UNIVERSIDADE ESTADUAL DE CAMPINAS

VERA LÚCIA CORRÊA FEITOSA

MATRIZ EXTRACELULAR DE DIFERENTES REGIÕES DE DOIS TENDÕES FLEXORES DIGITAIS DE PORCO: ESTUDO MORFOLÓGICO E BIOQUÍMICO

Tese apresentada ao Instituto de Biologia para obtenção do Título de Doutor em Biologia Celular e Estrutural na Área de Biologia Celular.

ORIENTADOR: PROF. DR. EDSON ROSA PIMENTEL

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Os riscos têm de ser corridos, pois o maior risco na vida é não arriscar nada. A pessoa que não arrisca nada, não faz nada, não tem nada, não é nada e não se torna coisa alguma. Pode evitar o sofrimento e a tristeza, mas não pode aprender, sentir, modificar-se, crescer, amar e viver. Acorrentado por suas certezas, é um escravo. Foi privado do direito de sua liberdade. Somente a pessoa que arrisca é verdadeiramente livre.

Leo Buscaglia, vivendo, amando e aprendendo.

"É muito melhor arriscar coisas grandiosas,
Alcançar triunfos e glórias,
Mesmo expondo-se à derrota,
Do que formar fila com os pobres de espírito
Que não gozam muito nem sofrem,
Porque não conhecem vitória e nem derrota."

T. Roosevelt

Bendize, ó minha alma , ao Senhor,
E tudo o que há em mim bendiga ao seu santo nome.
Bendize, ó minha alma, ao Senhor,
E não te esqueças de nem um só de seus benefícios.

Salmos 103: 1-2

Os que confiam no Senhor
São como o monte de Sião.
Que não se abala, mas firme
Permanece para sempre.

Salmos 125: 1

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ABREVIATURAS UTILIZADAS

CEC	Concentração eletrolítica crítica
COMP	Proteína Oligomérica da Matriz
DEAE -Sephacel	Dietilaminoetil – Sephacel
d	Distal
GAG	Glicosaminoglicano
i	Intermediária
kDa	Quilo Dalton
MEC	Matriz extracelular
Mr	Massa molecular aparente
p	Proximal
PG	Proteoglicano
TFDS	Tendão flexor digital superficial
TFDP	Tendão flexor digital profundo
t	Terminal

ÍNDICE

I – RESUMO

Os tendões flexores digitais superficial e profundo de porco foram estudados com o objetivo de identificar diferenças morfológicas e bioquímicas em regiões que exibem diferentes propriedades biomecânicas. O tendão flexor digital superficial foi dividido em três regiões, denominadas de proximal, que passa sob a articulação tibio-tarsal e recebe forças de compressão além das forças de tensão; intermediária, onde predominam apenas forças de tensão e a região distal que contorna a articulação metatarsofalângica onde também atuam forças de compressão. O tendão flexor digital profundo foi dividido nas regiões proximal com predomínio de forças de tensão; distal, região bifurcada em direção aos dígitos contornando a articulação metatarsofalângica e região terminal que se insere nos terceiro e quarto dígitos. Nestas duas últimas regiões atuam forças de compressão.

As análises estruturais dos tendões mostraram que nas regiões de compressão os fibroblastos apresentaram-se arredondados, semelhantes a condrócitos e os feixes de colágeno têm uma organização muito complexa, assumindo várias direções e se associando aos proteoglicanos. A distribuição dos feixes de colágeno nas regiões de compressão e tensão pôde ser observada através das análises aos microscópios de polarização e eletrônico de varredura. A morfologia apresentada pelas regiões de tensão é típica de tendão, os feixes de colágeno estão alinhados entre si e os fibroblastos se dispõem paralelamente entre estes feixes.

A birrefringência mostrou um alto nível de ordem molecular e grau de agregação dos feixes de colágeno em áreas onde houve um predomínio da força de tensão. O dicroísmo linear confirma que as moléculas dos glicosaminoglicanos (GAGs) são paralelo ao eixo maior dos feixes de colágeno. O padrão de "crimp" apresentou uma morfologia regular e mais definida para as regiões de tensão. As fibras elásticas foram encontradas em todas as três regiões, porém com distribuição diferente. Nas regiões de tensão, elas seguem a mesma direção dos feixes de colágeno e em regiões de compressão elas se dispõem em várias direções.

As diferentes regiões dos dois tendões foram extraídos com cloreto de guanidina (GuHCl). As dosagens de proteínas e GAGs apresentaram valores significativamente

diferentes para todas as regiões dos dois tendões, ocorrendo uma maior concentração nas regiões em que os tendões contornam a articulação. Fracionamento em DEAE-Sephacel de todos os extratos e análise em SDS-PAGE 4-16% com e sem 2-mercaptoetanol evidenciou a presença de componentes polidispersos com Mr em torno de 94 e 200 kDa. O componente de 67 kDa apareceu em todas as regiões dos dois tendões e, através da digestão enzimática com queratanase e coloração CEC-azul de alcian seguida de coomassie brilliant blue, apresentou um comportamento semelhante ao pequeno proteoglicano fibromodulim.

As propriedades de intumescimento apresentadas pelos dois tendões foram típicos de uma matriz colagênica fibrosa. Foi verificado que ocorreu um intumescimento maior em água, nas regiões onde predominam forças de compressão, enquanto as regiões de tensão intumesceram mais quando em contato com o ácido acético 3%. Estes dados de intumescimento foram confirmados pelas dosagens dos GAGs sulfatados após a digestão pela papaína, que apresentou uma maior quantidade de GAG/mg de tecido naquelas regiões sujeitas a forças de compressão. O GAG dermatam sulfato detectado em gel de agarose-propilenodiamino foi encontrado em todas as regiões nos dois tendões, enquanto o condroitim sulfato foi observado principalmente nas regiões de compressão, embora tenha sido verificada pequena quantidade de condroitim sulfato também nas regiões de tensão. A digestão com as condroitinases AC e ABC e posterior análise em gel de agarose-propilenodiamino indicou a presença do dermatam sulfato como único GAG.

Nossos resultados, mais uma vez vêm reforçar a teoria de que as forças mêcanicas podem contribuir para a definição da composição e organização da matriz extracelular.

II – ABSTRACT

The superficial and deep digital flexor tendons of pigs were studied to identify the morphological and biochemical aspects of different regions which have different biomechanical properties. The superficial digital flexor tendon was divided in: proximal, intermediate and distal regions. The proximal and distal regions bear compressive forces while the intermediate region withstands only tensional forces. The deep digital flexor tendon was divided in proximal, distal and terminal regions. The first bears only tensional forces while the distal and terminal regions are under compressive in addition to tensional forces.

The structural analysis in both tendons, in the regions under compression, showed the presence of round cells and collagen bundles distributed in several directions, giving to the extracellular matrix a structure similar to a basket-weave pattern. In contrast, in the tensional regions, typical elongated fibroblasts were found following bundles of collagen fibers in parallel arrays.

The birrefringence images showed the highest degree of molecular order and collagen molecules aggregation in areas where the tensional forces were dominant, compared with areas where compressive forces were also present. The crimp pattern was more regular and larger in regions under tension than in compressive regions. The linear dichroism confirmed that the glycosaminoglycans (GAGs) were parallel to the long axis of the collagen fibrils.

For biochemical analysis, the different regions were extracted with 4M guanidine-HCl. A larger quantity of protein and sulfated GAG were found in the compressive regions. Chromatography in DEAE-Sephacel and analysis in polyacrylamide gel, showed the presence of polydisperse components with Mr around 94 and 200 kDa. A component with 67 kDa was detected in all regions. After keratanase digestion, electrophoresis and staining with alcian blue under specific critical electrolyte concentration conditions, it was proved to contain keratan sulfate. The presence of dermatan sulfate in the 94 kDa component, after β-elimination and electrophoresis in agarose gel, indicated strongly to be the small

proteoglycan decorin. This component was found in all regions, but in regions under compression probably interacts strongly with other matrix components.

The swelling properties exhibited by the two tendons were typical for a fibrous collagenic matrix. A larger swelling in water was found for the regions under compression, while the tensional regions were more swollen when embedded in 3% acetic acid. These results were in accordance with the measurements of total GAGs after papain digestion, where a larger amount of GAG/mg tissue was found in compressive areas. Dermatan sulfate detected in agarose gel was found in all regions of the two tendons, while chondroitin sulfate was mainly observed in compressive areas. The presence of chondroitin sulfate indicates the presence of the large PG.

Our results confirmed anterior studies carried out in wrap around tendons of other specimens of mammals, and once more reinforce the theory that biomechanical forces can contribute towards the composition and organization of the extracellular matrix.

III - INTRODUÇÃO

1. Aspectos morfológicos do tendão

Os componentes da matriz extracelular (MEC) estão organizados num complexo esquema hierárquico para a formação do tendão (Jozsa et al. 1991). As moléculas de colágeno agregam-se formando fibrilas, estas então formam as fibras que constituem os feixes que formam os tendões. Externamente, o tendão é revestido por uma camada delgada de tecido conjuntivo, o epitendíneo, que se estende por entre os feixes de colágeno constituíndo o endotendíneo, que é formado por tecido conjuntivo frouxo. Em alguns tendões há um revestimento ao redor do epitendíneo, o paratendíneo (Jozsa et al. 1991). Quando ocorre aumento na fricção que é em função da mudança de direção, e o tendão necessita de uma maior lubrificação, o paratendíneo divide-se nas camadas visceral e parietal (Elliot, 1965; Merrilees and Flint, 1980). Estas camadas delimitam um espaço conhecido por mesotendíneo, que é preenchido por um fluído semelhante ao líquido sinovial (Nisbit, 1960). O desenvolvimento sistemático desta hierarquia é necessário para a integridade estrutural e funcionamento normal do tendão (Birk et al. 1997).

O tendão é um tecido conjuntivo denso modelado, cuja principal função é a de transmitir a força de tensão gerada pela contração muscular ao osso no qual ele se insere (Vogel and Koob, 1989; Cribb and Scott, 1995; Birch et al. 1997; Milz et al. 1998). Nem sempre os tendões se prendem ao esqueleto, podendo se prender em outros elementos como: cartilagem, cápsulas articulares, septos intermusculares, derme ou tendão de outro músculo (Dângelo, 1995).

Os tendões "wrap around" são os que passam próximo a um osso em uma articulação antes da inserção, porém em uma direção que difere da do músculo (Alexander and Dimery, 1985). Estes tendões desenvolvem uma estrutura semelhante a uma fibrocartilagem, caracterizada pela presença de feixes organizados de colágeno e depósitos de proteoglicanos (PGs), representando um sistema especial em que a estrutura recebe forças de compressão e fricção, sem contudo afetar a transmissão da força de tensão gerada pelo músculo (Vogel and Koob, 1989; Benjamin and Evans, 1990; Evanko and Vogel, 1990). Nestes tendões, a organização dos feixes de colágeno bem como a presença dos PGs

nas áreas sujeitas a forças de compressão, parecem ser os principais fatores envolvidos na habilidade do tendão para resistir a forças compressivas (Carvalho and Vidal, 1994).

Os tendões variam na forma e no tamanho, podendo ser achatados ou cilíndricos. São encontrados na origem, inserção ou nas intersecções tendinosas dentro do músculo. Pode dar origem a ossos, como ocorre em ossos sesamoides que se originam de tendões que passam sob uma superficie articular. O tendão pode passar também por mudanças graduais, como ocorre na junção osteotendinosa, onde o tendão sofre transição para estrutura fibrocartilaginosa e desta para osso lamelar (O'Brien, 1997).

Algumas estruturas colagênicas de tendão, derme, pericárdio e *chordae tendinae* apresentam uma característica morfológica denominada de "crimp", detectada à microscopia de polarização, desde que haja uma orientação macromolecular das fibras de colágeno (Vidal, 1995). Existem diferentes padrões de "crimp", que podem ser mais retos ou ondulados (O'Brien, 1997), exibindo um padrão regular sinusoidal com uma periodicidade e amplitude que pode ser própria para uma determinada região do tendão. A sua variabilidade morfológica, sugere uma diferenciação funcional ao longo da região de tensão e isto poderia ser atribuído tanto a diferentes propriedades biomecânicas como também ao grau com que as diferentes zonas se acomodam no estado de repouso (Carvalho and Vidal, 1994). O "crimp" funciona como um sistema de tampão, de modo que uma fraca elongação longitudinal pode ocorrer sem significativo estiramento das fibras (O'Brien, 1977).

Os tendões flexores digitais superficial e profundo de porco são estruturas esbranquiçadas, brilhantes e calibrosas com anatomia complexa, envolvendo a participação de vários ligamentos.

A figura 1 esquematiza a anatomia do membro esquerdo posterior, vista de um plano superficial, segundo Barone (1980). Pode ser visto o ligamento interdigital ligado de um lado às falanges digitais dos pequenos dedos, e do outro lado, funde-se, na região palmar, com os ligamentos anulares dos tendões flexores digitais, e às junturas metacarpofalângicas dos dedos principais. Os ligamentos interdigitais longitudinais centrais têm origem nas bases dos dedos pequenos, atravessam distalmente os tendões flexores de maneira oblíqua e central passando através do ligamento interdigital proximal e se funde

com o ligamento distal. Os dois feixes colaterais representados pelos ligamentos interdigitais colaterais longitudinais, estão unidos juntamente com os ligamentos interdigitais proximais às falanges distais dos dedos pequenos e, distalmente, eles estão fundidos com a parte externa do ligamento distal.

Estes tendões foram divididos em regiões segundo os seus aspectos anatômicos e prováveis propriedades biomecânicas: Para o tendão flexor digital superficial (TFDS) temos a região proximal (p), que contorna a articulação tibio-tarsal, e recebe forças de compressão e fricção além das forças de tensão; intermediária (i), que está sujeita quase que apenas forças de tensão, e a distal (d), que bifurca-se em direção aos dígitos e provavelmente forças de compressão atuam nesta região, quando passa perto da junta metatarsofalângica. Em alguns experimentos a região proximal foi dividida em porção superficial (sp), mais próxima da articulação tibio-tarsal, e porção profunda (dp), que está mais distante desta articulação. Quanto ao tendão flexor digital profundo (TFDP) a região proximal (p), se origina no músculo flexor digital profundo e está predominantemente sujeita às forças de tensão; região distal (d), que se bifurca em direção aos dígitos contornando a articulação metatarsofalângica, e região terminal (t) que se insere nos terceiro e quarto dígitos. Nestas duas últimas regiões atuam forças de tensão e compressão.

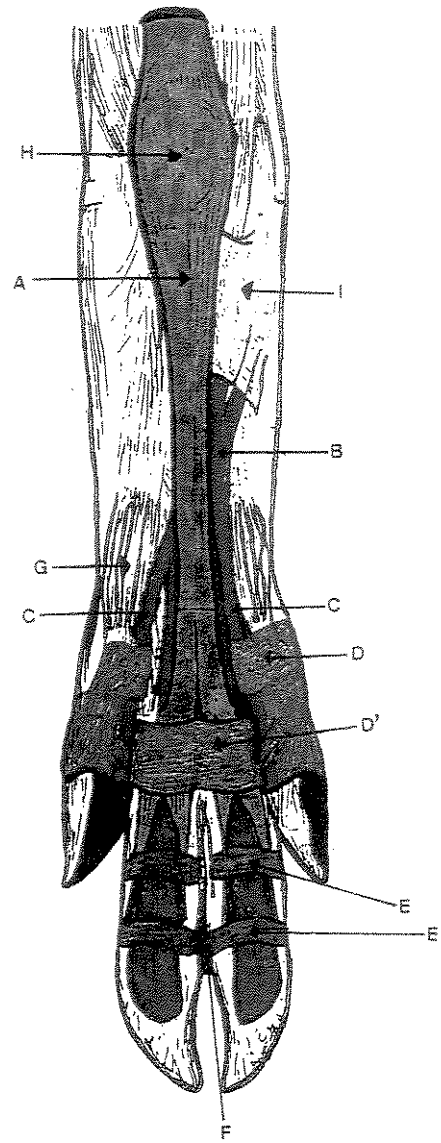

Figura 1. Aspectos anatômicos da face plantar do membro esquerdo posterior do porco, plano superficial segundo Barone, 1980. **A.** Tendão flexor digital superficial (TFDS). **B.** Tendão flexor digital profundo (TFDP). **C.** Ramos do TFDP para dedos pequenos. **D-D'.** Bainhas digitais ou ligamentos anulares plantares. **E.** Ligamentos anulares dos dedos. **F.** Ligamentos interdigitais distais. **G.** Músculo abdutor dos dedos pequenos. **H.** Calota calcanear do TFDS. **I.** Retináculo dos flexores e bainha plantar do tarso.

2. Bioquímica da matriz extracelular do tendão

A MEC de tendão é complexa sendo constituída por componentes fibrilares e não fibrilares. Entre os primeiros estão situados alguns componentes microfibrilares, as fibrilas colagênicas e fibras elásticas. Os componentes não fibrilares são representados pelos PGs e glicoproteínas não colagênicas. Essas moléculas interagem entre si através de ligações eletrostáticas, interações hidrofóbicas e pontes de hidrogênio, permitindo uma organização estrutural e funcional da matriz.

2. 1. Colágenos

O colágeno constitui a maior classe de proteínas fibrosas insolúveis da MEC de tecidos conjuntivos, sendo encontrado em diversos tipos de tecidos como, tendão, pele, ligamentos e ossos. Atualmente são conhecidos dezenove tipos de colágeno geneticamente distintos. Estas distinções são devidas à composição e sequência de aminoácidos das cadeias que compõem a molécula do colágeno, resultando em diferentes níveis de organização supramolecular. Os diversos tipos de colágeno apresentam diferentes arranjos que permitem agrupá-los de acordo com os aspectos estruturais (Eyre et al. 1987; Kwan et al.1991; van der Rest and Garrone, 1991; Mayne and Brewton, 1993; Birk and Mayne, 1997; Liu et al. 1995).

Os colágenos fibrilares dos tipos I, II, III, V e XI ocorrem na maioria dos tecidos conjuntivos de vertebrados, e se agregam em fibrilas apresentando estriações periódicas em torno de 67 nm.

Além desses tipos existem os colágenos não fibrilares, que estão presentes nos tecidos em menor quantidade desempenhando uma função de conexão entre os elementos do tecido conjuntivo. Estes tipos de colágenos fazem parte da família FACIT e compreendem os colágenos dos tipos IV, VI até o X e XII até o XIX. Os colágenos dos tipos IX, XII, XVI e XIX não formam estruturas supramoleculares entre eles próprios, mas se integram a fibras heteropoliméricas (LIU et al. 1995), ou na superfície dos colágenos fibrilares dos tipos I e II (Grässel et al. 1998). O colágeno tipo IX pode ser considerado também um PG, já que tem uma cadeia de glicosaminoglicano (GAG) covalentemente ligada. O colágeno do tipo VI forma finos filamentos em contas que podem associar-se com

fibrilas e células, e o tipo VII forma fibrilas de ancoragem ligando o epitélio e a membrana basal à derme (van der Rest and Garrone, 1991; Mayne and Brewton, 1993).

O componente mais abundante da MEC de tendões é o colágeno tipo I, cuja concentração nesse tecido pode atingir 90% do seu peso seco (Nimni and Harkness, 1988), mas o tipo III também foi detectado (Hermann et al. 1980; Brodsky and Eikenberry, 1985). A presença do colágeno V (Jimenez et al. 1978) e os da classe FACIT (colágenos associados às fibrilas com triplas hélices interrompidas), especialmente os tipos XII e XIV (Sugrue et al. 1989; Castagnola et al. 1992) foram detectados em tendões de feto bovino, e não em adultos. Estas fibras colágenas estão relacionadas diretamente com a função biomecânica (Vidal and Carvalho, 1990). Elas agem como transdutoras de energia, transformando energia mecânica em sinais capazes de estimular os fibroblastos a modularem o arranjo molecular do ambiente extracelular (Vidal, 1966; Vidal, 1969; Vidal, 1985). Além disso, o arranjo estrutural das fibrilas e fibras de colágeno, bem como sua associação com outros elementos da matriz tem reflexo nas propriedades biomecânicas dos tendões, que são influenciadas pelo diâmetro, o estado de agregação molecular e o espaço ocupado pelas fibras. Desse modo, as fibrilas são os elementos responsáveis pela resistência do tendão. A eficiência deste mecanismo depende do direcionamento paralelo destes elementos com a direção das forças a que estão sujeitas (Hukins and Aspden, 1985).

A interação das moléculas de colágeno aos PGs ocorre através das fibrilas, como também do colágeno tipo VI e das glicoproteínas da matriz interfibrilar. Estes componentes têm importante função na estabilização das fibrilas, na mediação das interações com outros componentes da matriz, estabilizando dessa forma o desenvolvimento e os tecidos maduros (Birk et al. 1996).

2. 2. Proteoglicanos

Outro componente da matriz fibrosa do tendão são os PGs que representam 1% do peso seco nos tecidos fibrosos (Vogel and Heinegård, 1985; Iozzo and Murdoch, 1996). São moléculas importantes na organização da MEC interagindo com o colágeno e outras glicoproteínas da própria matriz. Tais interações são realizadas através do esqueleto protéico central do PG, ao qual está ligada covalentemente no mínimo uma cadeia lateral de

GAG (Asundi et al. 1992; Hascal and Kimura, 1982; Heinegård and Oldberg, 1989; Ruoslahti and Yamaguchi, 1991).

Em tendões da cauda de ratos recém nascidos foram encontrados PGs contendo dermatam sulfato (Scott and Orford, 1981), mais tarde a presença de grandes e pequenos PGs foram encontrados em tendões bovinos adultos (Vogel and Heinegård, 1985). Outro GAG é o ácido hialurônico (que não é sulfatado), que se liga eletrostaticamente ao esqueleto protéico central de vários monômeros de PGs, formando vários agregados de alto peso molecular (Hardinghan et al. 1986; Heinegård and Sommarin, 1987; Oldberg et al. 1990). Estes agregados de PGs retém também grande quantidade de água devido a alta densidade de cargas negativas das cadeias de GAGs, sendo responsáveis pela manutenção do alto teor hídrico da matriz, gerando dessa forma uma pressão osmótica e um intumescimento que resulta numa espécie de um gel hidratado. Este intumescimento é contido pelas fibrilas de colágeno, possibilitando ao tecido suportar forças compressivas com um mínimo de deformidade (Hascall and Hascall, 1983).

Além dos grandes PGs, existe ainda um grupo classificado como pequenos PGs ou PGs de baixo peso molecular (Heinegård et al. 1986; Oldberg et al. 1990). Essas moléculas estão estruturalmente relacionadas mas geneticamente distintas, com ampla distribuição pelos tecidos de vertebrados (Fischer et al. 1987, 1989; Oldberg et al. 1989). Os pequenos PGs mais conhecidos são: decorim, fibromodulim, e biglicam.

2. 2. 1. Fibromodulim

O fibromodulim é um pequeno PG formado por um "core" protéico de aproximadamente 48 kDa, ao qual estão ligados quatro cadeias de queratam sulfato (Oldberg et al. 1989; Plaas et al. 1990), o que contribui para o caráter aniônico da molécula. Este pequeno PG é encontrado em tendão e cartilagem (Gallagher, 1989; Oldberg et al. 1990).

Trabalhos diversos têm evidenciado que pequenos PGs como decorim e fibromodulim inibem a agregação lateral das fibrilas colagênicas (Vogel et al. 1984; Vogel and Troter, 1987). Essa inibição provavelmente ocorre através da ligação do PG à molécula de colágeno, como detectado por Scott and Orford (1981). A associação entre os PGs e

colágeno ocorre de forma ordenada, de modo que os GAGs se dispõem paralelamente em relação ao eixo maior dos feixes de colágeno (Vidal and Mello, 1984), promovendo dessa forma a integridade e a manutenção das propriedades biomecânicas de diferentes estruturas (Scott, 1988).

2. 2. 2. Decorim

O decorim foi inicialmente purificado de osso, (Hocking et al. 1998), cartilagem articular de boi (Rosenberg et al. 1985; Heinegård et al. 1986; Fisher et al. 1989), tendão bovino adulto (Vogel and Heinegård, 1985) e isolado de pele (Choi et al. 1989). É um PG de baixo peso molecular, constituído de um esqueleto protéico com 45 kDa e apenas uma cadeia de GAG (Mann et al. 1990), que pode ser condroitim sulfato (osso) ou dermatam sulfato dependendo do tipo de tecido considerado (tendão, pele, esclera e cartilagem). O decorim é assim chamado porque decora as fibrilas de colágeno de um modo bem característico (Scott and Orford, 1981). Nos vários tecidos observados ele é caracterizado pelas diferentes quantidades de ácido idurônico presente na cadeia do GAG. O decorim *in vitro* regula a fibrilogênese do colágeno (Hedbom and Heinegård, 1989; 1993; Hedlund et al. 1994), sugerindo que ele desempenha importante função na organização das fibras de colágeno e na manutenção da integridade do tecido. Provavelmente o material cationicamente corado localizado nas bandas "d" das fibrilas de colágeno de tendão da cauda de rato (Scott and Orford, 1981; Scott, 1988), trata-se da cadeia de GAG do decorim, que também se estende ao longo das fibrilas de colágeno no espaço entre as bandas "d" e "e" (Pringle and Dodd, 1990). Recentemente foi constatado que o decorim se liga em um sítio da região C-terminal do colágeno I, justamente em uma região onde estão os principais sítios de ligação cruzada intermolecular em colágenos heterotriméricos (Keene et al. 200. A associação do PG rico em dermatam sulfato com fibras espessas de colágeno têm sido também observadas durante a decidualização do endométrio em camundongos (Greca et al. 2000). O decorim não está simplesmente envolvido com a fibrilogênese do colágeno *in vivo* (Birk, 1995), mas também ele modula a atividade do fator de crescimento TGF-β participando dessa forma da regulação do crescimento celular (Visser et al. 1994; Webber,

1996; Sini et al. 1997; Schonherr et al. 1998). É uma molécula altamente conservada através das espécies e possui um domínio central com leucinas repetidas (Webber, 1996).

2. 2. 3. Biglicam

Outro pequeno PG presente também na matriz de tendão é o biglicam. Este PG possui na sua constituição leucinas repetidas e dois conjuntos dipeptídicos, a cada um deles está ligado uma cadeia de condroitim sulfato ou dermatam sulfato, dependendo do tecido considerado (Neame et al. 1989). Este PG não se encontra associado com as fibrilas de colágeno, e sim a outros elementos dos tecidos conjuntivos como pode também estar associado às células endoteliais dos vasos sanguíneos. Tem sido encontrado em menisco, tendão, cartilagem, e MEC do sistema nervoso central e periférico (Oldberg et al. 1989; Fleischmajer et al. 1991).

2. 3. Proteínas não colagênicas

Além dos colágenos e PG, existem na MEC as proteínas não colagênicas, correspondendo cerca de 0,5% do peso úmido do tendão (Frank et al. 1987). Uma dessas proteínas estudadas em tendões é a proteína oligomérica da matriz (COMP), identificada pela primeira vez em cartilagem articular (Spitz-Fife and Brant, 1984; Spitz-Fife, 1985), com 524 kDa, acídica, oligomérica e composta de cinco subunidades (Efimov et al. 1994: Malashkevich et al. 1996). A sua função ainda não está esclarecida. Há indícios de que contenha resíduos de carboidratos, possivelmente condroitim sulfato (Hedbom et al. 1992). A presença da COMP em tendão foi identificada apenas na região metacarpofalângica do tendão flexor digital profundo de boi (DiCesare et al. 1994; Smith et al. 1995), uma área que recebe forças de compressão (Vogel and Koob, 1989; Evanko and Vogel, 1990). Segundo estes autores, a presença da COMP estaria relacionada à presença de fibrocartilagem desenvolvida mediante à presença de forças compressivas. No entanto, estudos posteriores mostraram que esta proteína teria uma distribuição muito mais ampla, tendo sido detectada também em regiões desprovidas de fibrocartilagem em bovinos (Smith et al. 1995). Recentemente, esta proteína foi encontrada em tendão flexor digital de cavalo, e quantificada em outros tipos de tendão de diferentes espécies e idades (Smith et al. 1997).

A maioria das proteínas não colagênicas são multifuncionais, devido à presença de domínios estruturais dentro da mesma molécula. Essas proteínas estão envolvidas em fenômenos que contribuem para a interação célula-matriz permitindo uma sinalização bidirecional (Johansson, 1996).

2. 4. Fibras elásticas

A MEC de tendão é formada também por um sistema elástico constituído por três tipos de fibras: elaunínicas; oxitalânicas e elásticas típicas que apresentam uma distribuição diferencial nos tecidos, promovendo diferenças nos graus de elasticidade.

As fibras elaunínicas contêm, proporcionalmente, muitas microfibrilas depositadas em feixes misturados com grumos de elastina, e apresentam propriedades intermediárias entre as elásticas e oxitalânicas. As fibras oxitalânicas são constituídas exclusivamente de microfibrilas sem elastina, livres de material amorfo e não se alongam sob tensão mecânica, portanto, atuariam impedindo o estriamento das estruturas presentes nas regiões onde elas ocorrem. Entretanto, o componente microfibrilar teria uma função similar nas fibras elásticas, ancorando a elastina amorfa à matriz circundante. Já as fibras elásticas típicas (maduras) apresentam material amorfo na parte central, circundado pelo componente microfibrilar. A elastina (90%) é o principal componente de uma fibra elástica madura e sua composição é rica em glicina (33%), prolina (10-13%), aminoácidos hidrofóbicos (44% dos resíduos totais) e 4% de lisina. Em nível estrutural, as fibras elásticas são formadas por um cilindro central sólido composto por abundante material amorfo e homogêneo (identificado como elastina) envolvido por microfibrilas (10 a 12 nm de diâmetro) formadas de glicoproteínas (Junqueira and Carneiro, 1995; Ross et al. 1995).

A presença de um sistema elástico desenvolvido permite ao tendão sofrer uma grande distensão antes de que as fibrilas de colágeno possam exercer qualquer efeito útil de resistência à tensão.

Carvalho e colaboradores (1994), analisaram a existência e distribuição dos componentes microfibrilares (fibras pré-elásticas) da MEC de tendão sujeito a forças de compressão em cães e coelhos, e concluíram que nestes tendões não ocorrem fibras elásticas maduras. No entanto foi observada a presença de fibras elaunínicas em tendão de

coelho, especialmente na zona de compressão. A existência das fibras pré-elásticas é importante na manutenção da arquitetura do tecido e na habilidade do tendão em suportar forças de tensão e compressão.

Em tendão de *Rana catesbeiana* a região sujeita a força de compressão apresentou fibras pré-elásticas e fibras elásticas maduras, às quais estavam associadas aos feixes de colágeno. O sistema elástico é importante para a organização supramolecular das fibras de colágeno na região de compressão e na morfologia do "crimp" na região de tensão (Carvalho and Vidal, 1995).

A presença das fibras elásticas foi estudada por Carvalho and Taboga (1996) em diversos tecidos de mamíferos, com o emprego do microscópio de fluorescência em cortes corados com a hematoxilina-eosina em tecidos ricos em elastina.

3. Características biomecânicas do tendão

O processo de evolução tem selecionado uma MEC com sua composição e estrutura organizada, acompanhando certas propriedades biomecânicas e fisiológicas bem definidas. As variações no tipo e concentração de macromoléculas na MEC podem relacionar-se com as características funcionais de cada tecido. Além disso, a orientação das fibras de colágeno, bem como o seu grau de organização exercem papel importante na propriedade mecânica do tecido (Vidal and Carvalho, 1990).

Vários trabalhos têm demonstrado que as moléculas de GAGs e de colágeno estão orientadas em tendões (Vidal, 1963; 1964) e na região de ossificação em cartilagem (Vidal, 1977).

Estudos baseados em análises de birrefringência e dicroísmo linear têm indicado que as moléculas de PGs não se encontram distribuídas ao acaso, mas estão dispostas de forma orientada e possivelmente associadas às moléculas de colágeno. Em tendões, o esqueleto protéico central dos PGs está inclinado em relação ao maior eixo dos feixes de colágeno, enquanto as cadeias laterais de GAGs estariam dispostas paralelamente ao referido eixo (Vidal, 1980; Vidal and Mello, 1984). Posteriormente, dados semelhantes foram encontrados em cartilagem articular (Vidal and Vilarta, 1988).

Os tendões têm como principal atividade transmitir forças de tensão, porém, podem receber forças de compressão quando contorna determinada articulação. Okuda e colaboradores (1987a; 1987b), estudaram a composição da matriz de cinco regiões anatômicas do tendão de cão, correlacionando a atividade metabólica e composição da matriz com forças mecânicas que supostamente atuam em cada região.

O tendão flexor profundo de boi, na região que passa junto à articulação próxima aos dedos está sujeito a forças de compressão, bem como a forças de tensão. O conteúdo de PG nesta região é sensivelmente maior, sendo que mais de 50% destes correspondem aos grandes PGs, que interagem com o ácido hialurônico (Vogel and Heinegård, 1985; Evanko and Vogel, 1990).

Os tendões não se adaptam somente às forças de tensão. Nas regiões, onde o tendão está sujeito às forças compressivas são encontradas estruturas típicas de cartilagem. Em tendões flexores digitais de coelho (Gillard et al. 1979; Merrilees and Flint, 1980), cães (Okuda et al. 1987a; 1987b), bois (Evanko and Vogel, 1990), e anfíbios (Carvalho and Vidal, 1994; Carvalho and Felisbino, 1999), são encontradas regiões sujeitas a intensas forças compressivas, exibindo nestas áreas placas fibrocartilaginosas. Essas placas apresentam uma matriz semelhante a cartilagem sugerindo que as células são capazes de responder a estímulos mecânicos e construir uma matriz que pode resistir a forças de compressão. A maior distinção biomecânica destas regiões de tendão é a presença de grandes quantidades de GAGs, primariamente na forma de PGs ricos em condroitim sulfato (Koob and Vogel, 1987), ao contrário das regiões de tensão do tendão adulto, que contém primariamente PGs ricos em dermatam sulfato (Vogel and Heinegård, 1985).

Os fatores que regulam a associação das moléculas de colágeno para formar fibrilas, bem como a função dos pequenos PGs, são questões ainda a serem desvendadas. De acordo com a literatura, pouco se sabe sobre a organização desses componentes em regiões de tendão sujeitas a diferentes forças biomecânicas. Vários autores têm demonstrado que tendões sujeitos a diferentes forças mecânicas, são capazes de ser remodelados dinamicamente pelas células em repostas a estas forças. No entanto, pouco se conhece a respeito destas respostas ao nível celular em decorrência das forças biomecânicas em regiões de tensão e compressão em tendão de porco.

Assim sendo, a intenção deste trabalho foi de realizar um estudo descritivo da composição bioquímica da matriz de tendão, utilizando como modelo experimental os tendões flexores digitais superficial e profundo de porco, considerando-se especialmente as regiões dos tendões que exibem diferentes propriedades biomecânicas. Além da abordagem bioquímica, para se obter um levantamento das moléculas da MEC presentes nas diferentes regiões dos respectivos tendões, foi realizado um estudo histoquímico, usando-se procedimentos recomendados para detectar proteínas e GAGs.

IV - OBJETIVOS

Geral

Identificar diferenças morfológicas e bioquímicas em regiões de tendão que estão sujeitas a diferentes forças biomecânicas.

Específicos

1 - Analisar a estrutura e ultraestrutura das diferentes regiões dos dois tendões.

2 - Identificar os tipos de GAGs presentes em cada região dos tendões.

3 - Identificar os pequenos proteoglicanos da matriz extracelular de tendões flexores digitais de porco.

4 - Avaliar a quantidade de GAG sulfatados e proteínas não colagênicas presentes em diferentes regiões de um mesmo tendão.

V – ARTIGOS

Durante a realização deste trabalho foram elaborados os seguintes artigos para serem submetidos à publicação:

1. FEITOSA, V. L. C.; ESQUISATTO, M. A. M.; JOAZEIRO, P. P.; GOMES, L. FELISBINO, S. L. and PIMENTEL, E. R. Variations in the glycosaminoglycan content, swelling properties and morphological aspects of different regions of the superficial digital flexor tendon of pigs. Submetido ao **Cellular and Molecular Biology**.

2. FEITOSA, V. L. C.; VIDAL, B. C. and PIMENTEL, E. R. Optical anisotropy of a pig tendon under compression. Submetido ao **The Anatomical Record**.

3. FEITOSA, V. L. C.; ESQUISATTO, M. A. M.; JOAZEIRO, P. P.; GOMES, L.; FELISBINO, S. L. and PIMENTEL, E. R. Physicochemical and structural analysis of three regions of the deep digital flexor tendon of pigs. Submetido ao **Annals of Anatomy**.

4. FEITOSA, V. L. C.; ESQUISATTO, M. A. M.; JOAZEIRO, P. P.; VIDAL, B. C. and PIMENTEL, E. R. Comparative ultrastructural analysis of different regions of two flexor digital tendons of pigs. Será submetido ao **Tissue and Cell**.

5. FEITOSA, V. L. C.; GOMES, L. and PIMENTEL, E. R. Biochemical study of a wrap around tendon of pigs. Será submetido ao **Brazilian Journal of Medical and Biological Research**.

6. FEITOSA, V. L. C.; GOMES, L. and PIMENTEL, E. R. Extracellular matrix components of different regions in wrap around tendon of the pig. Será submetido ao **Cell Biology International**.

VARIATIONS IN THE GLYCOSAMINOGLYCAN CONTENT, SWELLING PROPERTIES AND MORPHOLOGICAL ASPECTS OF DIFFERENT REGIONS OF THE SUPERFICIAL DIGITAL FLEXOR TENDON OF PIGS

V. L. C. FEITOSA[1]; M. A. M. ESQUISATTO[2]; P. P. JOAZEIRO[3]; L. GOMES[4] S. L. FELISBINO[4], AND E. R. PIMENTEL[4]

Department of Morphology[1], Federal University of Sergipe, Aracaju/SE, Brazil.

Department of Histology[2], Araras of University, Araras/SP, Brazil.

Departments of Histology[3] and Cell Biology[4], UNICAMP, Campinas, SP, Brazil.

Running title: Glycosaminoglycan content and swelling properties in a pig tendon.

Correspondence to:

Edson Rosa Pimentel

Dept. Cell Biology - Unicamp

CP 6109

13083 – 970 Campinas SP, Brazil

Tel: 55 019 788-7330

Fax: 55 019 788-7821

E-mail: pimentel@unicamp.br

ABSTRACT

Tendons which wrap around a bone or hard surface are subject to compression and frictional forces in addition to tensile forces. Normally this region, which experiences differential loading, exhibits a fibrocartilage-like structure, with collagen fibers in a non-uniform arrangement, elevated amount of glycosaminoglycan and cells resembling chondrocytes. The superficial digital flexor tendon of the pig is a wrap around tendon, and is an excellent model suitable for biochemical and structural studies. The superficial portion of the proximal region of this tendon, which is under compression as it is in direct contact with the bone, it exhibited greater swelling in water and presented a higher level of glycosaminoglycan as compared to the intermediate region, which is under tension forces. Electrophoresis in agarose gel showed the presence of dermatan sulfate in all regions of the superficial digital flexor tendon, while the chondroitin sulfate was prominent in the surface in direct contact with the bone. Intense metachromasy was observed in the sections stained with toluidine blue in the same regions submitted to compressive forces, but especially in the portion that is in contact with the bone. Chondrocyte like cells were observed in these areas. Crimp morphology, observed in the intermediate region, exhibited a clearly sloped aspect in relation to the main axis of the tendon. Elastic fibers were found in all regions, and were disposed in different directions in the regions subjected to compressive forces, while they were disposed in parallel to the collagen bundles in the region under tension. These results reinforce the idea that mechanical forces contribute to a differentiated composition and organization of the extracellular matrix of tendons.

Key words: proteoglycans, fibrocartilage, collagen bundles, crimp, elastic fiber, wrap around tendon.

Abbreviations used:

ECM – Extracellular matrix. SDFT – Superficial digital flexor tendon. sp – Surface of the SDFT in direct contact with the bone. dp – The opposite layer to the articulating surface of the proximal region. i – Intermediate region. d – Distal region. PG – Proteoglycan. GAG – Glycosaminoglycan. CS – Chondroitin sulfate. DS – Dermatan sulfate. PDA – Propylenediamine . HE – Hematoxylin/eosin. ANOVA – Analysis of variance.

INTRODUCTION

Tendon is a fibrous connective tissue with the role of transmitting forces from muscle to bone (Birk *et al.*, 1989; Cribb and Scott, 1995). They vary in shape and size and may be found at the origin of the muscle, at the insertion into the bone and in the form of tendinous intersections within a muscle (O'Brien, 1997).

Tendons have an abundant extracellular matrix (ECM) rich in type I collagen fibrils oriented along the major length of the tendon. Collagen fibers may be distributed in different patterns. Tendons subjected to compressive loads often have irregularly arranged collagen fibrils and are rich in proteoglycans (PGs). If tension is applied in only one direction, the fibers have an ordered parallel arrangement (O'Brien 1997).

PGs constitute about 1% of the tendon mass and are represented mostly by the small PGs decorin and fibromodulin (Vogel and Heinegård, 1985). These small PGs are of particular interest because both biochemical and morphological evidence indicate that they are able to interact with collagen fibrils, and may thus play specific and important roles in the structural and functional properties of the tissue (Vogel and Fischer, 1986). Large PG are also found in tendons, especially in the regions subjected to compression in the wrap around tendons (Vogel *et a*l., 1994). Besides collagen fibers and PGs, non- collagenous glycoproteins also occur, but in much smaller amounts. The distribution of these components along the tendon may not be uniform, due to the non uniform distribution of biomechanical forces.

Wrap around tendons pass around a bone in a joint before its insertion, in a direction that differs from the direction of the muscle (Alexander and Dimery, 1985). This aspect has been found in many species, and the tendon exhibits a fibrocartilaginous organization, where the tendon experiences compressive and frictional forces, in addition to tensile forces (Evanko and Vogel, 1990). In the fibrocartilaginous regions of a tendon, the collagen bundles are disposed in a basket-weave pattern, and large PGs are accumulated. The cells are rounded and disposed in lacunae (Vogel and Koob, 1989; Benjamin and Evans, 1990).

Some studies on rabbit (Gillard *et al.*, 1979; Merrilees and Flint, 1980), canine (Okuda *et al.*, 1987), bovine (Evanko and Vogel, 1990) and amphibian (Carvalho and

Vidal, 1994; Carvalho and Felisbino, 1999) tendons, have taken into account the presence of compressive forces in these fibrocartilaginous regions. The presence of a cartilage-like matrix in certain regions passing under a joint suggests that these tissues are capable of responding to mechanical stimuli and of building up a matrix which can withstand a compressive load (Evanko and Vogel, 1990; Carvalho, 1995). The major biochemical distinction of these regions is the presence of a large amount of GAGs, principally in the form of large chondroitin sulfate PG (Vogel and Heinegård, 1985; Koob and Vogel, 1987). In contrast, the tension region of the adult tendon contains mainly small PGs rich in dermatan sulfate (Vogel and Heinegård, 1985).

The purpose of this work was to study the correlations between PG content, tissue swelling properties and the morphological aspects of different regions of the SDFT of pigs. This tendon was divided in three regions, considering the presence of different mechanical forces: the proximal region (p) is a curved part which passes under the tibiotarsal joint; the intermediate region (i) is an extended region of the tendon, which experiences only tension forces, and the distal region (d), which passes close to the metatarsophalangeal joint. The results demonstrated differences in the composition and organization of the ECM in the different portions of the tendon as it receives a compressive load in addition to tension.

MATERIAL and METHODS

Animals: 45 day-old male pigs of the large White lineage were obtained from the Experimental Surgical Nucleus of the University Medical School.

Biological material: The superficial digital flexor tendon (SDFT) was dissected out of the hind limbs. It was divided into a proximal region, where there is the presence of compressive forces; an intermediate region, where only tension forces are present and the distal region, which slides against the metatarsophalangeal joint (Fig. 1A). The proximal region was separated into a surface portion (sp), which is in direct contact with the bone, and a deep portion (dp) which is further from the articulating surface (Fig. 1B), similar to that described by Koob and Vogel (1987) for the bovine tendon. The tendons were stored at –70°C until used for the biochemical analysis.

Swelling Test: The different regions of the SDFT were sequentially full-thickness cross-sectioned. The proximal region was divided into the sp and dp areas. Tendon fragments were equilibrated in 0.15 M NaCl, 50mM NaH_2PO_4 at pH 7.0 for 1h at room temperature, blotted on filter paper, and weighed. Each fragment was then equilibrated in a 1000-fold excess volume of water for 60 min with occasional stirring, and were then weighed again. The same fragments were subsequently equilibrated in 3% acetic acid at pH 2.5 (1000 - fold vol.) for 1h, after which the wet weight of each of them was determined again (Koob and Vogel, 1987).

BIOCHEMICAL ANALYSIS:

Papain digestion: 50 mg tendon fragments were digested with 500µL of a papain solution (40 mg/gram tissue) in 0.03M sodium citrate buffer containing 0.04M EDTA and 0.08M 2-mercaptoethanol at pH 3.5 for 24 h at 50 °C. Afterwards the material was centrifuged in a Fischer microcentrifuge mod. 235V, at 8,000 rpm for 3 min. The supernatant containing the GAGs was precipitated with 2 volumes of 95% ethanol for 24 h at 4°C. The GAGs were precipitated after centrifugation, washed with 80% ethanol followed by acetone

(Michelacci and Horton, 1989) and dried at 37°C. The GAGs were stored at 4°C for subsequent analysis. The GAGs, released by papain digestion, were identified by agarose gel electrophoresis in propylenediamine (PDA) buffer as described by Dietrich and Dietrich (1976).

Quantification of Sulfate-GAG: The sulfated GAGs were quantified using the dimethylmethylene blue method (Farndale *et al.*, 1986). The standard was whale chondroitin 4-sulphate (Sigma) at concentrations of 5, 10, 15, 20 and 25 µg/100µL. Absorbance was measured at λ=526 nm in a Hewlett Packard 8452 A Diode Array Spectrophotometer.

STRUCTURAL ANALYSIS:

Histology and Histochemistry: Tendon fragments were fixed in 4% paraformaldehyde in Millonig's buffer at pH 7.4 for 24 h at room temperature. The material was then dehydrated in a graded ethanol series, clarified in xylene and embedded in Paraplast Plus embedding medium. Serial 6 µm thick sections were cut longitudinally and stained with hematoxylin-eosin (Behmer *et al.*, 1976). Some sections were stained with 0.025% toluidine blue in McIlvaine buffer at pH 4.0 for 20 min (Mello and Vidal, 1980). Collagen fibers were observed after staining with picrosirius red solution (0.1% sirius red F3B 200 in a saturated picric acid solution) for 20 min (Junqueira *et al.*, 1979) and counter staining with Harris hematoxylin for 10 min. Elastic fibers were identified after oxidation with peracetic acid for 20 min and staining with Weigert's fuchsin-resorcin for 1 h, plus counter staining with picric acid for 5 min. Sections were mounted in Entelan mounting medium (Goldfischer *et al.*, 1983).

STATISTICAL ANALYSIS:

The data were statistically analyzed by the analysis of variance (ANOVA) with Fischer's distribution at the 5% level of significance (Beiguelman, 1991).

RESULTS

Swelling Properties

The swelling properties of the different regions of the SDFT are shown in Fig. 2. When the tendon fragments were transferred from PBS to distilled water (Fig. 2A), a noticeable increase in wet weight was observed for the sp portion. The dp portion and intermediate and distal regions actually shrank (represented by the negative values of relative swelling). When tendon fragments were soaked in 3% acetic acid (Fig. 2B), the wet weight increased differently for each region, but most noticeably for the intermediate region and dp portion. The aspects of the different regions after soaking in PBS and in 3% acetic acid can be seen in Fig. 3.

Sulfated GAG content and types

The total amounts of sulfated GAGs present in each region of the tendon are shown in Fig. 4. The presence of sulfated GAGs was higher in the sp portion of the proximal region. The lowest amounts were detected in the dp portion and intermediate region, while the distal region showed an intermediate value. The analysis in agarose gel using PDA buffer (Fig. 5), showed the marked presence of DS in all regions. CS was prominent in sp, but scarce in the dp portion, intermediate and distal regions.

Structural aspects

All the three regions were highly cellular (Figs. 6A, 6B, 6C). In the proximal region, specially in sp, collagen fibers seemed less organized and rounded cells were seen (Fig. 6A), unlike those found in the intermediate region, where the typically elongated fibroblasts were parallel to the long axis of the tendon (Fig. 6B). Similar aspects were observed in the distal region (Fig. 6C).

The differences in fiber organization in the three regions were better observed in the sections stained by picrosirius (Figs. 6D, 6F, 6G) and examined under polarized light. In the proximal region (Fig. 6D) the collagen bundles appeared much less organized and exhibited a shorter crimp period, as compared to the intermediate region (Fig. 6E). In the

distal region, the collagen bundles were less organized (Fig. 6F) than in the intermediate region, but with a crimp "pattern" similar to that of the proximal region.

Staining with toluidine blue revealed the presence of GAG in the three regions (Figs. 6G, 6H, 6I). However, the proximal region showed an intense metachromasy in the portion close to the bone (sp), (Fig. 6G). This metachromasy faded in the direction of the opposite portion (dp) (Fig. 6H). There was a weak basophily in the intermediate region (Fig. 6I), except in some areas where the fibroblasts formed clusters. A wavy aspect of collagen bundles was clearly observed in this region. In the distal region this aspect was much less visible (Fig. 6J). Also at the periphery of this region, the morphology was quite different when compared to that of the intermediate region, exhibiting rounded cells, like those observed in the proximal region, and collagen fibers that were not so organized.

Elastic fibers were detected in the three regions (Figs. 7A, 7B, 7C, 7D), but their distribution was different in each of them. In the proximal region (Fig. 7A), especially in the portion near the bone, the elastic fibers ran in different directions. The deeper layers of the proximal region (Fig. 7B) showed elastic fibers arranged predominantly parallel to the main axis of the tendon. A similar distribution was found in the intermediate region (Fig. 7C). In the distal region (Fig. 7D), besides those elastic fibers that were prominently distributed parallel to the tendon axis, there were some fibers which were arranged obliquely.

DISCUSSION

In this study, the composition, organization and the swelling capacity of different regions of the pig SDFT were analyzed, taking into account the presence of different mechanical forces acting on the tissue.

The swelling tests showed increased swelling in water for the superficial portion of the proximal region. That portion is close to the tibiotarsal joint and, as such, is subjected to compressive as well as tensile forces. The dp, intermediate and distal regions actually shrank. When the specimens were equilibrated in 3% acetic acid, the result was the opposite. The sp portion exhibited the lowest value of swelling and the intermediate region the highest, this being the typical behavior of a highly collagenous tissue, where only tensile forces were present. Similar results were found for bovine flexor tendon (Koob and Vogel, 1987).

The higher concentration of sulfated GAG found in the sp portion is in accordance with the swelling increments in water, showing a clear correlation between the two parameters.

The cell morphology and distribution followed a pattern typically observed in tendons withstanding compressive forces, (Merrilees and Flint, 1980; Okuda *et al.*, 1987; Vogel *et al.*, 1986; Ralphs *et al.*, 1991; 1992; Carvalho and Felisbino, 1999; Felisbino and Carvalho, 1999). The aspect exhibited by the cells in the proximal region of the SDFT, in contrast to the elongated fibroblasts present in the tension region, demonstrates that pig tendons wrapping around bones exhibit chondrocyte-like cells, as found in other mammals (Merrillees and Flint, 1980; Okuda *et al.*, 1987; Evanko and Vogel, 1990) and amphibians (Carvalho and Vidal, 1994). These observations also confirm earlier reports showing that connective tissue cells are sensitive to mechanical factors, such that under hydrostatic pressure, the cells enhance the production of PG, and when stretched, as in the tension regions, there is an increase in the production of collagen (Giori *et al.*, 1993). The less undulated aspect of the collagen bundles found in the proximal region, plus the greater amount of GAG and increased swelling, is clear evidence of the adaptation of this region to compressive forces.

Besides PG and collagen, other components of the ECM, such as tenascin and type XII collagen, may be regulated by mechanical stimuli in the tendon (Chiquet, 1999).

The distribution of elastic fibers in the pressure-bearing region was quite different to that in the region under tension. In the former, the elastic fibers were distributed in several directions and, as has been suggested (Carvalho *et al.*, 1994; Carvalho and Vidal, 1995), probably display an important role in the ability of the tendon to support tension and compressive forces. In the tension region, the elastic fibers were positioned parallel to the collagen fibers. In each region, elastic fibers are supposed to contribute to the initial distension of the collagen fibers and then to pull them back to this original situation when the tension force is no longer present (Oakes and Bialkower, 1977).

The less undulated aspect of the collagen bundles found especially in the proximal region, in association with the greater amount of GAG and the swelling test results, were a clear evidence of the adaptation of this region to additional compressive forces.

The analysis in agarose gel of the intermediate region GAGs, revealed a minor presence of CS, although DS was prominent, suggesting the presence of PG rich in DS in this tension region. These PGs were probably one or two of the small PGs, like decorin and biglycan. The presence of decorin is expected in a highly fibrous connective tissue, due its importance in regulating collagen fibrilogenesis (Fleischmajer *et al.*, 1991; Visser *et al.*, 1994; Weber *et al.*, 1996; Sini *et al.*, 1997 and Schönherr *et al.*, 1998). Although, the detection of CS discrete, in a region under tension, perhaps arise of another population of large PG, different of that found in the compressive region as discussed by Vogel and co-workers (1994). However cannot be discarded the possibility that the CS of the tensional region, is because the animals (45 days old) were young pigs, since there is a relation between immature tendon and presence of CS (Vidal and Mello, 1984). The high cellularity found in the tendons analyzed here is an indicative of its young nature. The CS found in the proximal region is probably due to aggrecan, a large PG detected in compressed regions of bovine tendon (Vogel *et al.*, 1994). It is important to emphasize the increased content of CS in the proximal region of the SDFT of pigs, in comparison to that in the intermediate region. The discrete presence of CS in the distal region, is probably due to a weaker compressive force in this region, as it passes under the metatarsophalangeal joint.

Our results, besides describing structural and compositional aspects of the SDFT of pigs, once more reinforce the idea that mechanical forces may contribute to the adjustment of the composition and organization of the ECM.

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FIGURE LEGENDS

Figure 1. Anatomical aspects of the SDFT. A - The tendon was divided into proximal (p), intermediate (i) and distal regions (d). The intermediate region experiences only tensional forces, while the proximal and distal regions receive compression in addition to tension. B - Diagramatic representation of the proximal region of the SDFT, showing the superficial portion (sp), which is close to the tibio-tarsal joint, and the deep portion (dp) which is opposite to the articulating surface.

Figure 2. Swelling properties of the three regions of the tendon. A – After transfer from PBS to distilled water, the swelling increment was higher for the sp portion. B – After transfer from distilled water to acetic acid, the swelling was higher for the intermediate region. The dp portion also exhibited great swelling . $P < 5\%$ using the ANOVA statistical analysis.

Figure 3. Appearance of the different regions of the tendon in water (A) and in acetic acid 3% (B).

Figure 4. Quantity of total sulfate GAG in the sp/dp portions, intermediate and distal regions after papain digestion. $P < 5\%$ using the ANOVA statistical analysis.

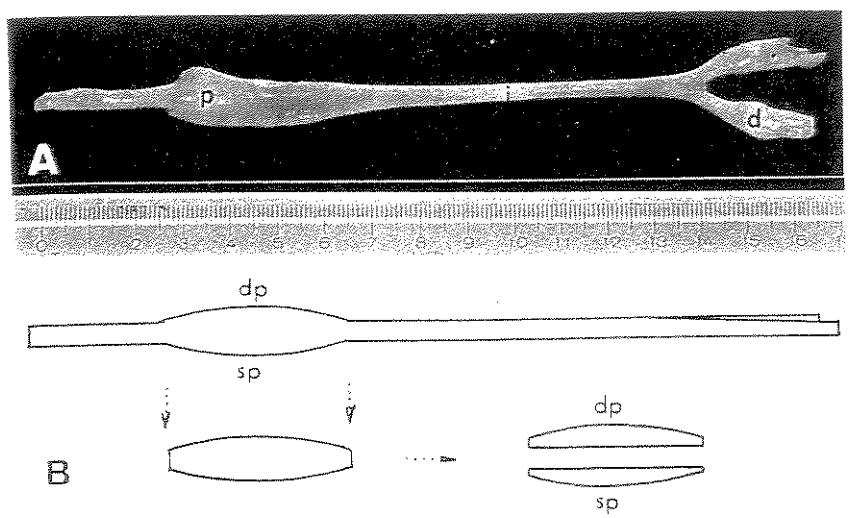
Figure 5. Agarose gel electrophoresis of glycosaminoglycans extracted by papain digestion of the tissue.
Dermatan sulfate (DS) was detected in all the regions of the SDFT, but it was much more expressive in the sp and dp portions. Observe that chondroitin sulfate (CS) predominates in the part close to the bone surface (sp) as compared to the opposite layer (dp), intermediate (i) and distal (d) regions. A standard mixture of CS and DS may be seen on the left. The arrow indicates the migration direction.

Figure 6. Aspects of the SDFT stained with hematoxylin-eosin. Longitudinal sections. A - The proximal region, especially that near the bone, exhibits rounded cells (→), which are randomly distributed. X 400. B - Aspect of the intermediate region. Observe the cells with elongated nuclei, aligned with the collagen bundles (→). X 400. C - Aspect of the distal region, exhibiting fibroblasts and collagen bundles arranged as in the intermediate region (→). X 400.

D-F - Photomicrographs of the compression (proximal and distal) and tension (intermediate) regions of the SDFT stained with picrosirius - hematoxylin. D - Compression region (proximal) showing fibers of collagen in different directions. The pattern of the crimp (➔) which appeared in the three regions is different. The proximal region presented a shorter crimp period when compared to the intermediate region. X 100. E - The tension region (intermediate) showed an intense birefringence of undulated fibers of collagen aligned side by side and along the main axis of the tendon. The crimp morphology seemed (➔) more regular in direction and period than in the proximal and distal regions. X 100. F - In the distal region, which probably receives some compression force, the collagen fibers were undulated, exhibiting a different crimp morphology (➔). X 100.

G-J - Aspects of the regions of the SDFT stained with toluidine blue. G - The articulating layer (sp) exhibits rounded cells (➔) and intense metachromasy (▶). X 400. H - In the nonarticulated layer (dp), typical fibroblastic cells were observed (➔). X 400. I - Morphological aspect of the intermediate region, where tension forces dominate. The basophily is not uniform, appearing abundant close to the vascular structures (▶) and adipocytes (➔). X 100. J - The distal region exhibits very weak metachromasy (▶) as compared to the intermediate and proximal regions. X 100.

Figure 7. Photomicrographs of the compression (sp, dp and distal) and tension (intermediate) regions of the SDFT stained by Weigert's fuchsin-resorcin method.
A - Aspect of the sp portion exhibiting elastic fibers in different directions, following the random orientation of collagen bundles (→). X 200. B - dp portion exhibiting the elastic fibers that follow the main direction of the tendon (→). X 200. C - Intermediate region, with the elastic fibers exhibiting the same distribution observed in the dp (→). X 100. D - The distal region exhibits a larger concentration of elastic fibers, than the proximal and intermediate regions (→). X 100.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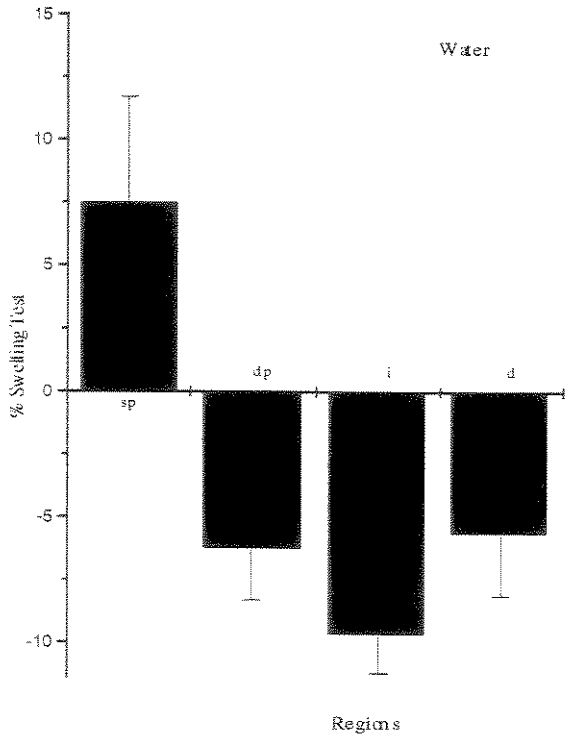
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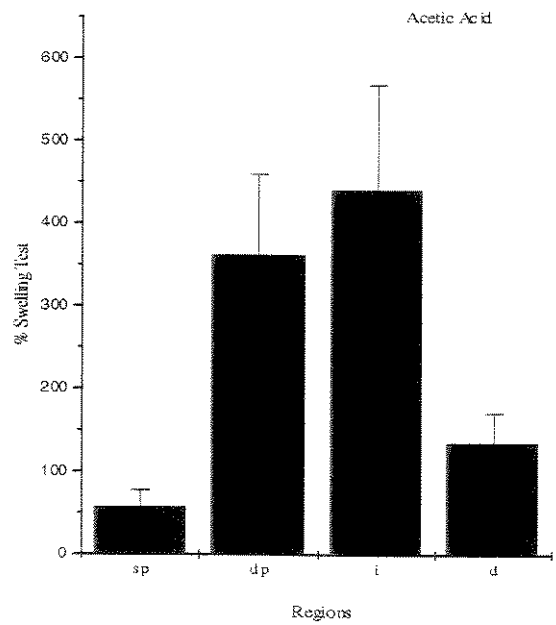
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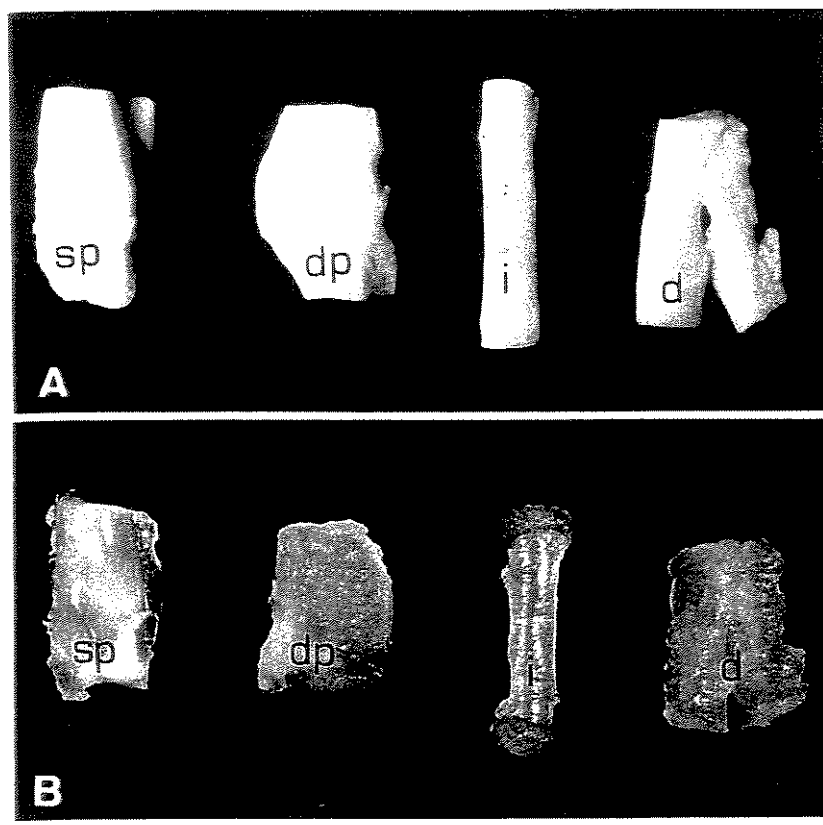

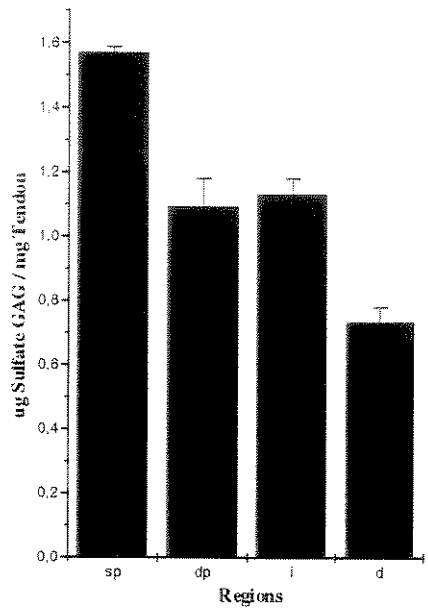
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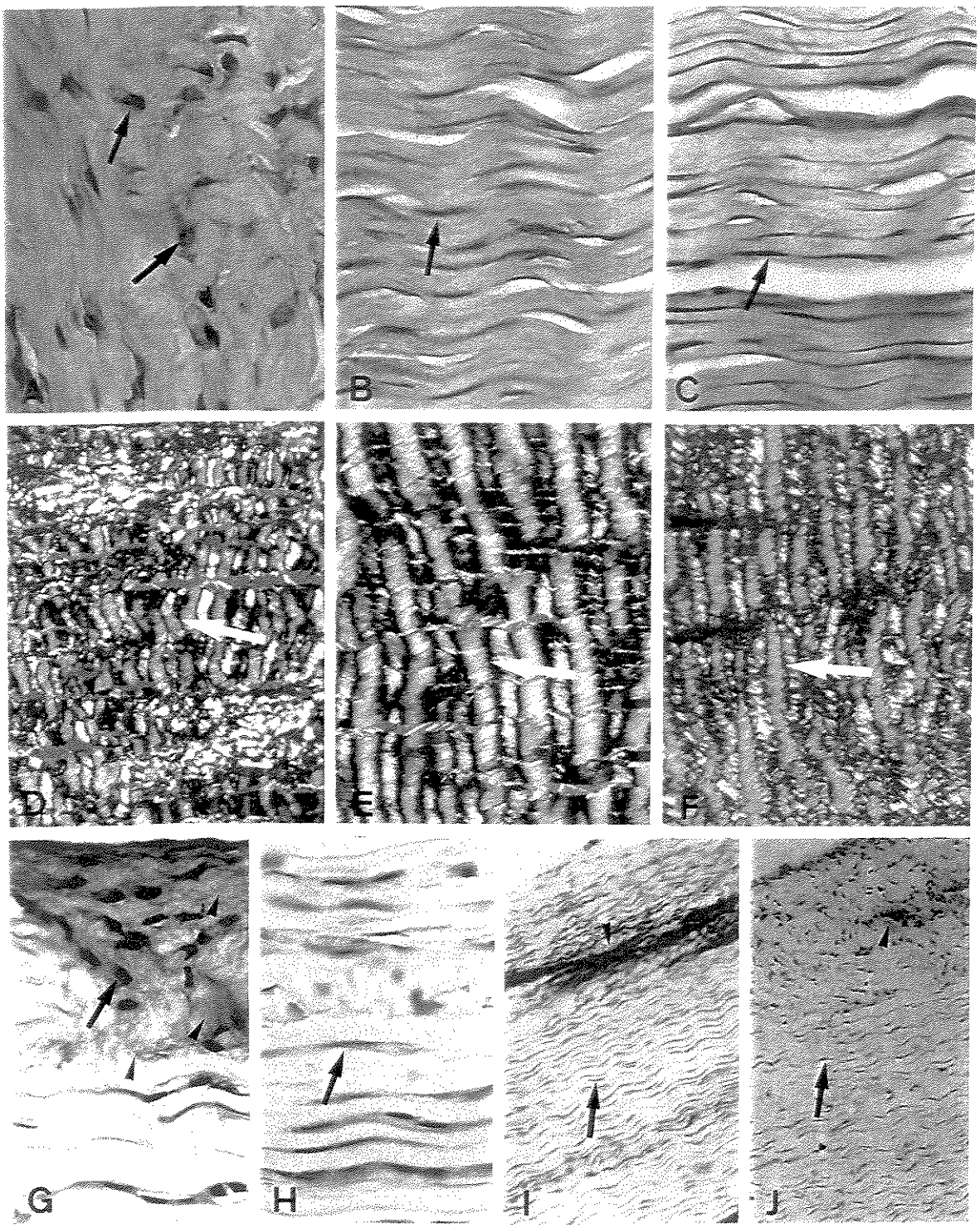
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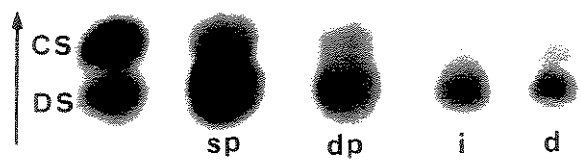
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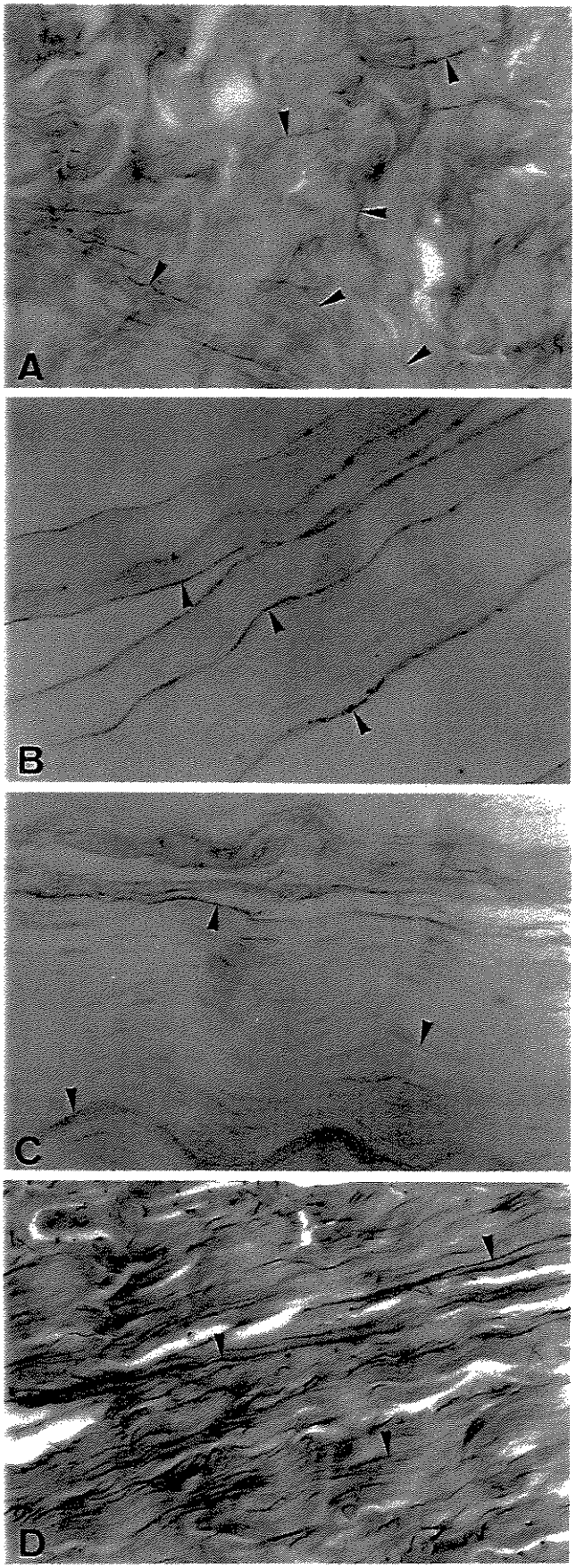
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D

OPTICAL ANISOTROPY OF A PIG TENDON UNDER COMPRESSION

Vera Lúcia C. Feitosa[1]; Benedicto C. Vidal[2] and Edson R. Pimentel[2]

Department of Morphology, Federal University of Sergipe – UFS[1], Aracaju, Sergipe, Department of Cell Biology - Estadual University of Campinas, UNICAMP[2], Campinas, São Paulo, Brazil.

Running title: Optical anisotropy of the pig tendon.

Correspondence to:

Edson Rosa Pimentel

Dept. Cell Biology - Unicamp

CP 6109

13083 – 970 Campinas SP, Brazil

Tel: 55 019 788-7330

Fax: 55 019 788-7821

E-mail: pimentel@unicamp.br

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ABSTRACT

The proximal region of the superficial digital flexor tendon of pigs passes under the tibiotarsal joint and receives compression in addition to tension forces. This region was divided into a surface portion (sp), which is in direct contact with the bone and into a deep portion (dp), which is the layer opposite the articulating surface. The purpose of this work was to analyze the distribution and organization of the collagen bundles and proteoglycans in the extracellular matrix in sp and dp. Toluidine blue stained sections were analyzed in a polarizing microscope. Strong basophilia and metachromasy were observed in sp, demonstrating accumulation of proteoglycan in a region bearing compression, but the intensity was reduced the further layers were from the bone. Linear dichroism confirmed that the glycosaminoglycan molecules were disposed predominantly parallel to the longest axis of the collagen fibrils. The birefringence analysis showed a higher molecular order and aggregation degree of the collagen bundles in areas where the tension was more prominent. The crimp pattern was more regular between sp and dp, probably to attend a major requirement for tendon stretching. The optical anisotropy exhibited by the collagen bundles, also confirmed the helical organization of the collagen bundles in the tendon. Hyaluronidase digestion caused a decrease in the basophilia, but it was not eliminated, supporting the idea that in the matrix, proteoglycans are not completely available to the enzyme action.

Abbreviations used:

SDFT – Superficial digital flexor tendon. p – Proximal region. i – Intermediate region. d – Distal region. sp – Superficial portion of the proximal region which is in direct contact with the bone. dp – The opposite layer to the articulating surface of the proximal region. ECM - Extracellular matrix. PG – Proteoglycan. GAG – Glycosaminoglycan. LD – Linear dichroism. EVPL – Electric vector polarized light.

Key words: pig, tendon, proteoglycan, collagen, anisotropic properties, molecular order, crimp.

INTRODUCTION

Tendons have the role of transmitting tension forces from the muscle to the bone (Vogel and Koob, 1989; Vidal and Carvalho, 1990; Cribb and Scott, 1995; Birch et al. 1997; Milz et al. 1998). Some tendons which pass around bony pulleys receive compressive forces in addition to the tension forces. They are distinguished by an extensive extracellular matrix (ECM) rich mainly in type I collagen fibrils oriented along the length of the tendon. Collagen corresponds to about 90% of the dry mass of this tissue (Nimni and Harkness, 1988). These collagen bundles act as transductors, transforming mechanical energy into signals which stimulate the fibroblast to modulate the molecular arrangement of the extracellular environment. The production of signals in the ECM is dependent on the levels of macromolecular organization of their components (Vidal, 1966; Vidal, 1969; Vidal, 1985).

Proteoglycans (PGs) constitute about 1% of the tendon and are represented mostly by the small PGs decorin and fibromodulin (Vogel and Heinegård, 1985). Non-collagenous glycoproteins are also present, but in a lesser amount. Large PGs are present especially in compressed regions (Vogel et al. 1994).

Studies based on the anisotropic properties of collagen fibers showed that the glycanic chains of the PGs would be positioned parallel to the collagen fibrils and displayed a helical conformation under certain experimental conditions (Vidal, 1963; 1964). Later it was proposed that the polypeptide core would be tilted with respect to the collagen fibrils, and the glycanic chains, with various helicity degrees, would be parallel to the collagen fibrils (Vidal and Mello, 1984). For a good comprehension on the optical anisotropy see Módis (1991).

The collagen fibers are distributed in different patterns. In tissue or tendon regions where tension is exerted in all directions, the collagen bundles are interwoven without regular orientation, while in regions where the tension is only in one direction, the fibers exhibit an orderly parallel arrangement (O'Brien, 1997).

Some collagenous fiber structures of tendons present a morphofunctional characteristic denominated as crimp (Gathercole and Keller, 1991). It is detected by

polarization microscopy since it is revealed when one observes the birefringence due to the macromolecular orientation of collagen fibers (Vidal, 1995). The variations in crimp in the different regions of the same tendon, as well as the distribution of the fibers and their bundles, could reveal some details about the fiber organization in tendons that might explain the crimp organization (Vidal, 1995).

Earlier studies carried out with tendons from rabbits (Merrilees and Flint, 1980), canines (Okuda et al. 1987), bovines (Vogel et al. 1986; Kook and Vogel, 1987; Robbins et al. 1997) and amphibians (Carvalho and Vidal, 1994; Carvalho and Felisbino, 1999; Felisbino and Carvalho, 1999), showed that areas under compression present an increase in the GAG content. Part of these PGs aggregate with hialuronan (Vogel and Heinegård, 1985; Evanko and Vogel, 1990). These authors have also demonstrated that the composition and organization of the extracellular matrix is not the same for the different regions of the tissue, depending on the presence of biomechanical forces.

Fibrocartilage has been found in areas of tendon subjected to compressive and frictional load regimes, in addition to tension. These areas exhibit not only a weave basket-like distribution of collagen fibers and rounded cells usually in lacunae, but also an increased amount of large PGs (Felisbino and Carvalho, 1999).

Considering that in the case of swines, the superficial digital flexor tendon (SDFT) is a tendon which experiences different mechanical forces throughout, the purpose of this work was to analyze the effect of compressive forces on the organization of the extracellular matrix in the proximal region of the SDFT of pigs. This region passes under the tibiotarsal joint and experiences compressive and friction forces in addtion to the tensional forces.

MATERIAL AND METHODS

Biological Material

Forty five day old male pigs, of the large White lineage, obtained from the Experimental Surgical Nucleus of the University Medical School – UNICAMP were used. The hind limbs were dissected to obtain the superficial digital flexor tendon (SDFT) (Fig. 1A). Only the proximal region, which passes under the tibiotarsal joint and is subjected to expressive compressive forces, was employed here. This region was divided into sp, which was near the bone, and dp, which was the layer opposite the articulating surface (Fig. 1B).

Polarization Microscopy

The tendon was fixed in 10% paraformaldehyde in Milloning's buffer, pH 7.4 for 24 h, washed in distilled water several times, dehydrated in ethanol, embedded in Paraplast Plus so that the segments were oriented to obtain histological sections parallel to the longest axis of the tendon, and then cut into 6-μm sections. The preparations were stained with a 0.025% toluidine blue solution in McIlvaine buffer at pH 4.0 (Mello and Vidal, 1980). The toluidine blue stained sections were studied visually to detect basophilia and metachromasy and determine their distribution. Linear dichroism (LD) was detected and visually evaluated in sections stained by toluidine blue. LD observations were carried out by alternating the orientation of the major axis of fibers parallel ($A_{\parallel}$), and perpendicular ($A\perp$) to the azimuth of the electron vector of the polarized light (EVPL). The Zeiss Pol Fotomicroscope was used for this purpose.

Birefringence and morphometric analyses

The birefringence and area measurements were performed under monochromatic (λ=546 nm) light and using a software from the Image-Global Lab Data Translation (USA). Areas of crimp were accurately segmented and were expressed in μm^2 after calibration with a micrometric slide using the objective neofluar Zeiss 16 x optovar 1.25. The birefringence was expressed as transmittance after transformation of the gray values into transmittance

values for the same segmented areas (Vidal, 2000 – personal communication). Statistic analysis was performed using the Mann-Whitney test (Minitab-Release 11).

Enzymatic treatment

Some sections of the proximal region of the SDFT were treated with hyaluronidase (1mg enzyme/mL of 0.9% NaCl solution) for 2 h at 37°C (Kiernan, 1981). The sections were then washed in distilled water and stained with 0.025% toluidine blue for 15 min, washed again, clarified in xylene, and mounted in Entelan (Merck).

RESULTS

Sections of sp and dp of the pig tendon were stained with toluidine blue and observed under polarized light. They exhibited LD with a stronger metachromasy, especially in sp, which is the portion nearest to the bone (Figs. 2A, B).

Selective absorption of polarized light may be observed when toluidine blue planar molecules bind to oriented macromolecules of the ECM. The difference in the absorbance of polarized light when the material is parallel (Fig. 2B) and perpendicular (Fig. 2A) to the plane of polarized light is known as LD.

In this work sections of the proximal region of the SDFT were analyzed, staining with the cationic dye, toluidine blue. A more intense staining may be observed in sp, near the bone, as compared to the opposite layer, dp, which is more distant from the bone. When observations were carried out using a polarizing microscope, LD was detected with a stronger metachromasy, especially in sp. When the fibers were perpendicular (Fig. 2A), to the azimuthe of the EVPL, a greater absorbance was detected. On the contrary, in the parallel position (Fig. 2B) less light absorbance was observed. The further the collagen bundles were from the bone, and thus closer to the opposite layer, so the metachromasy and LD were clearly diminished (Fig. 2B).

When the toluidine blue stained sections were observed with a crossed analyzer and polarizer, at 45° with respect to the polarizers (⊕), a strong birefringence was detected in the median portion of the proximal region of the SDFT (Fig. 3). A greenish color was observed in sp, representing the less compacted collagen fibers present in that area. When the major axis of the tendon was positioned parallel to one of the polarizers, the collagen bundles, which were disposed at 45° in relation to the polarizers (Fig. 4), exhibited a striking birefringence and a well defined wavy like pattern (crimp). Changes in the crimp were observed in the collagen bundles nearest the bone. When the tendon was positioned parallel to one of the polarizers and a first order red gypsy compensator was used, birefringent colors could be seen (Fig. 5). The fibers that appear in blue are in the addition position with respect to the compensator direction (γ), which means that the fiber electron oscillation is at 45° to the polarizer axis. On the contrary, the yellow color means that the

same source of birefringence is in the subtraction position. The regularity of the collagen bundles was evident in the portion distant from the bone, but not in sp, and the crimp patterns seemed to be more regular in dp where tension dominated and compression was reduced. The standard deviations of the measurements (sp standard deviation=17, dp standard deviation=14), confirmed this assunption. The size of the crimp as well as the birefringence values were larger in dp than in sp (Table I).

The sections treated with hyaluronidase, stained by toluidine blue, and observed under polarized light (Figs. 6A, B), showed some metachromasy when the tendon was perpendicular (Fig. 6B) to the polarizer, in the region far the bone. In sp no difference with respect to the dichroism was observed.

DISCUSSION

Tendons which pass under a joint are an excellent biological model for studying the biomechanical and morphological adaptations as a function of the presence of different mechanical forces.

The evolutive process has selected, a special composition and organized structure for the ECM, to attain certain biomechanical and functional properties (Vidal and Carvalho, 1990). The hypothesis of this work was that for the two different portions of the tendon, respectively under pressure and tension, there were different assemblies of macromolecules to supraorganize those regions in order to respond to their biomechanical exigencies.

The collagen molecules are the most abundant component of the tendon, arranged in fibrils, forming fibers that are assembled into bundles of collagen fibers (Jozsa et al. 1991). The mechanical properties of tendons depend on the orientation of the fibrils and collagen bundles, on the diameter of the fibrils and on the level of organization, which plays an important role in the mechanical properties of this tissue (Birk et al. 1989; Vidal and Carvalho, 1990).

Studies based on the birefringence analysis in tendon sections stained with toluidine blue, have shown that the protein core of the PG are tilted with respect to the long axis of the collagen fibrils, while the glycosaminoglycans (GAGs) are statistically parallel (Vidal and Vilarta, 1988). Similar results were found in the articular cartilage (Vidal and Vilarta, 1988; Vilarta and Vidal, 1989).

Here the dichroism observed in sections of the compressive portion of the swine tendon, besides exhibiting greater light absorption when the tendon was perpendicular to the polarized light plane, once again confirming that the GAGs are disposed parallel to the long axis of the tendon (Vidal and Mello, 1984), also showed a stronger metachromasy in the sp portion, where the compressive forces were more intense than in the dp. This result is similar to results obtained with rabbit (Merrilees and Flint, 1980), bovine (Vogel et al. 1994) and frog (Carvalho and Vidal, 1994) tendons.

Topological differences with respect to the organization of the ECM inside a region under compression were observed in rabbits (Merrilees and Flint, 1980), and bovines (Koob

and Vogel, 1987) in agreement with the histochemical and histophysiological data obtained in the present research.

The crimp patterns in the sp and dp portions were quite different, and denote the importance of the arrangement and organization of the collagen fibers to attend the biomechanical requirements of the tissue.

The greater degree of compactness of the collagen fibers observed in the dp portion as compared to the sp portion, was indicative of the differential organization of the ECM in these two portions, which experience tension and compressive forces respectively.

The different patterns of crimp organization in the tension and pressure regions, i.e., greater birefringence and larger areas in the dp region, can be related to the stress and strain to which the collagen bundles are subjected in the dp portion. Collagen fibers and bundles in the dp portion exhibited molecular order and three-dimensional-helical superstructure that satisfy the thermodynamical exigencies in response to the biomechanical demands of that area.

In evolutionary terms, the physicochemical characteristics of the tendon macromolecules favour the specific self assembly process, to generate the tendon supraorganization (Vidal, 1995). Natural selection specified a special composition and organized structure in the tendon.

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Table I. Non-parametric test (Mann-Whitney) of the birefringence (transmitance) and area measurements for the dp and sp portions. η_1 and η_2 correspond to the median values. W represents the rank sum (Bhattacharyya and Johnson, 1977) obtained for the sp and dp portions. The test is significant at 0.0000 (adjusted for ties), (Farthofer and Lee, 1995).

	η_1-dp	η_2-sp	η_1-η_2	W
Areas/μm^2	92.57	59.09	23	136229.0
Birefringence	41.23	35.8	6.11	139156.0

FIGURE LEGENDS

Figure 1. Dorsal view of the hind limb of the pig showing the superficial digital flexor tendon (SDFT). A - The SDFT was divided into a proximal region (p) which bears compression in addition to tension, intermediate (i) where only tension forces are present and distal (d) region, which also withstands compressive force. B - Diagrammatic representation of the tendon, showing the sp portion in direct contact with the tarsic bones of the tibiotarsal joint. The dp portion is the layer opposite the non-articulating layer.

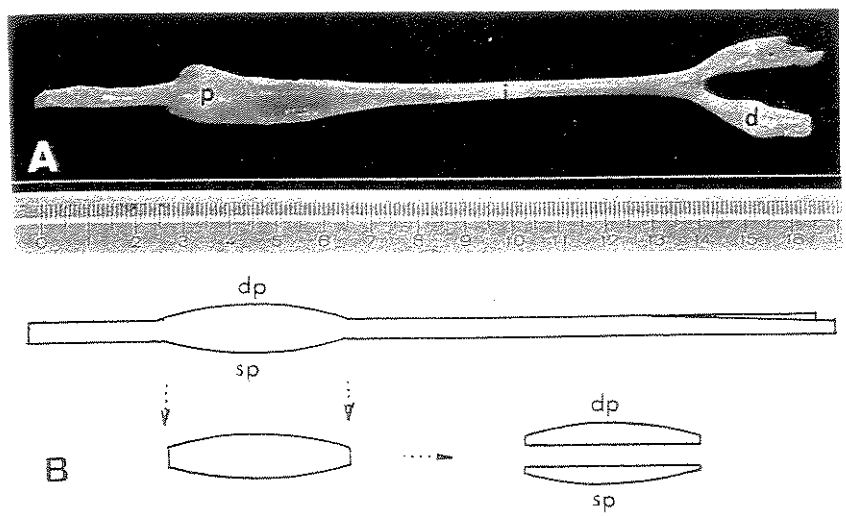
Figure 2. Linear dichroism of the toluidine blue stained sections. A and B correspond to the absorbance when the longest axis of the tendon is perpendicular and parallel respectively to the azimuth of the polarizer. Comparison of images A with B reveal higher absorption when the fibers are perpendicular to the polarized light plane. Observe the stronger metachromasy in sp, which is near the bone. In the deep layer (dp), the staining and even metachromasy is noticeably reduced. X 42. B - Same section, but with fibers positioned parallel to the polarized light plane. X 42.

Figures 3-4. Birefringent images of sections of the proximal region of the SDFT, stained by toluidine blue pH 4.0. The main axis of the tendon was positioned at 45° in relation to the polarizers ⊕. **Fig. 3** - Stronger birefringence is observed in the layer opposite (dp) the bone. A weaker birefringence and a greenish staining were observed in the superficial portion (sp), near the bone and where compression forces were present. X 42. **Fig. 4** - Detail of the crimp aspect of the dp layer. In this case the tendon was positioned parallel to the polarization plane. Dark regions (→) indicate areas of extinction where the fibers are positioned parallel to one of the polarization planes. X 268.

Figure 5. Similar image to those in figures 3 and 4, but with no staining and using a 1st order red compensator. The tendon is positioned parallel to one of the polarizers. The blue color represents the fibers oriented in additive position with respect to the compensator, and the yellow color correspond to the subtractive position. The different crimp patterns in sp

and dp portion are clearly visible. On the right may be seen a draft representing the direction of the crimps. X 42

Figure 6. Distal portion of the sp and dp regions treated with testicular hyaluronidase and stained with toluidine blue pH 4.0. Observations were made under polarized light. A - The fiber was positioned parallel to the plane of polarized light. X 42. B - The fiber was perpendicular to the plane of polarized light. X 42. Observe that even with enzymatic digestion, glycosaminoglycans were not completely removed, indicating that they are not totally available to the enzyme attack *in situ*, probably due to their interactions with other components of the extracellular matrix. The linear dichroism ($A\perp > A_{||}$) reinforce the idea that proteoglycans are oriented along the axis of the tendon.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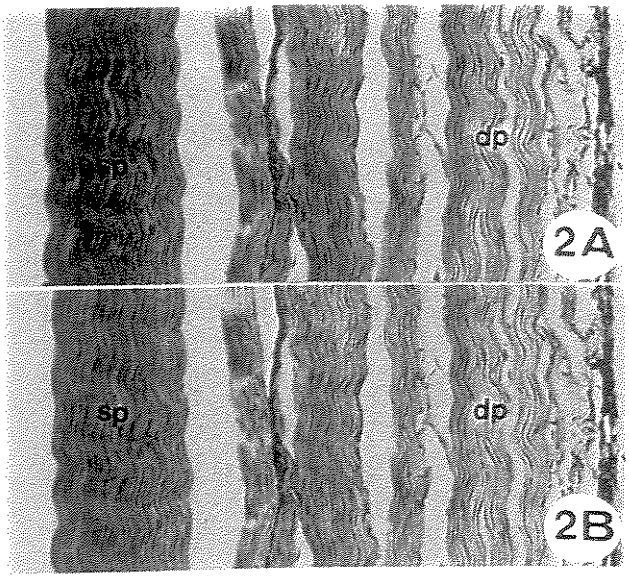
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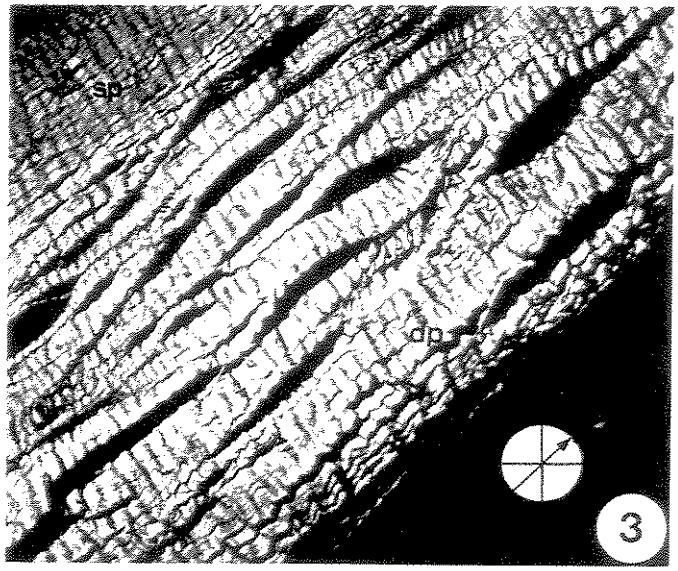
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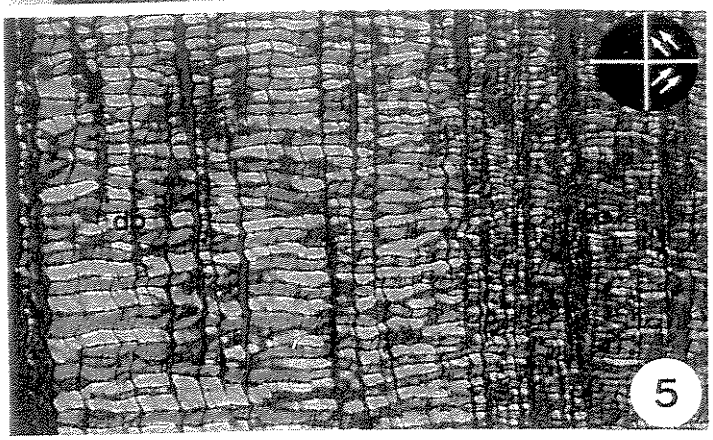
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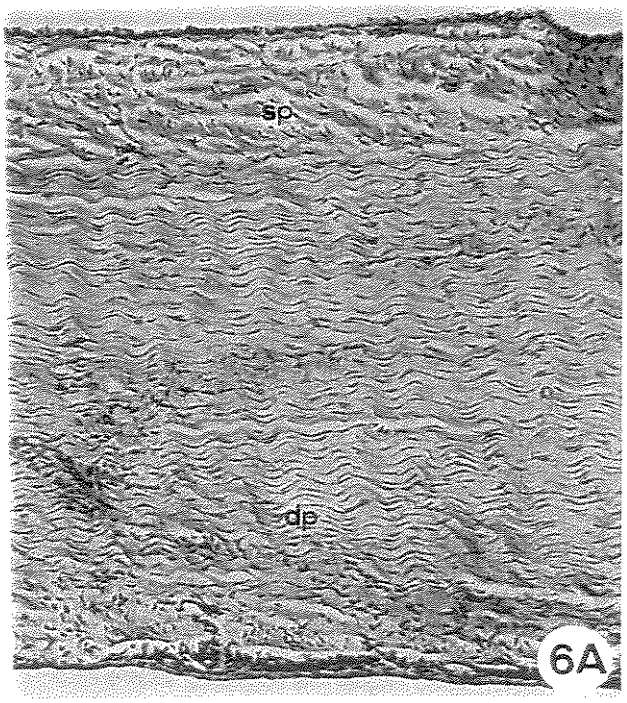
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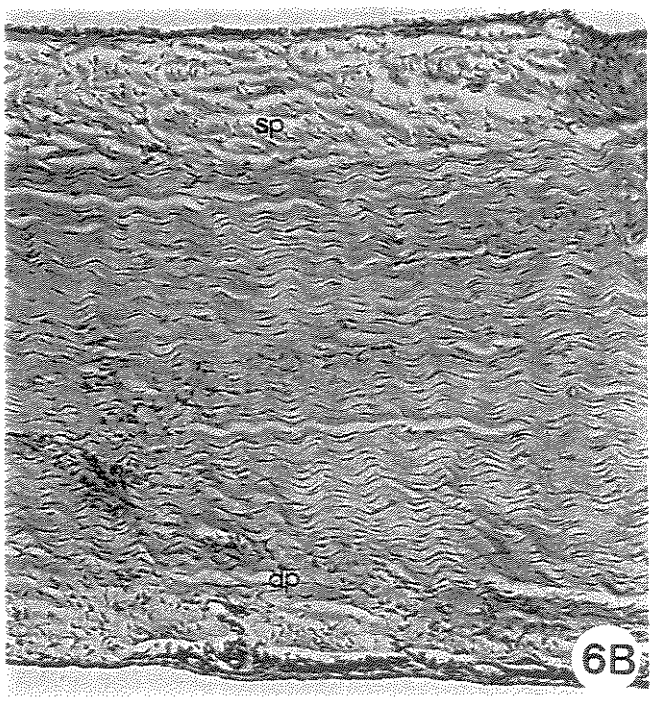
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Physicochemical and structural analysis of three regions of the deep digital flexor tendon of pigs

Vera Lúcia Corrêa Feitosa[1]; Marcelo Augusto Marretto Esquisatto[2]; Paulo Pinto Joazeiro[3]; Laurecir Gomes[4], Sérgio Luis Felisbino[4] and Edson Rosa Pimentel[4]

Department of Morphology[1], Federal University of Sergipe, Aracaju/SE, Brazil.

Department of Histology[2], University of Araras, Araras/SP, Brazil.

Departments of Histology[3] and Cell Biology[4], UNICAMP, Campinas, SP, Brazil.

Runing title: Physicochemical and structural analyses of a pig tendon.

Correspondence to:

Edson Rosa Pimentel

Dept. Cell Biology - Unicamp

CP 6109

13083 – 970 Campinas SP, Brazil

Tel: 55 019 788-7330

Fax: 55 019 788 7821

E-mail: pimentel@unicamp.br

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SUMMARY

In this work we characterized the extracellular matrix of the deep digital flexor tendon of pigs, which experiences tensional (proximal region) and compressive (distal and terminal regions) forces. The distal and terminal regions were shown to swell more than the proximal region, when soaked in water. More intense metachromasy after toluidine blue staining was observed in the distal and terminal regions, indicating an accumulation of proteoglycans in these regions. An analysis of the glycosaminoglycan in agarose gel showed dermatan sulfate in all regions, while chondroitin sulfate was found only in the compressive regions. The shape of the fibroblasts also changed along the tendon. Rounded cells appeared in regions under compression, while in the region under tension, only elongated cells were seen. The organization and distribution of the collagen bundles were different for each region. The birefringence analysis showed a more regular crimp pattern in the region under tension, than in the regions withstanding compressive forces. The elastic fibers also showed different distribution in each region. Our results reinforce earlier studies showing that the compositional and organizational aspects of tendons depend on the type and intensity of the forces to which they are subjected.

Abbreviations used:

ECM – Extracellular matrix. DDFT – Deep digital flexor tendon. p – proximal region. d – Distal region. t – Terminal region. PG – Proteoglycan. GAG – Glycosaminoglycan. CS – Chondroitin sulfate. DS – Dermatan sulfate. HE – Hematoxylin/eosin. ANOVA – Analysis of variance.

Key words: pig tendon, compressive forces, pressure-bearing tendon, proteoglycan, collagen, elastic fibers.

INTRODUCTION

Tendon is a dense regular connective tissue consisting of spindle-shaped fibroblasts aligned between densely packed bundles of type I collagen fibers (Bloom and Fawcett, 1994) and a very small amount of proteoglycans (PG). They transmit the tension forces, generated by muscle contraction to the bone on which they are inserted (Vogel and Koob, 1989). This function is correlated with the great tensile strength provided by collagen fibers (Benjamin and Ralphs, 1998). Some tendons which pass under pulleys or a bone extremity have the ability of withstanding compressive forces in addition to transmitting tension forces. In these tendons, the areas under compressive forces develop a fibrocartilage (Benjamin and Evans, 1990; Carvalho and Vidal, 1994), in which the remarkable amount of PGs is assumed to be the major factor involved in the ability of the tendon to resist compression forces (Vogel and Koob, 1989). A distinct collagen fiber arrangement and the presence of system elastic fibers also contribute to this property (Carvalho, 1995).

Furthemore, type VI collagen and other microfibrils are enriched in the pressure-bearing regions of the rabbit, dog, chicken, bullfrog and rat tendon (Carvalho et al. 1994; Felisbino and Carvalho, 1999). These microfibrils are important in the microarchitecture and supramolecular organization, especially in the maintenance of the convoluted state of the collagen fibers in the compression region, and their crimp morphology in the tensional region (Carvalho and Vidal, 1994; Carvalho and Vidal, 1995; Felisbino and Carvalho, 1999).

Studies correlating the extracellular matrix (ECM) composition and the biomechanical properties of tendons were accomplished in different mammals such as rabbits (Gillard et al. 1979; Merrilees and Flint, 1980), dogs (Okuda et al. 1987a; 1987b), and bovines (Evanko and Vogel, 1990), and in amphibians (Carvalho and Vidal, 1994; Carvalho and Felisbino, 1999). The deep digital flexor tendon (DDFT) of pigs is a wrap around tendon which presents three regions, supporting different biomechanical forces. The proximal region, is subjected only to tension forces; the distal region, bifurcates into two branches towards the fingers and is probably submitted to compressive forces as it passes close to the metatarsophalangeal joint. The terminal region extends to the fingers and

presumably withstands some compressive load.

The purpose of this work was to correlate the PG content, tissue swelling properties, structural features and macromolecular organization of the ECM, to the presence of different biomechanical forces in the DDFT of pigs.

MATERIAL and METHODS

Animals: Forty five day old male pigs of the large White lineage, were obtained from the Experimental Surgical Nucleus of the University Medical School - UNICAMP.

Biological material: The hind limbs were dissected to obtain the deep digital flexor tendon (DDFT). This tendon was divided in to the proximal region (p), which withstands only tension forces; the distal region (d), which branches in two toward the fingers, and is submitted to compressive forces as it passes close to the metatarsophalangeal joint, and the terminal region (t), which extends to the fingers and also receives some compressive load (Figure 1).

Swelling test: The different regions of the DDFT were sequential full-thickness cross-sectioned. Specimens were equilibrated in 0.15 M NaCl, 0.05 M NaH_2PO_4, pH 7.0 for 1h at room temperature, blotted and weighed. Each fragment was then soaked in a 1,000-fold excess volume of water for 60 min and weighed again. The fragments were subsequently equilibrated in 3% acetic acid, pH 2.5 (1,000 - fold vol.) for 1h, after which the wet weights were again determined (Koob and Vogel, 1987).

BIOCHEMICAL ANALYSIS:

Papain digestion: Tendon fragments (0.05 gram) were incubated with 500 μL of a papain solution (40 mg/gram of tissue) in 0.03 M sodium citrate buffer containing 0.04 M EDTA and 0.08 M 2-mercaptoethanol at pH 3.5, for 24 h at 50 °C. Afterwards the material was centrifuged in a Fischer microcentrifuge, at 8,000 rpm for 3 min. The precipitation of glycosaminoglycans (GAGs) was effected with 2 volumes of 95% ethanol, for 24 h at 4°C. After centrifugation, the GAGs were washed sequentially in 80% ethanol and in acetone (Michelacci and Horton, 1989). The GAGs liberated after papain digestion, were dried at 37°C and identified by agarose gel electrophoresis as described (Dietrich and Dietrich, 1976).

Determination of sulfated GAG: The quantification of the GAGs was effected using 100 μL of each sample for 2.5 mL of dimethylmethylene blue (Farndale et al. 1986), that was measured by reference to a whale chondroitin 4-sulfate calibration curve with concentration of 5, 10, 15, 20 and 25 μg/m100μL. The dosages were effected in a Hewlett Packard 8452 A Diode Array spectrophotometer, at 526 nm.

STRUCTURAL ANALYSIS:

Histology and Histochemistry: Tendon fragments were fixed in 4% paraformaldehyde in Millonig's buffer pH 7.4 for 24 h at room temperature. The material was then dehydrated in a graded ethanol series, clarified in xylene and embedded in Paraplast Plus. Serial longitudinal sections (6μm-thick) were stained with hematoxylin for 4 min and eosin for 1 min., and them differentiated in ethanol 70% for 1 min (Behmer et al. 1976). Some sections were stained with 0.025% toluidine blue in McIlvaine buffer at pH 4.0, for 20 min, (Mello and Vidal, 1980), to detect PGs. The collagen fibers were observed after staining with picrosirius solution (0.1% sirius-red F3B 200 in saturated picric acid solution), for 20 min (Junqueira et al. 1979), and counter stained by Harris hematoxylin for 10 minutes. To observe elastic fibers, the sections were oxidized in peracetic acid for 20 min, stained with the Weigert's fuchsin-resorcin for 1 h and counterstained by picric acid for 5 min (Goldfischer et al. 1983). After dehydration, the slices were mounted in Entelan.

STATISTICAL ANALYSIS:

The data were analyzed statistically by the analysis of variance (ANOVA) with the distribution of Fischer, at the 5% level of significance (Beiguelman, 1991).

RESULTS

Swelling Properties

The swelling increments for the different regions of the DDFT are shown in figure 2. When tendon fragments were transferred from PBS to distilled water (Figure 2A) a noticeable increase the wet weight was observed in the t region. The d region showed an intermediate value while the p region shrank. The opposite behavior was noted when the fragments were soaked in acetic acid (Figure 2B), the wet weight increased most prominently in the p region.

Analysis of sulfated GAGs

The total amount of sulfated GAG/gram of wet tissue was determined after papain digestion of the p, d and t regions of the tendon. The amount of sulfated GAGs was higher in the t region, followed by the d region (Figure 3). A lower amount was detected in the p region.

The identification of the GAG types was achieved by electrophoretic separation in agarose gels, using a PDA buffer (Figure 4). Dermatan sulfate (DS) was detected in all the regions. Chondroitin sulfate was more evident in the compressive regions, than in the tensional region.

Structural aspects

Sections of the p, d and t regions stained by HE showed the presence of cells with different morphology and distribution. In the p region (Figure 5A), where only tension forces are present, typical fibroblasts are disposed parallel to the collagen bundles. In the d region (Figure 5B), many rounded cells, which were similar to chondrocytes, were disposed between the elongated fibroblasts. The t region (Figure 5C, subjected to compressive and tensional forces, accumulated mainly chondrocyte-like cells especially in the area in contact with the bone.

When the sections were stained with toluidine blue pH 4.0, the presence of basophilic material was detected in the three regions. In the p region, staining was observed in certain

areas, preferentially around groups of fibroblasts (Figure 5D). The metacromasy was not spread out, but it was restricted to some areas around groups of cells. In the d region (Figure 5E) metacromatic material was more spread out than in the p region. In the t region, the metacromasy was dramatically increased in the superficial portion, which is in contact with the bone (Figure 5F). The wavy aspect of the collagen bundles was clearly observed, and the ECM was not so organized as in the p and d regions (Figures 5D and 5E). Rounded cells were more frequently found below the articulating surface (Figure 5F).

The differences in the organization of the ECM of the three regions were better observed by the polarized light analysis of the sections stained by picrosirius. A strong birefringence was observed in the three regions. The crimp structure was detected in all regions, but its pattern was different for each of them. In the p region, the collagen bundles were arranged in a highly ordered way, exhibiting crimp morphology with constant size and periodicity (Figure 5G). Different arrays were observed in the d (Figure 5H) and t (Figure 5I) regions. In the d region there was no periodicity in crimp structure even through the fibrilar components were strongly stained by the picrosirius-hematoxylin. Yet in the t region, a predominant greenish interference color and a non-uniform crimp morphology were observed.

The elastic fibers found along the tendon, exhibited different distributions for each region. In the tension region (Figure 6A) they were disposed in the same direction as the collagen bundles. In the d region (Figure 6B), the elastic fibers were parallel and oblique with respect to the longest axis of the tendon. A similar distribution was observed in the t region (Figure 6C).

DISCUSSION

Wrap around tendons bear compressive and frictional forces in addition to the tension forces originating from the muscle (Vogel and Koob, 1989). To withstand the compressive forces, the tendons develop a fibrocartilage, which shows structural and functional properties intermediate between a dense fibrous connective tissue and hyaline cartilage (Benjamin and Evans, 1990).

The DDFT of the pig is a wrap around tendon presenting: a proximal (p) fibrous region that is subjected only to tension forces, a distal (d) region, which bifurcates and articulates with the metatarsophalangeal joint and a terminal (t) region, which inserts into the fingers. These two regions experience compressive forces.

Physicochemical and biochemical procedures as well as structural analyses were carried out in this work for each region. The highest percentage swelling in water was found for the d and t regions, indicating the presence of PG, which was confirmed by the quantification of GAGs after papain digestion. On the other hand, when the tendon was soaked in acetic acid, the highest swelling increment occurred for the p region. The highest value of swelling in acetic acid may also be correlated to the lowest amount of PG, as demonstrated by the quantification of sulfated GAG. Similar results were found for the flexor tendon of bovines (Koob and Vogel, 1987). The increased presence of PG in the d and t regions in relation to the p region is an evidence of the importance of PG to provide osmotic resistance to compressive loading (Koob and Vogel, 1987).

An analysis by agarose gel electrophoresis showed a marked presence of DS in all regions of the DDFT, while CS was detected in the compressive d and t regions, and appeared only as a discret band for the tensional region. Our results confirm former data showing the presence of a small PG containing DS in a highly collagenous tissue that is under constant tensional forces, and the presence of CS in regions under compression in the rabbit (Gillard et al, 1979; Merrilees and Flint, 1980), bovines (Vogel et al. 1994) and amphibians (Carvalho and Vidal, 1994; Carvalho and Felisbino, 1999).

The detection of CS in a region under tension, probably corresponds to the presence of another population of PG, different from that found in the compressive region, as discussed by Vogel and co-workers (1994). However we need to correlate the presence of CS in the tensional region to the immaturity of the animals, since there is a correlation between age and the presence of CS in tendons (Vidal and Mello, 1984). The high cellularity found for the tendons analyzed here is also an indicative of its immaturity.

Structural analysis showed the presence of groups of rounded cells and a strong metachromatic basophilia in the d and t regions, as opposed to the elongated fibroblasts of the tension region. The metachromasy found in the two pressure-bearing areas is consistent with the higher content (~ 3-fold) of GAG detected by the DMMB procedure as compared to the p tensional area.

Polarized light analysis showed that the organization of the collagen fibers is not uniform in the three regions, demonstrating that they are designed to adapt to different biomechanics. The crimp morphology was more uniform in the p region. The d and t regions showed collagen fibers disposed in a three dimensional network, being disposed in different directions and then showing a less uniform crimp morphology.

The differences in elastic fiber content and distribution is a further factor which distinguishes the three regions of the pig tendon. They were aligned to the collagen fibers in the p region and showed no preferrence orientation in the d and t regions, where they can accomodate deformation in several directions. A similar distribution of elastic fibers in relation to the different areas of a specific tendon was also observed in wrap around tendons of the bullfrog (Carvalho and Vidal, 1994).

In conclusion, our results show that the DDFT of the pig presents compositional and structural differences within the different regions, and these differences are directly related with the capacity to support compression and still transmit tension forces.

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FIGURE LEGENDS

Figure 1. Dorsal view of the pig hind limb showing the location of the DDFT. The tendon was divided into the proximal (p) and distal (d) regions, which pass under the metatarsophalangeal joint, and terminal (t) region which inserts into the digits.

Figure 2. Swelling properties of the three regions of the tendon. A - After soaking in distilled water for 1h. B - After incubation for 1h in acetic acid. $P < 5\%$ using ANOVA statistical analysis.

Figure 3. Content of sulfate GAG in the p, d and t regions. $P< 5\%$ using ANOVA statistical analysis.

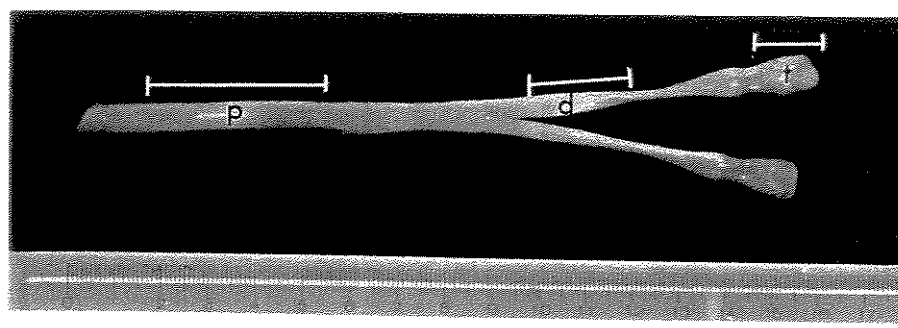
Figura 4. Agarose gel electrophoresis of glycosaminoglycans obtained after papain digestion of the tissue. DS was present in every region of the DDFT, while CS was present mainly in the distal and terminal regions (observed only in the gel, but not in the figure). DS and CS standards are seen on the left. Proximal (p), distal (d) and terminal (t) regions. The arrow indicates the direction of the run.

Figure 5. Longitudinal sections of the DDFT stained by HE (A-C), toluidine blue (D-F) and picrosirius (G-I).

A - Proximal region showing fibroblasts (→) aligned with the collagen bundles. X 200. B - Aspect of the d region exhibiting typical fibroblasts and rounded cells (→) that are similar to chondrocytes. X 200. C – The terminal region showing tendinocytes and rounded cells (→). X 200. D – The proximal region showing weak (→) staining which was restricted to the areas with a higher concentration of cells. Fibroblast nuclei (➔) are stained and demonstrate the organized distribution of cells in this region. X 200. E - Distal region, showing rounded cells (➔) and a more intensely stained ECM (→), especially in regions with a greater amount of cells. X 200. F - Terminal region exhibiting rounded cells (➔)

and metachromatic ECM (→). Observe the presence of fibril bundles (▶) distributed in several directions. X 200. G - Proximal region exhibiting intense birefringence. The undulated collagen fibers are organized side by side along the main axis of the tendon. The crimp seemed (→) to be more regular than in the distal and terminal regions. X 50. H - The distal region which bears some compression force. Note that the collagen fibers were undulated, exhibiting a different crimp pattern (→) compared with the proximal region. X 50. I - Compression region showing the arrangement of the collagen fibers, which are less organized. Different birefringence colors (yellow, green and red) indicate three levels of compactation of the collagen bundles. X 50.

Figure 6. Distribution of elastic fibers in the p, d and t regions of the DDFT. A – In the p region, the elastic fibers (→) follow the undulated morphology of the collagen bundles. X 200. B and C - Correspond to the d and t regions, respectively, where the elastic fibers (→) are distributed in several directions. X 200.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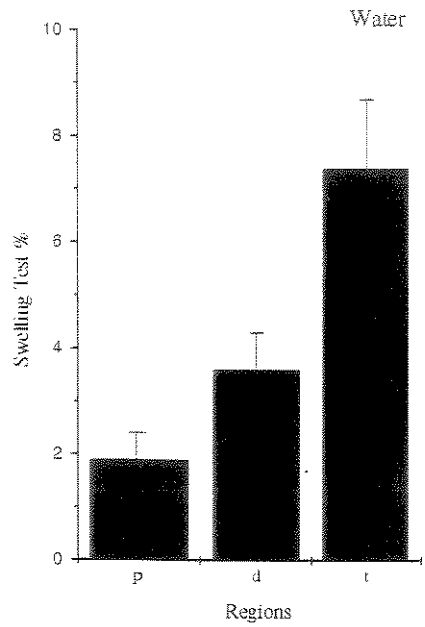
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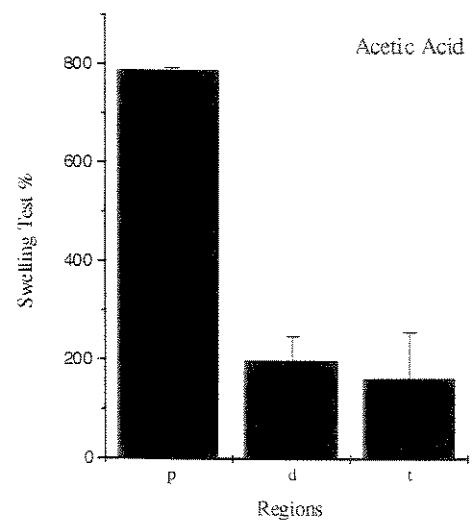
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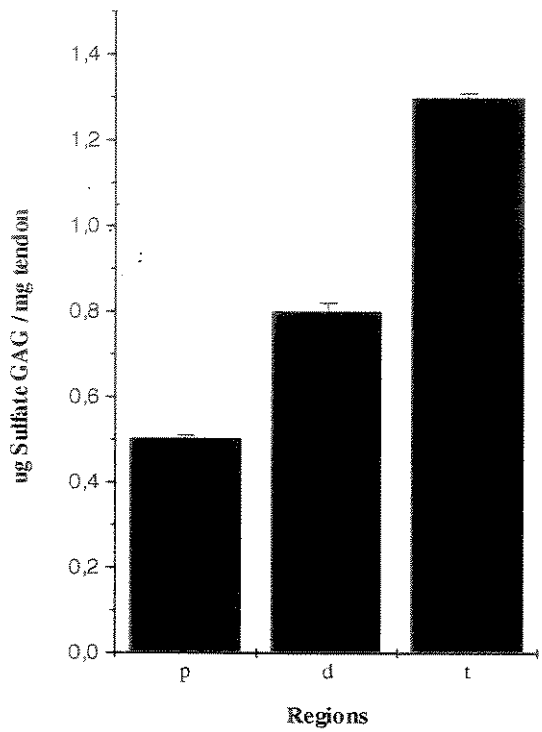
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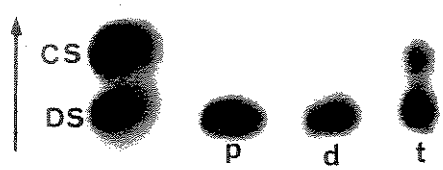
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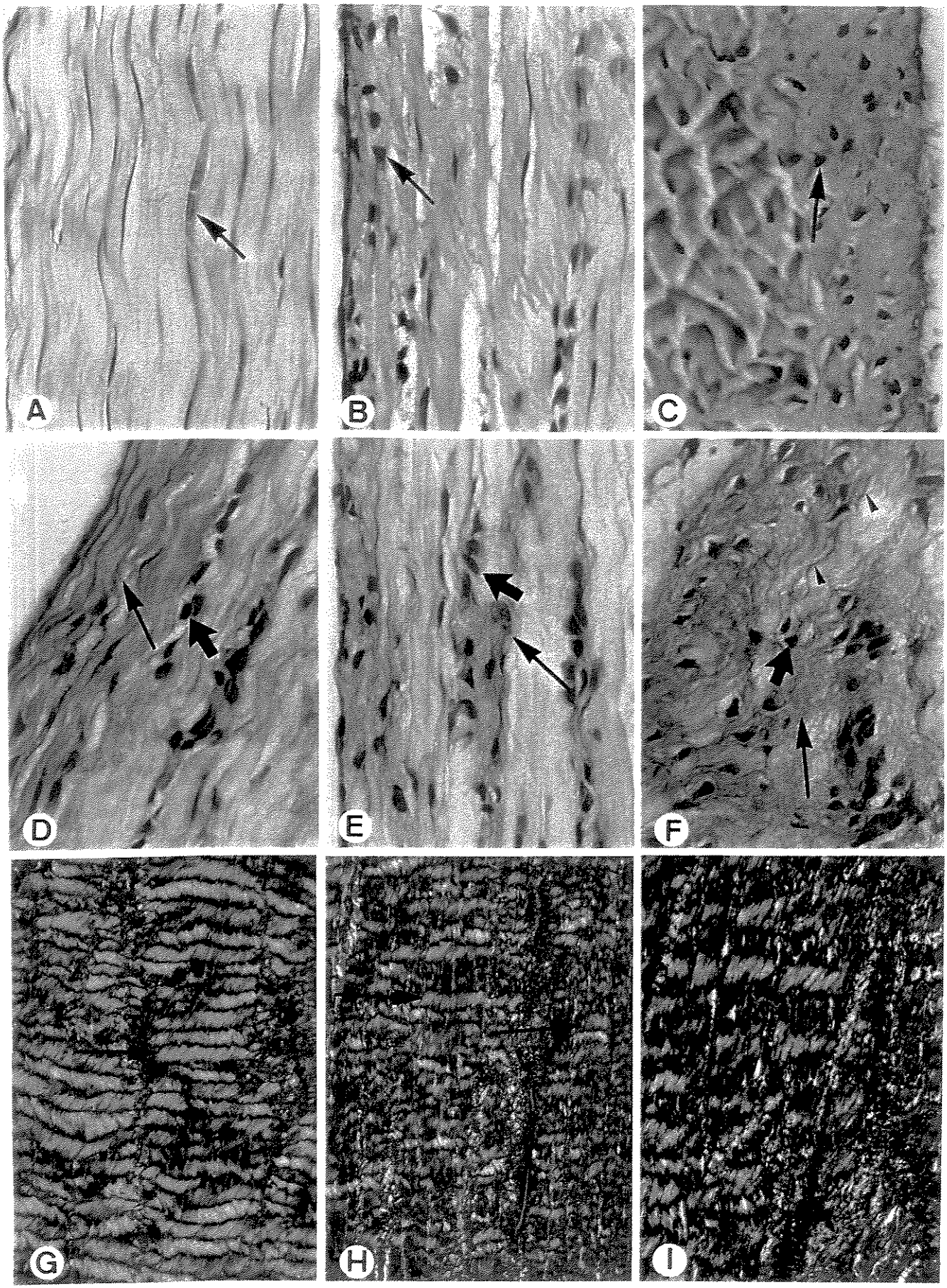
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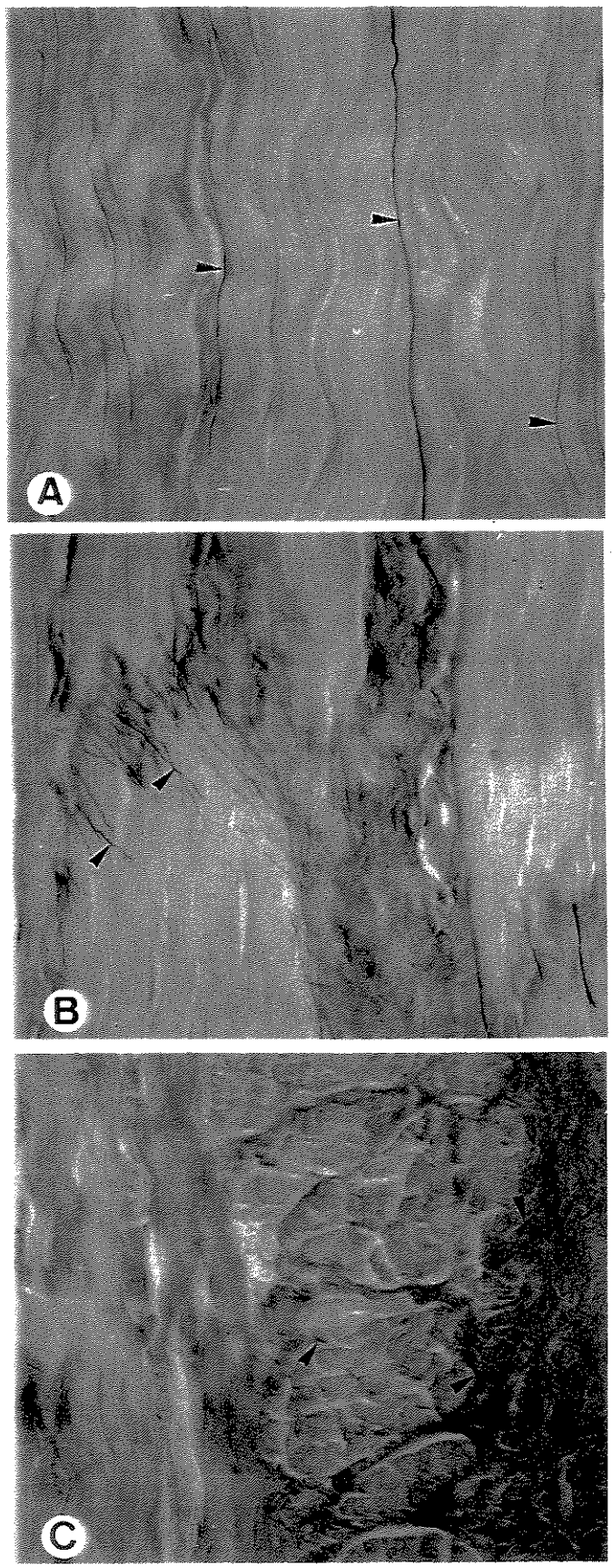
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COMPARATIVE ULTRASTRUCTURAL ANALYSIS OF DIFFERENT REGIONS OF TWO FLEXOR DIGITAL TENDONS OF PIGS

Vera Lúcia Feitosa[1]; Marcelo Esquisatto[2]; Paulo Joazeiro[3]; Benedicto Vidal[4], and Edson Pimentel[4]

Department of Morphology[1], Federal University of Sergipe, Aracaju/SE.

Department of Histology[2], University of Araras, Araras/SP.

Departments of Histology[3] and Cell Biology[4], UNICAMP, Campinas, SP, Brazil.

Running title: Ultrastructure of pig tendons.

Key words: pig, biomechanical forces, proteoglycans, collagen, fibrocartilage, tendon.

Correspondence to:

Edson Rosa Pimentel. Dept. Cell Biology – Unicamp. CP 6109. 13083 – 970 Campinas SP, Brazil, Tel: 55 019 788 - 7330, Fax: 55 019 788 - 7821. E-mail: pimentel@unicamp.br

ORIGINAL

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ABSTRACT

Tendon has the role of transmitting the tensional force generated by contraction of its muscle of origin to the bone upon which it inserts. They are parallel arrays of collagenous fibers which are specialized in resisting and transmitting tensile forces. The superficial digital flexor and deep digital flexor tendons are considered "wrap around" tendons. In these tendons fibrocartilaginous areas were observed in the regions subjected to compressive plus frictional loading regimen. Structural and ultrastructural analyses in the tensional region showed an extracellular matrix rich in collagen bundles, all in the same direction, and typical fibroblasts closely associated with collagen bundles. In the compressive regions besides typical fibroblasts, chondrocyte-like cells and elevated levels of glycosaminoglycans were present. The collagen bundles assumed several directions and were associated with proteoglycans. The crimp pattern detected in the tension region, demonstrated that collagen fibrils are ordered aggregates forming helical structures and crimps are part of such a helical arrangement.

Abbreviations used:

ECM – Extracellular matrix. SDFT – Superficial digital flexor tendon. DDFT – Deep digital flexor tendon. p – Proximal region. i – Intermediate region. d – Distal region. t – Terminal region. PG – Proteoglycan. GAG – Glycosaminoglycan. RR-alcian blue – Ruthenium red with alcian blue. HE – Hematoxylin/eosin. SEM – Scanning electron microscopy.

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INTRODUCTION

Tendons are specialized structures fitted to the function of transmitting the tension forces exerted by the muscles (Birk *et al.*, 1989; Cribb and Scott, 1995). However some tendons pass around bony pulleys and receive compressive forces in addition to the tension forces (Merrilees and Flint, 1980; Vogel and Koob, 1989). These tendons are characterized by exhibiting in the region under compression, a higher amount of proteoglycans (PGs), the presence of type II collagen, and random distribution of type I collagen bundles, which can be associated in heterotypic fibrils with type III and V collagens (van der Rest and Garrone, 1991). Immunocytochemistry, after testicular hyaluronidase or pepsin digestion, revealed the presence of type VI collagen in the tensional and compressive areas of the plantaris longus tendon of the bullfrog, in the deep flexor tendon of dogs and rabbits, calcanear tendon and suprapatela of rats and in the gastrocnemius tendon of chickens (Felisbino and Carvalho, 1999). Collagen architecture plays an important role in optimal functioning of various extracellular matrix (ECM) rich tissues such as skin, tendon, muscle, cartilage and cornea (Chakravarti *et al.*, 1998). Collagen corresponds to about 90% of the tendon dry mass (Nimni and Harkness, 1988) mainly in the regions under tension.

Proteoglycans are less than 1% of the dry weight of the tendon, and are represented mostly by the small PGs containing dermatan sulfate (Vogel and Heinegård, 1985). PGs are seen as filaments regularly attached to the collagen fibrils in electron micrographs of tendon sections stained with cupromeronic blue under critical electrolyte concentration (CEC) conditions. CEC methods ensure that only sulfated PGs are stained (Scott, 1985). Other components detected amongst the collagen bundles in tendon are the preelastic and elastic fibers, which may play a role in the ability of the tendon of bearing tension and compressive forces (Carvalho *et al.*, 1994; Carvalho and Vidal, 1995).

The cells in tendons and ligaments are capable of detecting physicochemical changes occurring in the ECM and coordinating their responses to alter the composition of this matrix. One of the most obvious ways in which the ECM of tendons is modified in response to compressive load is by the formation of a fibrocartilaginous matrix at sites where the tendons are under compression. This occurs with tendon passing around bony

pulleys, threading through fibrous retinacule and where they attach to the bone (Benjamin and Ralphs, 1998).

Fibrocartilage is a transitional tissue with intermediate structural property between dense fibrous connective tissue and hyaline cartilage (Benjamin and Evans, 1990). It is found in tendon areas subjected to compressive and frictional load regimes in addition to tension. These areas show not only a weave basket-like distribution of collagen fibers and rounded cells usually in lacunae, but also an increased amount of large PGs (Vogel *et al.*, 1994). Compressive fibrocartilage resists to the pressure of the bone against the tendon (Merrilees and Flint, 1980) and provides articulating surfaces. It was detected in regions under compression of the tendon of bovines (Vogel *et al.*, 1986; Vogel and Koob, 1989; Robbins *et al.*, 1997), and rabbits (Gillard *et al.*, 1979; Merrilees and Flint, 1980), but in amphibians a different arrangement of the convoluted and undulated collagen fibers has been observed allowing the tendon to undergo great distension before exerting resistance (Carvalho and Vidal, 1994a).

Some collagenous tissues as tendons present a morphofunctional characteristic named as crimp. As a broad generalization, crimp seems to be present in all those instances where the tissues are subjected to tensile forces (Gathercole and Keller, 1991). Vidal (1995) states that the variations of crimp in the different regions of the same tendon, as well as the distribution of the fibers and their bundles, could reveal details of the fiber organization in tendons.

Structural analysis carried out in our laboratory, unveiled organizational differences for the regions loaded with different mechanical forces. In addition observations using the polarizing microscope have shown different arrangements of the collagen bundles in the compressive and tensional regions of both tendons. So, in this work we intended to show ultrastructural differences between specific regions of the SDFT and DDFT, taking into account their biomechanical properties.

MATERIAL AND METHODS

Animals: 45 days old male pigs of the large White lineage were obtained from the Experimental Surgical Nucleus of the University Medical School – UNICAMP.

Biological material: Hind limbs were dissected to obtain the superficial (SDFT) and deep (DDFT) digital flexor tendons. Considering the presence of different mechanical forces, the SDFT was divided in the proximal (p) region, which wraps around the tibiotarsal joint and experiences compressive and frictional forces in addition to tension forces, the intermediate (i) region, an extended region of the tendon withstanding only tension forces, and the distal (d) region, which passes close to the metatarsophalangeal joint, bearing also compressive forces. The DDFT was divided in the proximal (p) region, which undergoes only tension forces, the distal (d) region, which splits in two branches towards the fingers, and presumably is submitted to compressive forces when it passes close to the metatarsophalangeal joint and the terminal (t) region, that extend into the fingers and also withstands compressive load.

Histology: Tendon fragments were fixed in 4% paraformaldehyde in Millonig's buffer at pH 7.4 for 24 h at room temperature. The material was then dehydrated in a graded ethanol series, clarified in xylene and embedded in Paraplast Plus embedding medium. Serial 6 μm thick sections were cut longitudinally and stained by hematoxylin for 4 min and eosin for 1 min. Differentiated in 70% ethanol for 1 min. After dehydration, the slices were mounted in Entelan resine (Behmer *et al.*, 1976).

Transmission electron microscopy:

The tendons were dissected out and fixed with either 2.5% glutaraldeyde and 1% tannic acid in 0.1 M cacodylate buffer at pH 7.3 for 2 h (Cotta-Pereira *et al.*, 1976) or, fixed with 2.5% glutaraldeyde containing 0.2% ruthenium red and 0.2% alcian blue in 0.1 M cacodylate buffer at pH 7.3 for 4 h, for the detection of the PGs (Tsuprun and Santi, 1996). After the fixations, the tendons were rinsed in cacodylate buffer and post-fixed with 1%

osmium tetroxide in the same buffer for 1 h. The impregnation was with 1% uranyl acetate in 1.2% NaCl and 7.3% sucrose in water, overnight at 4°C. The dehydration was carried out in graded ethanol and embedding was in Epon 812 resin. Ultrathin sections were stained with 1% uranyl acetate and lead citrate (Reynolds, 1963), and observed in a Leo 906 transmission electron microscope, operating at 40 or 80 kV.

Scanning electron microscopy:

The tendons were dissected out and immediately fixed by immersion in 2.5% glutaraldeyde and 1% tannic acid in 0.1 M phosphate buffer at pH 7.4. They were treated with 2 M NaOH, post-fixed with osmium tetroxide for 2 h at 4°C, dehydrated with ethanol and immersed in liquid nitrogen. The material was then fractured sagitally with the help of a stainless steel blade, dried to the critical point and sputter-coated with gold (Ohtani *et al.*, 1988, modified by Goranova *et al.*, 1996). Observations were in a JEOL JSM 5800 LV scanning electron microscope.

RESULTS

For a general histological viewing of two regions withstanding tensional and compressive forces, of the SDFT and DDFT, sections of those regions were stained with HE (Figs. 1-4). A large amount of cells was observed for the two tendons. In the tensional areas of both tendons (Figs. 1, 3), the elongated fibroblasts are parallel to the longest axis of the tendon. The unidirectional and undulated aspect of the collagen fibers was observed. In the compressive areas, many cells changed the morphology to rounded chondrocyte-like cells and were randomly distributed (Figs. 2, 4). In contrast to the tensional regions, the collagen fibers extracellular matrix (ECM) ran in several directions, arising an amorphous aspect.

Ultrastructural analysis:

The ultrastructural features of different regions of each tendon, representing areas under tension and compression were analyzed. In the tension region of the DDFT (Fig. 5) and SDFT (Fig. 6), the elongated cells appear closely associated to the collagen fibers. Some cells exhibited well developed rough endoplasmic reticulum and were surrounded by a large quantity of collagen fibers (Fig. 6). The nuclear envelope delimitates a slightly dilated perinuclear space. All cells presented the condensed chromatin in association with the inner side of the envelope nuclear. In the extracellular environment, no morphological distinction between a pericellular and an intercellular matrix was found. The ECM exhibited a more uniform organization, featured by closely packed and longitudinally aligned collagen fibrils. In contrast, in the p region of the SDFT (Fig. 7) and d region of the DDFT (Fig. 8), which are pressure-bearing zones, the collagen fibers assumed several directions and a wider interfibrilar space as compared to the tensional region was seen (Figs. 5, 6). In the d region of the DDFT (Fig. 9), chondrocyte-like cells with an abundant cytoplasm, that contained large amount of intermediate filaments cytoplasmic processes extending amongst the collagen fibrils.

Analysis of the RR-alcian blue stained material showed presence of a pericellular matrix rich in PGs (Fig 10), represented by globular precipitates. These precipitates were

linked to each other, and to the cell coat (Fig. 11). They were also found in the widered spaces between bundles of collagen fibrils (Fig. 12) and associated with them.

Straps representing PG were seen regularly distributed on the collagen fibrils of tension (Fig. 13) and compression (Fig. 14) regions of both tendons.

Analysis at the scanning electron microscope showed that the collagen fibers in the p (Fig. 15) and d (Fig. 16) compression regions of SDFT and DDFT, respectively, were disposed in several directions in a weave basket-like distribution. These regions were shown to posses filamentous material interspersed with thin fibers. These fibrils exhibited aspects of kinking or folding and a large number of microfilaments were observed around them. In the tension region of the SDFT (Fig. 17), can be observed a well defined wave like pattern denominated of crimp, typical of this region, while in the tension region of DDFT (Fig. 18), these fibers were more uniform and parallel the long axis of the tendon.

DISCUSSION

The SDFT and DDFT of pigs present two well defined organizations: The arrangement of classic tendons, designed to resist tensional forces and the fibrocartilaginous array that appears in areas under compression. Tendons are adapted to receiving and transmitting biomechanical loads generated from muscles, but some of them also withstand perpendicular compressive forces as they pass under a joint.

Sections of tension regions of both tendons stained by HE presented elongated fibroblasts parallel to the long axis of the collagen bundles, in contrast to the regions under compression which exhibited chondrocyte-like cells and collagen distribution as observed in tendons withstanding compressive forces, in other mammals (Merrilees and Flint, 1980; Okuda *et al.*, 1987a; Vogel *et al.*, 1986; Evanko and Vogel, 1990; Ralphs *et al.*, 1991; 1992; Carvalho and Felisbino, 1999; Felisbino and Carvalho, 1999), and amphibians (Carvalho and Vidal, 1994b).

The ultrastructural analyses described in this work demonstrated marked differences in cell morphology, matrix organization, collagen bundles arrangement and distribution of PGs in the different regions of the two tendons. The ultrastructure of these regions under pressure and frictional forces exhibited fibrocartilaginous features, showing collagen bundles disposed in several directions and an increased content of PGs, besides the presence of chondrocyte-like cells. Similar aspects were found in other wrap around tendons which receive perpendicular compressive forces (Vogel and Koob, 1989; Benjamin and Evans, 1990; Carvalho, 1995; Carvalho and Felisbino, 1999). On the other hand, in the tension regions of these same tendons, the fibrous matrix was closely associated with the fibroblasts with their wide cytoplasmic flanges conformed to the spatial limitations of the surrounding closely packed collagen fibrils.

In our observations, RR-alcian blue stained granules, representing PGs, appeared interconnected among themselves through thin filaments as observed in the rat tail tendon (Vidal and Mello, 1984). The PGs were also found joining collagen fibrils each other, as observed in of rat and mouse tendons, stained by cupromeronic blue (Cribb and Scott, 1995). The interaction of PG with the collagen fibers and other PG, are non-covalent and

therefore reversible (Scott, 1988, 1992). According to Hascall and Sajdera (1970), the PGs appear as granules due to the preparative procedures, mainly dehydration. Later, Vidal and Mello (1984), studying Achilles tendon of newborn rats, demonstrated the presence of the PGs as dense globules interconnected and attached to the cell coat through fibrillar formations. The authors also observed an intimate association of collagen fibrils with the ruthenium red stained cell coat.

The cell-matrix interactions observed in our work indicates that the morphology and metabolism of cells are closely related to the distribution of biomechanical forces. The correspondence of the structural and ultrastructural results confirm the organizational differences found between the tension and compression regions in both tendons.

The aspect exhibited by the compressive region of both tendons was typical of fibrocartilages, which are described as an intermediate structure between a dense fibrous connective tissue and the hyaline cartilage (Benjamin and Evans, 1990). A more pronounced presence of PG, found in compressive areas as compared to the tension regions, was expected as based on quantitative analysis and swelling tests carried out for these tendons (Feitosa *et al.*, 2000 – submitted), as well as on results obtained for other tendons (Koob and Vogel, 1987; Evanko and Vogel, 1990; Carvalho and Vidal, 1994).

The fibrilar arrangement detected the pericellular matrix of the compressive regions of both tendons may correspond to an accumulation of type VI collagen microfibrils, which may associate with PG forming the structure present in the pericellular environment of cells of regions under compression (Felisbino and Carvalho, 1999).

Structural and biochemical studies carried out with these two tendons (Feitosa *et al.*, 2000 - submitted), showed the compressive regions to posses a greater amount of PG, as characterized by the intense metachromatic basophilia after staining with toluidine blue, whereas in the regions under tension forces this feature could not be observed. Similar results were found for the bovine flexor tendon (Koob and Vogel, 1987; Koob, 1989) and digital flexor tendon of rats (Covizi-manuscript in preparation). Part of the GAGs found in compressive regions is chondroitin sulfate and probably correspond to large PGs. In bovine tendons under compression, 40% of them are able to aggregate with hyaluronan (Vogel and Heinegård, 1985; Evanko and Vogel, 1990), providing the tendon with ability for

withstanding compressive forces (Vogel and Heinegård, 1985; Vogel *et al.*, 1986; Vogel and Koob, 1989).

The pattern of crimp in the tension regions observed in this work by scanning electron microscopy, represent a three-dimensional helical superstructure of the collagen fibers and bundles in response to the biomechanical demands of that area (Vidal, 1966; Vidal, 1969; Vidal, 1995). Recently, studies on the optical anisotropy of the SDFT of pigs (Feitosa *et al.*, 2000 - submitted), showed that the crimp patterns were quite different for the tension and compression regions, which denote the importance of the arrangement and organization of the collagen fibers to attend the biomechanical requirements of the tissue.

Carvalho (1995) suggested that the capacity of resisting compression observed in fibrocartilages cannot be attributed solely to the existence of large aggregating PGs in the tissue, on the basis of direct analogy with the hyaline cartilage, but also to specific arrangement of the collagen fibers. The importance of these fibrocartilages is outstanding and the establishment of specific models for studying the macromolecular interactions and biomechanics of tendon fibrocartilages are currently needed for better understanding of the function of each of their components.

Our results once more indicate that mechanical forces influence the composition and structure of fibrous connective tissues.

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FIGURE – LEGENDS

Figures 1-4 – Longitudinal sections of the SDFT and DDFT stained by HE. Intermediate region of the SDFT presenting elongated fibroblasts (arrowheads) aligned with the collagen bundles (Fig. 1). X 270. Proximal region of the SDFT exhibiting tendinocytes (arrowheads) and rounded cells (arrows) (Fig. 2). X 400. Proximal region of the DDFT exhibiting typical fibroblasts with elongated nuclei, aligned with the collagen bundles (arrowheads) (Fig. 3). X 270. Terminal region of the DDFT presenting rounded cells (arrows), which are randomly distributed (Fig. 4). X 400.

Figure 5 - Elongated fibroblast of the tension region of the DDFT, associated with collagen bundles which are parallely arranged to the longitudinal axis of the tendon. Observe that in tension region the collagen fibrils (❋) follow the same direction, and are densely packed in the matrix. Most of the condensed chromatin is associated with the inner surface of the nuclear envelope. Nucleus (N). X 23,000.

Figure 6 - Detail of a fibroblast in the tension region of the SDFT. The cell is very active in protein synthesis, as suggested by the density of endoplasmic reticulum in the cytoplasm. There is no distinct pericellular matrix and coarse collagen fibers (❋) abut directly on the cell. X 16,000.

Figure 7 - Chondrocyte-like cell and extracellular matrix of the compression region of the SDFT after treatment with RR-alcian blue. Observe the presence of rounded nucleus (N), and a larger cytoplasm, compared to the cell in the tension region. There are just a few organelles which are embedded in this intermediate filament meshwork. Its matrix presented collagen fibrils in several directions (arrows) with RR-alcian blue granules corresponding to collapsed PGs (arrowheads) interacting with fibrils dispersed in the extracellular matrix. X 13,000.

Figure 8 - Aspect of the distal region of DDFT after treatment RR-alcian blue, presenting collagen fibers in several directions (arrows) and collapsed PG associated to collagen fibers and also randomly distributed in the cytoplasm. X 6,700.

Figure 9 - Ultrastructure of the DDFT of pigs after treatment RR-alcian blue. A typical cell of the compression region showing rounded nucleus (N) with condensed chromatin associated with the inner surface of the nuclear envelope. The cytoplasm dispersed with few extensions contain large quantities of intermediate filaments (arrowsheads) and lipid droplets (★). A amount considered of rough endoplasmic reticulum (arrows) dispersed in the cytoplasm. Occurred also the presence of PGs in the matrix pericellular (short arrow). These cells are in association with collagen fibers (❁). X 14,300.

Figures 10-12 - Aspects of the extracellular matrix after treatment with RR-alcian blue. **10** – Distal region of the DDFT showing PGs interconnected among themself (arrowheads) and interaction of PG with collagen fibrils (arrows). X 14,360. **11** – Detail of compression region of the SDFT exhibiting granules of PG (arrowheads) interconnected to each other, and also connected with microfibrils (mfl) in the pericellular matrix. X 48,200. **12** - PGs in the proximal region of the SDFT detected among the collagen fibrils (arrowheads). Note the collagen fibrils of this pressure region assuming several directions (arrows). A cellular process (❁) with some vesicles and dilated endoplasmic reticulum may be seen. X 72,000.

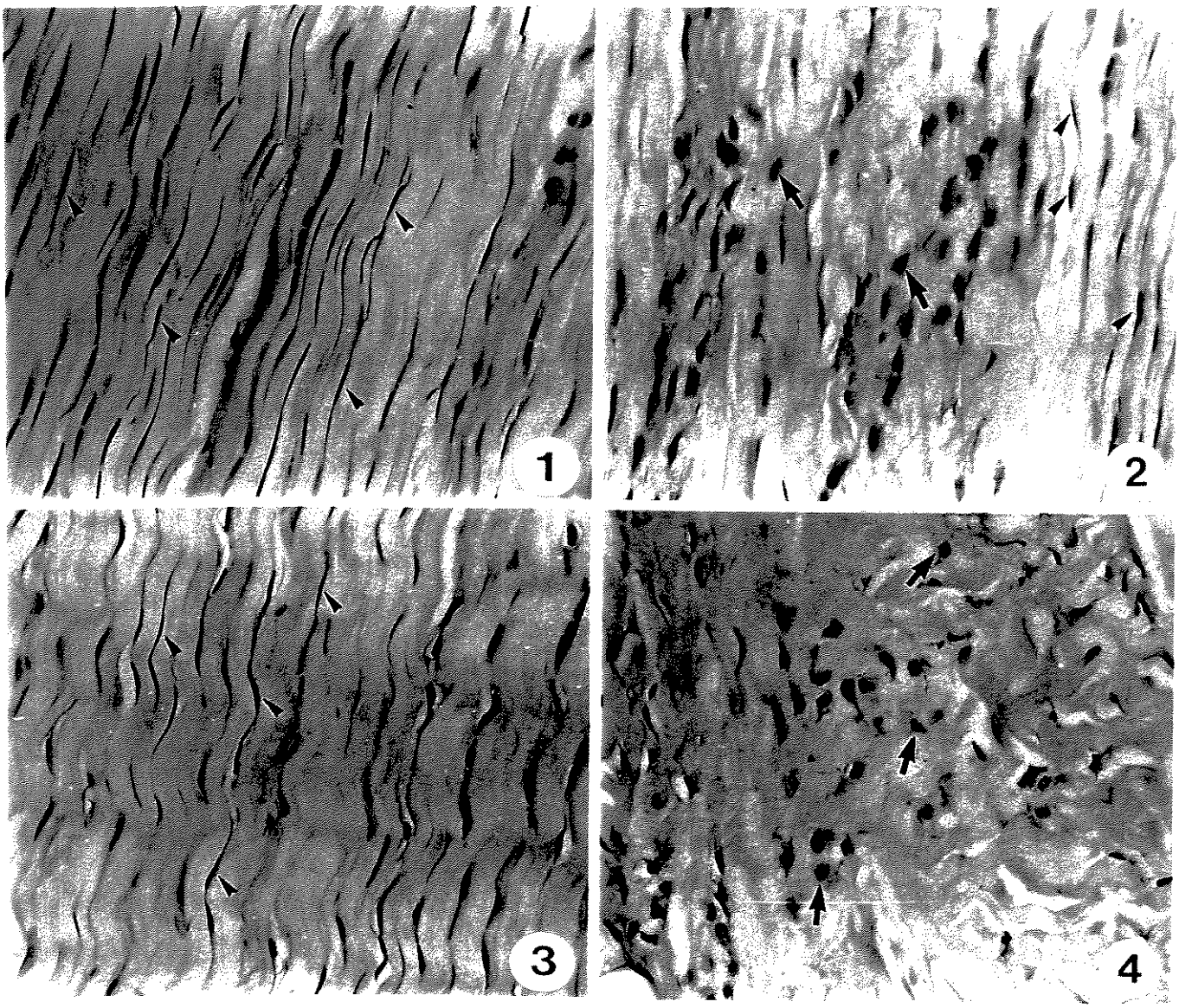
Figures 13-14 – Aspects of the fibrous portions of the tension (Fig. 13) and compression (Fig. 14) regions of the SDFT stained with RR-alcian blue. PG appear associated with the collagen fibrils, and may be bridging the collagen fibrils (arrows). Note the kinkins of the collagen fibrils (Fig. 14) in the region under compression. Fig. 13, X 46,300; Fig 14, X 72,000.

Figures 15, 16 – SEM of the proximal and terminal regions of the SDFT (Fig. 15) and DDFT (Fig. 16) respectively, both under compression. The fibers of the two regions assume

several directions and are arranged in a basket-weave pattern. Large spaces between fibers can also be seen. Fig. 15, X 1,950. Fig. 16, X 2,000.

Figure 17 – SEM of the intermediate region (tension) of the SDFT showing the crimp structure particularly clear and conspicuous. Observed the fibrous structures which follow a zag path. X 3,840.

Figure 18 – SEM of the tensional region (proximal) of the DDFT which present the collagen bundles well uniform, more regular and all following the a unique direction. X 900.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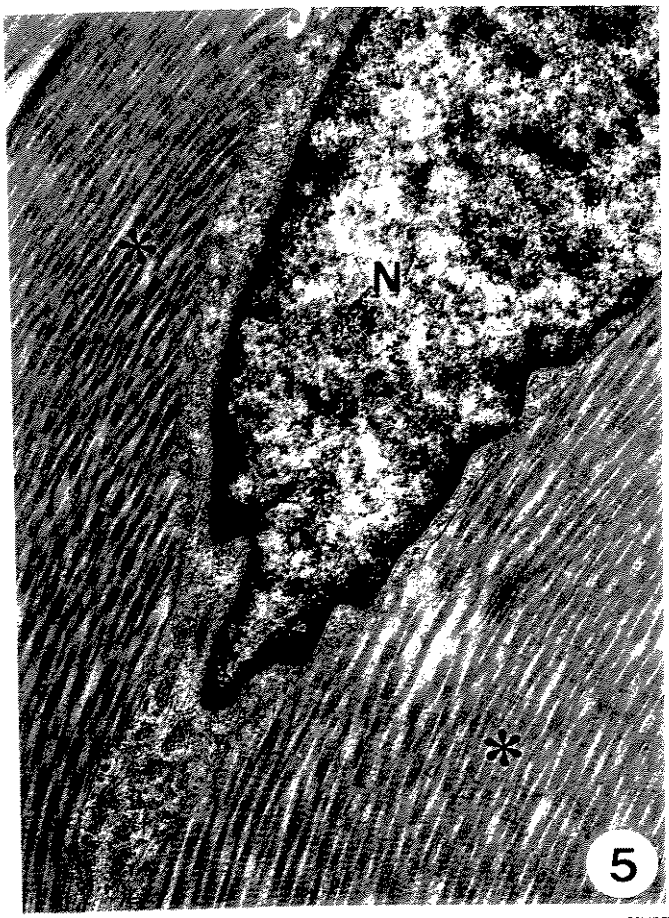

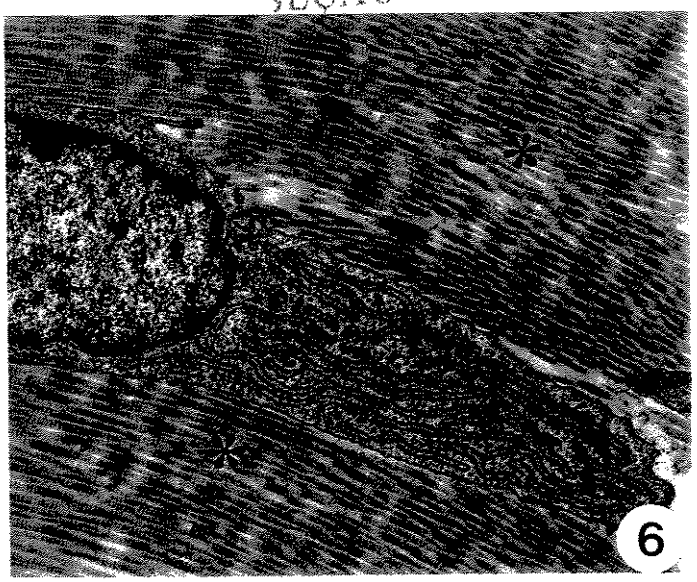

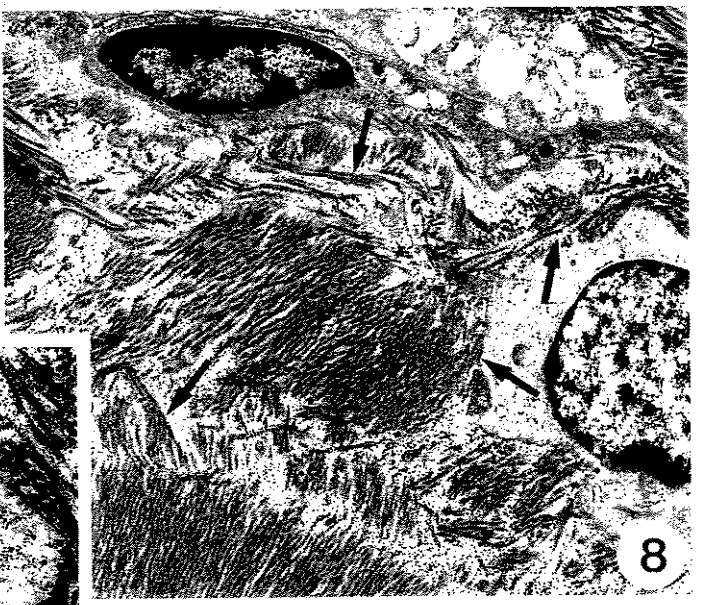

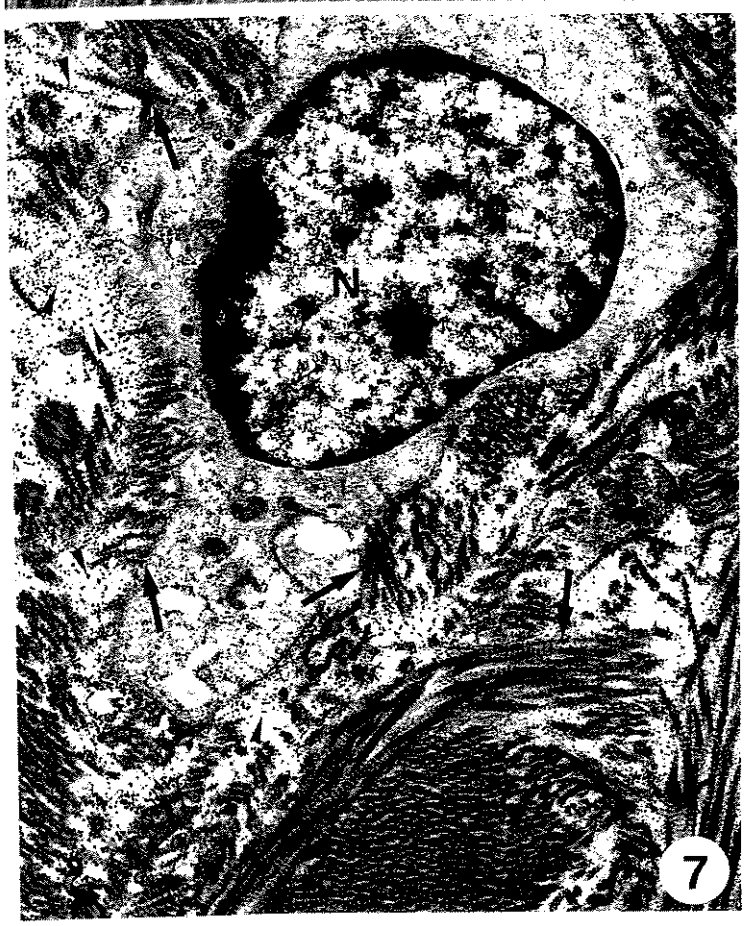

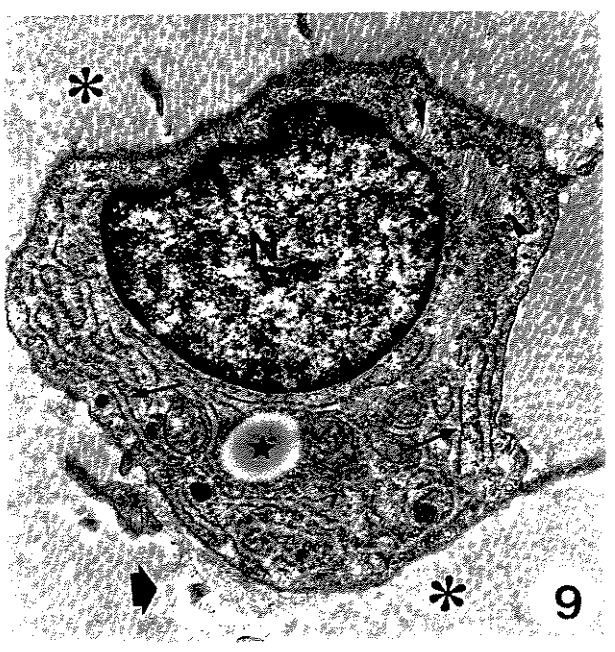

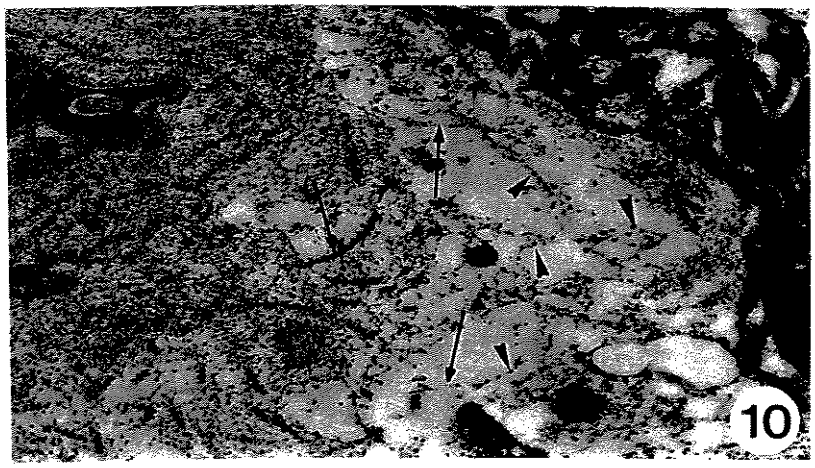
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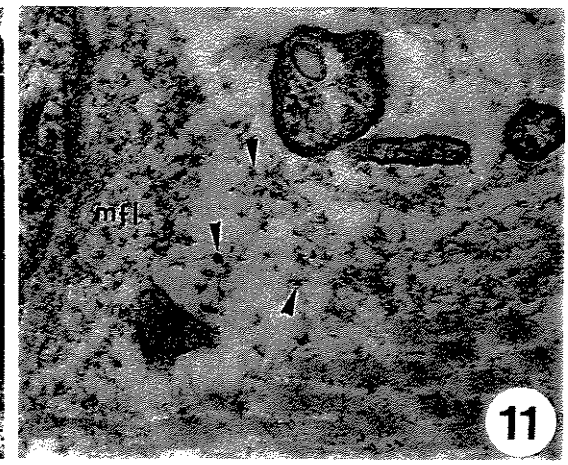
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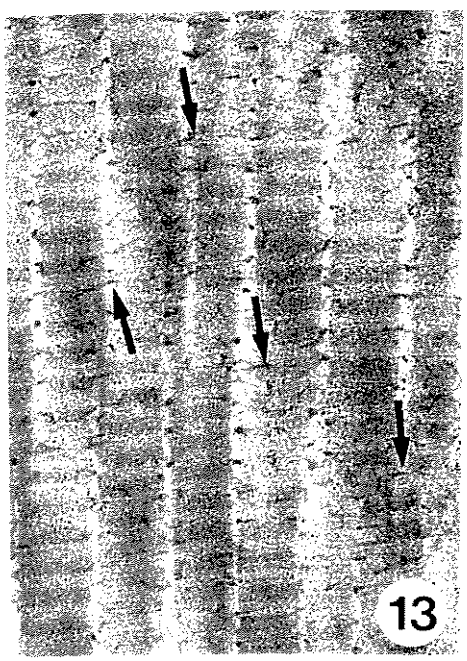
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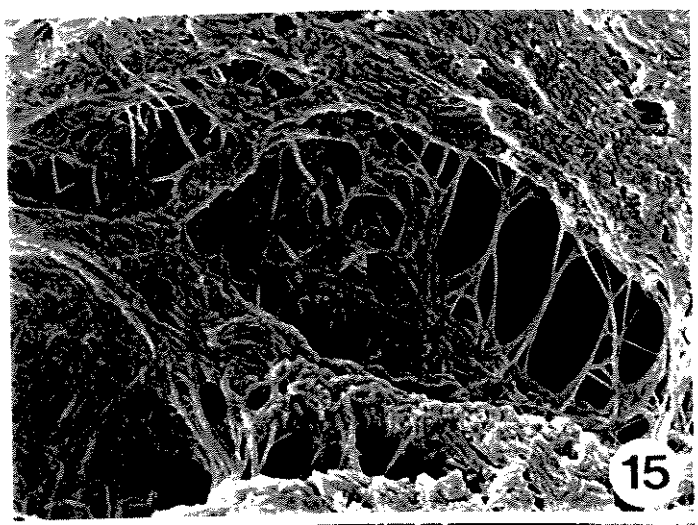
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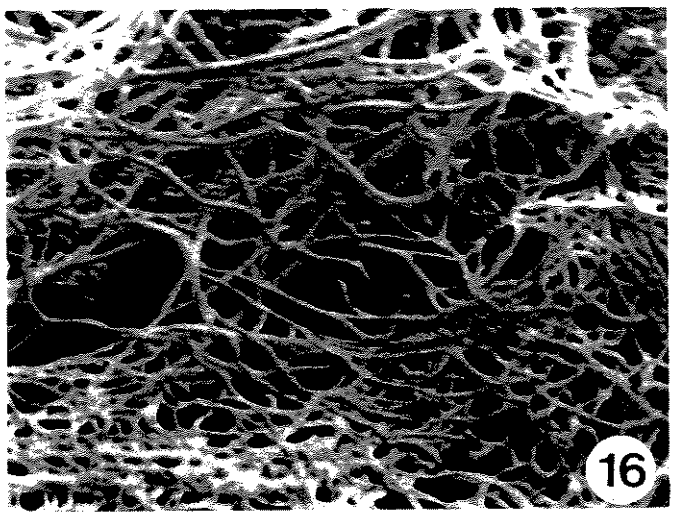
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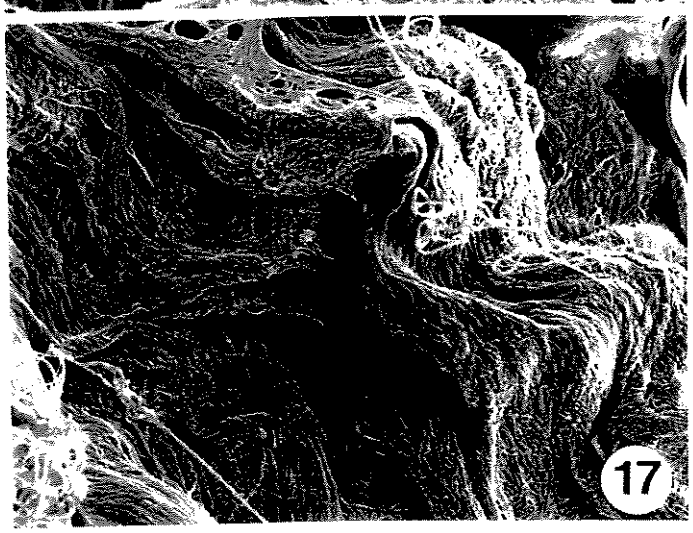
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BIOCHEMICAL STUDY OF A WRAP AROUND TENDON OF PIGS

V. L. C. Feitosa[1], L. Gomes[2] and E. R. Pimentel[2]

Department of Morphology[1], Federal University of Sergipe, Aracaju / SE, Brazil.

Department of Cell Biology[2], UNICAMP, Campinas / SP, Brazil.

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Correspondence:

Edson Rosa Pimentel

Dept. Cell Biology-UNICAMP

CP 6109

13083 – 970 Campinas SP, Brazil

Tel: 55 019 788-7330

Fax: 55 019 788-7821

E-mail: pimentel@unicamp.br

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ABSTRACT

Wrap around tendons have a high content of glycosaminoglycans (GAGs) and exhibit a fibrocartilagenous structure. Cells resembling chondrocytes are found at the sites where the tendon passes under bone and receive compressive and frictional forces in addition to tensiles forces. In this work was studied the extracellular matrix composition of different regions of the superficial digital flexor tendon (SDFT) of pigs, which experience different biomechanical forces. Analysis of GAG of the total extract showed that in the proximal and distal regions there is a larger quantity of GAG/mg tissue, compared with the intermediate region, probably due to the presence of compressive forces that occur in the proximal and distal regions. A similar result was obtained for the non-collagenous protein/mg of tissue. Samples of every extract were fractionated on a DEAE-Sephacel column. The bound material was eluted with a linear gradient of 0.1-1.0 M NaCl and the fractions analyzed in SDS-PAGE in reduced and non-reduced conditions. A polydisperse band (67 kDa) was detected in all regions. Analysis in SDS-PAGE with and without treatment with keratanase identified that protein as fibromodulin. Another polydisperse component with Mr around 94 kDa was detected in all regions of the tendon, but in the intermediate region, appeared in SDS-PAGE only under reducing conditions, suggesting some interaction with other extracellular matrix components. Analysis of the GAG in agarose gel with and without enzymatic treatment showed that the 94 kDa component contains dermatan sulfate. The presence of dermatan sulfate plus the electrophoretical behavior in SDS-PAGE, suggest it to be the small proteoglycan decorin.

Abbreviations used:

ECM – Extracellular matrix. SDFT – Superficial digital flexor tendon. p, i, d – proximal intermediate and distal regions. PG – Proteoglycan. GAG – Glycosaminoglycan. CS – Chondroitin sulfate. DS – Dermatan sulfate. HS – Heparan sulfate. KS – Keratan sulfate. Gu-HCl – Guanidine hydrochloride. PMSF – Phenylmethylsulfonyl fluoride. SDS-PAGE – Electrophorese in polyacrylamide gel with sodium dodecyl sulfate. 2-Me – 2-mercaptoethanol. Mr – Relative molecular mass. Rf – Relative migration value. ANOVA – Analysis of variance.

INTRODUCTION

Extracellular matrix (ECM) of tendon is composed of type I collagen, that corresponds to about 90% of the tendon dry mass (1), proteoglycasn (PGs) and non-collagenous protein (2, 3). The structure and organization of the collagen fibers determinate the architecture, stability, mechanical properties and resistance to the tensional forces (4).

PGs accounts to about 1% of the dry mass of the tendon, and most of them are small PG (5). Large PG are also found in regions under compression (6). The small PGs consist of a core protein covalently bound to one or a few glycosaminoglycans (GAGs). They are known as biglycan (PG-SI), decorin (PG-S2) and fibromodulin (3), and represent a family of structurally related, but genetically distinct molecules present in different tissues.

The N-terminal portion of decorin and biglycan contains chondroitin sulfate (CS) or dermatan sulfate (DS) depending on the tissue. Their core protein are defined by leucine-rich tandem repeats of about 24 amino acids flanked by cystein clusters. Decorin it interacts with a variety of ECM proteins, and with TGF-β. The mature core protein is highly conserved amongst species. It binds to type I collagen inhibiting *in vitro* fibrillogenesis, modulates growth factor activity, and regulates cellular growth. Biglycan and decorin have similar structures, but biglycan does not interact with collagen fibrils. In contrast, biglycan is usually associated with the cell surface and pericellular matrices in some cell types such as skeletal myoblasts, endothelial cells and differentiating keratinocytes (7-11).

Fibromodulin is another small PG containing four keratan sulfate chains and some sulfated tyrosine residues which also contribute to the anionic character of the molecule. This small PG also regulates collagen fibrillogenesis *in vitro* (12-14).

Tendons which pass under bones of a joint are denominated wrap around tendons (15) and receive compressive and frictional forces in addition to tensile forces. These tendons pass under a joint and develop a fibrocartilaginous structure, characterized by an special system so that the structure receive compressive and frictional forces, without affecting the transmission of tension forces from the muscle to the bone (16-18). In these tendons, the organization of the collagen bundles and the presence of large PGs in areas

under compressive forces, seem to be the main factors involved in the ability of the tendon to resist compressive forces (19).

Considering the lack of informations about the ECM composition and its relation with the presence of biomechanical forces in wrap around tendons of pigs, we led a comparative biochemical study considering the regions of the SDFT of pigs, which experience different mechanical loading

MATERIAL AND METHODS

2. 1. Tissue Preparations:

45-day-old male pigs of the large White lineage were obtained from the Experimental Surgical Nucleus of the University Medical School. Hind limbs were dissected to obtain the superficial digital flexor tendon (SDFT), which was divided in three regions: proximal (p) region, that forms a calcanear cap and experience compressive forces, intermediate (i) region, subjected only to tension forces and distal (d) region which also bears compressive forces.

2 .2. Extraction of matrix components:

Samples (1 gram) of the p, i and d regions of the SDFT were extracted for 24 h at 4°C with constant shaking with 25 volumes of the extraction solution (4 M guanidine – hydrochloride in 50 mM acetate buffer, pH 6.0, containing 50 mM EDTA, 1 mM PMSF), (20). Extracts were centrifuged in a Beckman JA-20 rotor at 18,000 rpm, 4°C for 50 min. The supernatant was employed for biochemical analysis.

2. 3. Quantitative analysis:

Protein concentration was determined by the Bradford method (21) using bovine serum albumin as standard. Sulfated GAGs were determined as described by Farndale and co-workers (22) and CS was used as standard. The GAG standards were purchased from Sigma Chemical Co, (St. Louis, MO, USA). The data were statistically analyzed by the analysis of variance (ANOVA) with Fischer's distribution at the 5% level of significance (23).

2. 4. Ion enchange chromatography:

Samples (2 mg of protein) of the extracts were dialyzed against 7 M urea in 50 mM acetate buffer pH 6.8, and then applied on a DEAE - Sephacel column (1.5 x 2.7 cm), pre-equilibrated with the urea solution. After additional flushing with 7 M urea in 50 mM acetate buffer pH 6.8, the column was eluted with a gradient of sodium chloride (0.1-1.0

M) (70 mL). Fractions (2.8 mL) were collected and monitored by the absorbance at 280 nm. The conductivity of the fractions was determined with a Hanna model HI-8810 N condutivimeter.

2. 5. Electrophoresis:

For electrophoresis, 200 μL of the fractions eluted from DEAE-Sephacel were precipitated with 100 μL of 1.0 M acetate buffer pH 7.4 and 2,700 μL of absolute ethanol at –20°C for 24 h (24). The precipitated was resuspended in 50 μL of sample buffer (62.5 mM Tris-HCl, 2% SDS, 10% glicerol, 1 mM EDTA pH 6.8 and 0.01% bromophenol blue and incubated for five minutes at 96°C and loaded onto a 4-16% gradient SDS-polyacrilamide gel (25). Some samples were incubated in the presence of 5% 2-Me. The molecular weight standards used were fosforilase b (94 kDa), bovine serum albumin (67 kDa), ovalbumin (43 kDa), carbonic anhydrase (30 kDa), trypsin inhibitor (20.1 kDa), α-lactoalbumin (14,4 kDa). After the running, the gels were stained by the silver nitrate method (26). The determinate of the relative molecular mass (Mr) was obtained after plotting the relative migration values (Rf) against the known molecular mass of standards on semi-logarithmic paper.

2. 6. Enzymatic treatments:

The decorin rich fractions, eluted from DEAE-Sephacel, (200 μL) were precipitated with 100 μL of 1.0 M acetate buffer at pH 7.4 and 2,700 μL absolute ethanol at 4°C for 24 h (24). To obtain free GAG, part of those samples was β-eliminated with 0.5 M NaOH in 4°C for 24 h. Some samples after β-elimination were incubated with 0.04 units of chondroitinase AC in 50 mM sodium acetate, 50 mM Tris and 10 mM EDTA buffer at pH 6.0 (27), and chondroitinase ABC in 50 mM sodium acetate, 10 mM EDTA and 50 mM Tris buffer pH 8.0 (28), at 37°C for 6 h. To identify the kind of GAG, samples were analyzed by agarose-propilenediamine gel electrophoresis and stained with 0.1% toluidine blue (29).

Other fractions rich in the 67 kDa component were incubated with 0.001 unit keratanase in 50 mM sodium acetate, 50 mM Tris and 10 mM EDTA buffer at pH 6.0 (18), at 37°C for 6 h, and analyzed in 10% polyacrylamide gel with SDS (30). Gels were stained with 0.05% alcian blue in 0.2 M acetate buffer at pH 5.8 with 1 M $MgCl_2$, and coomassie brilliant blue (31). The enzymes were purchased from Seikagaku American, (Far mouth MO, USA).

RESULTS

Quantitative analysis

Analysis on the quantity of proteins and GAGs in extracts of the different regions of the SDFT, showed a larger amount of protein (Fig. 1A) and sulfated GAGs (Fig. 1B) in the p and d regions which are under compression as compared to the i region, only under tension. The quantity of proteins is related to the non-collagenous components, also including the core protein of PG. The results were significantly different at level of 5%.

Chromatography and SDS-PAGE

Proteins of the total extract of each region of the tendon, were fractionated in a DEAE-Sephacel and the anionic components were eluted during the NaCl gradient. Most collagen and other cationic proteins were eluted with the beginning fractions. The components eluted during the NaCl gradient were analyzed in SDS-polyacrylamide gel (Figs. 2A, 3A, 4A). Analysis of fractions of the proximal region showed a polydisperse component with Mr around 67 kDa, which was eluted within a range of 0.1-0.42 M NaCl, while another polydisperse, but less prominent component with Mr around 94 kDa was eluted with concentration around 0.4 M NaCl (Figs. 2B, C). In the intermediate region (Figs. 3B, C), was found an expressive amount of the 67 kDa component (Fig. 3B), eluted from DEAE with 0.2 to 0.5 M NaCl. A polydisperse band correspondent to a protein with 94 kDa was eluted within 0.5-0.6 M NaCl, but it was detected only in presence of 2-Me (Fig. 3C). Analyzing the distal region, were detected three polydisperse bands corresponding to the 67 kDa component, eluted within a 0.3-0.5 M NaCl; the 94 kDa eluted within 0.5-0.6 M NaCl and a 200 kDa component, eluted within 0.5-0.7 M NaCl (Figs. 4B, C).

Other proteins, less expressive in quantitative terms comparing with the polydisperse components above, were also detected.

Enzymatic digestion

Samples of fractions rich in polydisperse component (Mr ≅ 94 kDa), eluted from DEAE-Sephacel, were β-eliminated and analyzed in agarose gel (Fig. 5). Samples of the p and i regions, representing regions bearing compression and only tension respectively were β-eliminated. To identify the GAG obtained after β-elimination, samples were treated with chondroitinases ABC and AC. Analysis of the agarose gel demonstrated that the polydisperse component with Mr around 94 kDa, found in both regions contains DS (Figs. 5A, B).

Some fractions containing the 67 kDa component were pooled, ethanol precipitated, digested with keratanase and analyzed in 10% polyacrilamide gel containing SDS. The vanished band observed in the case of the sample treated with the enzyme, indicates the presence of keratan sulfate in that component (Fig. 6).

DISCUSSION

The biomechanical forces are differentially distributed along the SDFT. In the p and d regions which are under the tibiotarsal and metatarsophalangeal joints, compressive forces in addition to tensional forces are acting. Yet in the i region, only tensional forces are present. Studies with wrap around tendons, like the SDFT of pigs, have demonstrated the existence of a fibrocartilage structure in a region which wraps around a joint and receives perpendicular forces (16, 19, 32, 33, 34, 35). These fibrocartilaginous regions are featured by an increased amount of PG as compared with the rest of the tendon.

Here we also found a larger accumulation of PG in the region under compression, as shown by the quantitation of sulfated GAG. The larger amount of proteins in the regions under compression are due not only to the non-collagenous proteins, but may also be due to the core protein of large PG, which is abundant in compressive areas.

The results obtained after fractionation of extracts of the different regions, showed the presence of polydisperse components. The polydisperse 67 kDa component was detected in the fractions eluted during the gradient (0.2-0.4 M NaCl) for the three regions studied. The presence of KS in that component, demonstrated by using keratanase suggest that the 67 kDa component is the small PG fibromodulin.

The polydisperse component with 94 kDa, eluted at around 0,4 M NaCl, is a DS-PG, as demonstrated using a combination of chondroitinases ABC/AC and agarose gel electrophoresis. Considering the presence of DS, the polydisperse aspect and the electrophoretic behavior, it is highly probably to correspond to be the small PG decorin. In the i region, it was only detected under reducing conditions, and this is an indicative of the existence of either some interaction with other components or an auto-aggregation process, dependent on the stability of S-S bridges (36).

Ultrastructural studies have already suggested an interaction between decorin and collagen in different connective tissues (37-39). Some studies based on the inhibition of collagen fibril formation, have also indicated that decorin binds to collagen molecules (38). There are evidences showing interactions of GAGs and collagen type VI (40-41), and core protein of decorin with collagen type VI (42). The present results show no clear interaction

between decorin and collagen, or any other ECM molecule but it is quite interesting that decorin in tension regions seems to be interacting more strongly with another component when compared to the p and d regions. The presence of decorin as well as its interaction with collagen is very important in the i region, especially considering that decorin is a candidat molecule to regulate the collagen fibrillogenesis *in vitro* (12, 13, 14) and probably *in vivo* (43), and provide a possible mechanism for maintaining the collagen fibers organization.

The indistinct distribution of the 67 kDa component, probably fibromodulin, in the three regions, is due to its essential presence for the maintenance of the organization and normal morphology of the collagen bundles. The importance of the fibromodulin on the tissue organization, was recently demonstrated in fibrodulin-null mice (44). With respect to the 200 kDa component, which is probably biglycan, its presence in the distal region may be correlated with a possible higher compression in that region. This correlation was observed for the different zones of the porcine knee meniscus.

Our results once more reinforce the idea, that biomechanical forces contribute to the definition of the composition and organization of the ECM.

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FIGURE – LEGENDS:

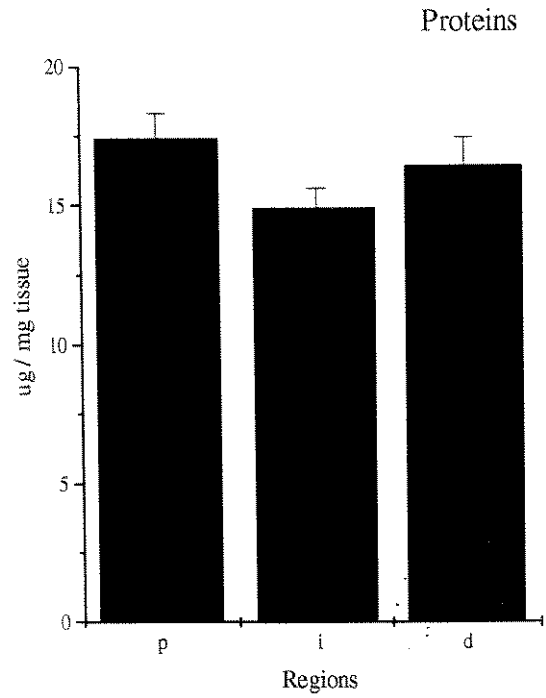
Figure 1. Concentration of proteins (A) and sulfated GAG (B) in the extracts of different regions of the SDFT. Observe a larger amount of proteins in the proximal (p) and distal (d) regions than in the intermediate (i) region. It was not found remarkable differences in terms of sulfated GAGs for the three regions. $P < 5\%$, Anova.

Figures 2-4. DEAE-Sephacel and SDS-PAGE. **2.** Proximal region. Small proteoglycans and other components were eluted with a gradient of 0.1-1.0 M NaCl (A). In the gels (B, C), may be seen polydisperse components with 67 (➞) and 94 kDa (➧). **3.** Intermediate region. Two peaks were eluted during the NaCl gradient (A). Analysis in SDS-PAGE in non reduced conditions showed the presence of a striking polydisperse band around the 67 kDa position (➞), (B). Only in reduced conditions a 94 kDa polydisperse component (➧) was observed (C). **4.** Distal region. Some fractions eluted with gradient of 0.1 – 1.0 M NaCl (A), were analyzed in SDS-PAGE and showed three polydisperse bands around 67 (➞), 94 (➧) and 200 kDa (▶), which have a behaviour similar to the small proteoglycans fibromodulin, decorin and biglycan respectively.
Most collagen was not bound to the DEAE, but some α chains were eluted with the NaCl gradient. Bands at the top of the gel may represent aggregated material.

Figure 5. Agarose gel electrophoresis of GAG of the fraction rich in the polydisperse component with 94 kDa, found in the compressive (A) and tensional (B) regions. 1 – Standards of CS, DS and HS. 2 – Sample. 3 – Sample with chondroitinase AC. 4 – Sample with chondroitinase ABC. The arrow indicates the direction of the running.

Figure 6. 10% SDS-polyacrylamide gel of fraction from DEAE-Sephacel of the d region containing the 67 kDa component, non digested and digested with keratanase. The 67 kDa protein was striking diminished when the sample was digested with keratanase (➞). Staining was by alcian blue with 1 M $MgCl_2$ (A) and coomassie brilliant blue (B). Similar

results were obtained for the proximal and intermediate regions. 1. Sample. 2. Sample with keratanase (A). 1. Keratanase. 2. Sample. 3. Sample with keratanase (B).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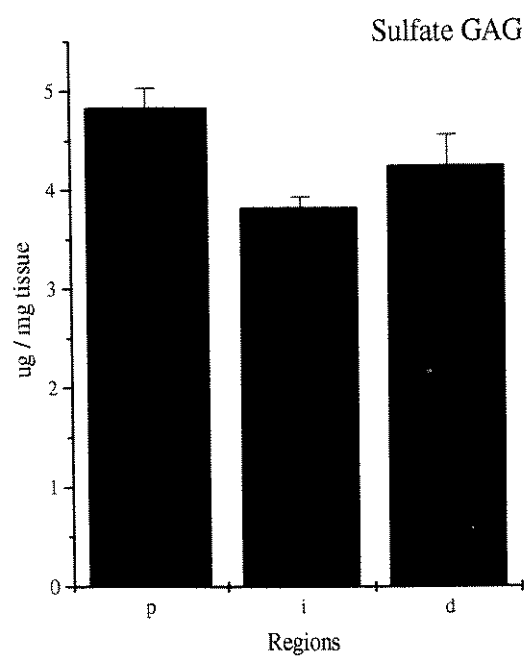
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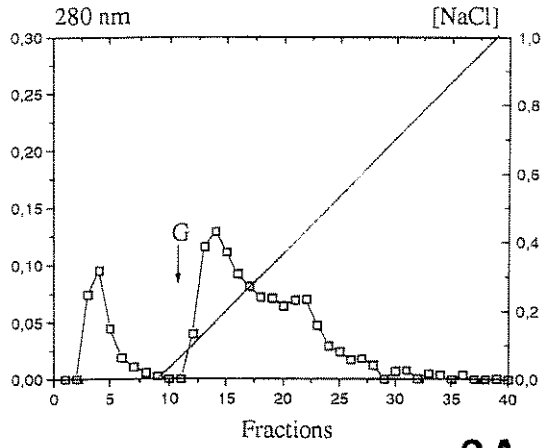

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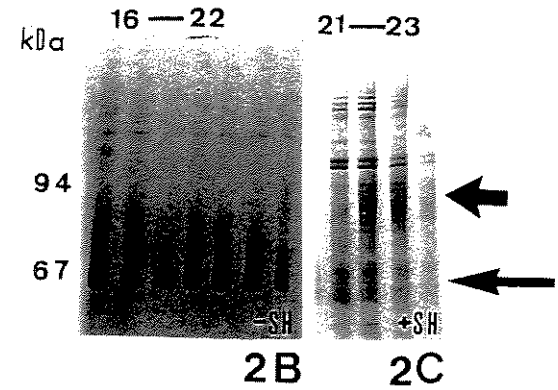

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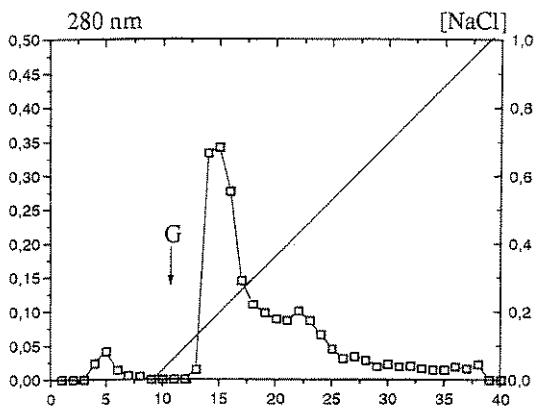

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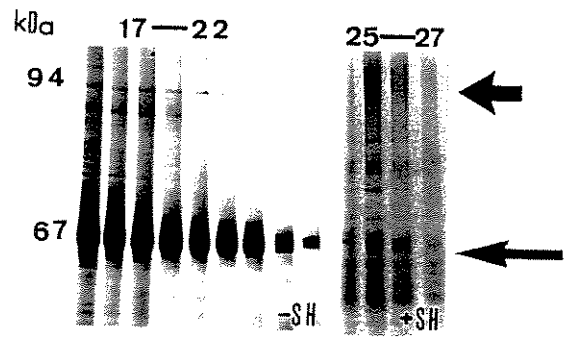

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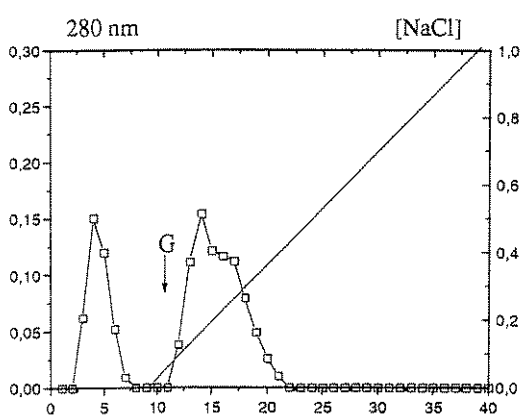

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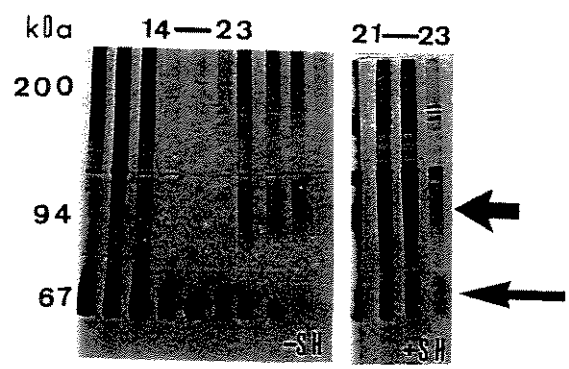

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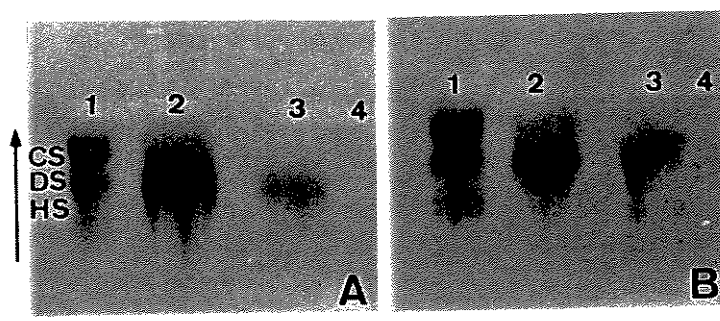
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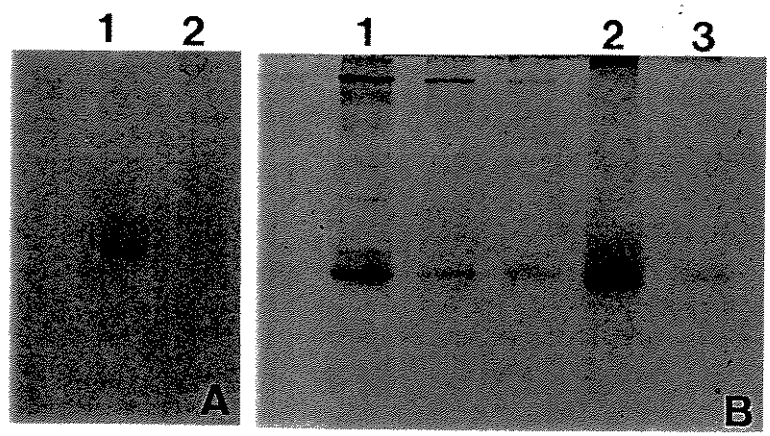
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EXTRACELLULAR MATRIX COMPONENTS OF DIFFERENT REGIONS OF THE DEEP DIGITAL FLEXOR TENDON OF PIGS

Vera Lúcia C. Feitosa[1], Laurecir Gomes[2] and Edson Rosa Pimentel[2]

Department of Morphology[1], Federal University of Sergipe, Aracaju, SE, Brazil.

Department of Cell Biology[2], UNICAMP, Campinas, SP, Brazil.

Key words: Tendon, decorin, fibromodulin, proteoglycans, biomechanics.

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Correspondence:

Edson Rosa Pimentel

Dept. Cell Biology – UNICAMP.

CP 6109. 13083 – 970 Campinas SP, Brazil. Tel: 55 019 788-7330. Fax: 55 019 788-782.

E-mail: pimentel@unicamp.br

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ABSTRACT

Wrap around tendon pass under bones and receive compression and frictional forces in addition to tensile forces. Normally, the region under compression exhibits a structure similar to a fibrocartilage with irregularly arranged collagen fibers, elevated levels of glycosaminoglycans (GAG), and cells resembling chondrocytes. In the present study was analyzed the deep digital flexor tendon of 45 days old pigs. The tendon was divided in three different regions: proximal region where only tensional forces are present, distal region which articulates with the metatarsophalangeal joint and withstands compression and friction forces, and the terminal region which is inserted into III and IV fingers and probably also receives compression and friction forces. Each region was extracted with 4 M guanidine chloride and the extract subjected to biochemical analysis. The GAG content was higher in the compression regions, and the analysis of the quantitation of proteins in each extract, showed that in the proximal and terminal regions there was a larger presence of non-collagenous protein/mg tissue than in the distal region.

A polydisperse component with 94 kDa was found in the three regions of the deep digital flexor tendon. Analysis of glycosaminoglycan in agarose propilenediamine gel and digestion with chondroitinases ABC and AC showed presence of dermatan sulfate in the 94 kDa component. Another polydisperse band (67 kDa) was detected in all regions. The use of keratanase digestion associated with critical electrolyte concentration/alcian blue staining of polyacrylamide gels, indicated the presence of keratan sulfate in the 67 kDa component. The electrophoretical behavior and results of enzymatic digestion suggest that the polydisperse components with 67, 94 and 200 kDa correspond to the small proteoglycans fibromodulin, decorin and biglycan, respectively. No striking differences were found with respect to the three polydisperse components, but it is interesting to mention that the 94 kDa component of the region only under tension, is more evident when the electrophoresis is in presence of the reducting agent, suggesting a strong interaction with other components of the extracellular matrix depends on the integrity of the disulfide bridges. The presence of small proteoglycans in fibrous tissue is an expected feature, considering the probable role of at least two of these components on the collagen

fibrillogenesis. Chondroitin sulfate detected in regions under compression may be an indication of the presence of large proteoglycans.

Abbreviations used:

ECM – Extracellular matrix. DDFT – Deep digital flexor tendon. p – proximal region. d – distal region. t – terminal region. PG – Proteoglycan. GAG – Glycosaminoglycan. Gu-HCl – Guanidina - hydrochloride. PMSF – Phenylmethylsulfonide fluoride. SDS-PAGE – Electrophoresis in polyacrylamide gel with sodium dodecyl sulfate. 2-Me – 2-mercaptoethanol. Rf – Relative migration value. Mr – Relative molecular mass. CS – Chondroitin sulfate. DS – Dermatan sulfate. HS – Heparan sulfate. DMMB – 1, 9 Dimethyl-methylene blue. ANOVA – Analysis of variance.

INTRODUCTION

Tendon is a dense connective tissue and have the role of transmitting tension forces from the muscle to the bone. Some tendons pass around a bone in a joint before insertion in a direction that differs from the direction of the muscle. They were described as wrap around tendons (Alexander and Dimery, 1985) and receive compressive forces in addition to the tension forces. In the sites where the tendon is adapted to withstand compressive loads, it is characterized as fibrocartilage by showing an extracellular matrix (ECM) with collagen fibrils irregularly arranged and an increased content of glycosaminoglycans (GAG) (Koob and Vogel, 1987).

The GAGs are covalently bound to a core protein forming proteoglycans (PG), and imparts to the molecule a high density of negative charges (Hardinghan and Fosang, 1992). In the compressive fibrocartilages the large PG are accumulated (Vogel *et al.*, 1994), but small PG represented by fibromodulin, decorin and biglycan may also be found (Vogel *et al*, 1994). Such fibrocartilages were found in wrap around tendons of rabbits (Gillard *et al.*, 1979; Merrilees and Flint, 1980), dogs (Okuda, 1987a, 1987b), bovines (Evanko and Vogel, 1990) and amphibians (Carvalho and Vidal, 1994; Carvalho and Felisbino, 1999). Structural analysis with the DDFT of pig (Feitosa *et al.*, 2000 – submitted) showed that in areas under compression, the collagen bundles assume a different crimp pattern, with smaller crimp lenght and a random organization of the collagen distribution, as compared with the extended areas which are only under tension.

Considering the absence of biochemical data on the DDFT of pigs and the morphological differences observed in the regions supporting different biomechanical forces, the purpose of this work was to evaluate the quantity of PG and non-collagenous proteins, and to analyze the presence of non-collagenous components with special attention to the small PG, in the different regions of that tendon.

MATERIAL AND METHODS

Material

Hind limbs of 45 days-old, large White lineage pigs, provenient from the Experimental Surgical Nucleus – UNICAMP, were dissected out to obtain the deep digital flexor tendon (DDFT). This tendon was divided into the proximal (p) region that is subjected to only tension forces; the distal (d) region, which splits into two branches towards the fingers, and is presumably submitted to compressive load as it passes close to the metatarsophalangeal joint and the terminal (t) region, which extends into the fingers and also withstands some compression.

Isolation of matrix components

Extraction of tendon samples (1 gram) was carried out with 25 volumes of 4 M guanidine-hydrochloride (Gu-HCl) in 50 mM sodium acetate buffer, pH 6.0 containing proteases inhibitors (50 mM EDTA and 1 mM PMSF) (Heinegård and Sommarin 1987). The extracts were separated from the residual tissue by centrifugation, using a Beckman rotor JA-20 at 18,000 rpm 4°C for 50 min. The Gu-HCl of the supernatants of each sample was changed to 7 M urea in 50 mM sodium acetate buffer, pH 6.0 using a Sephadex G-50 column (13 x 1.0 cm). Proteins and PG present in the first peak were separated in a DEAE – Sephacel column (1.5 x 2.7 cm), previously equilibrated with 7 M urea in 50 mM sodium acetate buffer pH 6.0. Unbound material was washed out with buffered 7 M urea and proteoglycans/anionic proteins were eluted with a linear gradient of NaCl (0.1 – 1.0 M; 35 mL each) in the same buffer. Eluant was monitored by absorbance at 280 nm. Conductivity and presence of GAGs (Farndale *et al.*, 1986) was evaluated for each fraction.

Analytical procedures

Proteins were determined by the Bradford method (1976) and sulfated GAG by the method of Farndale and co-workers, (1986). Bovine serum albumine and chondroitin sulfate (CS) used as standards were purchased from Sigma Chemical (Co, St. Louis, MO, USA).

Quantitative results were analyzed statistically by analysis of variance (ANOVA) with distribution of Fischer at the 5% level of significance (Beiguelman, 1991).

Electrophoresis

Samples (200 µL) of the fractions eluted from DEAE-Sephacel column were precipitated with 100 µL of 1.0 M acetate buffer pH 7.4 and 9 volumes of absolute ethanol at –20°C for 24 hours. The precipitate was resuspended in 50 µL of sample buffer (62.5 mM Tris HCl, 2% SDS, 10% glicerol, 1mM EDTA and 0.01% bromophenol blue at pH 6.8) incubated for five minutes at 96°C, and loaded onto a 4–16% gradient SDS–polyacrylamide gel (Zingales, 1984). Some samples were incubated in the presence of 2-mercaptoethanol. The molecular weight standards used were fosforilase b (94 kDa), bovine serum albumin (67 kDa), ovalbumin (43 kDa), carbonic anydrase (30 kDa), trypsin inhibitor (20.1 kDa), α - lactoalbumin (14.4 kDa). After the running, the gels were stained by the silver nitrate method (Blum *et al.*, 1987). The determination of the relative molecular mass (Mr) was obtained after ploting the relative migration values (Rf) against the known molecular mass of standards on semi-logarithmic paper.

Enzymatic treatments

Sections of the p and t regions were digested with 40 mg papain/gram of tissue (Michelacci and Horton, 1989). The samples after digestion were incubated with 0.04 units of chondroitinases AC in 50 mM sodium acetate, 50 mM Tris and 10 mM EDTA buffer at pH 6.0 (Beeley, 1989), and chondroitinase ABC in 50 mM sodium acetate, 10 mM EDTA, and 50 mM Tris buffer pH 8.0 (Koob, 1989), at 37°C for 6 hours. GAG types were separated by agarose-propilenediamine gel electrophoresis and stained with 0.1% toluidine blue (Dietrich and Dietrich, 1976).

Two - hundred µL of the fractions rich in the 67 kDa component, eluted from DEAE-Sephacel column were precipitated with 100 µL of 1.0 M sodium acetate buffer at pH 7.4 and 2,700 µL of absolute ethanol at 4°C for 24 h (Heinegård *et al.*, 1986). Some samples were incubated with 0.001 unit keratanase in 50 mM sodium acetate, 50 mM Tris

and 10 mM EDTA buffer at pH 6.0 (Evanko and Vogel, 1990), at 37°C for 6 hours, and analyzed in 10% polyacrylamide gel with SDS (Hames and Rickwood, 1990). Gels were stained with 0.05% alcian blue in 0.2 M acetate buffer at pH 5.8 with 1 M $MgCl_2$, and coomassie brilliant blue (Wall and Gyi, 1988). The enzymes were purchased from Seikagaku American, (Far mouth MO, USA).

RESULTS

Quantitative analysis

Each region of the DDFT was treated with 4 M Gu-HCl and the concentrations of sulfated GAG and proteins were determined for the different extracts. The quantity of sulfated GAG was different for each region. In the p region, which is only under tension, less sulfated GAGs (1.8 ± 0.1) were detected when compared with the d (2.3 ± 0.2) and t (2.6 ± 0.2) regions (Fig 1A). With respect to proteins, a larger amount was found in the p and t regions, in relation to the d region (Fig. 1B).

Fractionation and electrophoresis of ECM components

Extracts of the p, d and t regions were fractionated initially in the Sephadex G-50 to exchange Gu-HCl to 7 M urea, and afterwards the content of the peak with proteins and PG was applied onto DEAE-Sephacel column (Figs. 2A, 3A, 4A). The bound material was eluted with a NaCl gradient (0.1-1.0 M). Aliquots of fractions eluted during the NaCl gradient were precipitated and analyzed by SDS-PAGE in presence or absence of the 2-Me. Two polydisperse bands were detected in the three regions (Figs. 2B, 3B, 4B). The 67 kDa component was eluted in the earlier fractions during the NaCl gradient. Its presence was more prominent in the p region (Fig. 2B), as compared with the d and t regions (Figs. 3B, 4B). Another polydisperse component with 94 kDa was easily seen in SDS-PAGE of each region (Figs. 2C, 3C, 4C), but in the p and t regions, it was more prominent if reducing conditions were used (Figs. 2C, 4C). A third polydisperse band migrating at about 200 kDa was detected in fractions of the d and t regions (Figs. 3B, 4B, 4C). Analysis in SDS-PAGE showed that in the p and t regions, the polydisperse bands migrating around 67 and 94 kDa, are prominent when the electrophoresis is under reducing conditions.

Enzymatic digestion

The fractions containing the 94 kDa component from the compressive and tensional regions, were digested with papain and analyzed in agarose gel electrophoresis for the

identification of the composing GAG. Using digestion with chondroitinases ABC/AC it was demonstrated that DS was the main component (Figs. 5A, 5B). Fractions rich in the 67 kDa component found in all the three regions of the DDFT, were treatment with and without keratanase and analyzed in 10% polyacrylamide gel with SDS. Gels were stained with 0.05% alcian blue in 0.2 M acetate buffer at pH 5.8 with 1 M $MgCl_2$ and comassie brilliant blue. The presence of keratan sulfate was showed in its composition (Figs. 6A, 6B).

DISCUSSION

The DDFT is not uniform lengthwise, showing anatomically distinct regions. It has a proximal region originating from the deep digital flexor muscle, and is wrapped by a flexor retinaculum. It is only subjected to tension forces. The distal region splits into two branches and passes under the metatarsophalangeal joint and so withstands compressive forces besides the usual tensional forces. The t region is inserted in the distal phalanx of the third and fourth digits, and as so experiences compressive forces from the distal sesamoid bones in addition to the tensional forces (Barone, 1980; Sisson and Grossman, 1981).

To resist compressive forces, tendons may develop a fibrocartilaginous structure that is characterized by possessing thinner collagen fibrils, an accumulation of PG and chondrocyte-like cells (Carvalho, 1995).

Similar aspects were found in the DDFT of pigs (Feitosa *et al.*, 2000 – submitted). The larger amount of PG, as deduced from the sulfated GAG quantitation, and the presence of CS in the d and t regions, may be related to the action of compressive forces such an aspect was previously found for the compression regions present in the flexor tendon of rabbits (Merrilees and Flint, 1980), canines (Okuda *et al.*, 1987a, 1987b), bovines (Evanko and Vogel, 1990) and amphibians (Carvalho and Vidal, 1994; Carvalho and Felisbino, 1999). It is important to observe that the accumulation of PG in compressive areas, are in agreement with the fibrocartilaginous structure also described for those areas. The presence of CS in the p region, which is solely under tension forces, may be related to the age of the animals, which were relatively immature (45 days old).

The presence of polydisperse components with 67, 94 and 200 kDa in the d and t regions, both bearing compression and tension was evident. Curiously, the 94 kDa was barely detected in SDS-PAGE of the p region, unless reducing conditions were employed. Under these conditions a more prominent band appeared. It is suggestive that this behavior is due a strong interaction of that component with other components possibly collagen, in a manner that depends on the integrity of the S-S bridges. In the t region being a region supposedly under compression, also experiences tension forces due its insertion in the phalanx of the third and fourth digits. Although, there is no information about the ECM

composition in a region of insertion of the DDFT of pigs, on basis at the insertion of the human Achilles tendon (Wagget *et al.*, 1998), it is expected to find a complex structure indicating more possibilities of interaction amongst some ECM components. The increased presence of the 94 kDa component in reducing conditions may be due a dissociative process of the aggregate state of that component with collagen or with other ECM components as discussed for the p region.

The electrophoretic behavior of the 94 kDa component in SDS-PAGE plus the evidence of DS in its composition suggest it to be the small PG decorin, which is the major PG in the tensional regions of tendons (Vogel and Heinegård, 1985). Its presence is essential for the tendon development, an alteration in its gene result in abnormal collagen morphology (Danielson *et al.*, 1997).

With respect to the polydisperse 67 and 200 kDa components, they migrated in SDS-PAGE like fibromodulin and biglycan, respectively. Even though immunoassays with specific antibodies are missing, their behavior during in ion-exchange cromatography and SDS-PAGE is highly suggestive that they correspond fibromodulin and biglycan.

The presence of fibromodulin and decorin in both tension and compression regions of tendons is expected, because they have role in the regulation of the collagen fibrillogenesis (Hedbom and Heinegård, 1989; Oldberg *et al.*, 1989). In relation to the 200 kDa component, that probably is biglycan, it appears only in region under compression, as observed for the deep digital flexor tendon of bovines (Evanko and Vogel, 1990). Robbins and co-workers (1997) have postulated that there is a relationship between a larger expression of biglycan and the presence of compressive forces. However, biglycan was also detected in tensile region of deep flexor tendon of bovine (Vogel and Meyers, 1999). The actual function of biglycan in tendons remains to be elucidated.

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FIGURE - LEGENDS

Fig. 1. Concentration of sulfated GAG and proteins in the extracts of different regions of the DDFT. The larger amount of GAGs was detected in the t region (2,6±0,2) which was followed of the d (2,3±0,2) and p (1,8±0,1) regions (A). The concentration of non collagenous proteins (B) was more expressive in the p (12.9±0.2) and t (12.06±0,3) regions than in the d (11.5±0.6) region. P<5%, ANOVA).

Fig. 2. Proximal region. DEAE-Sephacel and SDS-PAGE. The fractions eluted from DEAE were monitored by absorbance in 280 nm and 526 nm (DMMB). The first peak corresponds to collagen and other cationic proteins. Small proteoglycans and other proteins were eluted with a gradiente of 0.1-1.0M NaCl (A). In the gel (B), under non reducing conditions, the two polydisperse bands represents the small proteoglycans fibromodulin (➞) and decorin (➡). Some aggregated material remained at the top of the gel (→). Observe that the 94 kDa band is more prominent under reducing conditions (C).

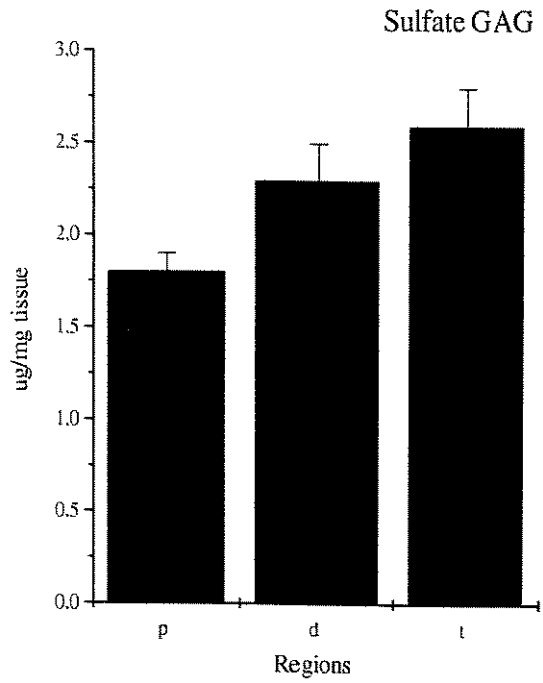
Fig. 3. Distal region. DEAE-Sephacel and SDS-PAGE. The fractions eluted from DEAE were monitored by absorbance in 280 nm and 526 nm (DMMB). Small proteoglycans and other non-collgenous glycoproteins were eluted within a gradient of 0.1-1.0M NaCl (A). Analysis of the fractions in SDS-PAGE under non reducing conditions showed the presence of three polydisperse bands with about 67 (➞); 94 (➡) and 200 (▶) kDa, which may represent the small proteoglycans fibromodulin, decorin and biglycan (B). Notice the presence of aggregated material at the separating top of the gel (→).

Fig. 4. Terminal region. DEAE-Sephacel and SDS-PAGE. The fractions eluted from DEAE were monitored by absorbance in 280 nm and 526 nm (DMMB). Polydisperse components and other proteins were eluted from DEAE with a gradient of 0.1-1.0 M NaCl (A). In the gel (B), the three polydisperse bands around 67 kDa (➞), 94 kDa (➡) and 200 kDa (▶), were observed and likely represent the small proteoglycans fibromodulin, decorin and

biglycan, respectively. Note that under non reducing conditions, some aggregated material remained at the top of the separating gel (→). The 94 kDa component was more prominent under reducing conditions (C).

Fig. 5. Agarose/PDA electrophoresis of GAG delivered by the papain digestion. Fragments of the p (A) and t (B) regions were digested by papain and the GAGs were analyzed in agarose gel with and without chondroitinases AC/ABC digestion. The complete digestion with chondroitinase ABC and resistence to digestion with chondroitinase AC, showed the GAG to be DS. 1. Standards of CS, DS and HS. 2. Sample. 3. Sample with chondroitinase AC. 4. Sample with chondroitinase ABC. The arrow indicates the direction of the runing.

Fig. 6. SDS-PAGE of the 67 kDa component without and after prior treatment with keratanase. The gel was stained with CEC-alcian blue (Fig. 6A) and coomassie brilliant blue (Fig. 6B). 1. Standard of keratan sulfate. 2. Sample. 3. Sample treated with keratanase (A). 1. Standard of keratan sulfate. 2. Sample. 3. Sample treated with keratanase. 4. Keratanase (B).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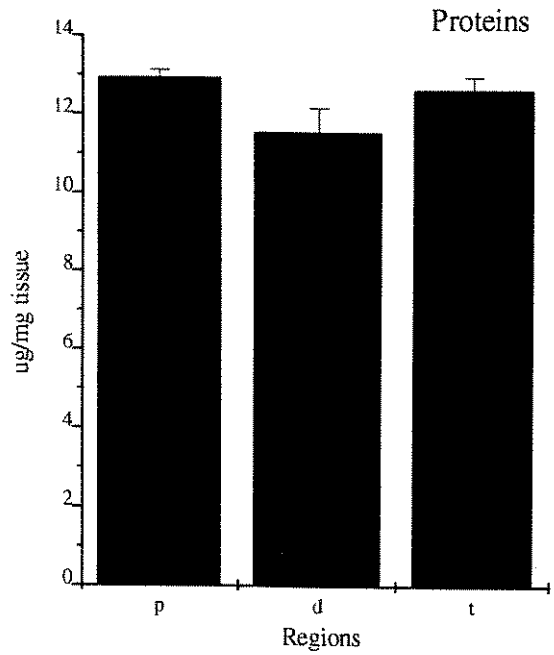
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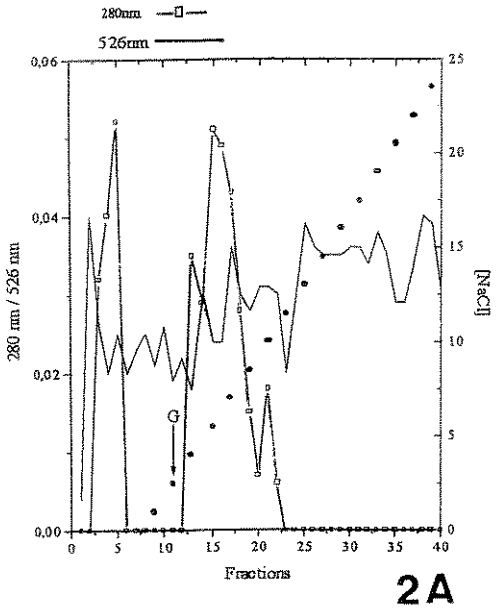

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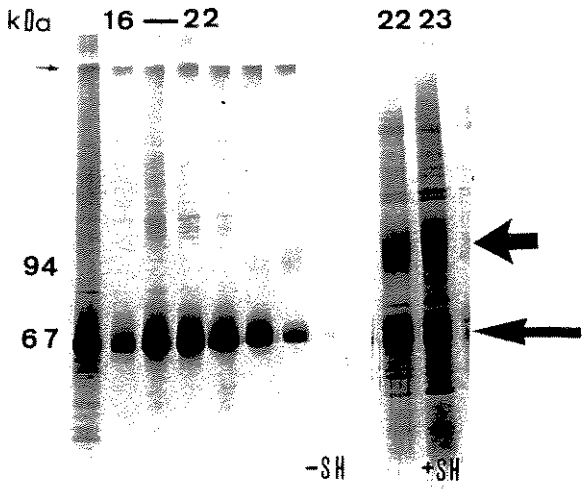
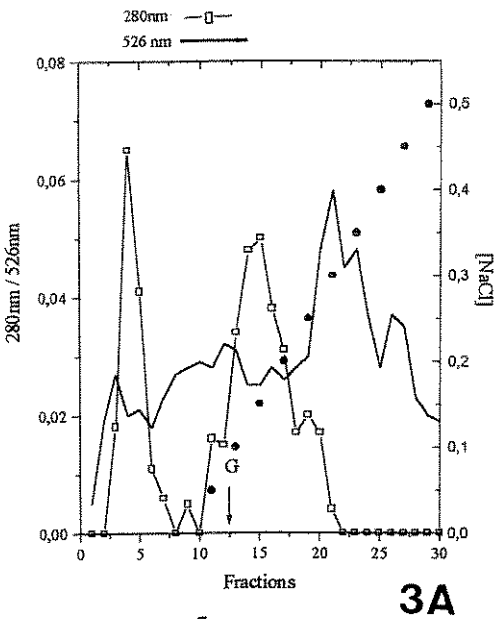


2B 2C



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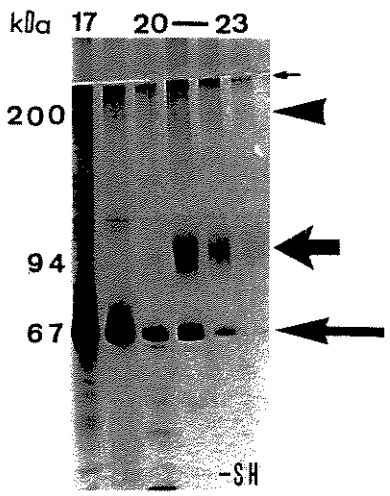

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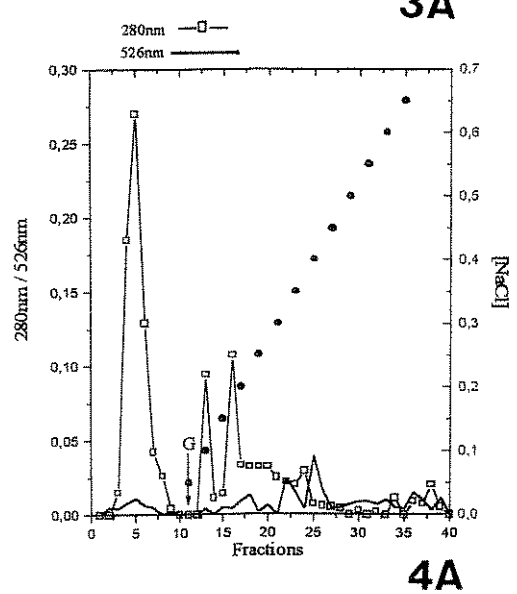

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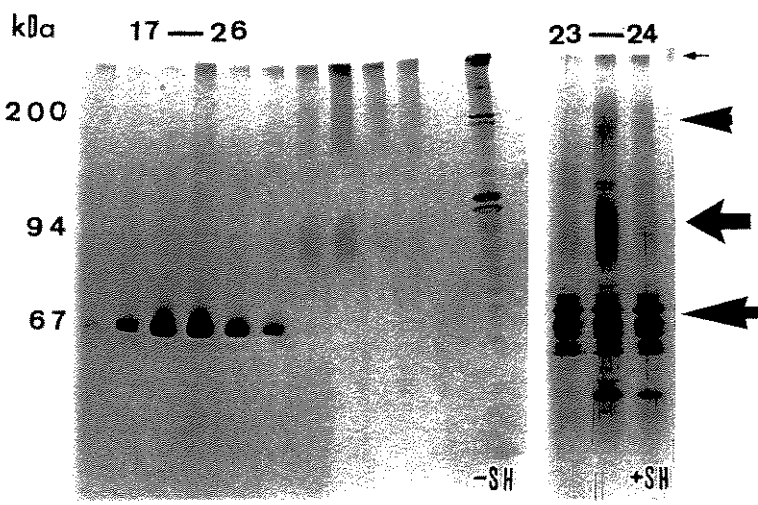
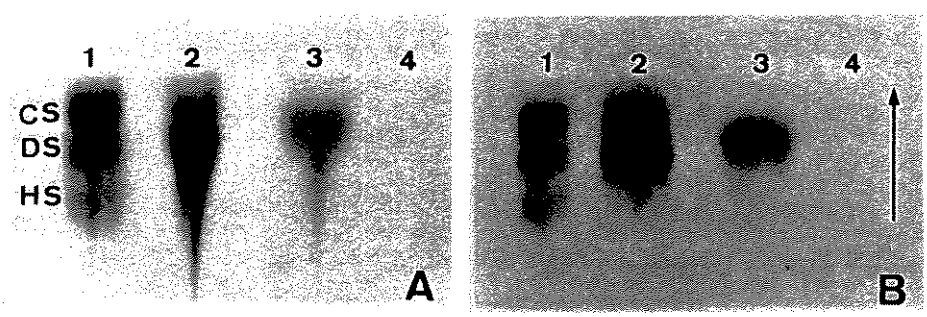


4B 4C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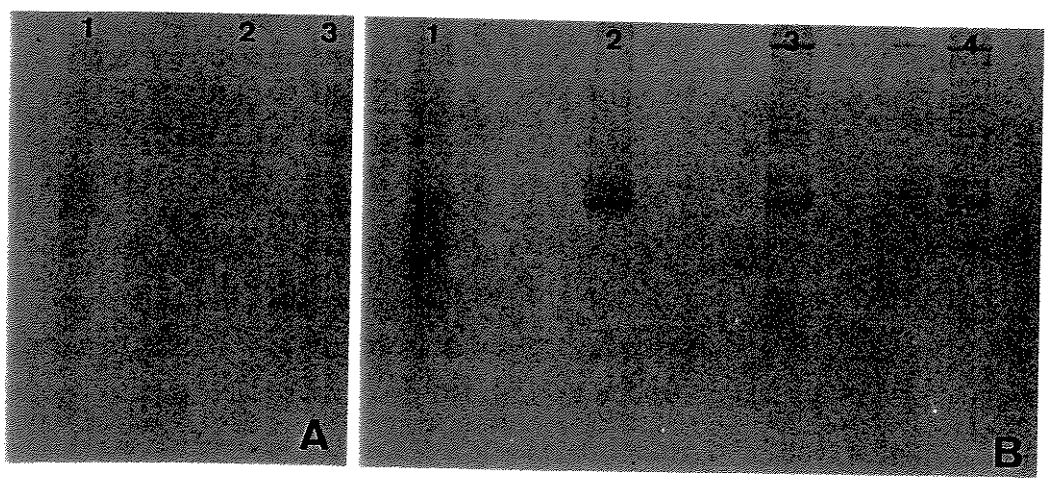
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VI - CONCLUSÕES GERAIS

1. Nas regiões onde atuam forças biomecânicas o tendão apresentou uma estrutura fibrocartilaginosa com células arredondadas semelhantes a condrócitos, e basofilia metacromática após coloração por azul de toluidina, ao contrário das regiões onde predominam forças de tensão, que apresentaram características típicas de tendão.

2. O "crimp" apresentou padrões diferentes entre as regiões de tensão e compressão, porém foi muito mais definido e uniforme na região de tensão.

3. A ultra-estrutura da região de compressão dos tendões revelou que a matriz pericelular dos tendinócitos apresenta uma grande concentração de proteoglicanos, além de uma rede de fibrilas finas de colágeno.

4. Através das análises ultraestruturais foi observado para os dois tendões, que nas regiões onde atuam forças de compressão, os feixes de colágeno assumem várias direções, enquanto que nas regiões onde ocorrem somente forças de tensão, estes feixes são mais uniformes e arranjados em uma única direção.

5. Nas regiões de compressão ocorreu uma maior concentração de glicosaminoglicanos sulfatados totais, em relação às regiões que estão só sob tensão.

6. O glicosaminoglicano dermatam sulfato predominou em todas as regiões dos dois tendões, e naquelas regiões onde forças de compressão estão atuando foi também detectado o condroitim sulfato.

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