topical applications of the plant extract for 7 days and sacrificed on the 7th, 14th and 21st days after surgery, respectively. This study was performed according to the Institutional Committee for Ethics in Animal Research of the State University of Campinas – Unicamp (protocol n° 1621-1).

Hydroxyproline quantification

After washing in PBS (Phosphate-Buffered Saline – 5 mM phosphate buffer, 0.15 M NaCl and 50 mM EDTA), fragments from the tendons (n = 5) were immersed into acetone for 48 h and then into chloroform:ethanol (2:1) for 48 h. Fragments were weighed and hydrolyzed in 6 N HCl (1 mL for each 10 mg of tissue) for 16 h at 110°C. The hydrolysate was neutralized with 6 N NaOH, and 20 µL of each sample was treated with 50 µL/well of chloramine T solution (282 mg chloramine T, 2 mL *n*-propanol, 2 mL distilled water, and 16 mL citrate acetate buffer, pH 6.0) for 20 min at room temperature. Next, 50 µL/well of Erlich's solution [2.5 g 4-(dimethylamino) benzaldehyde, 9.3 mL *n*-propanol, and 3.9 mL 70% perchloric acid] was added and incubated for 15 min at 60°C, as described by Stegemann and Stalder (1967)[39] and Jorge et al. (2008)[7], with some modifications. The 96-well plate was cooled until it reached room temperature, and the absorbance was measured at 550 nm in a spectrophotometer. Hydroxyproline concentrations from 0.2 to 6 µg/mL were used for a standard curve.

Extracellular matrix extraction

The transection region of the Achilles tendon from each group and the tension region of normal tendons (n = 4) were analyzed after a rapid washing in PBS (Phosphate-Buffered Saline – 5 mM phosphate buffer, 0.15 M NaCl, and 50 mM EDTA). The segments were cut into pieces and were immersed in 50 volumes of 4 M guanidine hydrochloride supplemented with 0.05 M EDTA and 1 mM PMSF in 0.05 M acetate buffer, pH 5.8 [38] (Heinegård and Sommarin, 1987). The extraction mixture was gently stirred for 24 h at 4°C, followed by centrifugation at 27,000 x g for 50 min. The supernatant (total extract) was used in the western blot and SDS-PAGE analyses.

Quantification of non-collagenous proteins (NCPs)

Samples from the total extract of the tendons were used to quantify NCPs according to the Bradford Method,[41] with some modifications, using bovine serum albumin as a standard (from 0.3 to 5 µg/mL were used for a standard curve). In the 96-well plate, 10 µL of each sample was treated with 200 µL/well of the reagent (Bio-Rad Protein Assay, #500-0006). The absorbance was measured at 595 nm in a spectrophotometer.

Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE)

The total extracts (50 µg) of the tendons from animals of all experimental groups were precipitated with 100 µL of 1 M acetate buffer (pH 7.4) and 9 volumes of absolute ethanol for 24 h at 4ºC. These samples were analyzed by SDS-PAGE following the method of Zingales (1984)[42] using gradient gels (4-16%) at 30 mA for 3.5 h. The relative molecular masses were estimated by comparisons with protein standard molecular mass markers.[43] Gels were stained with Coomassie Brilliant Blue R-250 (Sigma).

Western blotting

Samples from each group containing 50 µg of protein (for collagen type I) and 80 µg of protein (for collagen type III) were separated by electrophoresis in SDS-polyacrylamide gels under reducing conditions and non-reducing conditions, respectively.[44] After electrophoresis, the proteins were transferred to Hybond-ECL nitrocellulose membranes

(Amersham, Pharmacia Biotech, Arlington Heights, USA) at 70 V for 3 h. The membranes were blocked with Chemiluminescent blocker (WBAVDCH01, Millipore) for 20 s, rinsed with basal solution (0.01 M Trisma base, 0.15 M NaCl and 0.05% Tween 20) and incubated at 4°C for 10 min with the following primary antibodies: collagen type III (C7805, Sigma) diluted in basal solution with 1% BSA (1:500), collagen type I (C2456, Sigma) diluted in basal solution with 1% BSA (1:500) and glyceraldehyde 3-phosphate dehydrogenase (GAPDH: sc-25778, Santa Cruz), used as an endogenous control, diluted in basal solution with 1% BSA (1:100). The membranes were incubated for 10 min with goat or mouse secondary HRP-conjugated antibody (A0545 and A0412 respectively, both Sigma) diluted (1:100 and 1:500) in basal solution with 1% BSA. After washing in basal solution, peroxidase activity was detected by incubation with a diaminobenzidine (DAB) chromogen (Sigma Chemical Company, St Louis, USA) for 5 min. GAPDH was used as an endogenous control for the comparison among groups. The intensity of the antigen bands in each experimental group was determined by densitometry using the Alpha 4.0.3.2 software (Scion Corporation, USA) and was expressed in arbitrary units.

Zymography for gelatinases

The transection regions of the tendons (n = 4) were washed in PBS (Phosphate-Buffered Saline – 5 mM phosphate buffer, 0.15 M NaCl, and 50 mM EDTA) and cut into pieces. The segments were immersed in a solution of 50 mM Tris-HCl (pH 7.4), 0. 2 M NaCl, 10 mM $CaCl_2$, 0.1% Triton and 1% protease inhibitor cocktail (Sigma) for protein extraction (100 µL of extraction solution for each 30 mg of tissue) at 4°C for 2 h.[45,46,47] After this first extraction, the samples were incubated using 1/3 of volume of the same solution described previously at 60°C for 5 min. For the MMP-2 and MMP-9 analyses, 1 µg and 20 µg of proteins per sample, respectively, were applied to the gel after the quantification of the total proteins. Ten percent polyacrylamide gel electrophoresis containing 0.1% gelatin was performed at 4°C. After electrophoresis, the gels were washed with 2.5% Triton X-100 and incubated for 21 h in a solution of 50 mM Tris-HCl (pH 7.4), 0.1 M NaCl and 0.03% sodium azide at 37°C. The gels were stained with Coomassie Brilliant Blue R-250 for 1 h. The gels were then washed with a solution containing 30% methanol and 10% acetic acid for observation of the negative bands of protein

corresponding to gelatinolytic activity. Additionally, 20 mM EDTA, which inhibited the activity of gelatinases, was used in the incubation buffer as a positive control, confirming the identification of MMPs in the gels. The intensity of the isoforms bands in each experimental group was determined by densitometry using the Alpha 4.0.3.2 software (Scion Corporation, USA).

Histology and Morphometry

After dissection, the tendons (n = 4) were fixed using a 4% formaldehyde solution in Millonig's buffer (0.13 M sodium phosphate, 0.1 M NaOH - pH 7.4) for 24 h at 4°C. The tendons were then washed in water, ethanol dehydrated, diaphanized with xylene and paraffin-embedded. Longitudinal serial sections of 7 μm were obtained for microscopy analysis. For observation of the general morphology of the tendons, the sections were deparaffinized and then stained with hematoxylin-eosin (HE).[48] The sections on the slides were immersed in xylene before embedding in entellan (Merck). For observation of the morphology of the tendons, tissue sections were analyzed under an Olympus BX 60 light microscope.

For morphometry, images from longitudinal sections of the transection region of the tendons were captured and digitized using a Leica DM 2000 photomicroscope. Digitized images were used for the determination of the morphometric parameters, which included the total number of fibroblasts and inflammatory cells ($n/10^4$ μm^2), and the number of blood vessels ($n/10^4$ μm^2). For this purpose, three 10^4 μm^2 samples were collected from the central region of the transection in each animal using a virtual Leica Image Measure™ grid.

Evaluation of the Max Contact Intensity of the rat paw after partial transection

The CatWalk system (Noldus Inc., The Netherlands) was used to analyze the gait recovery of the animals (n = 5). In this protocol, the rats crossed a walkway (100 cm length x 15 cm width x 0,6 cm thickness) with a glass floor illuminated from the long edge in a dark room. Data acquisition was performed with a high-speed camera (Pulnix TM-765E CCD), and the paw prints were automatically classified by the software. The paw prints were obtained during the 3 days before the partial transection of the tendons to assess the

normal standard gait of the animals, and they were collected again after the lesions. Post-operative data were assessed on the 1st, 3rd, 5th and 7th days following surgical lesion for the groups that were sacrificed 7 days after surgery; on the 1st, 3rd, 5th, 7th, 9th, 11st, 13rd and 14th days following surgical lesion for the groups that were sacrificed 14 days after surgery; and finally, on the 1st, 3rd, 5th, 7th, 9th, 11st, 13rd, 15th, 17th, 19th and 21st days following surgical lesion for the groups that were sacrificed 21 days after surgery. The parameters used herein were "Max Contact Intensity", corresponding to the pressure exerted by the paw on the glass floor during gait. The intensity of magnification can vary from 0 to 255 pixels.

Statistical Analyses

Data from different experimental groups were analyzed by *t*-test student ($p < 0.05$), using the GraphPad Prism® (Graph-Pad Software, La Jolla, CA, USA), version 3.0.

Results

The results observed for the quantitation of hydroxyproline (Fig. 2.), which estimates the total collagen content in the tissue indicated higher concentrations (mg/g dry weight) in the A7 (91.5 ± 18.9) and A21 (95.8 ± 12.0) groups when compared with their respective controls (S7: 75.2 ± 7.2; and S21: 72.0 ± 7.9). However, no group reached a value equivalent to the normal tendon (124.0 ± 17.2).

The SDS-PAGE gel analysis (Fig. 3.) showed some differences between the populations of proteins extracted from the tendons at 7, 14 and 21 days after transection. Under reducing conditions (Fig. 3A.), we observed an increase in the amount of non-collagenous proteins (molecular weight between 14.4 and 67 kDa) in all transected groups, but with no difference between the saline- and plant-treated groups. Regarding the presence of collagen, it is worth mentioning the presence of more prominent bands related to the α and β chains of collagen compared to NCPs. Because type III collagen forms co-fibrils with type I collagen, there is an overlap of bands corresponding to these two types of collagen in the gel. Therefore, only the Western blotting analyses report the exact amounts of type I and type III collagen.

Densitometry analysis of the bands (arbitrary units) obtained from the type I collagen western blots (Fig. 4. and Table 1) showed that group A7 (103.8 ± 15.9) had less of this type of collagen than groups S7 (165.2 ± 31.1) and N (188.4 ± 31.1) during the 7 first days of the healing process. On days 14 and 21, no significant differences were observed between the groups. We observed less type III collagen in group A7 (206.3 ± 8.1) compared to groups S7 (338.6 ± 48.8) and N (462.3 ± 56.8). No significant difference was observed between groups S14 and A14.

With relation to the NCPs, group A7 had lower amounts of NCPs (36.3 ± 4.7 mg/g tissue) compared to group S7 (59.5 ± 5.9) (Fig. 5.). No differences were found between groups A14 (47.5 ± 5.3) and A21 (44.2 ± 5.9) and their controls S14 (49.3 ± 4.9) and S21 (42.0 ± 5.8), although all transected groups showed greater amounts in relation to the group N (23.3 ± 3.9).

In the MMP-9 zymography, we observed bands corresponding to the latent (92 kDa) and active isoforms (83 kDa) in groups S7, A7, S14 and A14. In the 21 day groups, neither isoform was evidenced (Fig. 6A.). Densitometry of the bands (pixels) detected significant differences between the MMP-9 isoforms only on the 14th day of injury, due to the smaller amount of latent and active isoforms in group A14 compared to group S14 (Table 2). The MMP-2 zymography showed the presence of latent (72 kDa), intermediate (68 kDa) and active isoforms (62 kDa) in all groups except the normal group, which did not show the active isoform of the enzyme (Fig. 6B.). Densitometry of the bands did not detect significant differences in the three MMP-2 isoforms between groups S7, A7, S14 and A14 (Table 2). However, on day 21, group A21 showed higher amounts of latent and intermediate isoforms in relation to group S21. With respect to the active isoform, there was no significant difference between groups S21 and A21.

Overall, the sections stained with HE showed marked differences between the periods analyzed (Fig. 7.). We observed a high cellularity in the region of repair of the transected tendons, with total disorganization of the matrix of groups S7 and A7 (Fig. 7B. and 7C.). In the 14 day groups, cellularity was also high, and the ECM was in the process of remodeling, with greater orientation of the matrix than at 7 days post-injury, although there were no differences between the saline- and plant-treated groups (Fig. 7D. and 7E.). We did not observe differences between the 21 day groups, which were characterized by a more

organized matrix compared to the other periods, with some regions more heavily stained at the site of repair and smaller cellularity. However, our data showed that after 21 days following injury, the remodeling of the tendon ECM is not finished (Fig. 7F. and 7G.), as can be verified by a comparison with a section of a normal tendon (Fig. 7A.). Collagen bundles oriented in only one direction are characteristic of the tension region of a normal tendon, with few fibroblasts arranged between this dense and organized collagen matrix.

In morphometry, our analyses showed that all transected groups had a higher number of fibroblasts, inflammatory cells and vessels than the normal tendon, but with no significant differences between the saline- and plant-treated groups. Note that 7 days following injury, there is a clear excess of inflammatory cells in groups S7 and A7 compared to other periods. After 14 days of injury, there was a significant reduction in the number of these cells (Table 3).

Our results obtained through the CatWalk system showed positive functional response to topical treatment with the *A. chica* extract. Group A7 had a higher value corresponding to the maximum intensity of contact of the paw (pixels) on the platform during gait than group S7, corresponding to the greater pressure from the paw of the animal at 5 and 7 days after injury, with values close to the tendons without transection. This result suggests that the plant treatment was effective in recovering the gait of the animals in this group after tendon injury, especially in the acute phase of healing. After 14 days of injury, group A14 had higher values than the S14 group only at 11 days of injury, obtaining values equivalent to the S14 group on the 13^{th} and 14^{th} days. Group A21 showed a trend towards higher values in relation to group S21, although this was not significant (Fig. 8.).

Discussion

The healing of tendons does not occur through a regenerative process, but by a slower healing process, with the formation of fibrous scarring that can cause significant dysfunction and restriction of joint movement.[49,50,51] After a tendon injury, there are changes in the proportion and organization of ECM components, and these changes may be dependent on the type and extent of the injury. Because collagen is the main constituent of the ECM of tendons and its hierarchical organization into bundles is essential for maintaining the biomechanical strength of this tissue, it is extremely important to utilize strategies that stimulate collagen synthesis and its reorganization into fiber bundles during

healing processes. Because of the traditional use of *A. chica* during wound healing and its effect on *in vitro* collagen synthesis,[7] our hypothesis was that *A. chica* extract may improve tendon repair.

Our results showed that the topical application of *A. chica* extract on the tendon increased the total collagen content at the 7th and 21st days of the healing process. These results corroborate the data in the report by Jorge et al. (2008)[7], which demonstrated that the crude plant extract stimulated collagen synthesis with an effect that can be compared to vitamin C, which has an important role in the hydroxylation of proline and lysine residues during collagen synthesis; the authors additionally showed that the plant extract also stimulated *in vitro* collagen synthesis. Tendon injuries initiate signaling mechanisms that increase the synthesis of some types of collagen by fibroblasts to reconstitute the collagen fibers.[37,52,53,52,55] Considering the results obtained in the western blots, which showed a reduction of type I collagen in group A7 and an absence of a significant difference between the groups in the 14th and 21st days, we believe that the increase in the hydroxyproline concentration in the groups treated with the plant extract 7 and 21 days after the lesion can be related to the synthesis of other types of collagen. Although they are present in small amounts in the tendons, some collagens, such as types IV,[54] V and XII,[35] have their synthesis increased after lesions. We should also consider the possibility of the existence of hydroxyproline from previously degraded collagen fragments as a result of the process of matrix remodeling.

As previously described, *A. chica* extract treatment affected type I collagen synthesis during the inflammatory phase. A similar result was observed with type III collagen, as there was a lower amount of this protein observed in the group treated with the plant extract 7 days after injury. Several studies have reported increased synthesis of type III collagen, mainly during the first 7 days (the acute phase of tendon healing), with irregular organization of the collagen fibrils.[52,53,55] We believe that our results did not show an increase in this type of collagen in any of the three periods analyzed during the healing process due to our injury model. The changes in the proportions of ECM components may be dependent on the type and extent of the lesion.

The NCPs are important components during the healing of the tendon and are therefore present in increased amounts after injuries.[56,57] NCPs have multiple roles, such as

cell differentiation,[58] degradation of the extracellular matrix,[35,59] chemotactic action for neutrophils to the site of injury, stimulus for fibroblast proliferation, and vessel formation.[60] Our results showed that all transected tendons had a higher concentration of NCPs than the normal tendon, although the plant extract decreased the concentration of NCPs on the 7th day after injury compared to the saline group. Our analysis does not allow us to pinpoint which proteins are decreased in this group, but our hypothesis is that inflammatory mediators may be present in smaller quantities. This hypothesis is based on the functional result achieved by the catwalk system, in which animals more strongly supported the injured paw during gait, possibly indicating decreased pain severity, especially in this acute healing phase, in which there is a high level of inflammation.

The increased production of inflammatory mediators leads to pain and swelling,[61] hindering the movement of the talocrural articulation and the support of the paw during gait after injury. According to Zorn et al. (2001)[4], the lipophilic extract of *A. chica* leaves contains a natural phenolic compound, the carajurin (3-desoxyanthocyanidin), which has anti-inflammatory action in synergy with other components yet to be identified. This result is quite interesting considering the fact that during healing processes, it is important to use strategies that minimize pain and discomfort for the patient in the shortest possible time.[8,62] However, further studies need to be conducted to prove the anti-inflammatory effect of *A. chica* crude extract in tendon injuries. It is still worth noting that in the groups treated with *A. chica* showed, at 14 and 21 days after lesion, a tendency towards an increase of intensity of the support for the injured paw during gait was observed, although not a significant one.

During the inflammatory phase that begins immediately after injury, there is a migration of inflammatory cells that participate in the phagocytosis of necrotic material and cell debris to the lesion site.[55] Macrophages also participate in the recruitment of fibroblasts and in the release of factors that promote angiogenesis to initiate the growth of the capillary network within the injured area.[63,64] Growth factors such as platelet-derived growth factor (PDGF), epidermal growth factor (EGF), transforming growth factor (TGF)-β1[65,66,67] and vascular endothelial growth factor (VEGF) show increased synthesis after injuries and have a probable role in the revascularization of the tendon.[67,68,69] These cellular events explain why all of the transected groups have higher amounts of vessels than the normal group but there are no differences between the groups treated with saline and plant extract. We did

not observe any difference in the number of fibroblasts and inflammatory cells after the utilization of *A. chica* in any of the three periods studied, although, again, all transected tendons showed higher amounts than the normal tendon. According to the results of Jorge et al. (2008)[7], there was an increase in the number of fibroblasts after the utilization of the plant; we attribute our result to the fact that our study was performed *in vivo*, where various factors act directly on signaling to control cell division and death.

MMPs are a family of calcium- or zinc-dependent proteases that degrade various ECM components and exhibit altered levels during tendon healing, thereby acting as regulators of the matrix remodeling.[57,70] MMP activity can be regulated at both the gene transcription level and by the synthesis of pro-MMPs, and the activation of these proenzymes and their inhibition by TIMPs (tissue inhibitors of metalloproteinases) are important regulatory processes.[34] It is noteworthy that an imbalance of these elements may cause a disturbance in the production of collagen during the remodeling of the tendon.[71] Thus, due to the importance of MMPs in the reconstitution of the ECM after injury, this study also aimed to investigate the effect of the *A. chica* extract on the synthesis and activation of MMPs-2 and -9.

MMP-2 (gelatinase A) and MMP-9 (gelatinase B) seem to play different roles during tissue repair, with peaks of activities at different stages of this process.[35] We observed the presence of latent and active isoforms of MMP-9 during the first 7 days after tendon injury in the *A. chica* and saline groups. This period can be characterized as inflammatory,[72] and MMP-9 is synthesized predominantly by inflammatory cells, as it has an important role in the degradation of denatured collagen and other matrix components.[59] MMP-9 may also have a similar role in the degradation of pre-existing collagen bundles that MMP-13, a type of collagenase, has in the degradation of fibrillar collagen.[59,73,74] A remarkable result was observed on the 14th day, when tendons treated with *A. chica* showed smaller amounts of the two MMP-9 isoforms when compared to the saline group. These data once again imply an anti-inflammatory activity of the *A. chica* extract. According to Oliveira et al. (2009),[75] the aqueous *A. chica* extract has substances with inhibitory activity on the inflammatory effects of the poisons of the *Bothrops atrox* and *Crotalus durissus* ruruima, which induce edema.

The presence of the active MMP-2 isoform is indicative of the matrix remodeling process.[76] The *A. chica* extract appears to not have stimulated the synthesis or activation of MMP-2 until the 14th day after the injury, as we did not observe differences in relation to the saline group until that time. On the 21st day, the plant extract seems to have stimulated the synthesis of the enzyme, although it has not stimulated its activation. Some studies suggest that MMP-9 participates only in collagen degradation, whereas MMP-2 participates in both the degradation and the remodeling of the collagen bundles, with peak production between the 7th and 14th day. After this period, there is a reduction in MMP-9 and a high prevalence of MMP-2 until the 28th day[35,59] when compared to the tendon without transection. These data agree with ours, as we observed a decrease in the amount of MMP-2 from the 21st day after injury, the MMP-9 peak on the 14th day after injury and MMP-9 absence on day 21.

The results presented in this study justify the traditional medicine utilization of *A. chica* in healing processes. We observed that the topical application of the plant extract during the process of tendon healing stimulated total collagen and MMP-2 synthesis, decreased MMP-9 synthesis and activation, and improved the gait recovery of animals after injury. Further studies should be conducted to investigate the effect of the *A. chica* extract on the inflammation and reorganization of the collagen bundles after tendon injury.

Figures and Tables

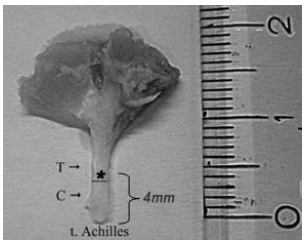


Fig. 1. Image of the Achilles tendons of rats with regions submitted to compression (C) and tension (T) forces. The region of the partial and transversal transection (*) is located 4 mm from the insertion in the calcaneus bone (modified from Aro et al., 2008).[38]

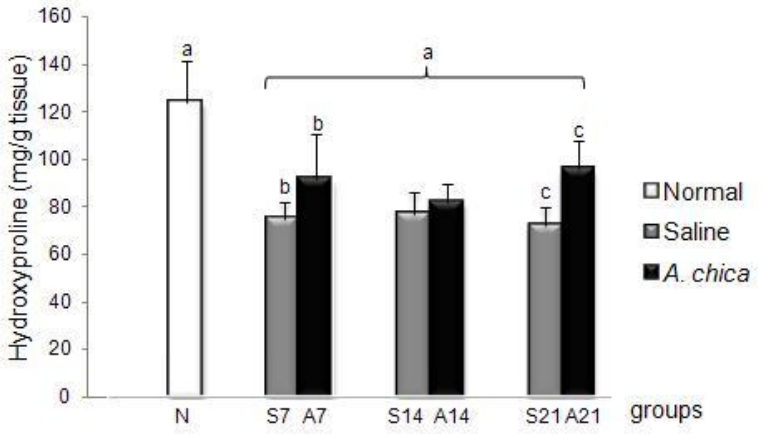


Fig. 2. Concentration of hydroxyproline (mg/g dry weight) in the transection region of the tendons from the different groups. Significant differences ($p<0.05$) can be observed between the groups treated with saline and *A. chica* at 7 and 21 days after the transection of the tendon. Note that, at these times, the tendons treated with the plant extract had higher concentrations of hydroxyproline than the control group treated with saline. It is noteworthy that the hydroxyproline levels of the transected tendons were less than that of the normal tendon. The same letters between the groups corresponds to a significant difference between them.

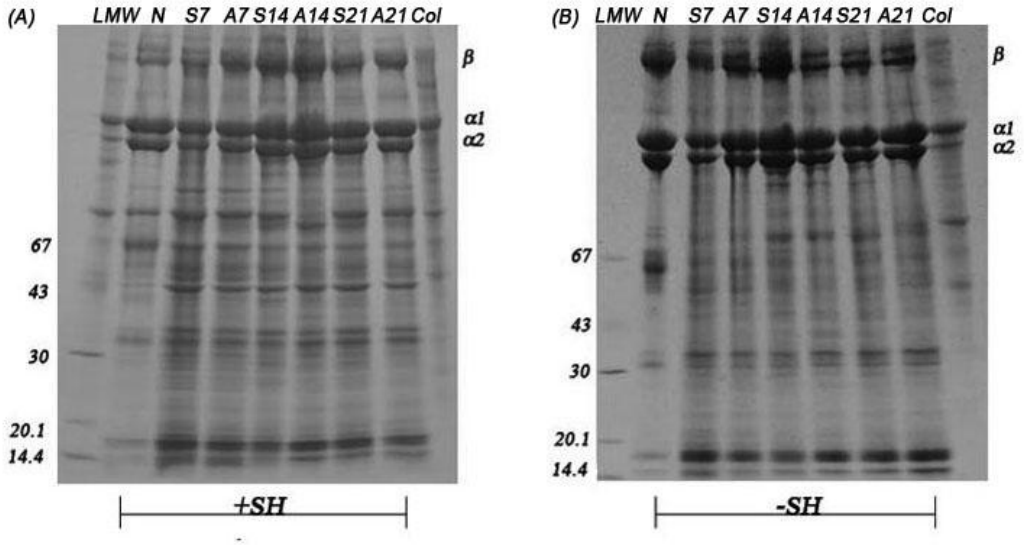


Fig. 3. SDS-PAGE (4-16%) of total proteins in the tendons. (A) Electrophoresis under reducing conditions (+SH), that is, with β-mercaptoethanol. (B) Electrophoresis under non-reducing conditions (-SH), without β-mercaptoethanol. Note that in both gels, the collagenous proteins represented by bands corresponding to the α and β chains of collagen are more prominent than the bands of non-collagenous proteins with molecular weights between 14.4 and 67 kDa. No significant difference between the saline and *A. chica* groups was observed at any time point.

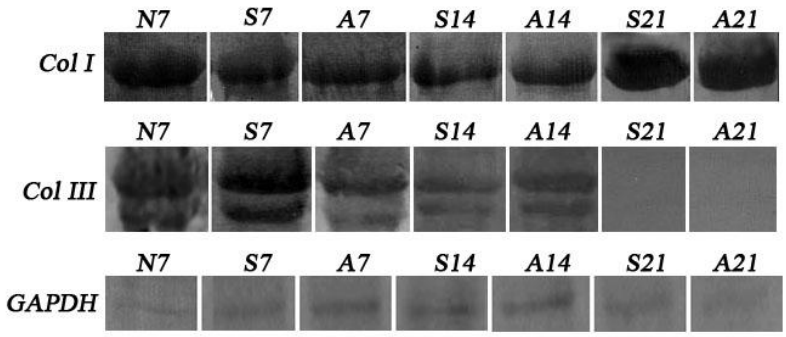


Fig. 4. Western blotting showing type I and type III collagen: 50 and 80 µg of total protein extract, respectively, were used from the tendons of the different experimental groups. Type I collagen is present in all groups. Type III collagen is absent 21 days after injury. GAPDH was utilized as an endogenous control. For the significant differences between the groups, see the band densitometry analysis in Table 1 below.

Parameters	N	S7	A7	S14	A14	S21	A21
Collagen type I	195.7 ± 40.0	165.2 ± 31.1 [a]	103.9 ± 15.9 [a,*]	230.8 ± 99.7	203.2 ± 51.7	285.0 ± 72.4	199.9 ± 23.9
Collagen type III	462.3 ± 56.8	338.6 ± 48.8 [b]	206.3 ± 8.1 [b,*]	232.0 ± 120.1	212.7 ± 135.8	-	-

(*) Significant differences ($p<0.05$) between the transected and normal tendons; ([a, b]) Significant differences ($p<0.05$) between the groups marked with the same letter.

Table 1: Densitometry of the bands (arbitrary units) corresponding to type I and type III collagen at 7, 14 and 21 days after injury. Considering the type I collagen, group A7([a]) had less of this type of collagen than groups S7 ([a]) and N (*). No significant differences were observed between the groups on days 14 and 21. With relation to the type III collagen, group A7 ([b]) had less of this type of collagen compared to groups S7 ([b]) and N (*). No significant difference was observed between groups S14 and A14. Note the absence of the type III collagen on day 21.

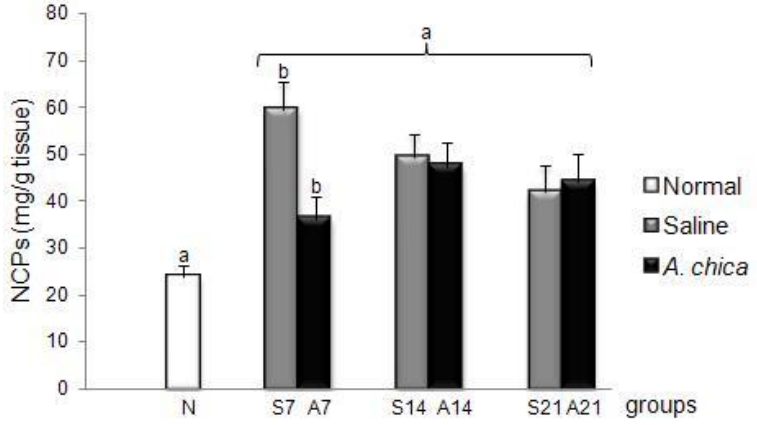


Fig. 5. Non-collagenous protein concentration (mg/g of tissue) in the tendons. The transected tendons had high values compared to the normal tendon. Observe the marked decrease in the concentration of these proteins in the group treated with *A. chica* compared to the control treated with saline (b) 7 days after injury. There was no significant difference ($p<0.05$) between the experimental groups in the remaining periods. The same letter between groups corresponds to the significant difference between them.

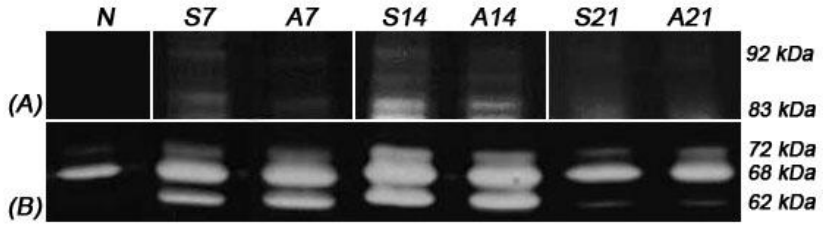


Fig. 6. A) Zymography of MMP-9 for the analysis of the latent (92 kDa) and active (83 kDa) isoforms. Observe the presence of MMP-9 in groups S7, A7, S14 e A14. The more prominent bands corresponding to the latent and active isoforms in S14 group compared to the A14 group. **B)** Zymography for the analysis of MMP-2 latent (72 kDa), intermediate (68 kDa) and active isoforms (62 kDa) in the experimental groups. Observe the presence of the three isoforms of the enzyme in all groups, with the bands corresponding to the latent and intermediate isoforms in the A21 group being more intense than the S21 bands. The densitometry below shows significant differences between the groups (Table 2).

Parameters	N	S7	A7	S14	A14	S21	A21
MMP-9 latent (92 kDa)	0	2132 ± 824*	2047 ± 801*	4726 ± 1002 a,*	1547 ± 530 a,*	0	0
MMP-9 active (83 kDa)	0	3311 ± 1552*	3454 ± 1989*	16,058 ± 2052 b,*	5088 ± 2672 b,*	0	0
MMP-2 latent (72 kDa)	3898 ± 45	24,468 ± 3688*	18,956 ± 4175*	51,380 ± 9140 *	47,450 ± 15745*	8545 ± 596 c,*	24,710 ± 2867 c,*
MMP-2 intermediate (68 kDa)	38,990 ± 876	84,250 ± 31,956*	77,810 ± 28,155*	103,100 ± 10,390*	99,490 ± 20,558*	56,220 ± 3489 d,*	62,530 ± 1371 d,*
MMP-2 active (62 kDa)	0	56,890 ± 6109*	49,550 ± 20,313*	62,070 ± 23,197*	42,620 ± 10,652*	2934 ± 657*	3798 ± 265*

(*) Significant differences ($p<0.05$) between the transected and normal tendons;
(a, b, c, d) Significant differences ($p<0.05$) between the groups marked with the same letter.

Table 2: Densitometry of the bands (pixels) corresponding to MMP-9 and MMP-2 at 7, 14 and 21 days after injury. We did not observe significant differences of MMP-9 between the experimental groups 7 days after injury. On the 14th day after injury, lower values were found in group A14 than in group S14 for the latent ([a]) and active isoforms ([b]). No isoform of MMP-9 was observed in the S21 and A21 groups. Higher values of the MMP-9 isoforms

were found in all the transected tendons when compared to the normal tendons (*). We did not observe significant differences in MMP-2 levels between the saline and plant groups for the three MMP-2 isoforms at 7 and 14 days after injury. On day 21, differences were found between the saline and plant groups for the latent ([c]) and intermediate isoforms ([d]), with higher values for group A21. Higher values for the three MMP-2 isoforms were found in the transected tendons when compared to normal tendons (*).

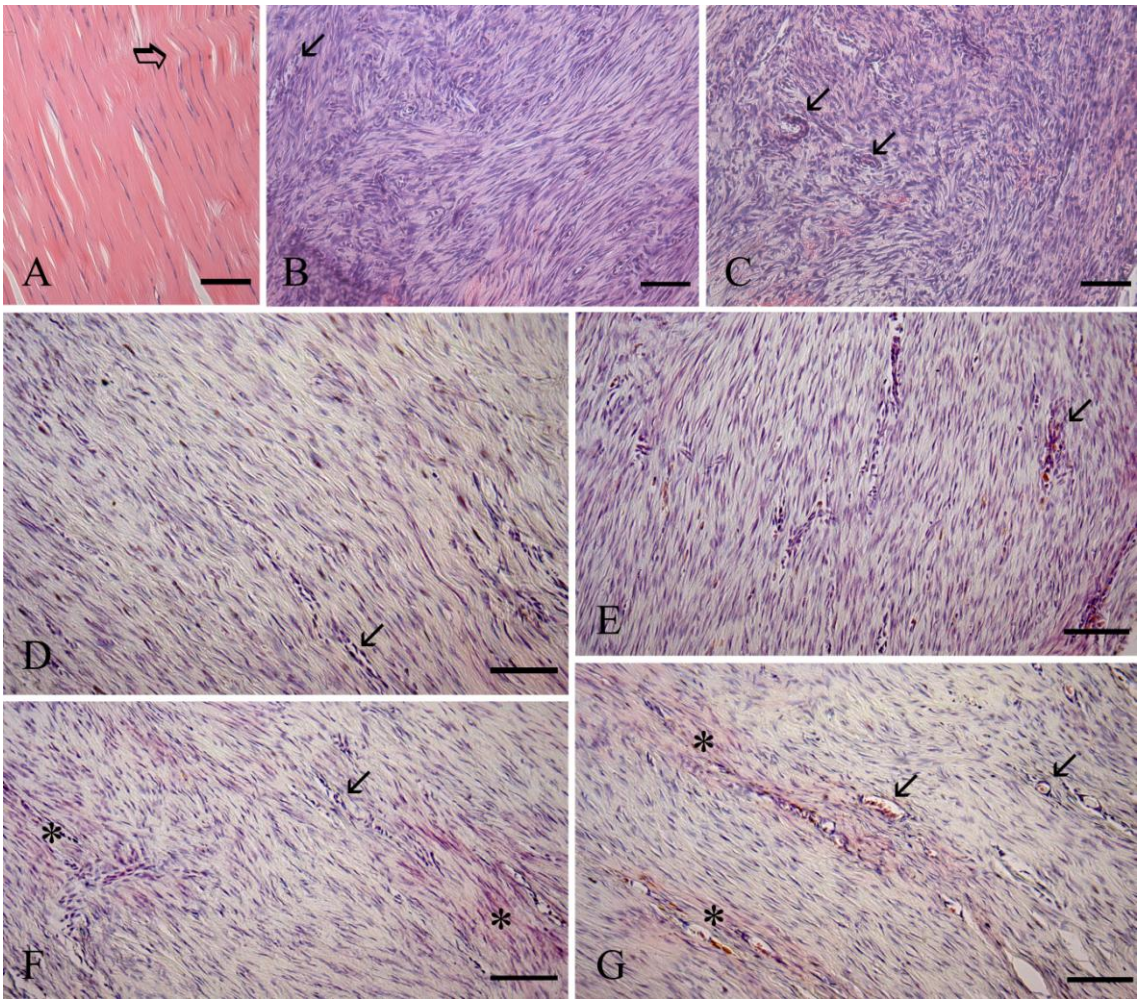
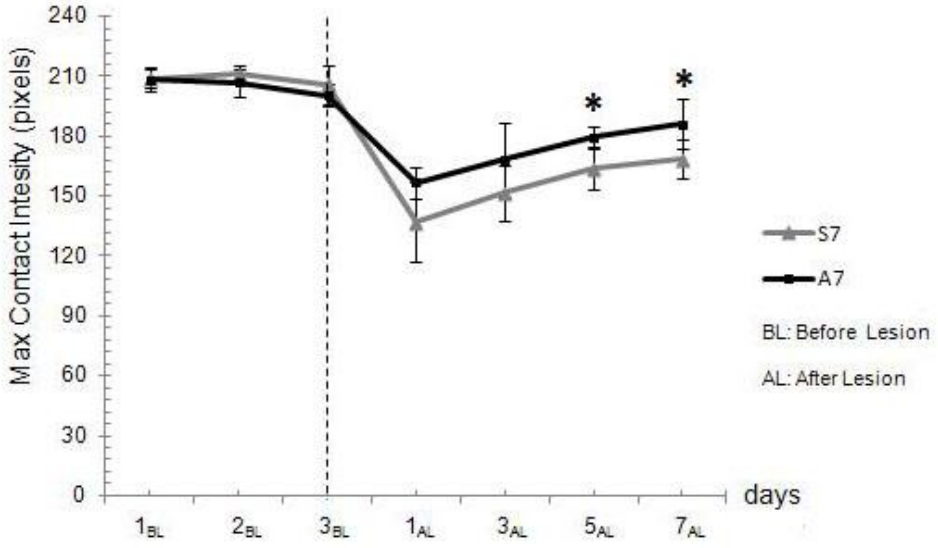


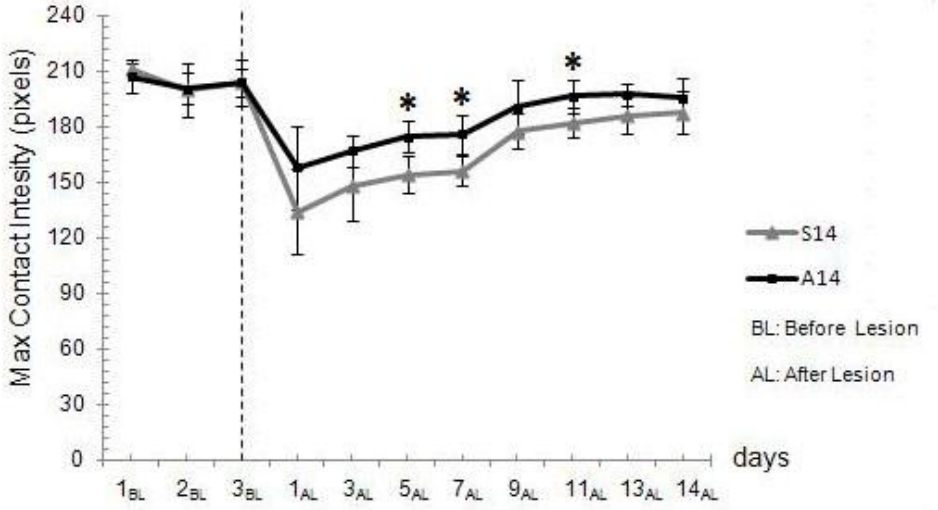
Fig. 7. Tendons sections stained with HE. **A:** normal tendon without transection; **B:** group S7; **C:** group A7; **D:** group S14; **E:** group A14; **F:** group S21; **G:** group A21. The remodeling of the ECM that initiated on day 7 after the lesion was extended until day 21, without complete reorganization of the matrix, as compared to the normal tendon **(A)**, in which there are characteristic fibroblasts located between the collagen bundles oriented in one direction. Note the cells in the matrix with waves (⇨) called "crimp", characteristic of the collagen bundles. In the groups at 7 days **(B and C)**, note the high cellularity and complete disorganization of the ECM. Observe the high cellularity in the groups at 14 days **(D and E)** and the better organization of the matrix compared to the groups at 7 days, although with the apparent absence of differences between groups S14 and A14. In the

groups at 21 days **(F and G)**, a general but unfinished remodeling of the tendons was evident with a high number of cells. Observe that the ECM was slightly more intensely stained in some regions (*). The presence of vessels was observed on the transected groups (↙). Bar = 20 µm.

Parameters	N	S7	A7	S14	A14	S21	A21
Fibroblastic cells ($n/10^4$ µm^2)	22.9 ± 4.8	117.8 ± 23.8*	108.2 ± 29.2*	122.4 ± 31.4*	112.1 ±21.8*	126.1 ± 29.7*	121.7 ± 19.8*
Inflamatory cells ($n/10^4$ µm^2)	0.00	53.8 ± 11.8*	52.4 ± 9.8*	17.8 ± 8.4*	12.4 ± 5.4*	12.1 ± 4.7*	10.1 ± 3.7*
Number of blood vessels ($n/10^4$ µm^2)	2.1 ± 0.4	3.4 ± 1.1	2.9 ± 1.0	2.9 ± 1.3	3.1 ± 0.8	3.1 ± 1.2	3.0 ± 0.9

Table 3: Morphometry of the tendons represented by the mean ($n/10^4$ µm^2) and standard deviation. All transected tendons had a higher number of fibroblasts and inflammatory cells in relation to the normal tendon (*), but with no significant differences ($p<0.05$) between the saline- and plant-treated groups.


Max Contact Intesity (pixels)
240
210
180
150
120
90
60
30
0
1BL 2BL 3BL 1AL 3AL 5AL 7AL
days
S7
A7
BL: Before Lesion
AL: After Lesion


Max Contact Intesity (pixels)
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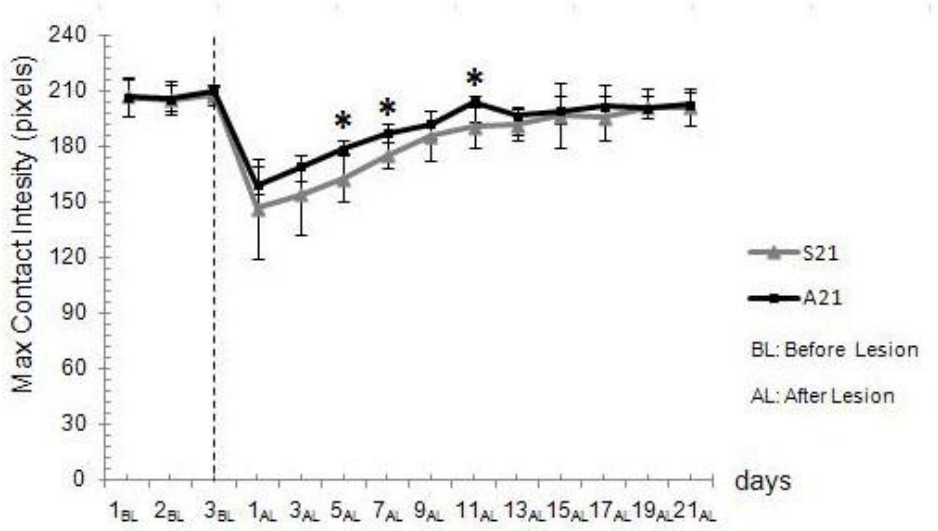
1BL 2BL 3BL 1AL 3AL 5AL 7AL 9AL 11AL 13AL 14AL
days
S14
A14
BL: Before Lesion
AL: After Lesion


Max Contact Intesity (pixels)
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1BL 2BL 3BL 1AL 3AL 5AL 7AL 9AL 11AL 13AL 15AL 17AL 19AL 21AL
days
S21
A21
BL: Before Lesion
AL: After Lesion

Fig. 8. Max Contact Intensity during gait of the rats obtained by the catwalk system. **A)** Measurements performed on animals 7 days after injury. Observe the higher value ($p<0.05$) of paw pressure during the gait of the animals in group A7 when compared to group S7 (*). Measurements were made on the 1st, 3rd, 5th and 7th days after injury. Although the action of the plant extract has been effective in the recovery process of the gait, the values in these groups are still inferior to that of normal animals. **B)** Measurements performed on animals 14 days after injury. The A14 group had a higher value at 11 days after injury than the S14 group, obtaining values equivalent to the saline group at 13 and 14 days after injury. Measurements were made on the 1st, 3rd, 5th, 7th, 9th, 11st, 13rd and 14th days after injury. **C)** Measurements performed on animals 21 days after injury. There was no significant difference ($p<0.05$) between the S21 and A21 groups at the end of this period. Measurements were made on the 1st, 3rd, 5th, 7th, 9th, 11st, 13rd, 15th, 17th, 19th and 21st days after injury.

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MANUSCRITO 3

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Structural and biochemical alterations during the healing process of tendons treated with *Aloe vera*

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ABSTRACT

Aims: The tendon is composed of highly organized collagen fibers that form a complex supramolecular structure. After lesions, the organization and composition of the tendon is not completely restored. Our purpose was to evaluate if the application of *Aloe vera* (L.) Burman f. improves the tendon healing, considering the effectiveness in the stimulus of collagen synthesis.

Main methods: The calcaneal tendon of male Wistar rats was partially transected with subsequent topical application of *A.vera* ointment at the injury. The animals were separated into groups with tendons treated with the *A.vera* extract for 7 days and excised on the 7th, 14th and 21st days after surgery; control rats received only ointment base without plant extract.

Key findings: Morphological analysis using polarization microscopy showed that the entire tendon undergoes a remodeling process, with disorganized collagen fibers by days 7 and 14 in plant-treated and non-treated groups, and with a higher birefringence in tendons of the plant-treated group on 21st day. A higher concentration of hydroxyproline was found in plant-treated tendons on days 7 and 14 compared with their controls. Western blots showed lower amounts of type I collagen in the plant-treated group on day 14 compared with the control. MMP-9 diminished 14 days after lesion and the active isoform of MMP-2 increased on day 21 in plant-treated groups.

Significance: The present study indicates a beneficial effect of *A. vera* in the tissue reorganization in the transected region of the tendon 21 days after injury and is supported by an increment of active MMP-2.

Keywords: tendons, collagen, tissue reorganization, *Aloe vera*, healing process

Introduction

The calcaneal tendon is a dense connective tissue that transmits tension originating from the soleus and gastrocnemius muscles to the calcaneus, enabling articular movement. This tissue is composed of an abundant extracellular matrix (ECM) and highly organized collagen fibers that form a complex supramolecular structure (Vidal and Mello, 2010). Other macromolecules, such as proteoglycans (PGs) (Vidal and Mello, 1984; Vogel and Heinegård, 1985), non-collagenous proteins (PNCs) (Eyre et al., 1984; Riley et al., 1996; Józsa and Kannus, 1997; Muller et al., 1998), cells and water are present in tendons, resulting in an oriented architecture (Vidal and Mello, 2010). In addition to type I collagen (Kjaer, 2004), types II (Benjamin et al., 1991; Rufai et al., 1992), III (Hanson et al., 1983), IV (Taylor et al., 2011), V and VI (Felisbino and Carvalho, 1999; Józsa and Kannus, 1997; Carvalho et al., 2006) are also present in lesser amounts. Collagen and PGs are arranged to form supramolecular structures to attend to the biomechanical properties in different regions of the calcaneal tendon (Vidal and Mello, 1984). Matrix metalloproteinases (MMPs) are endopeptidases that are present in tendons and are responsible for homeostasis of the tissue under normal conditions (Magra and Maffulli, 1995). The amount of MMPs increase during tendon healing because they play an important role in regulating matrix remodeling during tendon repair (Agren, 1994; Madlener, 1998; Oshiro et al., 2003).

The human calcaneal tendon can support loads up to 5000 Newtons (Gerdes et al., 1992), although a slight change in the composition can be a determining factor in the occurrence of injuries. Total or partial rupture of the tendons may occur during a chronic degenerative processes, sportive practice, and recreational activities and even during daily activities (Woo et al., 1999; Butler et al., 2008). Other factors, such as age (Dudhia et al., 2007; Esquisatto et al., 2007; Caroll et al., 2008; Nakagaki et al., 2010), diabetes (Bedi et al., 2010; de Oliveira et al., 2010; Fox et al., 2011) and hormones (Cook et al., 2007; Soo et al., 2011), can alter the metabolism of the connective tissue by modifying the functional properties of collagen fibers. Lesions in the human calcaneal tendon often occur in a hypovascular region located 2-6 cm above the enthesis region due to a maximum rotation of the collagen fibers that affect blood flow in this area (Lesic and Bumbasirevic, 2004). Although several treatments for tendon lesions have been proposed in recent years, tendon lesions are still a serious clinical issue because the site of the injury becomes a region with

a high incidence of recurrent rupture. After lesions occur, the structural organization and composition of the tendon is not completely restored and becomes biomechanically weakened (Mello et al., 1975; Tomiosso et al., 2009; Ni et al., 2011). Despite appropriate management, the disability that tendon lesions cause may last for several months (Almekinders and Almekinders, 1994).

Tendon healing occurs in three phases that are dynamic and overlapping called the inflammatory, proliferative and remodeling phases. In the inflammatory phase, which peaks approximately on the 7^{th} day after the injury, there is a proliferation of fibroblasts, a migration of inflammatory cells, erythrocytes and macrophages to the site of the injury, and then, a release of cytokines and vasoactive and chemotactic factors. Angiogenesis and type III collagen synthesis are increased during this phase (Murphy et al., 1994; Oakes, 2003). During the proliferative phase, which starts after the injury and peaks on approximately the 14^{th} day, the peak of collagen type III synthesis occurs. At this stage, the type III collagen fibers form thinner fibers in the injured area compared with the type I collagen fibers (Pajala et al., 2009). The water content and the concentration of GAGs remain high during the proliferative phase (Oakes, 2003). In the remodeling phase, which starts approximately 21 days after the injury, there is a decrease of cellularity, collagen and GAGs synthesis (Sharma and Maffulli, 2005). The remodeling of the ECM starts immediately after the injury, concomitant with the events of the inflammatory and proliferative phases. However, remodeling is markedly increased a few weeks after the injury, with a change from cellular tissue to fibrous tissue. The fibroblasts and collagen fibers become aligned in the direction of the biomechanical stress (Hooley and Cohen, 1979). After approximately 10 weeks, a gradual change from fibrous tissue to scar-like tissue begins and continues over the course of one year (Aro et al., 2008). Concomitant to these events, MMP-2 and MMP-9 participate in collagen degradation and in the remodeling of collagen bundles. Peak production occurs between the 7^{th} and 14^{th} day for MMP-9, and then, they experience a subsequent reduction after this period. There is a high prevalence of MMP-2 until the 28^{th} day after the lesion occurs (Armstrong and Jude, 2002; Oshiro et al., 2003).

Plant extracts have been employed to treat diseases for many centuries and because their active compounds possess great pharmacological potential, they have been widely studied by the scientific community. *Aloe vera* (L.) Burman f., (Liliaceae) is a tropical plant

that is easily grown in hot and dry climates (Maenthaisong et al., 2007) and is widely distributed in Asia, Africa and other tropical areas. The long and pointed leaves of *Aloe vera* are composed of two parts: an outer green layer and an inner, clearer pulp, which occupies most of the volume of the leaf. The outer layer produces exudate, a bitter yellow liquid formed mostly by phenolic compounds, such as anthraquinones (Turner et al., 2004). The pulp, also called mucilaginous gel, has been used for therapeutic purposes due to the rich concentration of polysaccharides (Ni et al., 2004). The scientific interest in identifying the biological activity of these components that are mainly extracted and described in *Aloe vera* is because many studies have demonstrated their effectiveness in the treatment of diabetes (Okyar et al., 2001; Bolkent et al., 2004; Huseini et al., 2012), cholesterol (Lim et al., 2003; Huseini et al., 2012), cancer (Saini et al., 2010; Tomasin and Gomes-Marcondes, 2011), ulcers (Eamlamnam et al., 2006; Park et al., 2011), arthritis (Davis et al., 1992), dermatitis (West et al., 2003; Kim et al., 2010) and inflammation (Chithra et al., 1998a,b,c; Somboonwong et al., 2000; Eamlamnam et al., 2006; Prabjone et al., 2006; Habeeb et al., 2007; Takahashi et al., 2009; Takzare et al., 2009; Atiba et al., 2011; Tarameshloo et al., 2012). However, the effect of *Aloe vera* on tendon healing has not been previously reported.

New strategies for tendon healing should be tested and developed to achieve a better restoration of the structural organization of the ECM. Many studies have successfully demonstrated the positive effect of the *Aloe vera* extract, especially during the healing process of skin. Because of this effect, we hypothesize that the use of this plant could improve tendon repair, especially considering the crude extract´s effectiveness in the stimulus of collagen synthesis. In the present study, we evaluated the effect of *Aloe vera* on the organization and concentration of collagen and MMPs during the three phases of the healing process in the calcaneal tendon.

Materials and methods

Plant material and extraction

The lyophilized extract of the *A. vera* (L.) Burman f. utilized in our study was provided by CPQBA (Multidisciplinary Center for Chemical, Biological and Agricultural Researches) - UNICAMP. Leaves of the plant were collected at CPQBA – UNICAMP

experimental field, located in the city of Paulínia (22º45'00" South and 47º10'21" West), a voucher specimen is deposited at CPMA collection under supervision of Dr. Glyn Mara Figueira. Mature *Aloe vera* leaves were cut and the exudate was removed by gravity. After removal of the rind, the colorless parenchyma (pulp) was ground in a blender and centrifuged at 10,000 x g for 30 min at 4°C to remove fibers, according to Chithra and colleagues (1998a,b,c). The supernatant rich in polysaccharides previously isolated and characterized in a study of Ni and colleagues (2004), was freeze-dried and stored at room temperature. The lyophilized *Aloe vera* powder was added to an ointment base (constituted by anhydrous lanolin and Vaseline solid 30:70) in a proportion of 1:1. The ointment composed of 50% *Aloe vera* was used (32 mg) for each topical application, and 32 mg of the ointment base was used for the control tendons.

Protocols for the partial transection of the calcaneal tendon and topical application of the A. vera ointment

The animals were anesthetized with intraperitoneal injection of Ketamine (80 mg/kg) and Xylazine (10 mg/kg) for surgical procedures, and the lower right paws underwent trichotomy and antisepsis with iodine alcohol. A longitudinal incision was made in the skin to expose the calcaneal tendon, and the transverse partial transection was performed in the tension region of the tendon that is located at an approximate distance of 4 mm from the tendon's insertion into the calcaneus bone (Murrell et al., 1997; Tomiosso et al., 2009). We applied 32 mg of the 50% *A. vera* ointment at the site of the tendon transection prior to the suture of the skin. After application of the extract, the skin was sutured with nylon suture (Shalon 5-0) and needle (1.5 cm). From day 1 after surgery, we performed a daily topical application of the *A. vera* ointment in the region of the sutured skin, gently massaging for its absorption. This procedure was repeated until the 7^{th} day after the surgery for a total of 7 topical applications. This same procedure was utilized for the transected tendons of controls that received applications of 32 mg of the ointment base. For the immobilization of the animals only during the daily topical applications, a retainer rat was used (Request Privilege of Utility Model in Brazil, titled: Retainer of rats for laser beam and biocompounds application, deposited in National Institute of Industrial Property – INPI, on 04.16.10, under n° MU9000622-4, authored by researchers Andrea Aparecida de Aro and Edson Rosa Pimentel, from Institute of Biology, UNICAMP).

Experimental groups

Sixty eight-day-old male Wistar rats (n = 119, weighing 250 ± 20 g), with free access to food and water, were used in the present study. The animals were separated into 7 experimental groups: Normal (N) **-** rats with tendons without transection and sacrificed at age eighty-two-days-old; Base-treated groups (B7, B14 and B21) **-** rats with tendons treated with topical applications of ointment base for 7 days and sacrificed on the 7^{th}, 14^{th} and 21^{st} days after surgery, respectively; and *Aloe vera*-treated groups (Av7, Av14 and Av21) **-** rats with tendons treated with topical applications of the *Aloe vera* ointment for 7 days and sacrificed on the 7^{th}, 14^{th} and 21^{st} days after surgery, respectively. This study was performed according to the Institutional Committee for Ethics in Animal Research of the State University of Campinas – UNICAMP (protocol n° 1621-1).

Birefringence: Image Analysis and Measurements

The tendons (n = 4) were fixed using 4% formaldehyde solution in Millonig's buffer (0.13 M sodium phosphate, 0.1 M NaOH, pH 7.4) for 24 hr at 4°C. Then, the tendons were washed in water, ethanol dehydrated, diaphanized with xylene, and paraffin embedded. Longitudinal serial sections of 7 μm were obtained and after deparaffinization were subjected to microscopy analysis. Image analyses of the tendons were evaluated to detect differences in its morphology, considering the aggregation and organization of the collagen bundles, which reflect in the variation of the birefringence intensity. Birefringence properties were studied by using an Olympus BX51-P BX2 polarizing microscope and an image analyzer (Image-Pro Plus 6.3, Media Cybernetics, Inc. – Silver Spring, MD, USA). The λ/4 Senarmont compensator was used for visual compensation of the birefringence in order to determine the variation of the collagen fiber direction comparing the compensated (dark) and non-compensated (brightness) images.

Since birefringence appears visually as brilliance, this phenomenon was measured with the image analyzer and expressed as gray average (GA) values in pixels, after its calibration (8 bits = 1 pixel). The largest axis of the tendon was positioned at 45° to the crossed analyzer and polarizer during the measurements. Considering that collagen bundles exhibit two kinds of birefringences: intrinsic birefringence (Bi) and form or textural birefringence (Bf) (Vidal, 1980; Vidal, 1986), total birefringence (sum of Bi and Bf) was

used in this study. One hundred GA measurements of the transected and contiguous adjacent regions of the tendons in each experimental group were made after immersing the sections in water, a condition in which total birefringence is highly detectable (Mello et al., 1979; Vidal, 1965; Vidal et al., 1975; Vidal, 1980; Vidal, 1986; Vidal and Mello, 2010). The measurements of GA were represented as arithmetic mean and standard deviation.

Hydroxyproline quantification

After washing in PBS (Phosphate-Buffered Saline – 5 mM phosphate buffer, 0.15 M NaCl and 50 mM EDTA), fragments from the tendons (n = 5) were immersed into acetone for 48 h and then into chloroform:ethanol (2:1) for 48 h. Fragments were weighed and hydrolyzed in 6 N HCl (1 mL for each 10 mg of tissue) for 16 h at 110°C. The hydrolysate was neutralized with 6 N NaOH, and 20 µL of each sample was treated with chloramine T solution, as described by Stegemann and Stalder (1967) and Jorge et al. (2008), with some modifications. The absorbance was measured at 550 nm in a spectrophotometer. Hydroxyproline concentrations from 0.2 to 6 µg/mL were used for a standard curve.

Extracellular matrix extraction

The transected region of the calcaneal tendon from each group and the tension region of normal tendons (n = 4) were analyzed after a rapid washing in PBS (Phosphate-Buffered Saline – 5 mM phosphate buffer, 0.15 M NaCl, and 50 mM EDTA). The segments were cut into pieces and were immersed in 50 volumes of 4 M guanidine hydrochloride supplemented with 0.05 M EDTA and 1 mM PMSF in 0.05 M acetate buffer, pH 5.8 (Heinegård and Sommarin, 1987). The extraction mixture was gently stirred for 24 h at 4°C, followed by centrifugation at 27,000 x g for 50 min. The supernatant (total extract) was used in the western blot analysis, after total protein quantification performed according to the Bradford Method (Bradford, 1976), with some modifications.

Western blotting

Samples from each group containing 50 µg of protein (for collagen type I) and 100 µg of protein (for collagen type III) were separated by electrophoresis in SDS-polyacrylamide gels under reducing conditions and non-reducing conditions, respectively

(Montico et al., 2011; Towbin and Gordon, 1979). After electrophoresis, the proteins were transferred to Hybond-ECL nitrocellulose membranes (Amersham, Pharmacia Biotech, Arlington Heights, USA) at 70 V for 3 h. The membranes were blocked with Chemiluminescent blocker (WBAVDCH01, Millipore) for 20 s, rinsed with basal solution (0.01 M Tris, 0.15 M NaCl and 0.05% Tween 20) and incubated at 4°C for 10 min with the following primary antibodies: collagen type III (C7805, Sigma) diluted in basal solution with 1% BSA (1:100), collagen type I (C2456, Sigma) diluted in basal solution with 1% BSA (1:500) and glyceraldehyde 3-phosphate dehydrogenase (GAPDH: sc-25778, Santa Cruz), used as an endogenous control, diluted in basal solution with 1% BSA (1:100). The membranes were incubated for 10 min with goat or mouse secondary HRP-conjugated antibody (A0545 and A0412 respectively, both Sigma) diluted (1:100 and 1:500) in basal solution with 1% BSA. After washing in basal solution, peroxidase activity was detected by incubation with a diaminobenzidine (DAB) chromogen (Sigma Chemical Company, St Louis, USA) for 5 min. GAPDH was used as an endogenous control for the comparison among groups. The intensity of the antigen bands in each experimental group was determined by densitometry using the Alpha 4.0.3.2 software (Scion Corporation, USA) and was expressed in arbitrary units.

Zymography for gelatinases

The tension region of normal tendons and the transacted region of the tendons after lesion (n = 4) were washed in PBS (Phosphate-Buffered Saline – 5 mM phosphate buffer, 0.15 M NaCl, and 50 mM EDTA) and cut into pieces. The segments were immersed in a solution of 50 mM Tris-HCl (pH 7.4), 0. 2 M NaCl, 10 mM $CaCl_2$, 0.1% Triton and 1% protease inhibitor cocktail (Sigma) for protein extraction (100 µL of extraction solution for each 30 mg of tissue) at 4°C for 2 h (Marqueti et al., 2006; Silva et al., 2008; Aro et al., 2012a). After this first extraction, the samples were incubated using 1/3 of volume of the same solution described previously at 60°C for 5 min. For the MMP-2 and MMP-9 analyses, 1 µg and 20 µg of proteins per sample, respectively, were applied to the gel. Ten percent polyacrylamide gel electrophoresis containing 0.1% gelatin was performed at 4°C. After electrophoresis, the gels were washed with 2.5% Triton X-100 and incubated for 21 h in a solution of 50 mM Tris-HCl (pH 7.4), 0.1 M NaCl and 0.03% sodium azide at 37°C.

The gels were stained with Coomassie Brilliant Blue R-250 for 1 h. The gels were then washed with a solution containing 30% methanol and 10% acetic acid for observation of the negative bands of protein corresponding to gelatinolytic activity. Additionally, 20 mM EDTA, which inhibited the activity of gelatinases, was used in the incubation buffer as a positive control, confirming the identification of MMPs in the gels. The intensity of the isoforms bands in each experimental group was determined by densitometry using the Alpha 4.0.3.2 software (Scion Corporation, USA).

Statistical Analyses

For the biochemical analysis, data from different experimental groups were analyzed by *t*-test student ($p < 0.05$). The Mann-Whitney test ($p < 0.05$) was used only for analysis of the birefringence measurements, also using the GraphPad Prism® (Graph-Pad Software, La Jolla, CA, USA), version 3.0.

Results

For biochemical analysis, we used the transected region of the tendons with a small extension of the proximal and distal contiguous region that was located adjacent to the transected region (Fig. 1), because only the transected region was not possible to accurately delimit. In the interface of these regions, there is an imbrication between the collagen bundles that were not cut and newly formed fibrils. For morphological analysis, the total length of the calcaneal tendon was used.

Our data related to the collagen fiber relative amount and molecular organization of collagen were obtained from images and measurements of birefringence using polarization microscopy. Our results showed intense birefringence in normal tendons due to the high aggregation and organization of collagen bundles in the proximal region, with the collagen fibers predominantly orientated only in one direction (Fig. 1a). In the transected tendon that was analyzed 7, 14 and 21 days after injury, we observed three distinct regions along the largest axis of the tendon. The areas were characterized as follows: transected region (TR), where the transection of collagen bundles was performed (Fig. 1b); proximal and distal contiguous region of transition (T1), which bordered the ends of TR (Fig. 1b); and transition region (T2), located in the adjacencies of T1 proximal and distal, where the

organization is similar to a normal tendon (Fig. 1f). T1 and T2 are both regions of transition.

In the transected groups 7 days after lesion, we observed the presence of relatively dense fibrils that ran perpendicular to the largest axis of the tendon in both the treated and non-treated groups (Fig. 1b). No birefringence measurements were performed during this period due to very low birefringence observed in both groups. Through image analysis, it was possible to observe that the low birefringence represented by the dark area indicated a complete disorganization of collagen bundles in the transected region. After compensation of the birefringence, fragmentation of collagen fibers was more evident in T1 (Fig. 1c).

On the 14th day after lesion, the birefringence measurements detected higher value in TR and lower value in T1 of the B14 (TR: 6.5; T1: 18.0) compared to Av14 group (TR: 6.3; T1: 23.5) (Table 1). In TR, we observed a more organized collagen matrix compared with the 7th day, although with marked differences in the organization of collagen fibers in TR compared with T1 on the 14th day. Collagen fibrils arranged in various directions, but with crimps, were detected (Fig. g-i), with the addition of fiber fragmentation in T1 (Fig. 1d). There was an imbrication between TR and T1 in the collagen fibers that were not cut and newly formed fibrils throughout the adjacency of TR. The presences of newly formed fibrils in regions of fragmentation of collagen in T1 were observed after birefringence compensation (Fig. 1e). In T2, the organization of collagen was similar to a normal tendon, with presence of fragmentation of the fibers (Fig.1f).

Through image analysis of the groups at 21 days after injury, we found that there is greater organization and aggregation of collagen fibers compared with images of tendons 14 days after injury (Fig. j-l). Measurements showed a gradual increase of birefringence 21 days after injury in both groups B21 and Av21. At this time point, the Av21 group (TR: 9.1; T1: 40.4) presented higher significant value compared with the B21 group (TR: 7.3; T1: 31.8) in TR and T1. We observed more birefringent fibers between TR and T1 of the Av21 group; however, there was lower organization in relation to the normal tendon (77.2 ± 21.8). As observed in the images, fragmentation of the collagen fibers was detected in TR and T1. *Aloe vera* improved the organization of the collagen fibers at this time point, but smaller fibrils in TR were observed along with a non-homogeneous organization of collagen fibers (Fig. 1l).

For the quantitation of hydroxyproline (Fig. 2), which estimates the total collagen content in the tissue (mg/g dry weight), the results showed higher concentrations in the Av7 (68.3 ± 16.0) and Av14 (87.1 ± 8.7) groups when compared with their respective controls (B7: 57.9 ± 6.1; and B14: 48.8 ± 13.1). The Av21 group (56.3 ± 12.4) displayed a lower concentration of hydroxyproline compared with the B21 group (80.9 ± 14.6) 21 days after injury. No transected group reached a value equivalent to the N group (108.5 ± 15.5).

According to the results observed for the type I collagen western blots (Fig. 3) obtained from densitometry (arbitrary unit) analysis of the bands, the Av14 group (0.6 ± 0.3) had less type I collagen than the B14 (4.5 ± 0.9) and N (17.6 ± 3.5) groups. No significant differences were observed between the groups on days 7 (B7: 9.8 ± 2.6; and Av7: 8.8 ± 2.8) and 21 (B21: 1.3 ± 0.2; and Av21: 2.3 ± 0.7) after injury, although the transected groups presented less type I collagen compared with the N group. No significant differences of type III collagen levels were observed between the groups treated with the *Aloe vera* ointment and the non-treated groups. A noteworthy observation was that the transected groups had less type III collagen than N group (Table 2).

MMP-9 zymography showed bands corresponding to the latent (92 kDa) and active isoforms (83 kDa) in groups B7, Av7, B14 and Av14 (Fig. 4). In the densitometry of the bands (pixels), we observed significant differences between the MMP-9 isoforms on the 14^{th} day after injury; smaller amounts of latent and active isoforms in group AV14 (latent: 2287±820; and active: 11,682±124) compared with group B14 (latent: 5131±391; and active: 13,941±868). Neither MMP-9 isoform was present in the N and 21 days groups. When MMP-2 zymography was analyzed, latent (72 kDa), intermediate (68 kDa) and active isoforms (62 kDa) were observed in all groups except in the normal group, which did not show the active isoform of the enzyme. Densitometry of the bands only detected significant differences in the active isoform of MMP-2 between the groups treated with the *Aloe vera* ointment and the non-treated groups during the 3 phases analyzed. On days 7 and 14 after injury, groups Av7 (179,109 ± 42,996) and Av14 (218,850 ± 55,393) had lower values compared with groups B7 (256,276 ± 29,981) and B14 (384,328 ± 46,323). Group Av21 (49,149 ± 2410) displayed a higher value of the active isoform of MMP-2 compared with group B21 (14,741 ± 8380). Higher values of the latent and intermediate MMP-2 isoforms were found in the transected groups when compared with group N) (Table 3).

Discussion

In the present work, we studied the effect of *Aloe vera* on the concentration and organization of some components of the ECM of the calcaneal tendon, which have an important role during healing process. Considering that collagen is the main component in tendons and provides integrity, tensile strength and functionality to tendons, the synthesis, secretion and subsequent organization of collagen play an integral role in wound healing after lesions (Chithra et al., 1998a; Wang et al., 2012). Thus, new strategies that improve collagen synthesis and organization after tendon lesions could be interesting for clinical procedures. Our purpose was to demonstrate the effect of the topical application of *Aloe vera* ointment on the molecular orientation of collagen fibers during the inflammation, proliferative and remodeling phases, and to correlate differences in the organization with changes in the collagen and MMPs content during tendon repair.

Our morphological analysis, obtained through birefringence intensity, showed differences in the degree of aggregation of the collagen fibers along the largest axis of the tendon during the phases of healing. Birefringence is the result of molecular orientation and the fiber packing state and is a useful tool for detecting changes in collagen fiber arrangement (Vidal, 1980; Vidal, 1986; Vidal and Mello, 2010). Seven days after the lesion, our data showed total disorganization of the collagen fibers in the transected region of both plant-treated and non-treated groups, which was shown by the lower birefringence and justified the absence of measurement in these groups. As described in our results, the increment of the birefringence in the distinct regions of the tendon especially on 21st day compared to the 7th and 14th days after the lesion, indicates that the remodeling process is overlapping and more prominent in the remodeling phase. Furthermore, the entire tendon, not only the transected region, undergoes a general remodeling process after injury. This general remodeling process was characterized due to the presence of an imbrication between TR and T1 in the collagen fibers that were not cut and newly formed fibrils throughout the adjacency of TR.

The transected region of both plant-treated and non-treated groups analyzed on the 7th day after the lesion, was characterized as an area under a phagocytosis process and where the damaged collagen fibers are completely digested, as observed by Tomiosso and

colleagues (2009). These changes could have been caused by the inflammatory process that starts immediately after injury (Murphy et al., 1994; Oakes, 2003), by the change in the intensity of biomechanical stress that affects the tissue after injury or both. As our injury model was partial, the remaining portion of the tendon allowed gait execution by the animals, with consequent traction of the calcaneal tendon. Thus, due to traction acting on the tendon, there was a separation of the damaged collagen fibers at the lesion site, and a subsequent deposition of granulation tissue. Studies have shown that there is a formation of granulation tissue in the tendon to stabilize the injured area, which is followed by scar formation in the later stages of the healing process (Favata et al., 2006). The collagen is disorganized in the granulation tissue (Tomiosso et al., 2008), corroborating our results that showed complete disorganization of collagen fibers, especially in the acute phase of the repair process. Importantly, there are relatively dense fibrils that are helically intertwined in the transected area, whose function is related to the reorganization of newly formed collagen fibers in fascicles, as described in rat tail tendons and chordae tendineae (Vidal, 1995; Vidal and Mello, 2009).

As mentioned above, the acute phase of wound healing is characterized by an intense inflammatory process that is marked by the presence of a high amount of fragmented collagen. This collagen fragmentation observed in the transected region may primarily be a result of the action of gelatinases, MMP-2 and MMP-9, as we detected active isoforms of these enzymes 7 days after injury. The plant-treated groups had a higher concentration of hydroxyproline compared with the base-treated groups. We believe that this increment in the hydroxyproline can have relationship with the high amount of fragmented collagen due to the actions of MMPs in the plant-treated group. There is knowledge that collagen degradation starts in the ECM, mainly by gelatinases (Kjaer, 2004) that rapidly degrade denatured collagen and collagen fragments (Collier et al., 1998; Wilhelm et al., 1989) and participate in the ECM turnover induced by tendon injury (Agren, 1994; Madlener, 1998) is well known. Chithra and colleagues (1998c) also observed increment of collagen degradation during skin healing after treatment with the *Aloe vera* extract. Another hypothesis to explain the increment in the hydroxyproline content in groups treated with the plant is the synthesis of other types of collagen, such as types IV (Murphy et al., 2003) V and XII (Thomopoulos et al., 2002; Oshiro et al., 2003).

Although they are present only in small amounts in the tendons, the synthesis of these other types of collagen increases after lesions. Some studies have shown that *Aloe vera* stimulates collagen synthesis during the skin healing process (Chithra et al., 1998a,b,c; Takahashi et al., 2009).

A higher concentration of hydroxyproline was observed in Av14 group in relation to B14 group, which reflected in the birefringence measurements with lower values in the plant-treated group. The combination of these two results indicate that the higher concentration of hydroxyproline/collagen not always mean higher molecular organization, probably because the quantitation of hydroxyproline includes native and denatured collagen, and non-organized collagen fibers. The observation of Western blot suggests that in the time point of 14 days, less intact α-chains of collagen were present in tendons treated with *Aloe vera*. Although *Aloe vera* did not stimulate the activation of MMP-2, the detection of MMP-2 activity shows the involvement in matrix degradation concomitant with the probable action of other MMPs, such as collagenases, which degrade intact and fragmented collagen. Studies have shown that collagenase is increased after tendon injuries, with a peak on the 14^{th} day after injury (Oshiro et al., 2003; Berglund et al., 2011). Fourteen days after injury, the tissue reorganization was still low compared with the normal tendon, although the organization of the collagen fibers was better when compared with day 7 after the lesion. However, other characteristic events occur during the proliferative phase, such as high vascularization, cell migration, synthesis and degradation of matrix components, particularly collagen (Palma et al. 2006; Lin et al., 2004; Liu et al., 1995).

Aloe vera influenced the activation of MMP-9 14 days after injury, resulting in a smaller amount of MMP-9 at this time point, suggesting an anti-inflammatory effect of the plant during tendon healing. MMP-9 is synthesized predominantly by inflammatory cells and this MMP has an important role in the degradation of gelatin and other matrix components (Armstrong and Jude, 2002). According to the literature, MMP-9 is released by neutrophils after their activation by inflammatory mediators present at the site of inflammation (Hasty et al. 1990). Our result corroborates data from Vijayalakshmi and colleagues (2012), which showed an anti-inflammatory effect of the extract of *Aloe vera* that was obtained under conditions similar to those used in this study. We did not observe the presence of MMP-9 in either group 21 days after injury, corroborating studies by

Oshiro et al. (2003) and Armstrong and Jude (2002). These data suggest that MMP-9 participates only in collagen degradation, with peak production of MMP-9 between the 7th and 14th day after injury and a reduction after this period.

The collagen type III plays an important role during the initial phase of the healing process of the tendon. However, we did not observe differences in the concentration of type III collagen in the analyzed periods considering the plant-treated and non-treated groups. According to Chithra et al. (1998b), collagen type III forms scaffolding for an accumulation of collagen type I at the site of injury, participates in the signaling of inflammatory and fibroblasts cells, and participates in the regulation of fiber diameter and organization. After injury, collagen type III has the ability to rapidly form crosslinks to stabilize the repair site (Liu et al., 1995). In the later stages of the healing process, collagen type III is substituted for collagen type I (Abrahamsson, 1991; Oshiro et al., 2003).

While we did not observe quantitative differences in collagen type I between the groups during the time period 21 days after injury, the molecular organization of the collagen fibers was higher in the plant-treated group. The plant-treated group had a noteworthy increased amount of collagen type I on the 21st day, with values equivalent to the base-treated group, after a drastic reduction on the 14th day. The higher collagen organization in this group may have relationship with activation of MMP-2 by *Aloe vera* because there was a higher level of MMP-2 in this group. Previous studies from our laboratory have shown that the presence of active MMP-2 is related to an increased remodeling of the ECM in tendons during different processes (Aro et al., 2012a; Vieira et al., 2012). MMP-2 shows activity against several matrix components, including gelatin and native fibrillar and nonfibrillar collagens, laminin, aggrecan, and vitronectin (Aimes and Quigley, 1995; Woessner and Nagase, 2000; McCawley and Matrisian, 2001) during tissue remodeling.

According to Jung et al. 2009 (Jung et al., 2009), MMP-2 with other MMPs and their tissue inhibitors (TIMPs), participate in the linear and lateral growth of fibrils during the development of the tendon, removing small proteoglycans and fibril-associated collagens, allowing adjacent fibril association and fiber growth. The higher amount of MMP-2 in the plant-treated group compared with the non-treated group, contributed for the better reorganization of collagen bundles in the plant-treated group, in both TR and T1

regions. Probably this reorganization was due to a large number of crosslinks between the collagen fibrils. Chithra et al. (1998b) proved that *Aloe vera* stimulates the crosslink formation between collagen fibers during the tissue repair process (Chithra et al., 1998b,c).

The proximal region of the normal calcaneal tendon, closest to the myotendinous junction, has a strong birefringence caused by the extensive aggregation and high organization of collagen bundles (Vidal, 1995; Aro et al., 2008; Aro et al., 2012b). However, the lower degree of aggregation of collagen fibers along of the proximal region, even 21 days after injury in the *Aloe vera*-treated group, and the presence of regions with fragmented collagen fibers in T1 and T2 indicate an impairment of biomechanical resistance of the calcaneal tendon. During the repair process, the biomechanical resistance diminishes because the structural disorganization of the tendon is related to the inability of the tissue to fully recover collagen fibers with normal diameters (Mello et al., 1975; Matthew and Moore, 1991) and to the precise recovery of the stereo-arrangement of the GAGs in the newly synthesized collagen fibers (Mello and Vidal, 2003). Tomiosso and colleagues (2008) demonstrated that the lower resistance is a consequence of the lower organization of the collagen bundles in the transected tendons.

Conclusion

In conclusion, the present study demonstrates a beneficial effect of topical application of *Aloe vera* ointment on tissue organization, which was observed in the transected area of the tendon 21 days after injury. This effect is supported by an increment of active MMP-2. Thus, further studies will be realized by our laboratory to identify the effect of *Aloe vera* on the activity of collagenases and TIMPs and on the GAG content to find a better understanding of the higher matrix organization observed.

Figures and tables captions

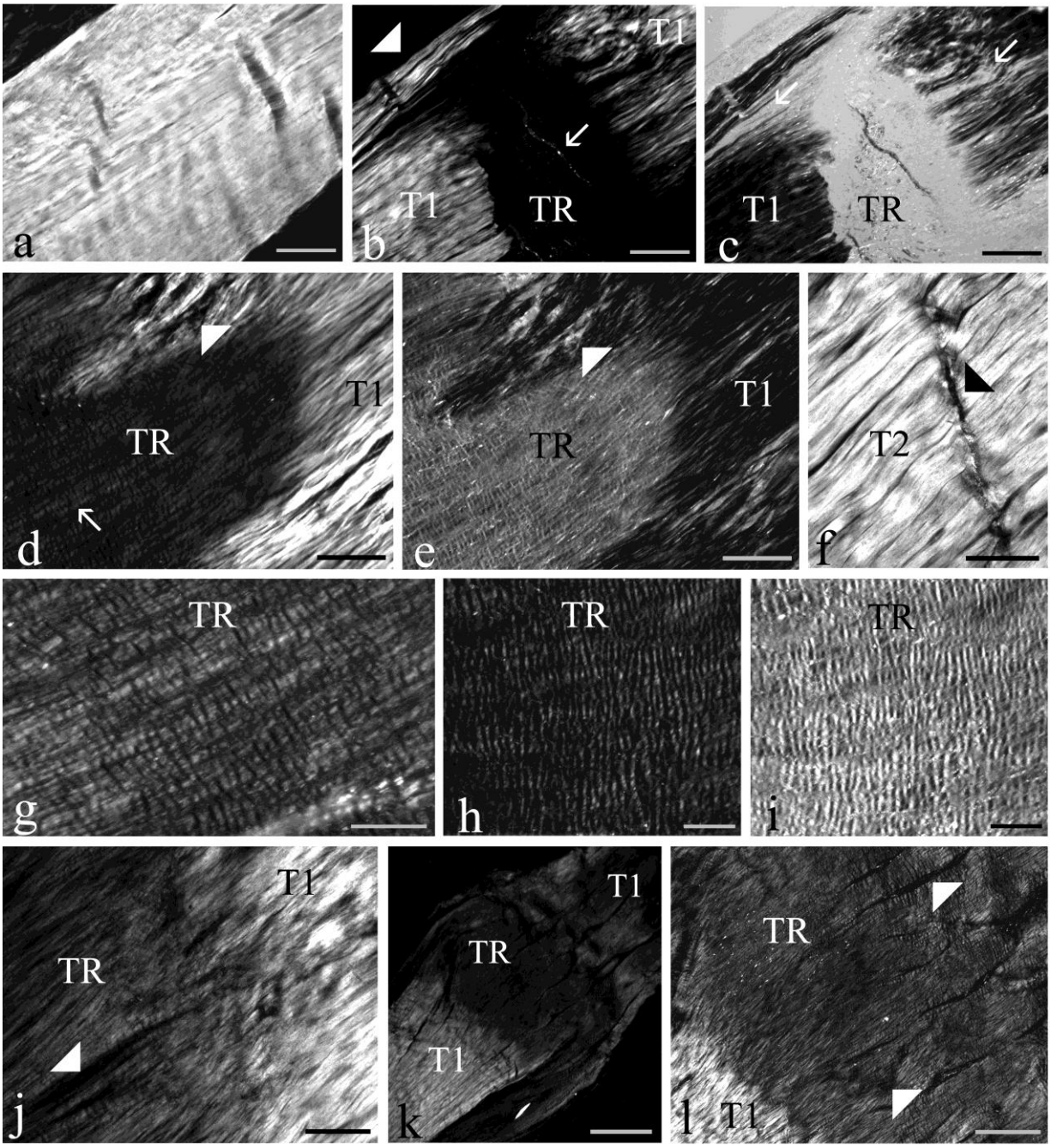

Fig. 1. Images of tendon sections using polarization microscopy. The tendon long axis was set at 45° to the polarizers, except in (h and i) which was set parallel to the polarizers. **(a)** N group: strong birefringence of the collagen fibers is observed; the variation in brilliance intensity (gray levels) indicates fibers presenting different orientations. **(b)** Av7 group: TR was represented by the dark area due a complete disorganization of the collagen fibers. Note the presence of relatively dense fibrils that were helically intertwined and running transversally to the largest axis of the tendon (→); observe T1, TR and the remaining portion of the tendon located below the sectioned region (▶). **(c)** After birefringence compensation, the same image as in (b) displays fragmentation of the collagen fibers in T1

(→). **(d)** Av14 group: observe a marked difference in the organization of collagen fibers in TR and T1. In TR, the collagen fibrils are arranged in various directions and display crimp (→). A fragmentation of the fibers in T1 (▶) and an imbrication between collagen fibers that were not cut as well as newly formed fibrils throughout the adjacency of TR was observed. **(e)** After birefringence compensation, the same image as in (d) shows the presence of collagen fibrils (▶) in the fragmented area indicated in (d). **(f)** Av14 group: T2 where the organization is similar to a normal tendon, with presence of fragmentation of the fibers (▶). **(g)** Av14 group: crimp from the new formed fibrils in the TR can be observed. The most intense birefringence corresponds to fibers that were at 45° to the polarizers, while differently oriented fibers show less intense birefringence brilliance. **(h)** Av14 group: distinct crimp images in the TR is shown when the largest axis of the tendon was set parallel to the polarizers, indicating that the orientation of the collagen fibers varies along the tendon axis. Therefore, the complete extinction of all birefringence was never achieved by rotating the largest axis of the tendon. **(i)** After birefringence compensation, the same image as in (h) shows a helical organization of the fibers. **(j)** B21 group: the presence of collagen fiber fragmentation in TR and less intense birefringence can be observed compared to the Av21 group (k and l). **(k)** Av21 group: a panoramic view of the tendon showing the non-homogeneous organization of collagen fibers along the TR and T1 regions. **(l)** Av21 group: the imbrication between newly formed collagen fibers in TR and T1, and the presence of fibrils more birefringent in both regions can be observed; note the presence of fragmentation of the collagen fibers (▶). Images of base-treated groups were not shown because they were similar to the plant-treated groups on the 7th and 14th day after injury. Bars = 300 µm (k), 150 µm (b, c), 120 µm (a, l), 60 µm (j), 30 µm (d, e, f, g, h and i).

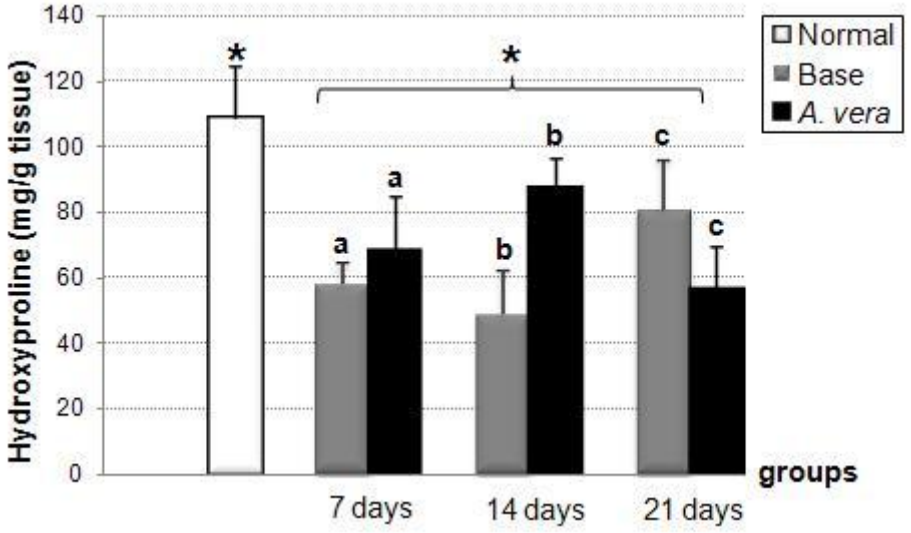

Fig. 2. Concentration of hydroxyproline (mg/g dry weight) in the tendons from the different groups. Significant differences ($p<0.05$) were observed between the groups treated with base and *A. vera* at 7, 14 and 21 days after the transection of the tendon. The hydroxyproline levels of the transected tendons were lower than the levels of the normal tendon (*). The same letters correspond to significant differences between the groups with transected tendons.

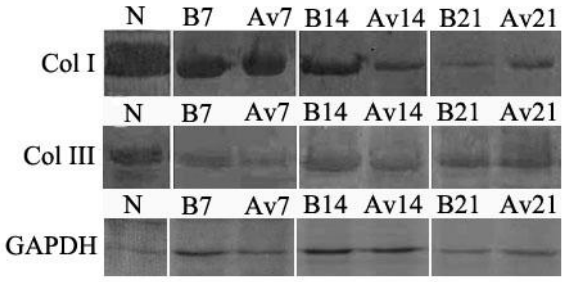

Fig. 3. A Western blot showing type I and type III collagen in the tendons from the different groups. GAPDH was used as an endogenous control. For the significant differences between the groups, see the band densitometry analysis in Table 2.

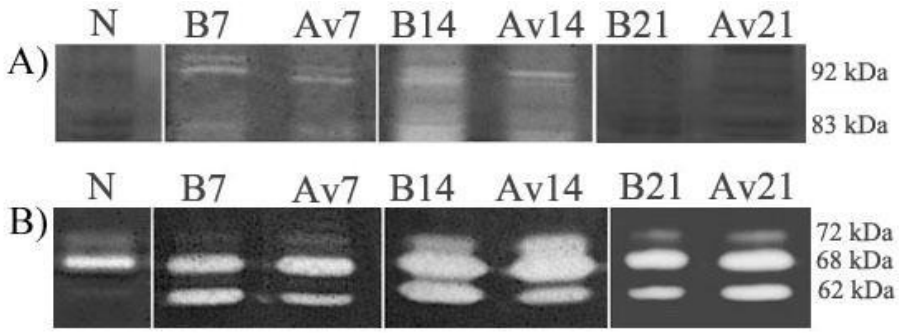

Fig. 4. A) Zymography of MMP-9 for the analysis of the latent (92 kDa) and active (83 kDa) isoforms. MMP-9 was present in all groups on days 7 and 14 after injury; more prominent bands corresponding to the latent and active isoforms in group B14 compared with the group Av14 were observed. B) Zymography for the analysis of MMP-2 latent (72 kDa), intermediate (68 kDa) and active isoforms (62 kDa) in the experimental groups. The three isoforms of the enzyme were present in all groups; the bands corresponding to the active isoform in the Av7 and Av14 groups were less intense than the B7 and B14 bands. On the 21st day after injury, the Av21 group displayed a band that corresponded to the active isoform and was more intense compared with the band of the B21 group. The densitometry below showed significant differences between the groups (Table 3).

Groups	TR (GA median)	T1 (GA median)	Comparisons	Mann-Whitney test (p)
N	73.8	73.8		
B14	6.5	18.0	B14 x Av14 (TR)	(0.000)*
Av14	6.3	23.5	B14 x Av14 (T1)	(0.000)*
B21	7.3	31.8	B21 x Av21 (TR)	(0.000)*
Av21	9.1	40.4	B21 x Av21 (T1)	(0.021)*

GA: Gray Average. The largest axis of the tendon was positioned at 45° with respect to the crossed polarizers. The number of measurements (100) chosen at random in 12 sections from four tendons of each group. (*) Significant differences between the transected groups (p<0.05).

Table 1 Birefringence measurements: GA (pixels) variability in collagen fibers organization on the TR and T1 regions of the calcaneal tendon. Observe on the 14th day after lesion, higher value in TR and lower value in T1 of the B14 compared to Av14 group. Note a gradual increase of birefringence 21 days after injury in both groups B21 and Av21. At this time point, the Av21 group presented higher significant value compared with the B21 group only in TR.

Parameters	N	B7	Av7	B14	Av14	B21	Av21
Collagen type I	17.6 ± 3.5	9.8 ± 2.6*	8.8 ± 2.8*	4.5 ± 0.9 a,*	0.6 ± 0.3 a,*	1.3 ± 0.2 *	2.3 ± 0.7 *
Collagen type III	9.4 ± 2.0	4.1 ± 2.2*	3.1 ± 1.2*	3.8 ± 1.1*	2.5 ± 0.8*	2.8 ± 1.2*	3.0 ± 0.4*

(*) Significant differences between the normal and the transected tendons ($p<0.05$); (a) Significant differences between the groups marked with the same letter ($p<0.05$).

Table 2 Densitometry of the bands (arbitrary units) corresponding to type I and type III collagen at 7, 14 and 21 days after injury. Group Av14 ([a]) had less type I collagen than the B14 ([a]) and N (*) groups. Groups B21 and Av21 had less type I collagen compared with group N. When compared with group N, the transected groups had a lower level of type III collagen.

Parameters	N	B7	Av7	B14	Av14	B21	Av21
MMP-9 latent (92 kDa)	0	3783 ± 1195*	2877 ± 1017*	5131 ± 391 a,*	2287 ± 820 a,*	0	0
MMP-9 active (83 kDa)	0	6044 ± 2061*	5286 ± 2150*	13,941 ± 868 b,*	11,682 ± 124 b,*	0	0
MMP-2 latent (72 kDa)	3746 ± 50	57,961 ± 15,327*	40,256 ± 4733*	132,622 ± 30,211 *	219,930 ± 48,830*	34,256 ± 9494 *	47,175 ± 8776 *
MMP-2 intermediate (68 kDa)	36,240 ± 976	269,000 ± 23,086*	281,437 ± 20,227*	434,907 ± 48,994*	453,262 ± 17,108*	110,809 ± 11,253*	95,198 ± 22,005 *
MMP-2 active (62 kDa)	0	256,276 ± 29,981 c,*	179,109 ± 42,996 c,*	384,328 ± 46,323 d,*	218,850 ± 55,393 d,*	14,741 ± 8380 e,*	49,149 ± 2410 e,*

(*) Significant differences between the normal and the transected tendons ($p<0.05$); ($^{a, b, c, d, e}$) Significant differences between the groups marked with the same letter ($p<0.05$).

Table 3 Densitometry of the bands (pixels) corresponding to MMP-9 and MMP-2 at 7, 14 and 21 days after injury. We did not observe significant differences between the MMP-9 experimental groups 7 days after injury. On the 14th day after injury, lower values were found in the Av7 group than in the B7 group for the latent (^{a}) and active isoforms (^{b}) of MMP-9. No isoform of MMP-9 was observed in the B21 and Av21 groups. Differences were found between the base- and plant-treated groups for the active isoform (*) of MMP-2 on days 7 and 14 after injury, with the lowest values for groups Av7 and Av14. On day 21 after injury, a higher value was observed for the Av21 group compared with the B21 group. Higher values for the three MMP-2 isoforms were found in the transected tendons when compared with normal tendons.

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Effect of the *Arrabidaea chica* extract on the collagen fibers organization during the healing of partially transected tendon

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Abstract

After lesions, tendons present decreased collagen fiber organization compared to undamaged tendons. The leaves of the *Arrabidaea chica* have great pharmacological potential due their healing properties. Thus, the aim of this study was to investigate the effect of topical application of crude *A. chica* extract during tendon healing. The extract was standardized according to the presence of carajurin and carajurone. The calcaneal tendon of male Wistar rats was partially transected with subsequent treatment with *A. chica* extract (2.13 g/mL in 0.85% saline solution) followed by excision on the 7th, 14th and 21st days; control rats received only saline treatment. Transmission electron microscopy analysis showed the presence of a large amount of small collagen fragments in the transected region of the tendons on the 7th day in the saline- and plant-treated groups. With respect to the organization of the collagen fibers, higher values of birefringence were observed under polarization microscopy in the tendons of the plant-treated group on the 14th day compared to the saline-treated group. A larger quantity of dermatan sulphate was also detected after plant treatment in the same period. However, on the 21st day, lesser dermatan and chondroitin sulphate were detected in the plant-treated group than in the saline-treated group. No differences were found in the birefringence between the groups. In addition, intense metachromasy was observed in both transected groups on the 21st day. In conclusion, the use of *A. chica* extract improves collagen organization and increases the quantity of DS on the 14th day of the healing.

Keywords: calcaneal tendon, healing, organization, *Arrabidaea chica*, collagen.

INTRODUCTION

Tendons are resistant structures that are constantly subjected to high biomechanical loads, thus becoming prone to acute and chronic injuries (Zhang and Wang, 2010). The incidence of tendon ruptures has increased dramatically due to an increase in sports participation by the general population (Moller et al., 1996; Jozsa et al., 1989). However, other factors can cause tendon lesions, such as age (Dudhia et al., 2007; Esquisatto et al., 2007; Caroll et al., 2008; Nakagaki et al., 2010), diabetes (Bedi et al., 2010; de Oliveira et al., 2010; Fox et al., 2011) and hormones (Cook et al., 2007; Soo et al., 2011). Studies have shown that healed tendons present decreased collagen fiber organization compared to intact tendons (Park et al., 2010), with a high prevalence of re-rupture due to the formation of a fibrotic scar, restrictive adhesions, and suboptimal functionality after treatment of tendon lacerations (Kleinert et al., 1973; Morberg et al., 1997; Józsa and Kannus, 1997).

Although several treatments for tendon lesions have been proposed in recent years, some clinical approaches remain controversial (Okamoto et al., 2010). Surgical treatment has been described with advantages including low re-rupture rates (Lea and Smith, 1972; Möller et al., 2001; Carden et al., 1987), good final range of motion (Gigante et al., 2008; Cetti et al., 1993), short recovery time (Möller et al., 2001; Gigante et al., 2008), and a good final outcome without muscle atrophy (Gigante et al., 2008; Cetti et al., 1993). Non-surgical treatment methods report better cosmetic appearance (Lea and Smith, 1972; Gigante et al., 2008), reduced risk of infection (Beskin et al., 1987; Carden et al., 1987) and no risk of suture granulomas (Beskin et al., 1987; Fierro and Sallis, 1995). Despite the existence of several treatments, tendon injuries are a considerable socioeconomic problem (Dyment et al., 2012) that can affect people of all ages.

Tendons are formed by an abundant extracellular matrix (ECM) composed mainly of type I collagen bundles (Birk et al., 1996), proteoglycans (PGs) (Vogel and Heinegård, 1995), non-collagenous proteins (NCPs) (Fredrick et al., 2007; Józsa and Kannus, 1997), cells and metalloproteinases (MMPs), which are responsible for tissue remodeling (Magra and Maffulli, 2005). These components, especially collagen and PGs, are arranged to form supramolecular structures (Vidal and Mello, 1984) that confer the biomechanical properties of different regions of this tissue. The presence of large amounts of cross-linked collagen molecules confers tensile strength to tendons and makes them highly resistant to

mechanical stress (Kjaer, 2004). These molecules have intermolecular interaction sites located at regular intervals along the collagen molecules and along the fibrils (Miller, 1985). These interactions are determined by the primary amino acid sequence, with the sites involved being rich in polar and hydrophobic residues (Hofmann et al., 1978). After tendon lesions, a mechanism called mechanotransduction is initiated wherein mechanical stress acts on the cell and starts an intracellular signaling program that promotes growth and cellular survival (Vidal, 1994). Thus, the structural, biochemical and even biomechanical properties of this tissue are not completely recovered after lesions (Park et al., 2010; Kleinert et al., 1973; Morberg et al., 1997; Józsa and Kannus, 1997). Clearly, alternative methods need to be developed to improve the efficacy of tendon repair.

For many centuries, plant extracts have been employed to treat diseases, and their active principles have great pharmacological potential. With the aim of improving tendon healing, some plants such as *Hippophae rhamnoides* (Fu et al., 2005), pineapple fruit parts (Aiyegbusi et al., 2010), safflower yellow (Liu et al., 2011) and bromelain and fresh pineapple juice (Aiyegbusi et al., 2011) have been studied. The *Arrabidaea chica* (Humb. & Bonpl.) B. Verlot. syn. *Bignonia chica* (Humb. & Bonpl.), (Bignoniaceae) is a liana widely distributed in the Neotropics (Borroso, 1986; Cronquist, 1988; Glyn et al., 2010). The leaves of this plant have been used in folk medicine for wound healing, the treatment of inflammation, intestinal colic, sanguine diarrhea, leucorrhoea and anemia (Zorn et al., 2001; Jorge et al., 2008). In the present study, the effect of the *A. chica* leaf extract was investigated during tendon healing. For this experiment, the crude *A. chica* leaf extract was standardized according to Jorge et al. (2008) and included the aglycones carajurin and carajurone. These compounds are anthocyanins, which are phenolic water-soluble pigments that are released after acid hydrolysis (Lima et al., 2006). Scientific interest in these pigments has recently increased due to the effective bioactivity of anthocyanins and their aglycones (Lima et al., 2006) as anti-inflammatories (Seeram et al., 2001), antioxidants (Kähkönen and Heinonen, 2003) and vaso-dilators (Burns et al., 2000), among other biological properties.

According to Jorge et al. (2008), the crude extract of *A. chica* improved skin healing, with increased collagen production. These data corroborate a previous study performed in our laboratory (Aro et al., 2012a) in which a higher total collagen

concentration was detected in injured tendons after treatment with this plant extract. Antimicrobial (Höfling et al., 2010) and anti-inflammatory (Zorn et al., 2001) potentials of the leaf extract of *A. chica* have also been identified. However, there is no study showing the effect of *A. chica* on collagen organization. Because tendon injuries are a clinical issue that requires innovative treatment solutions, we have proposed to improve the organization of collagen fibers by the topical application of *A. chica* extract during the tendon healing.

MATERIALS AND METHODS

Plant material and extraction

The lyophilized extract of the *A. chica* plant utilized in our study was provided by CPQBA (Multidisciplinary Center for Chemical, Biological and Agricultural Researches) – UNICAMP, after standardization of the aglycones according to Jorge et al. (2008). Leaves of *A. chica* (Humb & Bonpl) Verlot available in the Germplasm Bank (from acess 06) were collected from the Center for Chemical, Biological and Agricultural Researches of UNICAMP, located in the city of Paulínia (22º45'00" South and 47º10'21" West). The plant material was ground with dry ice in a grinder of knives, Stephen brand, model UM 40. The ground leaves were extracted three times with a mixture of 1:5 (v/v) ethanol/0.3% citric acid. The extracts were filtered, dried with anhydrous Na_2SO_4 and filtered, and the solvent was removed in a vacuum, followed by lyophilization. The lyophilized extract was dissolved in saline solution (0.85%) at a concentration of 2.13 g/ml for application to the lesion site on each tendon, as described below.

Protocols for the partial transection of the calcaneal tendon and topical application of the *A. chica* extract

For surgical procedures, the animals were anesthetized with intraperitoneal injection of Ketamine (80 mg/kg) and Xylazine (10 mg/kg), and the lower right paws underwent trichotomy and antisepsis with iodine alcohol. A longitudinal incision was made in the skin to expose the Achilles tendon, and the transverse partial transection was performed in the tension region of the tendon that is located at an approximate distance of 4 mm from the tendon's insertion into the calcaneus bone (Murrell et al, 1997; Tomiosso et al, 2009). We dissolved 32 mg of the *A. chica* in 15 µL of 0.85% saline solution and applied the solution at the site of the tendon transection prior to the suture of the skin. After application of the

extract, the skin was sutured with nylon suture (Shalon 5-0) and needle (1.5 cm). From day 1 after surgery, we performed a daily topical application of the plant extract in the region of the sutured skin, gently massaging for its absorption. Due to the high penetrating power of the *A. chica* extract, this procedure was repeated until the 7th day after the surgery for a total of 7 topical applications. This same procedure was utilized for the transected tendons of controls that received applications of 15 μL of 0.85% saline solution. For the immobilization of the animals during the daily topical applications, a retainer rat was used (Request Privilege of Utility Model in Brazil, titled: Retainer of rats for laser beam and biocompounds application, deposited in National Institute of Industrial Property – INPI, on 04.16.10, under n° MU9000622-4, authored by researchers Andrea Aparecida de Aro and Edson Rosa Pimentel, from Institute of Biology, UNICAMP).

Experimental groups

Sixty eight-day-old male Wistar rats (n = 154, weighing 250 ± 20 g), with free access to food and water, were used in the present study. The animals were separated into 7 experimental groups: the normal group (N) – rats with tendons without transection and euthanized at age eighty-two-days-old; the S7, S14 and S21 groups **-** rats with tendons treated with topical applications of saline for 7 days and euthanized on the 7th, 14th and 21st days after surgery, respectively; and the A7, A14 and A21 groups **-** rats with tendons treated with topical applications of the plant extract for 7 days and euthanized on the 7th, 14th and 21st days after surgery, respectively. This study was performed according to the Institutional Committee for Ethics in Animal Research of the State University of Campinas – Unicamp (protocol n° 1621-1).

Birefringence: Image Analysis and Measurements

The tendons (n = 4) were fixed using 4% formaldehyde solution in Millonig's buffer (0.13 M sodium phosphate, 0.1 M NaOH, pH 7.4) for 24 hr at 4°C. Then, the tendons were washed in water, ethanol dehydrated, diaphanized with xylene, and paraffin embedded. Longitudinal serial sections of 7 μm were obtained and after deparaffinization were subjected to microscopy analysis. Image analyses of the tendons were evaluated to detect differences in its morphology, considering the aggregation and organization of the collagen bundles, which reflect in the variation of the birefringence intensity. Birefringence

properties were studied by using an Olympus BX53 polarizing microscope and an image analyzer (Life Science Imaging Software, Version 510_UMA_cellSens16_Han_en_00). Since birefringence appears visually as brilliance, this phenomenon was measured with the image analyzer and expressed as gray average (GA) values in pixels, after its calibration (8 bits = 1 pixel). The largest axis of the tendon was positioned at 45° to the crossed analyzer and polarizer during the measurements. Considering that collagen bundles exhibit two kinds of birefringences: intrinsic birefringence (Bi) and form or textural birefringence (Bf) (Vidal, 1980; Vidal, 1986), total birefringence (sum of Bi and Bf) was used in this study. Sixty GA measurements of the transected regions of the tendons in each experimental group were made after immersing the sections in water, a condition in which total birefringence is highly detectable (Mello et al, 1979; Vidal, 1965; Vidal et al, 1975; Vidal, 1980; Vidal, 1986; Vidal and Mello, 2010). The measurements of GA were represented as arithmetic mean and standard deviation.

Transmission Electron Microscopy

The transected region of the calcaneal tendon from each group and the tension region of normal tendons (n = 3) were immersed into fixative solution containing 5% glutaraldehyde and 0.25% tannic acid in Millonig's buffer (0.13 M sodium phosphate, 0.1 M sodium hidroxide, 0.03 M glucose - pH 7.4) for 24 h at room temperature. The samples were washed with Millonig's buffer, post fixed in 0.5% osmium tetroxide in the same buffer for 1 h at room temperature. After washing in water rapidly, the samples were acetone dehydrated and embedded in Epon® resin. Ultrathin sections (60-80 nm) were stained with 3% uranyl acetate for 30 min. and 0.2% lead citrate in 0.1 N sodium hidroxide for 5 min. and observed under an LEO 906 transmission electron microscope (Zeiss, Jena, Germany) operated at 80 kV. The images were captured with a digital imaging system for documentation.

Light Mycroscopy Analysis

After dissection, the tendons (n = 4) were fixed using a 4% formaldehyde solution in Millonig's buffer (0.13 M sodium phosphate, 0.1 M NaOH - pH 7.4) for 24 h at 4°C. The

tendons were then washed in water, ethanol dehydrated, diaphanized with xylene and paraffin-embedded. Longitudinal serial sections of 7 µm were obtained for microscopy analysis. For observation of PGs, the sections were deparaffinized and then were stained with 0.025% toluidine blue (TB) in McIlvaine buffer (0.03M citric acid, 0.04M sodium phosphate dibasic - pH 4.0) (Mello and Vidal, 2003). The sections on slides were air dried and immersed in xylene, before embedding in entellan (Merck, Rio de Janeiro, Brazil). For observation of morphology of the tendons, tissue sections were analyzed under an Olympus BX53 microscope.

Quantitation and characterization of sulfated glycosaminoglycans

After washing in PBS (Phosphate-Buffered Saline – 5 mM phosphate buffer, 0.15 M NaCl and 50 mM EDTA), fragments from the tendons (n = 4) were immersed into acetone for 12 h and dried in a histological oven (37°C) for 12h. GAGs were extracted with papain solution (40 mg/g tissue) in 100 mM sodium fosfate buffer, pH 6.5, containing 40 mM EDTA and 80 mM β-mercaptoethanol for 24 h at 50°C. After precipitation using 90% TCA (trichloroacetic acid) for 10 min. at 5°C, the samples were centrifugated and submitted to precipitation with methanol during 12 h at 5°C. The precipitate was resuspended in water and used for the measurement of GAGs (Farndale et al., 1986). Before the electrophoresis, 5 µg of each sample was treated with DNAse (10 mg/mL) in 20mM Tris-HCl buffer, pH 7.4 for 30 min. at 37°C. After that, the sulfated GAGs chondroitin (CS), dermatan (DS) and heparan sulfate (HS) were separated by electrophoresis in agarose gel (0.5%) in 0.05M propylene diamine (Dietrich and Dietrich, 1976), at 0.1 mA for 45 min. The agarose gels were fixed in cetavlon and stained with 0.2% toluidine blue. The gels were washed with a solution containing 50% ethanol and 1% acetic acid for observation of bands. The identification of GAGs was confirmed by digestion with chondroitinases B and AC. In addition, the DS and CS were individually quantified in agarose gel by densitometry of bands using the Scion Image software Alpha 4.0.3.2 (Scion Corporation, Frederick,MD, USA) and by comparison with a standard of 5 µg of each GAG provided by Sigma (Sao paulo, Brazil).

Statistical Analyses

For the biochemical analysis, data from different experimental groups were analyzed by *t*-test student ($p < 0.05$). The Mann-Whitney test ($p < 0.05$) was used only for analysis of the birefringence measurements, also using the GraphPad Prism® (Graph-Pad Software, La Jolla, CA, USA), version 3.0.

RESULTS

Analyses of images obtained from polarization microscopy showed intense brightness in normal tendons due to the high aggregation and organization of collagen fibers (Fig. 1a). The transected region of injured tendons (TR) and the proximal and distal regions (T1) that border the TR were analyzed as shown in Figure 1. On the 7th day, no birefringence measurements were performed due to the low birefringence observed in the TR of groups S7 and A7, indicating complete disorganization of the ECM at this time (Fig. 1b and c).

On the 14th day after lesion, the birefringence measurements detected higher values in the TR of the A14 (88.6 ± 15.4) compared to the S14 (65.9 ± 15.0) groups (Table 1), indicating that *A. chica* improved the organization of the collagen fibers at this time. It is important to note the presence of fiber fragmentation in T1 (Fig. 1d and e). There was an imbrication between TR and T1 in the collagen fibers that were not cut, and there were also newly formed fibrils throughout the region adjacent to the TR (Fig. 1f). At 21 days after injury, a greater organization and aggregation of collagen fibers compared with images of tendons 14 days after injury was observed (Fig. 1g, h and i). At this time, measurements showed a gradual increase of birefringence compared to 14 days, with no differences between groups S21 (101.2 ± 20.0) and A21 (106.8 ± 26.0). As observed in the images, fragmentation of the collagen fibers was detected in TR and T1 even 21 days after lesion, with values of birefringence lower than the normal tendon (Table 1).

Ultrastructural analysis (Figure 2) showed highly organized longitudinal collagen fibers in the normal group, with the presence of striations in the form of uniform pattern of bands (Figure 2a). This pattern occurs because of the oriented aggregation of collagen molecules during the formation of each collagen fiber. In groups S7 and A7 on the 7th day

after the injury, the collagen fibers were completely disorganized, as shown by the fibrils arranged in various directions in TR (Figure 2b,c). It was possible to observe the presence of small collagen fragments in the ECM of TR in both groups on the 7th day; these were likely to be the remnants of old fragments (Figure 2b,c). Regions of matrix lacking fibers were observed in S7 and A7 groups. The cells presented well-developed endoplasmic reticulum and secretory vesicles in their cytoplasm, indicating a high level of matrix component synthesis. In A7 group, a endoplasmic reticulum with enlarged cisternae was observed, probably involved in the large amount of procollagen production (Figure 3a).

The micrograph analyses on 14th day (Figure 2d,e) revealed a greater number of newly formed collagen fibrils in TR, which were more organized in the plant-treated group than in the saline-treated group. It was still possible to observe the presence of small collagen fragments in A14 group (Figure 2e), as well as the presence of more elongated fibroblasts and fibers longitudinally and transversely oriented. On the 21st day after lesion (Figure 2f,g), the presence of small fragmented collagen was diminished compared to earlier phases. The longitudinally oriented collagen fibrils were more prominent, with no apparent differences between groups S21 (Figure 2f) and A21 (Figure 2g). Endoplasmic reticulum with enlarged cisternae was detected in S21 group (Figure 3b). In the A21 group, the plasma membrane cleft was evidenced (Figure 3c).

The longitudinal sections of the calcaneal tendons stained with toluidine blue showed a high cellularity and an increased number of blood vessels in the TR of groups S7 and A7, with low metachromasy in both groups (Fig. 4b and c). The cellularity was also high in the 14 day groups, with greater orientation of the matrix than at 7 days post-injury. Apparently, no differences in metachromasy were observed between the saline- and plant-treated groups on 14th day (Fig. 4d and e). On the 21st day, the metachromasy was higher in both groups compared to earlier phases, and the cellularity was still high (Fig. 4g and h). It is important to mention that in the N group, the matrix was not stained because of the absence of PGs. Only the nuclei were stained in the sections of normal tendons.

The total sulfated GAGs quantitation (mg/g tissue) showed that all groups of transected tendons showed higher concentration of these components compared to N group, but without significant differences between the saline and plant-treated groups (Tabel 2). Densitometry analysis of the bands (pixels) obtained from the electrophoresis (Fig. 5)

showed an increase of DS in A14 group (679.8 ± 95.1) compared to S14 group (403.7 ± 10.4). On the 21st day, A21 group had lower amounts of DS (747.8 ± 69.3) and CS (220.1 ± 18.3) compared to S21 group (SD: 958.9 ± 85.1; CS: 322.1 ± 54.6). Considering the amounts of DS and CS, with the exception of S21 group, all groups showed lower values than N group.

DISCUSSION

Tendon healing is a slow and complex process that occurs in three phases called the inflammatory, proliferative and remodeling phases (Dyment et al., 2012; Marsolais et al., 2003; Wojciak and Crossan, 1993; Garner et al., 1989; Lee et al., 2003; Chen et al., 2004; Hoppe et al., 20120; Bedi et al., 2012; Berglund et al., 2011). Our purpose was to study the effect of the topical application of the *A. chica* extract on the collagen fibers organization during these three phases of the tendon repair. On the 7th day after injury, the tendon sections showed remarkable increases of cellularity and an increased number of blood vessels at the transected area in both the saline- and plant-treated groups. It is known that the inflammatory phase that begins immediately after injury and peaks on day 7 is characterized by an increased number of blood vessels and the migration of synovial and inflammatory cells and monocytes/macrophages to the site of tendon repair (Sahin et al., 2012; Marsolais et al., 2003; Wojciak and Crossan, 1993). Fibroblasts and myofibroblasts that participate in tendon healing may arise from the tendon, epitendon, paratendon, or from a combination of these (Garner et al., 1989; Le et al., 2003). This cellular migration and proliferation are due to the action of cytokines and growth factors produced from platelets and macrophages (Berglund et al., 2011; Chen et al., 2004; Hope et al., 2012; Bedi et al., 2012). These events in the early phase of tendon healing have an important role in increasing the metabolism of cells, as evidenced by the presence of well-developed rough endoplasmic reticulum and of secretory vesicles observed in the micrographs of both groups on the 7th day. The increase in cellular metabolism at this phase is targeted towards the synthesis and degradation of matrix molecules to provide a temporary fragile structure at the repair site (Dyment et al., 2012).

The ultrastructural results showed the presence of small collagen fragments in the ECM of the transected region in both groups on the 7th day. The fragmentation of collagen can be directly related to degradation by the metalloproteinases (MMPs) that are increased in this phase (Oshiro et al., 2003; Sahin et al., 2012; Choi et al., 2002; Berglund et al., 2011). Recent work performed by our laboratory showed similar quantities of the active isoform of MMP-2 and MMP-9 in both the saline- and plant-treated groups, indicating the participation of these MMPs in the collagen fragmentation observed in this period (Aro et al., 2012a). MMP-2 and MMP-9 degrade several types of collagen, gelatin and other non-collagenous components of the ECM (Chakraborti et al., 2003) and are involved in remodeling (MMP-2) and inflammatory (MMP-9) processes (Aro et al., 2012b; Vieira et al., 2012; Oshiro et al., 2003). Therefore, concomitant with the action of MMPs, inflammatory cells and macrophages act on the removal of cell debris (Siddiqi et al., 1992; Tomiosso et al., 2009) and fragmented and denatured collagen. Other matrix components are also removed in this early stage of the healing process, resulting in regions without the presence of matrix, as observed in the micrographs of both groups 7 days after lesion.

The proximal region of the calcaneal tendon which is predominantly subjected to tensional forces, is especially composed of collagen type I highly organized into fibrils, fibers, bundles of fibers and fascicles (Kjaer, 2004; Aro et al., 2008; Aro et al., 2008c), forming a complex supramolecular structure (Vidal and Mello, 2010). In this region, the collagen bundles are organized in a helical arrangement along the largest axis of the tendon (Vidal, 1986; Liu et al., 1995; Vidal, 2003) and the fibers are highly oriented and aggregated, exhibiting strong birefringence (Vidal and Carvalho, 1990). On the 7th day after lesion, our results showed a completely disorganized ECM and low orientation of collagen fibers, justifying the absence of birefringence measurements in this period. Our ultrastructural results showed that the low birefringence is due to the presence of a disorganized network composed of newly-formed thin fibrils and to a large amount of old remnant collagen fragments which were still not removed in this early phase of repair. It was also possible to observe the presence of two different populations of small collagen fibrils with different diameters and without a regular orientation in the saline- and plant-treated groups.

On the 14th day after the injury occurs the peak of the proliferative phase (Oshiro et al, 2003; Tomiosso et al, 2009). The morphological and ultrastructural analyses showed a more compact granulation tissue in this phase, with fibroblasts more regularly arranged along the largest axis of the tendon. According to previous results from our laboratory (Aro et al., 2012a), the granulation tissue is formed by collagenous and non-collagenous proteins, MMPs, PGs, cells and new capillaries that were initially formed during the inflammatory phase, as also shown by several other studies (Dyment et al., 2012; Wojciak and Crossan, 1993; Garner et al., 1989; Berglund et al., 2011; Choi et al., 2002; Lui et al., 2012; Thomopoulos et al., 2002). During the proliferative phase, the cells still have high metabolic activity, as evidenced by the presence of the well-developed rough endoplasmic reticulum observed in the micrographs.

Our birefringence measurement data showed differences in the degree of aggregation of the collagen fibers along the largest axis of the tendon on the 14^{th} day after lesion. The collagen fibers in the transected region of the group treated with the plant extract showed higher birefringence compared to the saline-treated group, indicating greater organization of these fibers. The higher molecular orientation of collagen fibers observed in this group may be related to differences in the amounts of PGs. Our results showed large quantities of chondroitin sulphate (CS) and dermatan sulphate (DS) after plant extract treatment. Fibromodulin and lumican are PGs containing keratan-sulfate (KS), while decorin and biglycan are both chondroitin/dermatan sulfate PGs belonging to the extracellular matrix family of small leucine-rich PGs (Esquisatto et al., 2007; Viola et al., 2007). The small PGs are altered during the healing process (Mello et al., 1975; Mello and VIdal, 2003; Thomopoulos et al., 2002; Lui et al., 2012; Liang et al., 2008) and are intimately associated with collagen fibers to regulate the fibrillogenesis process in the tendons (Zhang et al., 2006; Lo et al., 2004; Ezura et al., 2000; Liang et al., 2008) and restore the matrix structural organization. In addition to the small PGs that are characteristic of the regions subjected to tensional forces (Vogel and Heinegard, 1985; Waggett et al., 1998; Rees et al., 2000) as in the proximal region of the calcaneal tendon where the transection was performed in the present study, the large aggregating PG aggrecan may also be found in tensional regions of tendons (Vogel et al., 1994; Rees et al., 2000, 2005, 2007).

Thus, the higher amounts of DS observed in the group treated with the *A. chica* extract suggest alterations in the proportions of different PGs bearing DS.

During the beginning of the remodeling phase on 21 days after lesion, the recess in the fibroblast plasma membranes observed mainly in the group treated with the plant is called a cell plasma membrane cleft, which is a specialized extracellular compartment (Sprague et al., 2011). Within these clefts, newly synthesized procollagen is cleaved into tropocollagen (Birk and Trelstad, 1986), which will be incorporated into the expanding collagen fibers (Birk et al., 1989; Franchi et al., 2007). The collagen fibrillogenesis is remarkable during this period compared to the earlier phases and can be evidenced by the presence of regions with unidirectional organization of collagen fibers.

In this phase, the results showed that the *A. chica* extract did not influence the collagen fiber organization in the transected area. No significant differences were observed in the birefringence measurements between the saline- and plant-treated groups. This result is interesting because higher birefringence in this group was expected on the 21st day, as a higher value was observed 14 days after injury. However, this result corroborates with the lesser amount of DS and CS observed in this group after plant treatment on the 21st day. The small leucine-rich PGs regulate the fibrillogenesis of type I collagen due to their association with the collagen fibers and thereby regulate the structural organization of the tissue (Vogel et al., 1984; Linsenmayer et al., 1990; Hocking et al., 1998; Iozzo, 1999). Once again, differences in CS and DS quantities suggest possible alterations in the proportions of different PGs affecting the collagen organization. Further studies should be conducted to investigate whether the decrease in CS and DS after treatment with the plant is related to increased enzymes, which could have acted in the degradation of these components, or if there was a change in PGs turnover.

In the present study, we hypothesized that the topical application of *A. chica* extract could improve the organization of collagen fibers. In fact, the results reported herein demonstrated that the anthocyanins from crude *A. chica* extract acted in the proliferative phase of tendon repair, as evidenced by the improvement of the molecular organization of the collagen fibers. New strategies that improve collagen organization after tendon lesions could be interesting for clinical procedures, considering that collagen provides integrity and functionality to tendons. Thus, further studies should be conducted to identify which

molecular mechanism are involved in the better collagen reorganization, as well as the effect of the *A. chica* extract on the PGs turnover.

FIGURES AND TABLES CAPTIONS

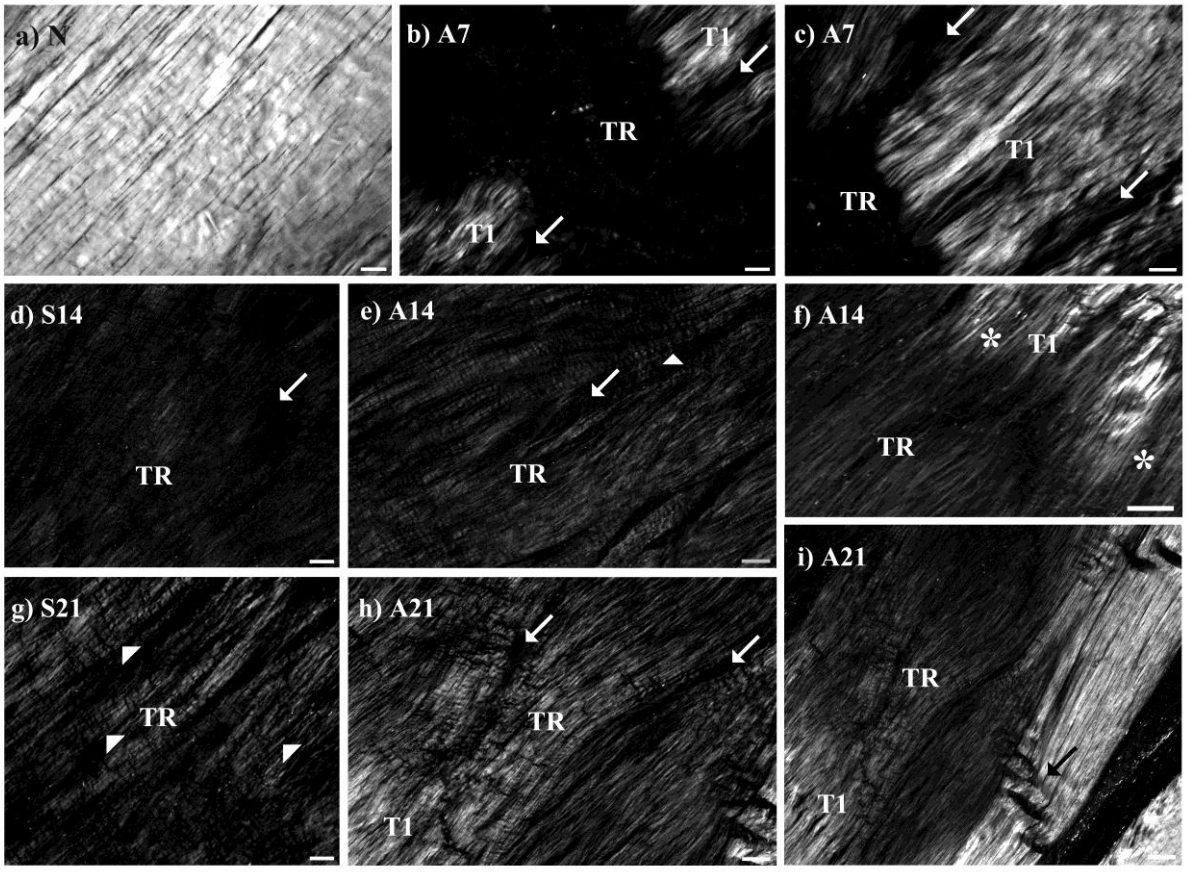

Figure 1. Images of tendon sections using polarization microscopy. The largest axis of the tendon was set at 45° to the polarizers. **(a)** group N: observe the strong birefringence of the collagen fibers in the proximal region of tendon; the variation in brilliance intensity (gray levels) is due to the collagen fibers waviness. **(b)** group A7: TR corresponds the dark area due a complete disorganization of the collagen fibers. Observe the proximal and distal regions (T1), which border the TR. **(c)** group A7: the detail of the same image as in (b) displays the fragmentation of the collagen fibers in T1 (→). **(d)** group S14: In TR, the collagen fibrils are more regularly arranged along the largest axis of the tendon, although with fragmentation of the fibers (→). **(e)** group A14: observe higher birefringence of the collagen fibers compared to the S14, with presence of crimp (▶) and fragmentation of the fibers (→). **(f)** group A14: observe an imbrication between collagen fibers that were not cut in T1 as well as newly formed fibrils (*) throughout the adjacency of TR. **(g)** group S21: the crimp from the new formed fibrils is more prominently in the TR, compared to the earlier periods (▶). **(h)** group A21: the birefringence is apparently higher than in the S21,

with several regions exhibiting fragmentation of the fibers (→) especially in TR. **(i)** group A21: a panoramic view of the tendon showing the non-homogeneous organization of collagen fibers along the TR and T1 regions. Note the presence of the fragmentation of the fibers in the remaining portion of the tendon located below the sectioned region (→). Images of the S7 group were not shown because they were similar to the A7 group. Bar = 60 µm (a, b, c, d, e, f, g, h) and Bar = 120 µm (i).

Groups	TR (GA median)	Comparisons	Mann-Whitney test (*p*)
N	228.5		
S14	62.9		
A14	85.0	S14 x A14 (TR)	(0.000)*
S21	105.0		
A21	104.8	S21 x A21 (TR)	(0.400)

GA: Gray Average. The largest axis of the tendon was positioned at 45° with respect to the crossed polarizers. The number of measurements (60) chosen at random in 12 sections from four tendons of each group. (*) Significant differences between the transected groups ($p<0.05$).

Table 1. Birefringence measurements: GA (pixels) variability in collagen fibers organization on the TR region of the calcaneal tendon. On the 14th day after lesion, higher value in TR of the A14 group was observed compared to S14 group. Note a gradual increase of birefringence 21 days after injury in both groups S21 and A21, although without significant difference between saline- and plant-treated groups.

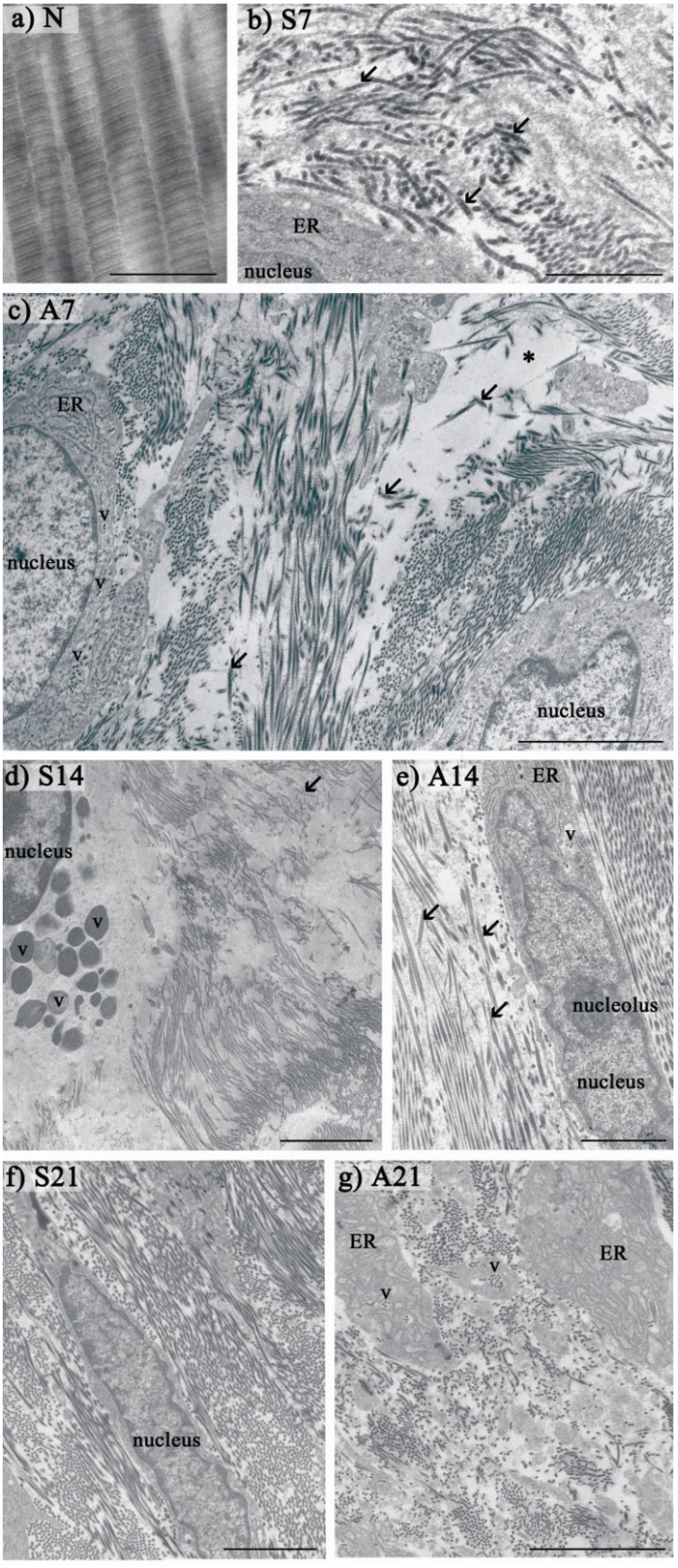
a) N
b) S7
ER
nucleus
c) A7
ER
v
nucleus
v
v
*
nucleus
d) S14
nucleus
v
v
v
e) A14
ER
v
nucleolus
nucleus
f) S21
nucleus
g) A21
ER
ER
v
v

Figure 2. Transmission electron micrographs of the tendons. A highly organized ECM may be seen in the group N, with the characteristic band pattern of collagen in longitudinal section (a). Observe in the TR of S7 and A7 groups (b,c) the collagen fibers completely disorganized, the presence of small collagen fragments (→) in the ECM, and regions of the matrix without the presence of fibers (*). Observe on the 14th day, presence of probably mastocyte with secretory vesicles (V) in S14 (d), the higher organization of collagen fibers in A14 (e) compared to S14 and presence of small collagen fragments (→). The fibroblasts were more elongated in S21 (f) and the collagen fibers longitudinally and transversely oriented in S21 (f) and A21 (g) groups. Observe the presence of a well-developed endoplasmic reticulum (ER) in the transected tendons, suggesting intense protein synthesis. v = secretory vesicles. Bar = 3 µm (c,f,g), Bar = 2 µm (d,e), Bar = 1 µm (b) and Bar = 0,5 µm (a).

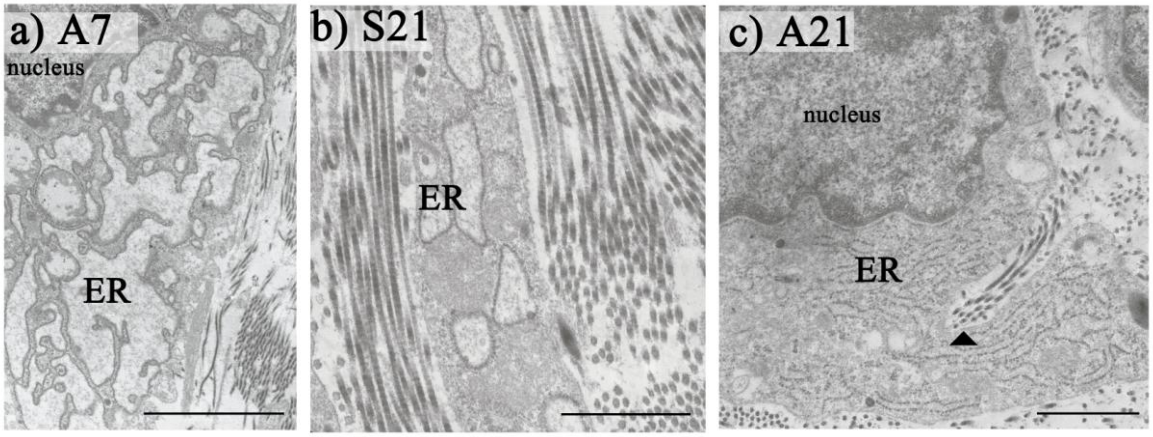

Figure 3. Transmission electron micrographs of the tendons. Observe the presence of ER (endoplasmic reticulum) with enlarged cisternae in A7 (a) and in S21 (b). Note the presence of plasma membrane cleft (▶) in A21 (c). Bar = 3 µm (a), Bar = 1 µm (b,c).

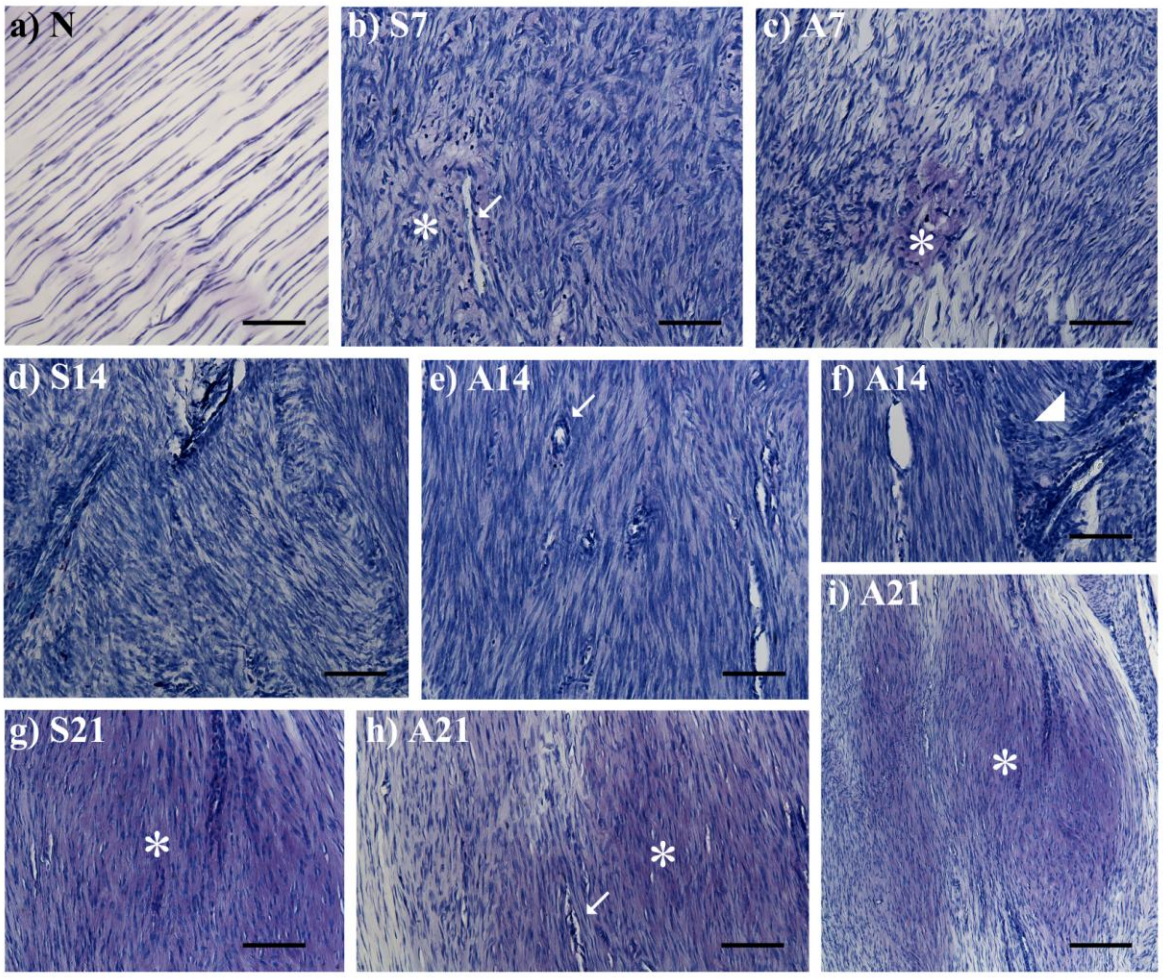

Figure 4. Images of tendon sections stained with toluidine blue. In group N, the matrix was not stained due the absence of PGs (a). Observe the numerous elongated fibroblasts highly willing in the ECM of the proximal region of the calcaneal tendon. In S7 and A7 groups, observe a high cellularity in TR, with metachromasy in the ECM (*) in both groups (b and c). Note that the cellularity was also high in the 14 day groups, but with greater orientation of the matrix in TR (d and e), compared to the 7 days groups. Apparently, no difference in metachromasy was observed between the saline- and plant-treated groups on 14th day (d and e). Observe the cell migration (▶) from the paratenon/epitenon to the TR (f). On the 21st day, the metachromasy was higher in both groups compared to the earlier phases with still high cellularity (Figures 4g and h). A panoramic view of the tendon showing the high metachromasy (*) in TR of the A21 group. (→) indicates presence of blood vessels. Bar = 120 µm (a, b, c, d, e, f, g, h) and Bar = 240 µm (i).

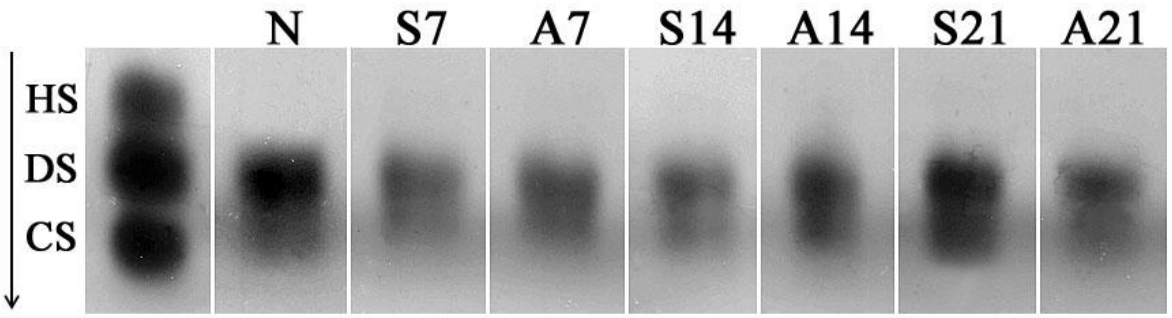

Figure 5. Electrophoresis in agarose gel with 5 µg of GAGs in each sample. Observe different proportions of DS and CS in the groups. Band densitometry is shown in table 2. HS, DS and CS standards are on the left.

Parameters	N	S7	A7	S14	A14	S21	A21
Concentration of GAGs							
GAGs (mg/g tissue)	3.4 ±1.2	9.7 ±1.3 *	9.9 ±1.3 *	10.5 ±2.0 *	11.1 ±3.0 *	11.7 ±2.1 *	10.5 ±2.9 *
Bands densitometry from the electrophoresis in agarose gel							
DS (pixels)	1092.0 ±104.5	608.6 ±169.8*	606.9 ±166.6*	403.7 ±10.4*,a	679.8 ± 95.1*,a	958.9 ±85.1b	747.8 ±69.3*,b
CS (pixels)	367.8 ±37.5	217.2 ±53.6*	242.1 ±34.4*	166.5 ±34.9*	249.2 ±48.9*	322.1 ±54.6c	220.1 ±18.3*,c

(*) Significant differences between the normal and the transected tendons ($p<0.05$); (a, b, c) Significant differences between the groups marked with the same letter ($p<0.05$).

Tabel 2. Concentration of GAGs of the tendons of different experimental groups, showed higher values for all transected tendons in relation to the normal tendon (*), with no difference between them. Band densitometry of the electrophoresis in agarose gel comparing plant- and saline-treated groups, showed higher value of DS in the A14 group, and lesser values of DS and CS in the A21 group, compared to the S14 and S21 groups respectively. All transected groups had lower values for DS and CS, except S21 group, in relation to the N group.

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MANUSCRITO 5

Efeito da *Aloe vera* sobre proteínas e glicosaminoglicanos durante o processo de cicatrização em tendão de rato

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RESUMO

As lesões tendíneas apresentam alta incidência e o reparo bem sucedido ainda é um grande desafio para a ortopedia. *Aloe vera* (L.) Burman f. tem sido utilizada no reparo de lesões principalmente de pele, com ação efetiva na síntese de diversos componentes de matriz extracelular. Entretanto, faltam estudos mostrando seu efeito em tecidos altamente fibrosos e organizados como os tendões. O objetivo do presente trabalho foi investigar o efeito da *Aloe vera* na síntese de alguns componentes da matriz extracelular, após transecção do tendão calcanear de ratos. Ratos Wistar adulto jovens foram distribuídos nos seguintes grupos: Av7, Av14 e Av21- animais com transecção parcial do tendão calcanear da pata direita seguido pela aplicação tópica de 32 mg de pomada de *A. vera*, sacrificados 7, 14 e 21 dias após a lesão respectivamente. Os grupos controles: B7, B14 e B21 receberam aplicação apenas do veículo. Nossos resultados mostraram aumento da concentração de glicosaminoglicanos (GAGs) e menor quantidade de dermatan sulfato (DS) no grupo Av14 em relação ao seu controle B14. Maior concentração de proteínas não colagênicas (PNCs) foi observada no grupo Av21 em relação ao controle B21. Análise do SDS-PAGE mostrou banda menos intensa referente ao colágeno no grupo Av14 comparado ao controle. Nos cortes corados com azul de toluidina, observou-se metacromasia principalmente na região de transecção dos tendões de todos os grupos. Com relação à organização dos GAGs, menor razão dicróica foi observada no grupo Av14 em relação ao grupo B14. Entretanto, não foi observada diferença significativa entre a razão dicróica dos grupos B21 e Av21. Em conclusão, após aplicação da pomada de *A. vera* nossos resultados indicaram aumento da concentração de GAGs, diminuição da quantidade de DS e da razão dicróica 14 dias após a lesão, assim como aumento das PNCs no 21° dia após a transecção tendínea.

Palavras-chave: cicatrização, proteína, *A. vera*, glicosaminoglicano, colágeno, tendão.

INTRODUÇÃO

A *Aloe vera* (L.) Burman f., também conhecida como planta da cicatrização, tem sido amplamente utilizada em várias doenças, com resultados efetivos para tratamentos de psoríase (Syed et al, 1996), herpes (Syed et al, 1996), hiperlipidemia (Nasiff et al, 1993), diabetes (Bolkent et al, 2004; Huseini et al., 2012), câncer (Tomasin e Gomes-Marcondes, 2011), artrite (Davis et al, 1992), inflamação e cicatrização (Grindlay e Reynolds, 1986; Chithra et al, 1998a,b,c,d; Somboonwong et al, 2000; Eamlamnam et al, 2006; Prabjone et al, 2006; Habeeb et al, 2007; Takahashi et al, 2009; Takzare et al, 2009; Atiba et al, 2011; Tarameshloo et al, 2012). Em tendões, de acordo com resultados de Filho et al. (2010), a aplicação tópica do gel de *Aloe vera* associada ao uso de ultrassom apresentou efeito benéfico no tratamento da inflamação causada por tendinite, com diminuição do infiltrado de células inflamatórias e aumento da resistência biomecânica do tendão. Durante o processo de cicatrização tendínea, estudo prévio realizado em nosso laboratório (Aro et al, 2012) foi o primeiro a analisar os efeitos da aplicação tópica da pomada de *Aloe vera*, com detecção de aumento de metaloproteinase-2, de colágeno total e maior organização das fibras de colágeno após transecção parcial do tendão. Várias substâncias tem sido identificadas na composição do gel da folha de *A. vera* com potencial efeito biológico, tais como polissacarídeos, taninos, substâncias antibióticas, enzimas, vitaminas, minerais, entre outros componentes (Filho et al, 2010). Embora amplamente utilizada, os mecanismos envolvidos na aceleração da cicatrização após uso da *A. ver*a não tem sido completamente descritos na literatura, principalmente em processos de cicatrização de tendão.

As lesões tendíneas apresentam alta incidência e o reparo bem sucedido ainda é um grande desafio para a ortopedia (Shearn et al, 2011). A cicatrização de tendão é um processo lento e complexo, que consiste na progressão ordenada de eventos bioquímicos e fisiológicos, para o restabelecimento da integridade do tecido danificado. Em tendões, devido à presença de uma matriz altamente organizada e com grande predomínio de proteínas colagênicas (Kjaer, 2005), além da produção dessas moléculas, é ainda fundamental a reconstituição da organização das fibras de colágeno no local da lesão. Portanto, uma maneira de avaliar a atividade cicatrizante de drogas ou extratos vegetais, pode basear-se também no estudo da sua influência na síntese de colágeno (Houghton et al,

2005), bem como de outros componentes que atuam durante a fibrilogênese do colágeno durante processos de remodelamento, tais como proteínas não colagências (PNCs) (Mackie e Ramsey, 1996) e proteoglicanos (PGs) (Derwin et al, 2001).

Os pequenos PGs ricos em leucina apresentam sua concentração alterada durante o processo de cicatrização tendínea (Mello et al, 1975; Mello e Vidal, 2003; Thomopoulos et al, 2002; Lui et al, 2012; Liang et al, 2008) e estão intimamente associados com as fibras de colágeno para regular o processo de fibrilogênese nos tendões (Zhang et al, 2006; Lo et al, 2004; Ezura et al, 2000; Liang et al, 2008) e restaurar a organização estrutural da matriz. Os pequenos PGs fibromodulin e lumican são constituídos de queratan sulfato (QS), enquanto que o decorin e o biglican são ambos constituídos por dermatan e condroitin sulfato (Esquisatto et al, 2007). O decorin interage ordenadamente com as fibras de colágeno (Scott, 1998), atuando na fibrilogênese (Scott, 1995; Scott et al, 1997) e provavelmente regulando o crescimento em diâmetro dessas fibras colagênicas (Iozo e Murdoch, 1996; Douglas et al, 2006). Além dos pequenos PGs característicos de regiões sujeitas às forças de tensão (Waggett et al, 1998; Rees et al, 2000), o grande PG agrecan também pode ser encontrado nessas regiões, sendo constituído por condroitin e queratan sulfato (Vogel et al, 1994; Rees et al, 2000, 2005, 2007).

Considerando o efeito da *A. vera* no estímulo da síntese de diversos componentes da matriz extracelular (MEC), o objetivo do presente trabalho foi investigar seu efeito sobre o conteúdo de PNCs e GAGs, assim como na organização dos GAGs após transecção parcial do tendão, nas fases inflamatória, proliferativa e de remodelamento do processo de cicatrização.

MATERIAIS E MÉTODOS

Grupos Experimentais:

Os animais foram mantidos no biotério do Departamento de Biologia Estrutural e Funcional, Instituto de Biologia – UNICAMP, e manipulados de acordo com as normas da Comissão de Ética na Experimentação Animal - UNICAMP (protocolo n°1621-1). Os animais foram distribuídos nos seguintes grupos: Av7, Av14 e Av21- animais com transecção parcial do tendão calcanear da pata direita e aplicação tópica de 32 mg de pomada de *A. vera* (contendo 16 mg de extrato liofilizado + 16 mg de base - uma mistura

de lanolina anidra e vaselina sólida (30:70)) sobre a lesão antes da sutura da pele, e nos próximos 6 dias após a lesão. Os animais foram sacrificados no 7°, 14° e 21° dias respectivamente. Os animais controles foram divididos nos grupos B7, B14 e B21, e tiveram aplicação tópica apenas de 32 mg de base (uma mistura de lanolina anidra e vaselina sólida (30:70)). Os animais do grupo normal (N) não tiveram os tendões transeccionados.

Protocolos para a transecção parcial do tendão calcanear e aplicação tópica da pomada de *A. vera*

Para os procedimentos cirúrgicos os animais foram anestesiados com injeção intra-peritoneal de Ketamina (80 mg/Kg) e Xylazina (10 mg/Kg) e as patas inferiores direitas submetidas à tricotomia e anti-sepsia com álcool iodado. Uma incisão longitudinal na pele foi feita para exposição do tendão calcanear e a transecção parcial transversal foi realizada na região de tensão do tendão, localizada a uma distância de 4 mm de sua inserção no osso calcâneo (Tomiosso et al, 2009). Os extratos de *A. vera* (32 mg) foram aplicados no local transeccionado do tendão antes da sutura da pele. Os tendões controles receberam apenas 32 mg de base, como já descrito anteriormente. Após a transecção do tendão, a pele foi suturada com fio de sutura de Nylon (Shalon 5-0) e agulha (1,5 cm). A partir do 1° dia após a cirurgia, foi feita a aplicação tópica diária da pomada sobre a região da pele suturada, massageando suavemente até sua completa absorção, durante os 6 primeiros dias após a lesão de acordo com cada grupo formado, contados a partir do dia seguinte da lesão. Os animais foram sacrificados por aprofundamento da anestesia.

Extração dos componentes MEC do tendão (n=4)

Os componentes da MEC foram obtidos após extração da região de transecção do tendão calcanear com cloreto de guanidina 4 M (contendo 0,05 M EDTA e 1mM de PMSF em tampão acetato de sódio 0,05 M - pH 5,8) durante 24h e a 4º C, em constante agitação, de acordo com o método de Heinergård e Sommarin (1987). Após centrifugação por 1h, a 10.000 rpm e a 4º C (centrífuga Beckman J2-21 - Rotor JA-20) o sobrenadante (extrato total) foi estocado em temperatura de -8°C para as diferentes análises bioquímicas.

Dosagens de proteínas não colagênicas (PNCs) (n=4)

O extrato total da região de transecção do tendão foi utilizado para quantificar

proteínas pelo método de Bradford (1976) com algumas modificações, utilizando o *kit* BioRad (BioRad *protein assay*) e albumina sérica bovina (BSA) como padrão, nas concentrações 1, 2, 4, 8 16 µg/ µL. A leitura das amostras foi feita a 595 nm em espectrofotômetro.

Eletroforese em gel de SDS-poliacrilamida (SDS-PAGE) (n=4)

Amostras provenientes do extrato total foram analisadas em gel de poliacrilamida contendo dodecil sulfato de sódio (SDS-PAGE), segundo Zingales (1984), usando um gel de gradiente de poliacrilamida (4-16%). O sistema tampão foi de acordo com Laemmili (1970), com gel de empacotamento contendo 3,5% de acrilamida. Após a dosagem de proteínas provenientes do extrato total segundo o método de Bradford (1976), 50 µg das diferentes amostras foram precipitadas em solução com tampão acetato de sódio 1M pH 7,4 (máximo de 150 µL) e 9 volumes de etanol (1350 µL), durante 24 horas à 4°C. As centrifugações foram realizadas em microcentrífuga Fischer Scientific Model 235 Va durante 5 minutos, a 8.000 rpm. Após duas lavagens (150 µL de tampão acetato de sódio 1M pH 7,4 e 1350 µL de etanol), o precipitado obtido foi seco à 37°C e ressuspendido em tampão de amostra não redutor, contendo Tris-HCL 62,5 mM, SDS 2%, glicerol 10%, EDTA 1 mM em pH 6,8 e azul de bromofenol 0,01 %. As amostras foram incubadas por 5 minutos a 96°C. O tampão utilizado na cuba de eletroforese foi Tris 2,5 mM, glicina 190 mM e SDS 0,1% em pH 6,8. A corrente elétrica aplicada foi de 30 mA, durante aproximadamente 4 horas. Os padrões de peso molecular empregados foram: fosforilase b (94 kDa), albumina sérica (64 kDa), ovoalbumina (43 kDa), anidrase carbônica (30 kDa), inibidor de tripsina (20,1 kda), α-lactoalbumina (14,4 kDa). Para o padrão de colágeno, foi empregado o colágeno tipo I extraído de tendão de cauda de rato. Após a corrida, o gel foi fixado em uma solução fixadora (metanol 50%, ácido acético 12 %) durante 1 hora, e em seguida corado por Coomassie brilliant blue-R (CBB R-250).

Quantificação de GAGs e análise por eletroforese em gel de agarose (n=4)

Fragmentos da região de transecção do tendão foram picados e desidratados, utilizando-se banho em acetona por 24 h. Após a desidratação, os fragmentos foram secos por 1h em estufa a 37°C, pesados e seguiram para a digestão com papaína (40 mg de papaína para cada 1g de tecido) em tampão fosfato de sódio 100 mM, pH 6,5, contendo

EDTA 40 mM e β-mercaptoetanol 80 mM, por 24h à 50°C. Após a digestão, as amostras foram tratadas com TCA 90% e seguiram para a dosagem de GAGs, através do método Azul de dimetilmetileno (Farndale et al, 1986), com leitura a 526 nm no espectrofotômetro Ultrospec, modelo 2100 Pro da Amersham Biosciences. Os GAGs sulfatados foram separados através da eletroforese em gel de agarose (0,5%) com tampão proprileno diamino (0,05M) (Dietrich e Dietrich, 1976), a 0,1 mA durante 2h. Foi utilizado um padrão consistindo de uma mistura de heparam sulfato, dermatam sulfato e condroitim sulfato (1mg/mL de cada). O gel de agorose foi fixado com cetavlon (0,1%), seco e corado com Azul de touluidina. O perfil eletroforético dos GAGs foi visualizado após tratamento com etanol (50%) e ácido acético (1%).

Microscopia ótica de luz comum (n=4) e morfometria (n=3)

Os tendões foram fixados usando formaldeído 4% em tampão Millonig (0.13 M fosfato de sódio, 0.1 M NaOH - pH 7.4), durante 24h a 4°C. Os tendões foram lavados em água, desidratados em etanol, diafanizados com xilol e embebidos em parafina. Cortes seriados longitudinais de 7 µm de espessura foram desparafinizados imediatamente antes do processo de coloração. Para observação dos PGs, os cortes foram corados com Azul de toluidina (AT) (0.025%) em tampão Mcllvaine (0.03M ácido cítrico, 0.04 M de fosfato de sódio dibásico - pH 4.0) (Mello e Vidal, 2003). Para análise da morfologia geral da região de transecção, os cortes foram corados com HE (Kiernan, 1981). Para contagem de células inflamatórias, foi utilizado o método de coloração de Dominici (Dominici, 1902). As lâminas foram tratadas com xilol e montadas com Entellan (Merck). Para a análise das lâminas, foi utilizado o microscópio Olympus BX 53. Para a morfometria, imagens de cortes longitudinais da região de transecção dos tendões foram capturadas e digitalizadas usando microscópio Leica DM 2000. As imagens digitalizadas foram usadas para a determinação do número de fibroblastos e de células inflamatórias ($n/10^4$ μm^2), assim como do número de vasos sanguíneos ($n/10^4$ μm^2). Sendo assim, três amostras de 10^4 μm^2 foram coletadas da região central da transecção de cada animal, utilizando-se uma grade virtual (Leica Image Measure™).

Medidas de dicroísmo linear (n=3)

Medidas de dicroísmo linear foram realizadas nos cortes corados com AT. De acordo com a literatura, a observação do dicroísmo linear tem mostrado que as cadeias de GAGs presentes nos PGs nas fibras de colágeno, estão distribuídas linearmente e predominantemente paralelas em relação ao maior eixo dessas fibras (Feitosa et al, 2002; Vidal e Melo, 1984). Neste caso, o dicroísmo linear é um fenômeno extrínsico resultando do arranjo helicoidal das moléculas de AT que estão eletrostaticamente ligadas aos sítios de ligações aniônicas orientadas do substrato (Vidal e Mello, 1984; Rodger e Nordén, 1997; Mello e Vidal, 2003). A razão dicróica (DR = $d_{\parallel}/d_{\perp}$) foi determinada com base na absorbância do AT medidas nas posições paralela ($d_{\parallel}$) e perpendicular ($d_{\perp}$) do maior eixo do tendão em relação ao plano de luz polarizada (Vidal e Mello, 1984; Mello e Vidal, 2003). As imagens foram capturadas utilizando o microscópio Olympus BX 53, e as medidas (1000) de dicroísmo linear foram realizadas utilizando o analisador de imagens (Image-Pro Plus 6.3, Media Cybernetics, Inc. – Silver Spring, MD, USA).

Análises estatísticas

Para as análises bioquímicas, os dados provenientes dos diferentes grupos experimentais foram analisados usando o Teste-*t* Student ($p < 0.05$), e para as análises de dicroísmo linear, foi utilizado o Teste de Mann-Whitney ($p < 0.05$). Para ambos os testes, foi utilizado o software GraphPad Prism® (Graph-Pad Software, La Jolla, CA, USA), version 3.0.

RESULTADOS

As análises morfométricas (Tabela 1) mostraram maior número de fibroblastos e de células inflamatórias em todos os grupos transeccionados comparados ao grupo N, embora sem diferenças significativas entre os grupos tratados e não tratados com a pomada de *A. vera* nos três tempos analisados. Nos cortes corados com AT e analisados sob a microscopia de luz comum, o tendão normal apresentou fibroblastos com núcleos alongados e matriz sem coloração. Nos grupos transeccionados, os tendões apresentaram duas regiões bem distintas: a região de transecção (RT), região onde foi realizada a

transecção dos feixes de colágeno; e região de transição contígua distal e proximal (T1), localizadas nas adjacências da RT (Figura 1).

Os grupos B7 e Av7 apresentaram metacromasia na matriz territorial da RT (Figura 1b). Nos grupos B14 e Av14 (Figuras 1 d, e) as células apresentaram-se mais orientadas na RT, com intensa metacromasia aparentemente maior no grupo Av14. Nos grupos transeccionados 21 dias após a lesão, não foram observadas diferenças entre os grupos B21 e Av21 (Figuras 1 f - h), embora marcante metacromasia tenha sido observada na matriz de ambos os grupos. Na região T1, também foi observada metacromasia em todos os grupos transeccionados no 14° e 21° dias, representada pela figura 1g, entretanto sem diferenças marcantes entre os grupos tratados e não tratados com a planta.

A análise dos cortes corados com AT e analisados sob a microscopia de luz polarizada mostrou a presença de dicroísmo linear (Figura 2) na RT dos grupos transeccionados no 14° e 21° dias após a lesão. Para detecção de diferenças na intensidade de dicroísmo linear entre os grupos, foram calculadas as razões dicróicos provenientes das medidas realizadas na RT. O grupo Av14 apresentou razão dicróica inferior ao seu respectivo controle B14, ao passo que não foram observadas diferenças significativas entre os grupos B21 e Av21 (Tabela 2).

Considerando a dosagem de GAGs sulfatados totais (mg/g de tecido), todos os grupos transeccionados apresentaram maiores concentrações desses componentes em relação ao grupo N. Entretanto, somente o grupo Av14 apresentou maior concentração de GAGs totais comparado ao seu respectivo controle B14 (Tabela 3). A densitometria de bandas (pixels) obtidas a partir da eletroforese em gel de agarose, mostrou variação nas quantidades de DS (dermatan sulfato) e CS (condroitin sulfato) presentes em 5 µg de GAGs de amostra de cada grupo experimental. Os grupos B7, Av7, B14 e Av14 apresentaram menores quantidades de DS comparados ao grupo N. Vale ressaltar que o grupo Av14 apresentou menor valor referente ao DS em relação ao seu respectivo controle B14. Com relação à presença de CS, apenas o grupo B7 apresentou maior valor em relação ao grupo N (Figura 3).

A análise do gel em SDS-PAGE (Figura 4) mostrou pequenas diferenças no perfil proteico total entre os grupos experimentais. Foi observado um pequeno aumento da quantidade de proteínas não colagênicas (peso molecular entre 14.4 e 67 kDa) nos grupos

transeccionados comparados ao grupo N, porém sem diferenças entre si. Com relação à presença de colágeno, o grupo Av14 apresentou banda menos intensa comparada ao grupo B14, ao passo que bandas mais proeminentes foram encontradas no grupo N em relação aos grupos transeccionados. Com relação à dosagem de PNCs (mg/g de tecido), todos os grupos transeccionados apresentaram maior concentração em relação ao grupo N, com maior concentração no grupo Av21 comparado ao grupo B21 (Tabela 3).

DISCUSSÃO

Na fase inflamatória que se inicia logo após a lesão tendínea, ocorre a liberação de citocinas e de fatores de crescimento provenientes de plaquetas e macrófagos que migram para a região lesionada (Berglund et al, 2011; Chen et al, 2004; Hope et al, 2012; Bedi et al, 2012), os quais induzem a neovascularização, proliferação e quimiotaxia de fibroblastos e miofibroblastos (Garner et al, 1989; Lee et al, 2003). Células sinoviais e inflamatórias também migram para o local da lesão (Sahin et al, 2012; Marsolais et al, 2003; Wojciak and Crossan, 1993). Nossos dados morfométricos mostraram aumento do número de fibroblastos e de vasos sanguíneos na RT em relação ao tendão normal, embora sem diferenças entre os grupos tratados e não tratados com a pomada de *A. vera*. Com relação à presença de células inflamatórias, nossos resultados também não mostraram redução no número dessas células após tratamento com a *Aloe vera*. Entretanto, resultados de Filho et al. (2010) mostraram redução do número de células inflamatórias na tendinite após tratamento com a *A. vera* associada à aplicação de ultrassom. De acordo com resultados de Atiba et al. (2011b) e Jettanacheawchankit et al. (2009), o tratamento com *A. vera* aumentou o número de vasos sanguíneos e fibroblastos, como sinal de aceleração do processo de cicatrização de pele, devido ao aumento de VEGF (fator de crescimento do endotélio vascular) e KGF-1 (fator de crescimento de queratinócito-1).

O alto metabolismo celular que se inicia na fase inflamatória e continua elevado durante algumas semanas após a lesão (Tillman e Chasan, 1996), resulta na síntese de vários componentes da MEC, tais como colágeno (Sharma e Maffulli, 2005), GAGs e PNCs, assim como de moléculas sinalizadoras e com função de degradação (Oshiro et al, 2003). Além do aumento de GAGs e de PNCs após a lesão observado no presente trabalho nos grupos tratados com a pomada de *A. vera*, estudo prévio realizado em nosso laboratório

mostrou aumento de MMP-2 e de MMP-9 no 7° dia após a lesão (Aro et al, 2012). O aumento da síntese desses componentes é importante para a formação do tecido de granulação, caracterizado como uma matriz provisória e frouxa, formada para preencher a região lesionada, a qual será posteriormente substituída por uma cicatriz (Dyment et al., 2012; Wojciak e Crossan, 1993; Garner et al., 1989; Berglund et al., 2011; Lui et al., 2012; Thomopoulos et al., 2002).

Após 14 dias da lesão, em que ocorre o pico da fase proliferativa, foi observada diminuição de colágeno no grupo tratado com a pomada de *A. vera* e aumento de GAGs sulfatados totais. Estudo prévio realizado em nosso laboratório detectou diminuição de colágeno tipo I (Aro et al, 2012), confirmando os dados observados no presente trabalho após análise do SDS-PAGE. Essa diminuição de colágeno tipo I pode estar relacionada com maior atividade de MMPs, principalmente de colagenases, as quais estão aumentadas nessa fase do processo de cicatrização (Oshiro et al, 2003). Com relação ao aumento da concentração de GAGs observada, esse dado corrobora com resultados de Chithra et al. (1998b), que detectaram aumento da quantidade desses componentes durante o processo de cicatrização.

As moléculas de GAGs são responsáveis pela metacromasia observada nos cortes corados com AT e são sintetizadas principalmente durante as fases iniciais do processo de reparo, mantendo-se elevadas ainda por algumas semanas (Mello et al, 1975; Mello e Vidal, 2003; Thomopoulos et al, 2002; Lui et al, 2012; Liang et al, 2008). Nossos resultados mostraram menor razão dicróica nos grupos tratados com a pomada de *A. vera* no 14° dia após a lesão comparado ao grupo B14, indicando mudanças no arranjo dos GAGs nas fibras recém formadas. As cadeias de GAGs são constituintes dos PGs presentes nas fibras de colágeno, e estão distribuídas linearmente e predominantemente paralelas ao maior eixo dessas fibras (Feitosa et al, 2002; Vidal e Melo, 1984). Resultados de Mello et al. (2003) também mostraram diferenças no arranjo dos GAGs até 110 dias após lesão do tendão calcanear. Sendo assim, nossos dados sugerem que nem todas as moléculas de GAGs presentes na maior concentração de GAGs totais observada no grupo Av14, encontram-se orientadas paralelamente em relação ao maior eixo das fibras de colágeno.

Nossos resultados mostraram ainda diminuição de DS no grupo Av14, sugerindo diferenças nas proporções dos tipos de PGs nesses grupos, que pode ter refletido na

orientação dos GAGs. Chithra et al (1998b), detectaram maior quantidade de hidrolases após tratamento com a *A. vera* durante a cicatrização de pele, tais como β-glicosidade, β-galactosidase, β-glicuronidase e N-acetilglicosaminidase. Essas hidrolases são enzimas de origem principalmente lisossomal e atuam na degradação de GAGs (Chithra et al,1998b). Estudos posteriores deverão ser realizados para investigação do efeito da *A. vera* na quantificação dessas hidrolases após transecção tendínea e sua possível relação com a menor quantidade de DS observada em nossos resultados.

No 21° dia após a lesão, caracterizada como fase em que o remodelamento é mais proeminente comparada às fases anteriores (Liu et al, 1995; Tomiosso et al, 2009), nossos resultados mostraram maior quantidade de PNCs após tratamento com a pomada de *A. vera*. Esse aumento pode estar relacionado com uma maior quantidade de proteínas estruturais de matriz que participam do processo de fibrilogênese do colágeno após lesão (Riley et al, 1996; Mackie and Ramsey, 1996), pois de acordo com resultados prévios (Aro et al. 2012), nesse período as fibras de colágeno presentes na RT apresentaram-se mais organizada. Entretanto, nossos resultados não são conclusivos com relação aos tipos de proteínas que apareceram aumentadas após uso da *A. vera*, criando novamente perspectivas para investigações futuras.

Ainda no 21° dia após a lesão, nossos resultados mostraram a presença de PGs através da intensa metacromasia observada na matriz territorial na RT, indicando processo de fibrilogênese do colágeno nesse período. Os PGs participam da fibrilogênese do colágeno após lesão tendínea, apresentando importante papel durante o remodelamento tecidual (Lui et al, 2012; Liang et al, 2008). Nesse período, não detectamos diferenças na concentração e disposição dos GAGs totais entre os grupos. Vale ressaltar que além do intenso remodelamento observado na RT, toda a extensão do tendão passa por acentuado remodelamento indicado pela presença de PGs nas regiões adjacentes à RT, embora sem diferenças entre os grupos tratados e não tratados com a pomada de *A. vera*. Resultados prévios (Aro et al, 2012) já tinham mostrado que as fibras de colágeno presentes em toda a extensão do tendão apresentaram alteração no grau de organização e agregação. Uma vez lesionado, há alteração no mecanismo de mecanotransdução, no qual as fibras de colágeno funcionam como transdutores através da transmissão de sinais elétricos para a superfície celular (Vidal, 1994). Após destruição das fibras devido à transecção do tendão, ocorrem

mudanças na intensidade dessa sinalização que reflete na atividade de síntese celular (Vidal, 1994). Análises futuras deverão ser realizadas com o objetivo de investigar o *turnover* de PGs e síntese de PNCs, visando um melhor entendimento do processo de remodelamento do tendão após aplicação tópica da pomada de *A. vera*.

Em conclusão a aplicação tópica da pomada de *A. vera* em tendões parcialmente transeccionados resulta em: 1. Aumento da concentração de PNCs e de GAGs sulfatados totais. 2. Redução da quantidade de DS e consequente diminuição da razão dicróica dos PG-DS após 14 dias da lesão.

TABELAS

Tabela 1: Morfometria dos cortes de tendões representada pela média (n/10^4 μm^2) e desvio-padrão. Os tendões dos grupos transeccionados apresentaram maior número de fibroblastos e de células inflamatórias (*) comparados ao grupo normal, sem diferenças significativas entre os grupos tratados e não tratados em cada período.

Parâmetros	N	B7	Av7	B14	Av14	B21	Av21
Fibroblastos (n/10^4 μm^2)	25.5 ± 2.1	110.7 ± 19.6*	105.3 ± 16.4*	118.3 ± 26.6*	123.7 ± 17.1 *	131.2 ± 26.8*	119.2 ± 21.3*
Células inflamatórias (n/10^4 μm^2)	0.00	54.8 ± 10.5*	59.8 ± 12.2*	16.3 ± 7.6*	12.7 ± 6.2*	12.5 ± 3.9*	9.8 ± 2.5*
Vasos Sanguíneos (n/10^4 μm^2)	2.2 ± 0.6	3.0 ± 0.9	2.8 ± 0.7	2.8 ± 1.4	2.7 ± 1.04	3.0 ± 0.9	3.0 ± 0.9

(*) Diferenças significativas entre os tendões normais e transeccionados ($p<0.05$).

Tabela 2: Valores de absorbância obtidos dos cortes corados com AT e razão dicróica.

Grupos	Mediana $A_{//}$ (RT)	Mediana $A_{\perp}$ (RT)	Razão dicróica (A_{PA} / A_{PE})	Comparações	Mann-Whitney test (p)
B14	0.75	0.92	0.86		
Av14	0.60	0.75	0.81	B14 x Av14	(0.000)*
B21	0.63	0.83	0.83		
Av21	0.51	0.62	0.82	B21 x Av21	(0.188)

$A_{//}$ e $A_{\perp}$: maior eixo do tendão na posição paralela e perpendicular em relação ao plano de luz polarizada, respectivamente. RT: Região de transecção. (*) Diferença significativa (p<0.05).

Tabela 3: Concentração de proteínas não colagênicas (PNCs) e de glicosaminoglicanos (GAGs) da região de transecção dos tendões. Observe marcante aumento de PNCs e de GAGs em todos os grupos transeccionados comparados ao grupo N. Observe maior concentração de PNCs no grupo Av21 em relação ao grupo B21, ao passo que o grupo Av14 apresentou maior concentração de GAGs em relação ao grupo B14. A densitometria de bandas das amostras após eletroforese em gel de agarose mostrou menor proporção de DS no grupo Av14 em relação ao seu controle B14. Considerando a presença de CS, somente o grupo B7 apresentou menor valor em relação ao grupo N. Observe a eletroforese em gel de agarose na figura 3.

Parâmetros	N	B7	Av7	B14	Av14	B21	Av21
Concentração de PNCs							
PNCs (mg/g tecido)	23.4 ± 4.0	42.2 ± 3.3*	44.0 ± 2.6*	35.2 ± 3.7*	33.7 ± 5.7*	28.3 ± 1.6*,a	32.6 ± 4.3*,a
Concentração de GAGs							
GAGs (mg/g tecido)	6.9 ± 1.8	12.3 ± 3.1*	14.2 ± 1.6*	10.2 ± 2.8*,b	15.8 ± 3.9*,b	11.7 ± 1.9*	12.6 ± 2.8*
Densitometria de bandas após eletroforese em gel de agarose							
DS (pixels)	639.6 ± 66.0	252.6 ± 66.5*	195.6 ± 18.0*	424.1 ± 74.4*,c	224.1 ± 26.2*,c	686.6 ± 266.2	494.1 ± 100.4
CS (pixels)	201.0 ± 20.2	325.9 ± 36.9*	269.4 ± 58.8	187.1 ± 8.0	153.3 ± 26.3	442.7 ± 169.0	240.3 ± 96.1

Diferenças significativas ($p<0.05$) entre os tendões normais e transeccionados (*) e entre os grupos marcados com a mesma letra (a, b, c).

FIGURAS

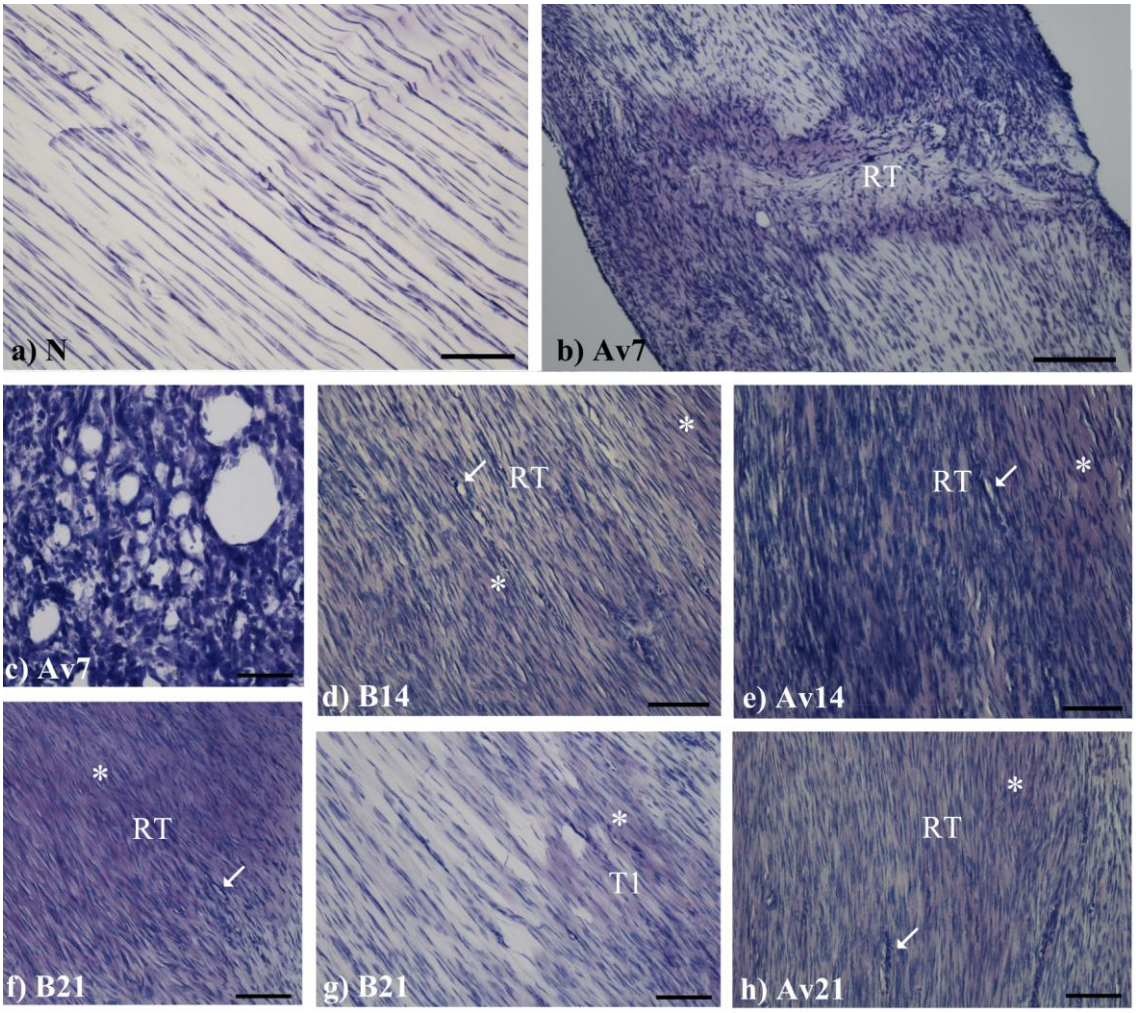

Figura 1: Imagens de cortes de tendões corados com AT. (a) Tendão normal sem transecção, com presença de fibroblastos com núcleos alongados e matriz sem coloração devido à ausência de proteoglicanos. Observe RT no grupo Av7 (b) com presença de metacromasia. Em (c), observe a presença de grande quantidade de vasos sanguíneos típicos dessa fase inicial do processo de reparo. Em B14 (d) e Av14 (e) as células estão mais orientadas, com metacromasia aparentemente maior em Av14. Observe metacromasia em RT e T1 dos grupos B21 e Av21 (f, g, h). (→) vasos sanguíneos. (Imagem do grupo B7 não foi mostrada porque é idêntica à imagem do grupo Av7). Barra = 60µm (c), Barra = 120µm (a, d, e, f, g, h) e Barra = 240µm (b).

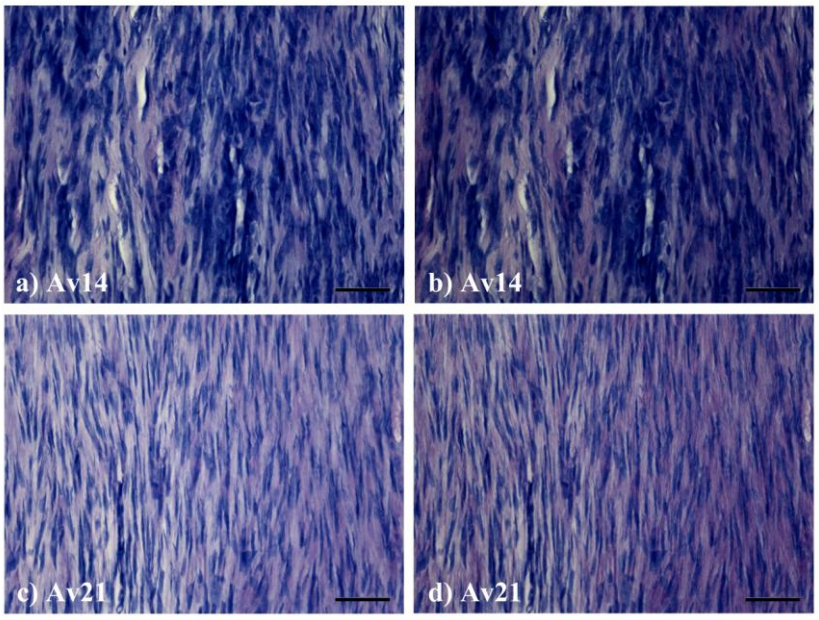

Figura 2: Imagens de cortes de tendões transeccionados corados com AT e analisados sob a microscopia de polarização. O eixo maior do tendão foi posicionado paralelo (a e c) e perpendicular (b e d) em relação ao plano de luz polarizada. Observe dicroísmo linear mais intenso em b e d. Imagens dos grupos B14 e B21 não foram mostrados porque são idênticas às imagens dos grupos Av14 e Av21. Para diferenças entre os grupos, observe a Tabela 2. Barra = 60µm.

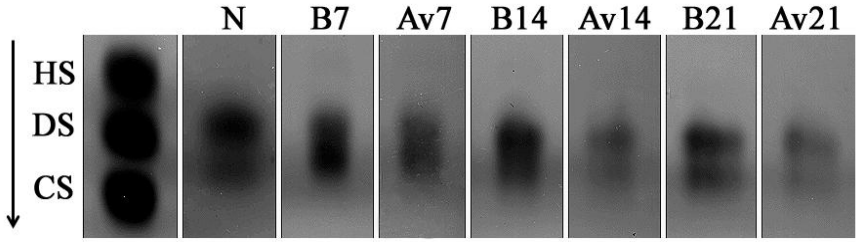

Figura 3: Eletroforese em gel de agarose com 5 µg de cada amostra. Observe diferentes proporções de DS e CS entre os diferentes grupos. Observe a densitometria de bandas na Tabela 1. Padrão de HS, DS e CS à esquerda.

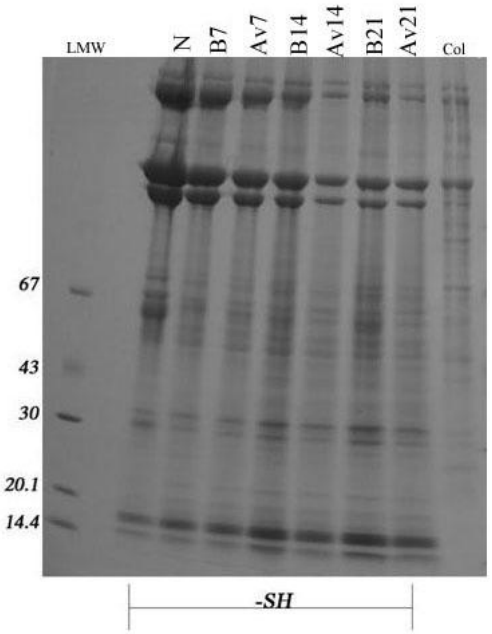

Figura 4: SDS-PAGE de proteínas totais provenientes dos tendões dos diferentes grupos experimentais, em condições não redutoras (-SH). Note que as proteínas colagênicas representadas pelas bandas referentes às cadeias α e β do colágeno, são mais proeminentes comparadas às bandas equivalentes às proteínas não colagênicas com peso molecular entre 14.4 e 67 kDa. Observe bandas menos intensas referentes ao colágeno no grupo A14 comparado ao grupo S14. LMW: Padrão de baixo peso molecular. Col.: Padrão de colágeno.

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8. CONCLUSÃO

Gerais:

A aplicação tópica da pomada de *A. vera* em tendões parcialmente transeccionados resulta em aumento nas concentrações de colágeno, proteínas não colagências e glicosaminoglicanos sulfatados e maior organização dos feixes de colágeno.

No caso de *A. chica* foram constatados: aumento da concentração de colágeno, aumento da organização dos feixes de colágeno e melhora da marcha durante a fase inflamatória.

Específicas:

A aplicação tópica da pomada de *A. vera* em tendões parcialmente transeccionados resulta em: 1. Aumento da organização tecidual e da atividade da MMP-2 no 21° dia após lesão; 2. Diminuição da síntese e ativação da MMP-9 no 14° dia do processo de reparo; 3. Aumento da concentração de colágeno total no 7° e 14° dias após a lesão, com subsequente diminuição no 21° dia; 4. Aumento da concentração de GAGs sulfatados totais e de proteínas não colagênicas, após 14 e 21 dias da lesão respectivamente; 5. Redução da quantidade de DS e consequente diminuição da razão dicróica dos PG-DS no 14° dia após a lesão.

A aplicação tópica da *A. chica* em tendões parcialmente transeccionados resulta em: 1. Aumento da concentração de colágeno total no 7° e 21° dias após a lesão; 2. Diminuição da síntese e ativação da MMP-9 no 14° dia do processo de reparo e aumento da síntese de MMP-2 no 21° dia; 3. Aumento da organização das fibras de colágeno no 14° dia após a lesão; 4. Melhora da marcha durante a fase inflamatória; 5. Diminuição da concentração de proteínas não colagênicas no 7° dia e aumento da quantidade de DS no 14° dia após a lesão.

ANEXO I:

DECLARAÇÃO

Declaro para os devidos fins que o conteúdo de minha **Tese de Doutorado** intitulada "Efeito dos extratos de *Aloe vera* e *Arrabidaea chica* sobre a cicatrização do tendão calcanear de ratos após transecção parcial":

( ) não se enquadra no § 3º do Artigo 1º da Informação CCPG 01/08, referente a bioética e biossegurança.

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

( ) CIBio - Comissão Interna de Biossegurança , projeto nº _____, Instituição: _____.

(x) CEUA - Comissão de Ética no Uso de Animais , projeto nº 1621-1, Instituição: _____.

( ) CEP - Comissão de Ética em Pesquisa, protocolo nº _____, Instituição: _____.

** Caso a Comissão seja externa ao IB/UNICAMP, anexar o comprovante de autorização dada ao trabalho. Se a autorização não tiver sido dada diretamente ao trabalho de tese ou dissertação, deverá ser anexado também um comprovante do vínculo do trabalho do aluno com o que constar no documento de autorização apresentado.*

Andrea Aparecida de Aro

Aluna: Andrea Aparecida de Aro

Orientador: Dr. Edson Rosa Pimentel

Para uso da Comissão ou Comitê pertinente:
(X) Deferido ( ) Indeferido

Ana Maria Aparecida Guaraldo

Carimbo e assinatura **Profa. Dra. ANA MARIA APARECIDA GUARALDO**
Presidente da CEUA/UNICAMP

Para uso da Comissão ou Comitê pertinente:
( ) Deferido ( ) Indeferido

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