UNIVERSIDADE ESTADUAL DE CAMPINAS

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MATRIZ EXTRACELULAR DE PERICÁRDIO FIBROSO PORCINO: ESTUDO MORFOLÓGICO E BIOQUÍMICO

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

Este exemplar corresponde à redação final da tese defendida pelo(a) candidato (a) Antonella Sachsida Braga Vilela e aprovada pela Comissão Julgadora.

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"O valor das coisas não está no tempo que elas duram, mas na intensidade com que acontecem. Por isso existem momentos inesquecíveis, coisas inexplicáveis e pessoas incomparáveis".

(Fernando Pessoa)

Índice

Resumo

Resumo

Tecido pericardial tem sido utilizado na confecção de biopróteses empregadas na reparação de diferentes lesões. Entretanto, calcificações e falência mecânica têm sido as principais causas de durabilidade limitada de biopróteses cardíacas fabricadas com pericárdio bovino. Nesse trabalho foi realizado um estudo do pericárdio fibroso porcino em sua estrutura microscópica e sua natureza bioquímica. A morfologia geral e a arquitetura das fibras colágenas e elásticas foram estudadas em tecido seccionado e também em montagens totais, corados por diferentes métodos histoquímicos e analisados em microscopia de luz convencional, de polarização, de fluorescência e confocal. O estudo bioquímico da matriz pericardial foram realizados de acordo com procedimentos bioquímicos pertinentes. O pericárdio mostrou-se um tecido altamente celularizado com características de tecido jovem. Os feixes colagênicos apresentaram-se arranjados em camadas multidirecionalmente orientadas, formando uma rede de trama fechada, com um número maior de fibras orientando-se obliquamente, partindo da região central inferior em direção súpero-lateral esquerda em relação ao coração. Uma discreta predominância de fibras colágenas no sentido base-ápice foi verificada na região anterior direita do pericárdio. A matriz extracelular apresentou-se pouco metacromática. Observaram-se finas fibrilas elásticas intimamente associadas às fibras colágenas, entrelaçadas em direções diversas ou paralelas entre si. Extração comparativa de componentes da matriz não revelou diferenças entre proteínas extraídas das regiões direita e esquerda e também entre material fresco e congelado. A análise em SDS-PAGE revelou dezesseis proteínas com valores de massa molecular aparente entre 11 e 109 kDa. Análises realizadas sugerem que o GAG encontrado possivelmente é o dermatam sulfato. Os dados obtidos podem subsidiar novos procedimentos direcionados à obtenção de biomembranas melhor preparadas e funcionalmente mais adequadas à construção de biopróteses.

Abstract

Abstract

Pericardial tissue has been used to construct bioprostheses employed in the repair different kinds of injuries. However, calcification and mechanical failure have been the main causes of limited durability of cardiac bioprostheses constructed with bovine pericardium. In the course of this work a study has been conducted on the porcine fibrous pericardium and its microscopic structure and its biochemical nature. The general morphology and the architecture of the collagen and elastic fibers have been studied in sectioned tissue and also total assemblies, stained by diverse histochemical methods and analyzed by conventional light, polarizing light, fluorescence and confocal microscopy. Biochemical study of the pericardial matrix was conducted according to pertinent procedures. The pericardium was shown as a highly cellularized tissue with a vascular tree very ramified and collagen fibers arranged in multidirectionally oriented layers. A predominant direction was verified, with a larger number of fibers obliquely oriented, starting at the lower central region towards high-lateral-left relatively to the heart. A discreet predominance of collagen fibers is the base-apex direction was verified in the right-front region of the pericardium. Extracellular matrix with little metachromasy was observed, indicating small quantities of acid glycosaminoglycans. Fine elastic fibrils were observed, intimately associated to collagen fibers, interlaced in various directions or parallel to themselves. No differences were found between proteins extracted from the right or left regions and between fresh or frozen material. The quantities of extracted GAGs were too small to be detected by the method used. The SDS-PAGE showed proteins with values of apparent molecular mass between 11 and 109 kDa included two polydisperse bands around 71 and 85kDa. The ectrophoretic analysis showed that the found GAG is possibly dermatan sulfate.

Introdução Geral

I - INTRODUÇÃO

1. Considerações gerais

O pericárdio é o saco fibrosseroso que envolve o coração e as raízes de seus grandes vasos, com o qual ocupa a maior parte do mediastino médio. É constituído principalmente de tecido conjuntivo fibroso (Hollinshead,1980; Moore & Dalley, 2001). Guarda relações anatômicas importantes com estruturas diversas como os pulmões, que o auxiliam em suas funções estabilizadoras topológicas.

Tecido pericardial tem sido utilizado na confecção de biopróteses que são empregadas na reparação de diferentes lesões, especialmente cardíacas (Olmos et al., 1997; Barros et al., 1999). Reparações de parede ventricular, de válvulas cardíacas e de parede de aorta para correção de aneurismas têm sido feitas com sucesso empregando biopróteses confeccionadas com pericárdio (Pires et al., 1997). Para tanto, pericárdios de diversas espécies têm sido testados na produção de biopróteses, sendo que em humanos, o bovino e o porcino têm apresentado bons resultados. A camada fibrosa do pericárdio porcino é mais uniforme nas suas diferentes regiões, com espessura intermediária entre a do humano e do bovino e, desta maneira é um dos biomateriais indicados na produção de biopróteses para uso humano (Fentie et al., 1986; Chanda et al., 1997).

Tratamentos diversos são aplicados a esses tecidos com a finalidade de evitar reabsorções dos implantes, manter sua estrutura original e sua integridade biomecânica, minimizar a degradação enzimática, melhorar suas propriedades biomecânicas e reduzir ou mesmo neutralizar suas propriedades antigênicas e

imunogênicas (Khor, 1997, Petite et al., 1995). Esses tratamentos podem ser de natureza física e/ou de natureza química. (Khor, 1997; Petite et al., 1995; Páez et al., 2001; Chang et al., 2002).

Tratamentos físicos, como exposição às radiações gama e ultravioleta, secagem e tratamentos térmicos têm como principal vantagem a não introdução de substâncias tóxicas ou seus resíduos no tecido. Entretanto, esses métodos parecem não promover ligações cruzadas com o colágeno em níveis desejáveis para a manutenção da integridade tecidual. A irradiação ultravioleta tem sido estudada como um método capaz de promover tais ligações (Khor, 1997) mas com resultados inconsistentes.

Os tratamentos químicos, que empregam agentes capazes de formar ligações cruzadas intra e intermoleculares com o colágeno desses tecidos, são preferidos pelo seu baixo custo, fácil obtenção e eficácia. Os agentes fixadores promotores de ligação cruzada mais utilizados são o formaldeído e glutaraldeído. Esses agentes, no entanto, podem ser altamente citotóxicos, mesmo em baixas concentrações, prejudicando a biocompatibilidade dos biomateriais por eles tratados (Petite et al., 1995; Duncan & Boughner, 1998). O glutaraldeído, que se liga a resíduos de lisina ou hidroxilisina da molécula do colágeno, é atualmente o agente fixador mais utilizado (Duncan & Boughner, 1998; Chang et al., 2002). Entretanto, há evidências cada vez maiores de que o glutaraldeído não estabiliza efetivamente todas as estruturas teciduais, especificamente os glicosaminoglicanos (GAGs). Perda de GAGs do espaço interfibrilar e da superfície das fibrilas colágenas são relatadas em tecidos tratados por

glutaraldeído (Simionescu et al., 2003) além de sua calcificação (Chanda et al., 1997). Compostos sintéticos como os de poliepoxi, a acilazida, as carbodiimidas e o diisocianato de hexametileno são reportados como uma alternativa ao glutaraldeído (Khor, 1997) e já vêm sendo empregados em estudos utilizando animais.

Calcificações e falência mecânica têm sido as principais causas de durabilidade limitada e perda de biopróteses cardíacas. Embora o mecanismo específico seja ainda desconhecido, considera-se que essa calcificação ocorra principalmente em resposta ao tratamento químico das biopróteses (Chanda et al., 1997; Páez et al., 2001).

Tratamentos químicos pós-fixação têm sido testados para extração da fração celular de tecidos biológicos, removendo desse modo antígenos associados à membrana, bem como proteínas solúveis, com o intuito de amenizar a resposta antigênica do organismo receptor ao mesmo tempo em que criam um microambiente natural para migração de células hospedeiras, acelerando assim o processo de regeneração tecidual (Petite et al., 1995; Chang et al., 2002; Bertipaglia et al., 2003). A descelularização por métodos enzimáticos ou com emprego de detergentes em combinação com fixação química podem controlar em parte a antigenicidade de transplantes (Stock & Vacanti, 2001) e reduzir a sua calcificação (Schmidt & Baier, 2000). A remoção química de componentes lipídicos também é utilizada, pois atribui-se a eles importante papel na precipitação de sais de cálcio em biopróteses (Páez et al., 2001). Tratamentos com diferentes agentes como o difenilfosforilazida e etildimetilaminopropil carbodiimida vêm sendo

testados numa tentativa de evitar efeitos indesejáveis, especialmente como aqueles que ocorrem com o glutaraldeído (Jorge-Herrero et. al., 1999). Implante de pericárdio porcino tratado com chitosan após a fixação com glutaraldeído resultou em uma redução significativa de reação inflamatória no organismo receptor. O chitosan presumivelmente previne a liberação lenta de resíduos de glutaraldeído reduzindo a toxicidade dos implantes assim tratados (Chanda et al. 1997). Um agente para ligações cruzadas de ocorrência natural - o genipin - foi testado por Sung e colaboradores (1999) e por Chang e colaboradores (2002) tendo apresentado citotoxidade e genotoxidade mais baixa e, conseqüentemente, biocompatibilidade superior à do glutaraldeído. Todavia, novos agentes e tratamentos devem ser buscados e testados para que se obtenha características teciduais mais adequadas (Jorge-Herrero et al., 1999).

Aspectos estruturais do pericárdio

O pericárdio é formado por dois componentes diversos: o saco fibroso e a membrana serosa. O pericárdio fibroso forma um saco com formato de frasco, fechado por sua aderência aos grandes vasos. Está preso ao centro tendíneo do diafragma pelo ligamento pericardicofrênico. Anteriormente está fixado ao esterno pelos ligamentos esternopericárdicos e posteriormente às estruturas situadas no mediastino posterior por tecido conjuntivo. O coração, desse modo, mantém-se posicionado e fixado no interior do saco fibroso (Moore & Dalley, 2001).

A membrana serosa reveste:

a) a face interna do saco fibroso onde juntos formam o pericárdio parietal (Ishihara et al., 1981; Fentie et al., 1986), e

b) externamente os átrios e ventrículos, onde denomina-se epicárdio, estendendo-se para além deles sobre os grandes vasos por 2 ou 3 cm (Gray & Goss, 1988; Moore & Dalley, 2001).

As duas partes da membrana serosa são contínuas uma com a outra e o ponto onde o epicárdio termina e se reflete para revestir internamente o saco fibroso é chamado reflexão do pericárdio (Figura 1). Suas características são tais que proporcionam a essas duas estruturas superfícies lisas e escorregadias, completamente livres e móveis, ainda que em contato uma com a outra.

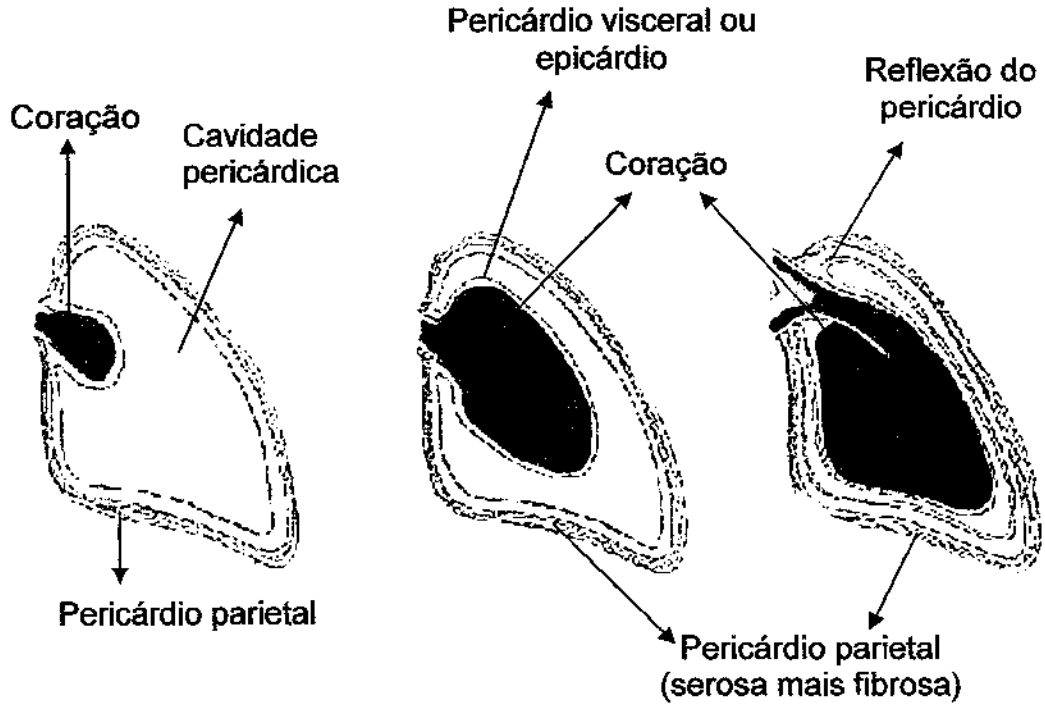

Figura 1. Aspectos morfológicos esquematizados do pericárdio no coração em desenvolvimento (Modificado de Moore & Dalley, 2001).

A cavidade pericárdica é assim completamente fechada como as outras grandes cavidades serosas pleurais e peritoneal. Embora ela envolva quase completamente o coração, na realidade contém apenas fluido em quantidade suficiente para manter as superfícies umedecidas (Gardner, 1978).

Histologicamente o pericárdio parietal é constituído por (figura 2):

a) Uma membrana serosa interna;

b) Uma camada de tecido conjuntivo, com matriz extracelular fibrosa, que se une com a camada adventícia dos grandes vasos que entram e saem do coração;

c) Uma camada de tecido adiposo, de espessura variável, cujas células podem se arranjar em grupos descontínuos ou em camadas e

d) Um mesotélio que reveste externamente o pericárdio fibroso freqüentemente denominado epipericárdio (Fentie et al., 1986).

Vasos arteriais correndo junto com suas contrapartes venosas, paralelamente à superfície, são numerosos na camada fibrosa, estendendo-se até a serosa (Ishihara et al., 1981). Eventualmente podem também se localizar na camada de células adiposas. A variação dimensional da camada adiposa contribui, pelo menos em parte, para a variabilidade na espessura do pericárdio que é característica de cada espécie. No porcino é grande a quantidade de tecido adiposo, mais regularmente arranjado em camadas e com espessura mais uniforme do que no humano e bovino (Fentie et al., 1986). Sob as células mesoteliais da serosa interna, na camada fibrosa, podem ser vistas fibras elásticas, mais grossas superficialmente e mais finas em regiões mais profundas,

interconectadas aos feixes de fibras colágenas (Ishihara et al., 1981). Esses feixes, com padrão ondulado, cruzam-se em ângulo agudo uns com os outros. Entre os feixes colágenos encontram-se as células do tecido conjuntivo além dos componentes vasculares e nervosos (Figura 2). As células encontradas são principalmente fibroblastos, macrófagos e mastócitos (Ishihara et al., 1981, Fentie et al., 1986).

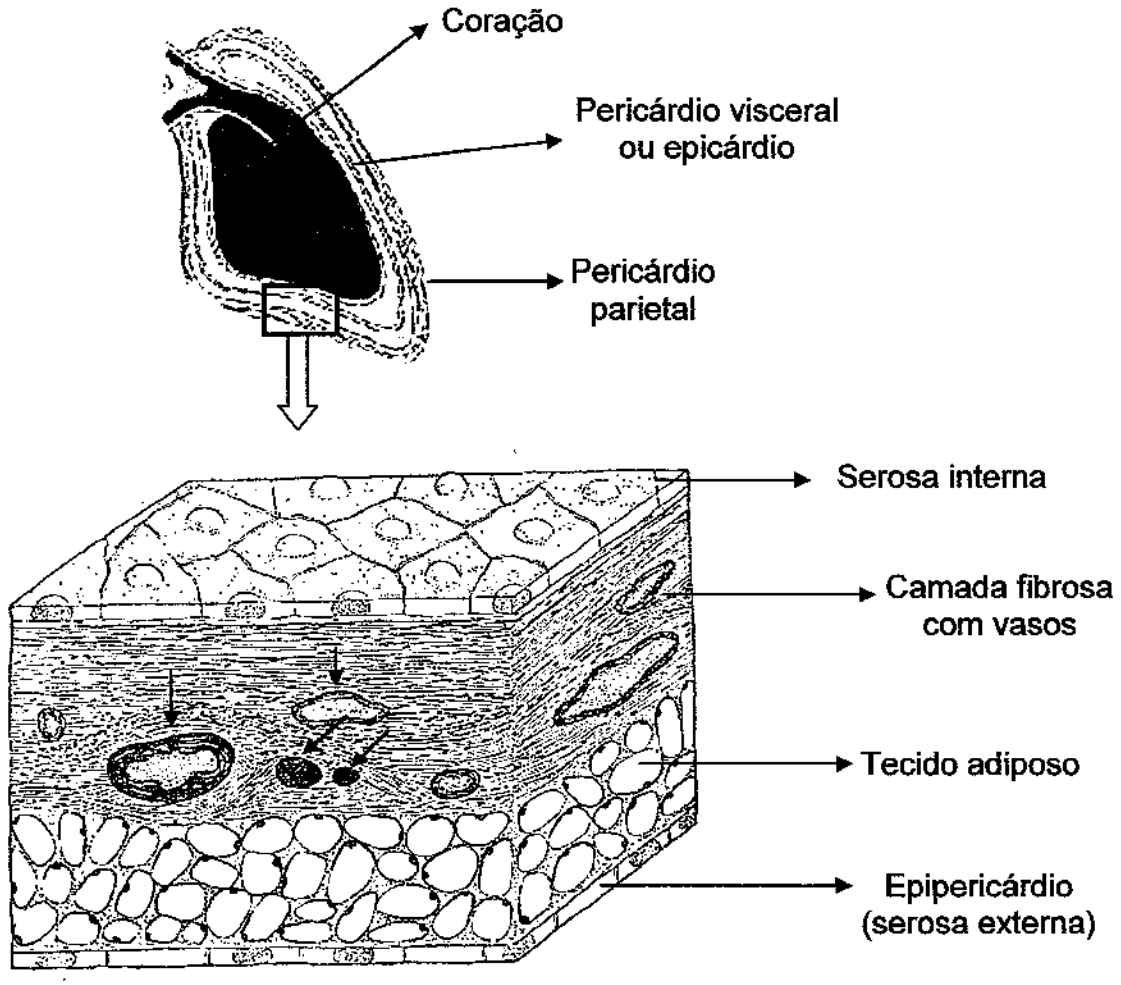

Figura 2. Representação esquemática ampliada do fragmento de pericárdio parietal, em corte transversal, destacado na figura superior. Camada fibrosa com vasos (setas pretas) e nervos (setas vermelhas) (Modificado de Fentie et al. 1986 e Moore & Dalley, 2001)

Segundo Sacks e colaboradores (1994), a região anterior esquerda do pericárdio bovino parece ser estruturalmente mais homogênea, com orientação geral dos feixes de fibras colágenas acompanhando, preferencialmente, a direção circunferencial do coração, enquanto que na região anterior direita esses feixes orientam-se preferencialmente no sentido base-ápice do coração.

As propriedades biomecânicas do pericárdio estão diretamente relacionadas com a distribuição e orientação dos feixes de fibras colágenas (Sacks et al., 1994; Castillo-Olivares, 2001), como também com a estrutura ondulada (EO) – "wave-like structures" - ou crimp dessas fibras (Loke et al., 1996; Langdon et al. 1999). A análise da EO revela a ordenação das moléculas de colágeno do tipo I nos feixes colagênicos e possíveis mudanças de direção desses elementos fibrilares (Ault & Hoffman, 1992). A variabilidade morfológica dos feixes colagênicos possivelmente reflete uma diferenciação funcional que pode ser resultado de diferentes demandas biomecânicas teciduais. Alterações na organização molecular colagênica que não são detectadas com a microscopia de luz comum podem ser observadas e quantificadas com o emprego da microscopia de luz polarizada (Whittaker et al., 1987) que tem sido recomendada como o método mais apropriado para detecção, descrição e interpretação da EO (Gathercole & Keller, 1991; Vidal, 2003). As propriedades anisotrópicas ópticas, birrefringência e dicroísmo, dos feixes de colágeno fornecem um modelo estatístico de sua organização molecular e, por conseguinte constituem um importante mecanismo de investigação de sua orientação (Vidal & Mello, 1972).

Enquanto o dicroísmo revela o arranjo molecular dos glicosaminoglicanos ácidos, a birrefringência revela, predominantemente, a estrutura cristalina das cadeias polipeptídicas do colágeno. Ambos os fenômenos estão intimamente relacionados uma vez que, em ratos jovens, em torno de 13% da birrefringência de forma do colágeno é devida aos glicosaminoglicanos a ele associados (Vidal, 1966, 1980a). A birrefringência, detectada pelo uso da microscopia de polarização, informa sobre a morfologia e direção das fibras, sua distribuição e seu grau de associação ou de empacotamento, ainda que nenhuma coloração do tecido tenha sido utilizada (Vidal, 1964,1987). A utilização de tecidos em montagens totais fornece subsídios adicionais ao estudo e estimativa do grau de formação de malha tridimensional de colágeno.

A arquitetura das fibras colágenas constitui-se em parâmetro de escolha e seleção de tecidos destinados à confecção de biopróteses (Sacks et al., 1994). Não foi possível, até o momento, encontrar na literatura dados referentes à arquitetura colagênica em pericárdio porcino.

Além dos componentes fibrosos estão presentes na camada fibrosa do pericárdio outros componentes típicos da matriz extracelular como os proteoglicanos, glicoproteínas estruturais e proteínas não colagênicas.

3. Considerações Bioquímicas

3.1 Colágeno

A molécula de colágeno tem forma de bastão, com cerca de 2.800Å de comprimento, 15Å de largura e peso molecular em torno de 300kDa. Sua estrutura alongada é formada por três cadeias polipeptídicas (van der Rest & Garrone, 1991) denominadas cadeias α, ricas em prolina e glicina, que formam uma tripla fita helicoidal orientada para a esquerda com três resíduos de aminoácidos por passo. Também contém uma seqüência repetitiva de Gly - X - Y, onde X ou Y podem ser qualquer aminoácido (Linsenmayer, 1985), mas freqüentemente X é prolina e Y é hidroxiprolina (van der Rest & Garrone, 1991).

Por apresentar quantidade expressiva de hidroxiprolina, o que não ocorre com outras proteínas animais, o colágeno pode ser quantificado pela dosagem desse aminoácido, uma vez que 13,5 a 14,5% dos aminoácidos do colágeno correspondem à hidroxiprolina (Slack et al., 1984; Riley et al., 1994; Stehno-Bittel et al., 1998; Reddy et al., 1998). O colágeno apresenta aproximadamente 35% de glicina, 11% de alanina e 21% de prolina e hidroxiprolina. As restrições estruturais características da hélice do colágeno estão relacionadas com seu conteúdo, não habitual, desses aminoácidos (Nelson & Cox, 2002). As moléculas de colágeno são glicosiladas, e a glicosilação ocorre nos resíduos de hidroxilisina (Hay, 1991). Os resíduos de prolina, por sua estrutura em anel, não apresentam rotação na ligação do nitrogênio com o carbono alfa e a rotação entre os carbonos alfa e a carbonila fica restringida. Assim, esses resíduos são os principais responsáveis

pela conformação estável do colágeno (Piez & Reddi, 1984) o que, juntamente com as várias ligações cruzadas intermoleculares, proporcionam uma resistência à tensão maior do que a de um fio de aço de idêntica secção transversal (Nelson & Cox, 2002). As cadeias α das moléculas do colágeno e as moléculas de colágeno das fibrilas são unidas por tipos não habituais de ligações covalentes cruzadas envolvendo resíduos de lisina, hidroxilisina ou histidina. Essas ligações criam resíduos de aminoácidos não-primários como a desidro-hidroxilisinonorleucina. O alinhamento específico e o número de ligações cruzadas variam de acordo com o tecido e produzem bandas características em uma micrografia eletrônica (Nelson & Cox, 2002).

O aumento da rigidez e a redução de elasticidade do tecido conjuntivo resultam de uma acumulação de ligações covalentes cruzadas nas fibrilas de colágeno com o passar do tempo. Um mamífero típico possui algo em torno de trinta variantes estruturais de colágeno que ocorrem em tecidos particulares (Nelson & Cox, 2002), sendo que a diferença entre eles reside na composição de aminoácidos, nos domínios de cada molécula e nos diferentes arranjos estruturais (van der Rest & Garrone, 1991). No pericárdio bovino cerca de 90% das proteínas extracelulares correspondem ao colágeno do tipo I (Simionescu et al.,1989)

Tradicionalmente o papel atribuído ao colágeno é estrutural. Entretanto, também funciona, direta ou indiretamente, como agente promotor de adesão e diferenciação celular, agente quimiotático para macrófagos e fibroblastos e como antígeno nos processos imunológicos (Linsenmayer, 1985). Além disso, *in vitro*,

substrato colagênico em sua forma hiperpolimerizada, induz diferenciação celular caracterizada por aberrantes alterações morfológicas, aumento no conteúdo de DNA e diminuição da área nuclear em células V79. Neste substrato, essas células apresentam períodos de latência proliferativa, levando a um atraso no processo de morte celular por apoptose (Maria, 1998). O colágeno tipo I hiperpolimerizado foi desenvolvido por Vidal e tem sido utilizado em implantes em humanos (Palma et al., 1996; Ricetto, 1997).

3.2 Proteoglicanos

Os proteoglicanos são constituídos basicamente por um esqueleto protéico central ao qual estão ligadas covalentemente uma ou mais cadeias de glicosaminoglicanos. No espaço intercelular existem duas classes principais de proteoglicanos: os grandes, representados pelo agrecam e versicam, e os pequenos proteoglicanos, onde se destacam o decorim, biglicam, fibromodulim (Kuc & Scott, 1997) além do lumicam, epificam, osteoglicina e queratocam (Chakravarti et al., 1998). A parte formada pelo glicosaminoglicano comumente é a maior fração (em massa) da molécula de proteoglicano e é freqüentemente o principal sítio de atividade biológica. A variabilidade molecular observada tanto no esqueleto protéico quanto nas diferentes classes de glicosaminoglicanos ligados, permite que os proteoglicanos desempenhem múltiplas funções no organismo (Hascall & Hascall, 1985). Em muitos casos a atividade biológica é resultante de múltiplos sítios de ligação, ricos em possibilidades de realizar pontes de

hidrogênio e interações eletrostáticas com outras proteínas da superfície celular ou da própria matriz extracelular (Vidal, 1963, 1964; Nelson & Cox, 2002).

Os heteropolissacarídeos chamados glicosaminoglicanos são uma família de polímeros lineares compostos de unidades repetidas de dissacarídeos. Um dos monossacarídeos que compõem os dissacarídeos é sempre a N-acetilglicosamina ou a N-acetilgalactosamina. O outro é geralmente um ácido urônico, usualmente o ácido D-glicurônico ou o ácido L-idurônico, exceto para o queratan sulfato que apresenta galactose substituindo o ácido urônico (Hascall & Hascall, 1985). Em alguns glicosaminoglicanos, uma ou mais das hidroxilas do açúcar aminado estão esterificadas com sulfato. A presença dos grupos sulfato e carboxila das unidades de dissacarídeos dá aos glicosaminoglicanos uma alta densidade de cargas negativas (Nelson & Cox, 2002). A presença dessas cargas garante a essas moléculas grande parte de suas características funcionais por se associarem a uma grande quantidade de cátions livres e com isso retêm água nos tecidos (Hay, 1991). Devido às forças repulsivas entre grupos vizinhos carregados, essas moléculas assumem uma conformação estendida quando em solução. O padrão característico das unidades sulfatadas e não-sulfatadas estabelece um reconhecimento específico por uma grande variedade de proteínas ligantes que se associam aos glicosaminoglicanos. As diferentes características apresentadas pelos glicosaminoglicanos estão relacionadas à composição dissacarídica, ao tipo de ligação glicosídica entre eles e ao número e a localização dos radicais sulfato. Com base nestas características temos os seguintes glicosaminoglicanos: ácido

hialurônico, condroitim sulfato, dermatam sulfato, heparam sulfato e o queratam sulfato (Nelson & Cox, 2002).

Os pequenos proteoglicanos decorim e fibromodulim estão envolvidos na fibrilogênese do colágeno, podendo influenciar na organização e no diâmetro dessas fibras (Kuc & Scott,1997). O decorim é rico em leucina, possui uma cadeia de glicosaminoglicano que pode ser o condroitim ou o dermatam sulfato, dependendo do tecido em que se encontra. Está presente provavelmente em todos os tecidos conjuntivos fibrosos (Comper, 1996),

O fibromodulim tem sua distribuição restrita em relação ao decorim e biglicam. É abundante em cartilagem, tendões e esclera e pouco encontrado em ossos e pele. Sua função na organização da matriz extracelular é semelhante à do decorim (Comper, 1996).

Os pequenos proteoglicanos presentes nos tendões participam de sua estabilidade estrutural, mantendo e regulando o diâmetro das fibrilas de colágeno (Fishbein *et al.*, 1982; Scott, 1996). Proteoglicanos de alto e baixo peso já foram evidenciados em tendões bovinos (Vogel & Heinegard, 1985) e na pele (Carrino *et al.*, 2000) dentre outros tecidos.

O biglicam, assim como o decorim, é rico em leucina, possui duas cadeias de glicosaminoglicanos, e dependendo do tecido considerado, estas cadeias podem ser condroitim ou dermatam sulfato. Sua função parece estar relacionada à adesão celular e estruturação da matriz (Neame et al., 1989).

O lumicam, também um proteoglicano rico em leucina, contendo queratam sulfato, está envolvido com a regulação e montagem de fibrilas de colágeno. Encontrado principalmente na córnea, também está presente na derme e no tecido conjuntivo dos músculos (Chakravarti et al., 1998).

Em pericárdio bovino foi encontrado um pequeno proteoglicano contendo uma única cadeia de glicosaminoglicano do tipo dermatam sulfato (Simionescu et al., 1989). Entretanto a literatura ainda é pobre em dados referentes a esse tecido.

Embora representem uma fração relativamente pequena da massa tecidual (menos de 1% do peso seco em tendão flexor bovino), os proteoglicanos contribuem significativamente para com as propriedades dos tecidos conjuntivos, como, por exemplo, com a pressão de intumescimento e resistência osmótica às forças compressivas. Tecidos conjuntivos com pequenas quantidades de proteoglicanos não intumescem ou intumescem pouco em água (pH neutro) comparados com aqueles que apresentam quantidades maiores de proteoglicanos (Koob & Vogel, 1987).

Material não fixado e cortado em congelação, além de material fixado e embebido em parafina, corados com Azul de Toluidina em pH 4,0 são indicados para estudos da orientação de glicosaminoglicanos ácidos e disponibilidade de seus grupos aniônicos (Vidal, 1964,1972). A molécula do azul de toluidina, que em solução comporta-se como cristal líquido, é privilegiada para esses estudos por ser pequena, podendo assim intercalar-se entre outras moléculas, como por exemplo, entre as bases do DNA (Mello & Vidal, 1973). Além disso, é uma

molécula que apresenta geometria planar, com ressonância de elétrons ao longo do seu eixo (Vidal, 1963). Suas moléculas ligam-se aos glicosaminoglicanos empilhando-se de modo ordenado, sendo que seus eixos mais longos orientam-se perpendicularmente ao maior eixo da molécula de glicosaminoglicano (Vidal, 1984). Adicionalmente, os cristais de azul de toluidina são dotados de excelentes propriedades anisotrópicas, de grande importância, como o dicroísmo linear, que é um fenômeno anisotrópico óptico resultante da absorção seletiva da luz polarizada (Mello & Vidal, 2003), e a dispersão anômala da birrefringência (Vidal, 1963 e 1987). É relevante notar que tais eventos ocorrem *in situ* quando o azul de toluidina se liga a poliânions orientados.

3.3 Elastina e Fibrilina

As proteínas elastina e/ou fibrilinas são encontradas como componentes das fibras oxitalânicas, elaunínicas e elásticas e também das lamelas elásticas da matriz extracelular. O conjunto de tais fibras e lamelas é por vezes chamado "sistema elástico do tecido conjuntivo" por alguns autores (Cotta-Pereira et al,1976, 1984). Esse conjunto interage com outros componentes da matriz determinando em parte as características biomecânicas do tecido como ocorre, por exemplo, nos tendões elásticos de aves. Nesses locais, fibras elásticas intimamente associadas às fibras colágenas, alongam-se a medida que o tendão é distendido por forças de tensão. Assim, as fibras colágenas, inicialmente onduladas, alinham-se com o eixo do tendão e passam a resistir às forças

deformantes. Uma vez cessadas as forças aplicadas ao tendão, as fibras elásticas retornam ao estado retraído, fazendo com que as fibras colágenas também retornem ao estado ondulado, de menor distenção. Um mecanismo semelhante parece se aplicar às paredes vasculares arteriais. Esse mesmo mecanismo pode estar atuando na matriz extracelular pericardial, contribuindo com as características biomecânicas do tecido.

Muitos métodos têm sido utilizados para descrição morfológica e topográfica de fibras elásticas. Muito embora seja possível a visualização de fibras elásticas por muitos deles, sua especificidade ainda hoje não está esclarecida.

Sondas fluorescentes como o ANS (1-anilino-8-naphtalene sulfonate) e Cloreto de Danzila (1-dimethylamino-naphthalene-5-sulfochloride) têm sido empregadas em estudos de polaridade, hidrofobicidade, reatividade e conformação molecular de proteínas (Stryer, 1965; Beyer, 1973; Radda, 1973; Yguerabide, 1973). Sulfonatos de anilinonaftaleno e corantes correlatos são relatados como sondas apropriadas para identificação de sítios moleculares não polares desde que as regiões não polares sejam estericamente acessíveis e suficientemente extensas para ligar o corante (Stryer, 1965). Ainda de acordo com esse autor ocorre uma transferência altamente eficiente de energia eletrônica de ativação de resíduos de aminoácidos aromáticos para a ligação do ANS. O ANS foi utilizado no estudo do grau de agregação de histonas (Laurence, 1966; Penzer,1972), na avaliação de alterações estruturais na rede de elastina (Gosline

1975 e 1976) e em histoquímica para colágeno e elastina (Vidal,1978 e 1980b; Carvalho & Vidal, 1995).

O cloreto de danzila foi inicialmente introduzido por Weber em 1952 no preparo de conjugados de proteínas (Bartzatt, 2001). É largamente utilizado em diversas áreas como farmacologia, toxicologia, bioquímica entre outras, em estudos estruturais e análises quantitativas de moléculas complexas que contenham aminas primárias, secundárias e terciárias, além de compostos aromáticos carboxilados (Bartzatt, 2001 e 2003). Segundo Rosselet & Ruch (1968), a solução de cloreto de danzila em acetona é específica para demonstração microfluorométrica de grupos α–NH_2 e ε– NH_2 e ainda, a reação é específica para lisina quando utilizada para resíduos de aminoácidos ligados a proteínas. Assim, o cloreto de danzila tem sido utilizado como sonda fluorescente na determinação da hidrofobicidade e do teor de resíduos de lisina em fibras e lamelas elásticas além de fibras colágenas (Vidal, 1980b; Timberlake et al., 2002).

O método desenvolvido por Fischer (1979) que utiliza o permanganato de potássio como agente oxidante e o azul de toluidina como corante, empresta metacromasia e birrefringência às fibras elásticas, indentificando-as *in situ* com clareza, ainda que associadas a fibras colágenas.

3.4 Proteínas não colagênicas

Na MEC, além de componentes como o colágeno e os proteoglicanos, estão presentes proteínas não-colagênicas como a fibronectina e laminina, esta última como componente de lâmina basal, que servem como mediadores da adesão celular com outros componentes da matriz (Comper, 1996). As fibronectinas são moléculas sintetizadas por fibroblastos, células endoteliais, condrócitos, células gliais e miócitos. São abundantes na matriz dos tecidos conjuntivos, membranas basais e fluidos corporais (Piez & Reddi, 1984). São importantes para alguns processos básicos, como migração e diferenciação celular, desenvolvimento embrionário, coagulação sanguínea e formação de metástases. Pode se ligar às integrinas, proteoglicanos e/ou ao colágeno (Comper, 1996). Outras proteínas não colagênicas têm sido identificadas como proteínas oligoméricas de matriz de cartilagem (COMP) (Hedbom *et al.*, 1992), proteína de matriz de cartilagem (CMP), proteínas de 58 e 36kDa (Heinegard & Pimentel, 1992), entre outras.

Assim, os vários componentes da MEC são importantes não só por constituírem estruturas que suportam diferentes tipos de forças biomecânicas, especialmente as moléculas de colágeno e proteoglicanos, mas por transmitirem para as células as várias alterações de natureza física ou química que ocorrem no meio extracelular.

O propósito deste trabalho foi o estudo do pericárdio porcino em sua estrutura microscópica e sua natureza bioquímica. O acréscimo de conhecimento

sobre a natureza físicoquímica e supraorganização molecular do pericárdio pode fornecer condições para que possam ser revistos os atuais métodos de preparo do pericárdio e, assim, novos procedimentos possam ser adotados com o intuito de serem obtidas biomembranas melhor preparadas e funcionalmente mais adequadas à construção de bioproteses, contribuindo para futuras intervenções médico-cirúrgicas.

Capítulo 1

Esse capítulo contém um artigo intitulado **"EXTRACELLULAR MATRIX OF PORCINE PERICARDIUM: BIOCHEMISTRY AND COLLAGENIC ARCHITECTURE"** a ser submetido à publicação.

EXTRACELLULAR MATRIX OF PORCINE PERICARDIUM : BIOCHEMISTRY AND COLLAGENIC ARCHITETURE

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Short Title : Extracelular Matrix of Porcine Pericardium

Summary

Pericardial tissue has been used to construct bioprostheses employed in the repair different kinds of injuries, mostly cardiac. However, calcification and mechanical failure have been the main causes of limited durability of cardiac bioprostheses constructed with bovine pericardium. In the course of this work a study has been conducted on the porcine fibrous pericardium and its microscopic structure and its biochemical nature. The general morphology and the architecture of the collagen have been studied under conventional light, polarizing light, fluorescence and confocal microscopy. The biochemical study of the pericardial matrix was conducted according to the following procedures: swelling test; dosage of hydroxyproline and collagen; quantification of aminoacids in soluble collagen; extraction of components of the extracellular matrix of the right and left ventral regions of fresh and frozen pericardium with different molarities of GuHCl; dosage of proteins and glycosaminoglycans (GAGs); SDS-PAGE and analysis of total GAGs. The pericardium was shown as a highly cellularized tissue. The microscopic analysis showed collagen fibers arranged in multidirectionally oriented layers, forming a closely-knitted web, with a larger number of fibers obliquely oriented, starting at the lower central region towards high-lateral-left relatively to the heart. No qualitative differences were found between proteins extracted from the right or left regions. Likewise, no differences were found between fresh or frozen material. The dosages of proteins from front-left and front-right regions of pericardium

showed no significant differences from each other. The quantities of extracted GAGs were too small to be detected by the method used. The enzymatic digestions and the electrophoretic analysis show that the found GAG is possibly dermatan sulfate. The proteoglycan showed a running standard very similar to the small proteoglycan decorim.

1. INTRODUCTION

The pericardium is a fibrous-serous sac enveloping the heart and the commencement of the large vessels, composed mostly of fibrous connective tissue (Hollinshead , 1980: Moore & Dalley, 2001). The pericardial tissue has been used for the construction of bioprostheses especially for the repair of cardiac injuries (Olmos et al., 1997: Barros et al., 1999). The repair of the ventricular wall, cardiac valves and aorta wall for the correction of aneurysm has been successfully performed using pericardium bioprostheses (Pires et al., 1997). Pericardium from different animals has been tested for the construction of bioprostheses. Good results have been attained using bovine and porcine pericardium. The fibrous layer of porcine pericardium possesses greater uniformity in its different regions and a thickness ranging between the human and the bovine pericardium. Thus, its use in the construction of bioprostheses appears to be a distinct possibility. (Fentie et al., 1986; Chanda et al., 1997).

The biomechanical properties of pericardium, as well as other collagenous tissues, are directly related to the distribution and orientation of the bundle of collagen fibers (Sacks et al., 1994), and also to the wave-like structures (WLS) or crimp of such fibers (Loke et al., 1996; Langdon et al., 1999). The analysis of the WLS shows the arrangement of the type-I collagen molecules in the collagen bundle and possible change of direction of those fibrillar elements (Ault & Hoffman, 1992). The morphological variability of the collagenic bundle possibly reflects a functional differentiation resulting from different biomechanical properties of the

tissues. Alterations in the collagenic molecular organization can be observed and quantified by polarized light microscopy (Whittaker et al., 1987), which has been recommended as the most appropriate method for the detection, description and interpretation of WLS (Vidal, 2003; Gathercole & Keller, 1991).

The anisotropic optical properties, birefringence and dicroism of the collagen bundle provide a statistical model of its molecular organization and consequently establish an important investigation mechanism of its structural pattern (Vidal & Mello, 1972). While dicroism, using toluidine blue pH 3,5-4,0, reveals the molecular arrangement of acid glycosaminoglycans (GAGs), birefringence reveals predominantly, the crystalline structure of the polipeptidic chains of collagen. Both phenomena are intimately related due to the fact that about 13% of the form birefringence of the collagen bundle is due to the GAGs associated to them, as seem in young rat tendon (Vidal, 1966 and 1980).

The use of *in totum* preparations of tissues provides additional subsidies for studies and appraisal of the degree of tri-dimensional grid formation of collagen. The architecture of collagen fibers establishes a choice and selection parameter concerning to the tissues to be used for construction of bioprostheses (Sacks et al., 1994).

Besides the fibrous components, other typical components of the extracellular matrix such as proteoglycans, structural glicoproteins and collagenic proteins are part of the fibrous layer of pericardium. A small proteoglycan containing a single chain of glicosaminoglycan of dermatan sulfate type was found in bovine pericardium (Simionescu et al., 1989). However, literature is still poor

concerning the data related to porcine pericardium. Proteoglycans, although amounting to a small fraction of the tissue mass (less than 1% of the net weight of bovine flexor tendon), contribute significantly for the physicochemical properties of connective tissues such as the phenomena of swelling and osmotic resistance to compression forces.

Tissue used for the construction of bioprostheses are submitted to several treatments in order to avoid implant reabsorption, to maintain its original structure and biomechanical integrity, to minimize enzymatic degradation, to improve its biomechanical properties and to reduce or even neutralize its antigenical and imunogenical properties (Khor, 1997, Petite et al., 1995). However, calcification and mechanical failure have been the main causes for limited durability and loss of cardiac bioprostheses. Thus, in order to obtain more adequate tissue characteristics for bioprostheses, new procedures must be searched and tested (Jorge-Herrero et al., 1999).

The purpose of this work was the study of porcine pericardium in its microscopic structure and biochemical nature. The surveyed data and the additional knowledge about its molecular supra-organization will become useful towards reviewing the methods of preparing pericardium in order to obtain membranes with a better preparation and exempt of antigenous matter.

2. MATERIAL AND METHODS

2.1 Animals

Six-months-old pigs of Large White lineage with an average weight of 95kg were used. Pericardia were collected immediately after slaughter at the abattoir and were fixed, kept fresh or frozen (3 days at –20ºC) for the following experimental procedures.

2.2 Morphology

The ventral surface of the pericardium (n=10) was used, being identified into regions: upper central (UC), lower central (LC), upper right lateral (URL), upper left lateral (ULL), lower right lateral (LRL) and lower left lateral (LLL). The fragments were fixed in paraformaldehyde 4% (in PBS pH 7,4 and NaCl 0,15M). Part of the fragments was put through the routine histological procedure for imbedding in Histosec/Paraplast Plus (Merck) and microtomy with a thickness of 7 μm and the remaining part was appointed for in totum preparations. Part of the in totum preparations and sections were stained with toluidin blue (Merck, Darmstadt, Germany) in McIlvaine buffer pH 4,0 and others were kept with no staining. The slides were analyzed under polarized light microscopy, using a Zeiss Polarizing Microscope equipped with Planachromatic objective and photographed using a Zeiss Axiophot 2 microscope, equipped with Pol-Neofluar infinitive objectives/infinitive focus.

2.3 Analysis of collagen aminoacids

Pericardial collagen (n=5) was obtained by extraction in acetic acid 5% for 72 hours at 4° C, precipitation in NaCl 2,5M for 24 hours at 4°C and dialyze against water for 96 hours at 4°C. The analysis of aminoacids was done by reverse phase HPLC in order to separate feniltiocabamoil derived from aminoacids. The aminoacids were obtained from collagen samples submitted to hydrolysis with HCl 6N in the presence of fenol 0,1% in vapor phase at 106°C for 24 hours. The content of aminoacids was evaluated by a PICO-TAG (Waters) aminoacid analyzer.

2.4 Swelling test

Pericardium fragments (n=5) were washed in water, compression–dried between paper-filter sheets and weighed. They were, then, immersed in water for 2 hours and again dried and weighed. They were subsequently immersed in acetic acid 3% for 1 hour and again dried and weighed. The volumes of water and acetic acid that were used corresponded to 500 times (v/v) the volume of the pericardium fragment (Koob & Vogel variant, 1987).

2.5 Dosage of hydroxyproline

In order to quantify hydroxyproline the fragments from five pericardia were weighed and submitted to hydrolysis in HCl 6N (1mL/10mg tissue) for 24 h at

106°C. The hydrolysate was then treated with a chloramine T solution for 20 minutes at 20°C, perchloric/aldehyde acid was then added and left in water-bath at 60°C for 15 minutes according to the description by Stegman & Stalder (1967). Absorbances were read in 550 nm in a Hewlett-Packard 845A spectrophotometer. For the standard curve were used different concentrations of hydroxyproline (Sigma, St. Louis, Mo, USA).

2.6 Extraction of components of the extracellular matrix

The samples of the left-front and right-front regions of both fresh and frozen pericardia (n=5) were cut into fragments of approximately 1mmx1mm which were submitted to extraction with 15 volumes of 3M, 4M, 5M or 6M guanidine chloride (GuHCl) containing 1mM-PSMF, 20mM EDTA, in 50mM sodium acetate buffer, pH 5,8 (Heinegard & Sommarin, 1987) at 4°C for 24 h. The mixture was then centrifuged (39.000 x g, 4°C, 50 min) and the supernatant of each extract was used for biochemical analysis.

2.7 Dosage of proteins and sulfated GAGs

Proteins were quantified according to the Bradford method (1976), using bovine serum albumin as standard. Sulfated GAGs were quantified by the dimethylmethylene blue (DMMB) method, according to Farndale et al. (1986), using chondroitin sulfate (CS) as standard.

2.8 Chromatography of Ionic Exchange

The total extracts in GuHCl were dialyzed against 20 volumes of 7M urea 0,05M Tris-acetate pH 8,0 buffer. After four changes of dialysis 3mL of dialyzed material was applied on ionic exchange column dietilaminoetil-Sephacel (DEAE-Sephacel 1,5 x 2,7 cm) equilibrated with 7M urea 0,05M Tris-acetate pH8,0 buffer. The fractions were eluted (flow rate 1,5mL/min) using a gradient of NaCl 0,1-1M in the same buffer with 7M urea. Fractions of 2.8 mL were collected and the elution of proteins was monitored by absorbance at 230 and 280 nm in a Hewlett Packard 8452 A spectrophotometer.

2.9 Electrophoresis in polyacrylamide gel with sodium dodecyl sulfate (SDS-PAGE)

SDS-PAGE was performed according to Zingales (1984) using gradient of 4-16% of acrylamide in the presence of SDS and stacking gel with 3,5% of acrylamide and buffer system according to Laemmli (1970). The proteins were precipitated in a mixture of acetate-ethanol, after 12h at a temperature of -4°C. The precipitate was suspended in a sample buffer containing 0,05M TrisHCl pH 6,8, 2% SDS , 10% glycerol , 0,002% bromofenol blue. β-mercaptoethanol 5% was used under reducing conditions. The staining of gels was done with Coomassie Brilliant Blue (CBB) or by silver impregnation (Blum et al., 1987). The relative

molecular masses were deduced from the Rf of molecular mass markers (Klaus and Osborn, 1969).

2.10 β-elimination

To release the GAG chains from the proteoglycans (PGs) obtained by chromatography, samples of fractions containing PGs were precipitated with acetate-ethanol and incubated for 20 hours in 0,5M NaOH at 4°C followed by precipitation with ethanol and washing with acetone (Michelacci and Horton, 1989). The GAGs were analyzed in agarose- propylene diamine gel.

2.11 Enzymatic treatment

2.11.1 Digestion with papain

For the extraction of GAGs from the tissue, pericardium fragments were dehydrated in acetone overnight at 4°C, dried at 37°C for 24 hours and treated with papain (40mg/g of tissue) in 0,03M sodium citrate buffer pH 5,5 , containing 0,04M EDTA and 0,08M β-Me (80ul/1ml) and incubated at 50°C for 24 hours (Michelacci & Horton, 1989). The GAGs obtained after precipitation with ethanol were analyzed in agarose-propylene diamine gel.

2.11.2 Digestion with chondroitinases ABC/AC

Samples containing glycosaminoglycans obtained from β-elimination and digestion with papain were treated with chondroitinase ABC and AC (Seikagaku). For chondroitinase ABC (0,04U), the sample was suspended in 10μL of 50mM sodium acetate buffer, 10mM EDTA and 50mM Tris pH 6,0. For chondroitinase AC (0,08U) the buffer was the same but with pH 8,0 (Beeley, 1985). The digestion was for 20 h at 37°C. After ethanolic precipitation the GAGs were analyzed in gel.

2.12 Electrophoresis in Agarose-propylene diamine gel (Agarose-PDA)

The GAGs obtained by enzymatic digestion were analyzed by electrophoresis in Agarose gel in 50mM acetate-propylene diamine buffer pH9,0 as described by Dietrich and Dietrich (1976), using CS, dermatan sulfate (DS) and heparan sulfate (HS) as standard.

2.13 Elution of proteins from SDS-PAGE gel

Bands corresponding to the proteins of 75 and 68 kDa, in SDS-polyacrylamide gel, were trimmed, chopped and placed for elution in 0,5%SDS with 50mM Tris-HCl buffer pH 7,4 at ambient temperature under constant agitation for 24h. The supernatant, containing the eluted protein was then precipitated in ethanol-acetate for 12h in the freezer. After centrifugation the precipitate was

suspended in a sample buffer in the presence and absence of β-mercaptoethanol. The eluted proteins were analyzed in SDS-PAGE.

3. RESULTS

3.1 Morphological analysis

The in totum preparations stained with Toluidin Blue in pH4,0 have only their polyanionic components stained. Examining these preparations by polarizing light microscope enabled the detection of the collagen fibers due to their birefringence, as well as their distribution, orientation and aggregation.

Morphological aspects of porcine pericardium can be seen in Figs. 1 to 3. A highly cellularized tissue can be seen, whose cells are mainly fibroblasts, besides endothelial and smooth muscle cells of vessels (Fig. 2). A highly ramified vascular tree can also be seen whose vessels can be classified as a transition among arterioles – capillaries – small veins (Fig. 3).

Extracellular matrix showing little or no metachromasy can be seen in total preparations stained with Toluidin Blue in pH 4,0 (Fig. 3 and 4) evidencing small amounts of acid GAGs.

A network with a close texture of relatively fine collagen fibers arranged in layers can be seen under a polarizing microscope. The analysis of tangential sections and of *in totum* preparations of pericardium, observed in different focal planes, allowed the viewing of multidirectionally oriented fibers (Fig. 5 and 6), frequently crossing each other at acute angles. It was also possible to observe a

predominance of direction, with a larger number of fibers obliquely oriented, from the lower central region (LC) towards left higher lateral (Fig. 6). A discreet predominance of fibers in the heart's apex-base direction was verified in the pericardium's right-front region (data not shown). Collagen fibers, with an apparently rectilinear course and showing typically undulant pattern (crimp) were observed in diverse pericardium regions (Fig. 7 and 8).

3.2 Analysis of aminoacids

The composition and percentage of aminoacids in the pericardial soluble collagen and the ratio hydroxyproline/proline are presented in Table 1.

3.3 Swelling test

Fragments of pericardium (n=5) submitted to the swelling test showed a small weight decrease after a 2-hour immersion in water. A significant increase was observed after a 1-hour immersion in acetic acid 3% as shown by the analysis of graphic 1.

3.4 Dosage of hydroxyproline

The average dosage of hydroxyproline and its conversion in quantity of collagen were respectively 42,21μg (SD±2,01) and 469,0μg per mg of tissue,

considering that hydroxyproline corresponds to 9% of the total amount of aminoacids of pericardial collagen.

3.5 Extraction of ECM components

The electrophoretic analysis of proteins of the ECM of the pericardium, obtanined after extraction with 3, 4, 5 and 6M GuHCl showed no apparent differences (Fig. 9A). Although no differences have been found in the extraction with different concentrations of GuHCl, extraction with 4M GuHCl chosen as standard procedure, as it has been most widely used concentration in extraction procedures of the extracellular matrix components especially to avoid any kind of molecular interactions. The analysis of electrophoresis in reducing (with β-mercaptoethanol) and non-reducing conditions (in the absence of β-mercaptoethanol) showed the same band pattern in both conditions. Also no differences were found in extract obtained from the right or left regions of pericardium (Fig. 9B). Likewise, no differences were detected between fresh and frozen tissue (Fig. 9B).

3.6 Dosage of proteins and sulfated GAGs.

The analyses of the dosage of proteins in the extracts obtained from the left and right regions of the pericardium showed no significant differences (α=0,05). For each gram of pericardium were found 20,94 mg (SD±2,7) of proteins for the right region and 21,08 mg (SD±3,4) for the left region. The dosage of sulfated

GAGs showed no presence of such polysaccharides. It is possible that the extracted amounts were too small to be detected by the method employed.

3.7 Chromatography of Ionic Exchange and SDS-PAGE

The extracted material was fractionated in DEAE- Sephacel column and the components bound to DEAE were eluted with a gradient of 0,1M to 1M NaCl (Fig. 10A). Samples of each fraction were analyzed in SDS-polyacrylamide gel. Analysis of the gel showed proteins with the following values of apparent molecular mass: 109, 105, 75, 71, 68, 63, 52, 48, 41, 35, 31, 22, 15, and 11 kDa. Two polydisperse bands were found averaging 71 and 85 kDa (Fig. 10B). The bands corresponding to the α1 and α2 chains of collagen were found only in the material not bound to DEAE-Sephacel.

3.8 Electrophoresis in Agarose-PDA gel of GAGs obtained by digestion with papain

The analysis of GAGs in Agarose-PDA gel after digestion of the tissue with papain showed a large polydisperse band in intermediate position between HS and DS. After treatment with chondroitinases AC and ABC was observed that the GAG was completely digested by chondroitinase ABC but not by chondroitinase AC, indicating it was dermatan sulfate (Fig. 11). The extense polydisperse band seen in the control probably contained some contaminant nucleic acid.

3.9 Electrophoresis in Agarose-PDA gel of GAGS obtained by β-elimination of fractions of chromatography

The polydisperse component with an apparent molecular mass of 80 to 100 kDa, probably a small proteoglycan, eluted from DEAE Sephacel was submitted to β-elimination and the liberated GAG was analysed in Agarose-PDA gel (Fig. 12). The band is in the same direction of the standard dermatan sulfate and was completely digested by chondroitinase ABC, and not by chondroitinase AC, indicating that the polydisperse component is a small proteoglycan containing dermatan sulfate.

3.10 Analysis of proteins with 75 and 68 kDa

The fractions 19 and 20 of DEAE-Sephacel showed two proteins of apparent mass of 75 and 68 kDa respectively, whose electrophoretic behavior was different in the presence or absence of β-mercaptoethanol (Fig. 13A). To analyze individually the two proteins, the bands were trimmed from the gel, eluted and submitted to a new electrophoresis in the presence and the absence of β-mercaptoethanol (Fig. 13B). Considering the protein with 75 kDa, in the presence of β-mercaptoethanol, another band can be seen, besides that one with 75 kDa. In the case of the protein with 68 kDa, in the presence of the reducing agent, it exhibited a polydisperse band instead the sharp band observed in the absence of reducing agent.

4. DISCUSSION

An expressive cellularity, such as the one found in our study with porcine pericardium is generally characteristic of tissues not completely mature, as it happens, for instance, in young tendons. In spite of its high cellularity, porcine pericardium behaves as a mature tissue regarding its swelling and the extractability of its collagen.

The presence of a network of relatively fine collagen fibers, in layers and multidirectionally oriented composing the fibrous layer of the porcine pericardium is in accordance to the pericardial function. As the pericardium suffers distension during the diastolic movements, the multidirectional arrangement of the collagen fibers guarantees the restraining of the movement helping to stabilize and preventing its superdilating.

The observation in this work of a discreet predominance of direction, with a higher number of fibers obliquely oriented, from the lower-central region towards higher-left lateral, could be the reflex of the continuity of pericardium fixation fibers, through the pericardicophrenic ligament, to the tendinous center of the diaphragm. Probably, these predominant fibers take a helical course, from the pericardicophrenic ligament towards the region of posterior fixation of the pericardium. Sacks and coworkers (1994) noted a circunferential predominance of fibers in the left-front region of bovine pericardium while in the right-front region the apex-base direction was predominant, in agreement with our findings. According to the reports of Langdon and coworkers (1999), the observation of WLS in the collagenic pericardial bundles was also frequent in our study. However, what

Langdon and coworkers called crimp -WLS- are apparently undulations in the course of collagen fibers seen through electronic scanning microscopy, which gradually unwind as the tissue is submitted to tension forces. According to our study the occurrence of WLS is due to the change in the molecular orientation in the collagen bundles, even though they may appear rectilinear. These structures are detected by the change of birefringence of the fibers under polarized light microscopy. Even if tension forces are applied to the tissue, changes is the molecular organization of the bundles can still be detected by observation under polarized light. Sections or in totum preparations of porcine pericardium stained with Toluidin Blue in pH4, showed a pale matrix with little or no metachromasy, reflecting very small quantities of available anionic groups. In pH4 toluidin Blue bound with carboxil radicals and sulfate of acid GAGs as well as to phosphate radicals DNA and RNA. That is a fact as the nuclei are stained by the phosphate groups of nucleic acids. The staining with Toluidin Blue enables the analysis and quantification of available anionic radicals and of electrostatic combinations which are important for the supramollecular organization and self-assembling (Vidal, 1995).

The molecule of Toluidin Blue, which in solution behaves as liquid crystal is small, has a planar geometry and possesses excellent optical anisotropic properties (Vidal, 1987). In the extracellular matrix its molecules combine with the glycosaminoglycans, piling ordenately and showing metachromasy (Vidal, 1984). Our topochemical findings which suggest a low content of available anionic radicals which are able in the production of metachromasy, are coherent with the

behavior of pericardium swelling conditions. The proteoglycans contribute significantly to the physico-chemical properties of the extra-cellular matrix. That is especially due to the great number of negatively charged groups – carboxil and sulfate – which form their chains of glycosaminoglycans. Thus, the proteoglycans in the cartilage, for instance, exert swelling pressure on the collagenic matrix, promoting osmotic resistance to compression loads (Urban et al., 1979). A hypothesis to be considered is the possibility that water fixed by the acid radicals could concomitantly create a hydrostatic pressure. Swelling tests performed with bovine flexor tendon showed that regions with relatively low levels of glycosaminoglycans lose weight in water and swelling in acetic acid (Koob & Vogel, 1987) as well as it has been verified with pericardium. On the contrary, tendon regions which swelling more in water and less in acetic acid have relatively high glycosaminoglycan levels. Low levels of proteoglycans are typical in tendons predominantly subjected to tension forces (Koob & Vogel, 1987). However, the content of negative charges is but one of the factors which contribute to the swelling properties of the tissue. We must, therefore, still consider the type and ratio of collagen, size and organization of fibrils, amount of inter-molecular crosslinking, inter-fribrilar interactions, among other factors (Grodzinsky, 1983). The behavior of pericardium during the swelling test was typical of highly collagenous tissues with response dominated, in acid pH, by the collagen matrix, as described by Viswanadhan et al., (1976) and Yannas & Grodzinsky (1973). Our topochemical and physico-chemical findings enables us to state that the pericardial extracellular matrix shows small quantities of proteoglycans.

Despite the apparently small diameter of its bundles, pericardial collagen proved clearly more insoluble in solutions most frequently used for its extraction, when compared to collagen from rat tails and bovine flexor tendons. It has been reported that cross links deriving from hydroxylisine are more stable than those deriving from lisine, and that the great amount of these crosslinks might be responsible for collagen's high insolubility which also occurs in cardiac valves (Bashey et al., 1978). These authors demonstrated that collagen in the cardiac valves possesses higher quantities of hydroxylisine if compared to skin collagen, suggesting that the increase in numbers and in the extension of crosslinks might represent an evolutive adaptation of collagen, making it able to stand the constant mechanical stress towhich it is submitted in the cardiac valves, which can also be applied to pericardial collagen which is similarly submitted to continuous mechanical stress resulting from systolic and diastolic movements.

About 47% of pericardium weight corresponds to collagen, as estimated by the converting the amounts of hydroxyprolin obtained into amounts of sample collagen. Variations concerning the percentage corresponding to hydroxyprolin in the molecule of type I collagen can be found in literature. The analysis of aminoacids in collagen I of porcine pericardium showed that around 9% of them correspond to hydroxyprolin, differing from the reports of Bashey et al (1978), Slack et al. (1984), Riley et al. (1994), Reddy et al.(1998), and Stehno-Bittel et al. (1998) concerning collagen I of other tissues. Such finding may suggest a functional adaptation of the collagen molecule, possibly with a consequent change of its biomechanical properties, adequating them to the mechano-physiological

needs of pericardium. The percentages of the remaining aminoacids, as well as the ratio hydroxyprolin/prolin of 0,76 verified in the soluble pericardial collagen are compatible to those of type I collagen (Bashey et al., 1978).

The dosage of proteins and GAGs in the total extracts of pericardium showed a large amount of proteins and only traces of GAGs, agreeing totally with the topochemical and physico-chemical findings which have been accomplished.

The analysis in SDS-PAGE of the material eluted from DEAE-Sephacel revealed sixteen anionic proteins present in the pericardial matrix showing apparent molecular mass between 11 and 109 kDa. These proteins perform structural and probably regulating functions as they interact with the various matrix components.

The polydisperse band of 85kDa found in pericardium exhibited an electrophoretic behavior very similar to that of decorin found in skin (Kuc & Scott, 1997) and cartilage (Heinegård & Pimentel, 1992). The hypothesis of the presence of the small proteoglycan decorin is strengthened by the result of electrophoresis in Agarose-PDA gel, after β-elimination of the fractions of chromatography containing that polydisperse component. The presence of DS was confirmed after treatment with chondroitinases AC and ABC, when the GAG was completely digested by chondroitinase ABC but not with chondroitinase AC. It is known that decorin in the soft tissues possesses DS and not CS as in bone decorin (Fisher, 1999). The presence of small proteoglycans like decorin should be expected in the pericardial

matrix, since it probably takes a part in the regulation of fibrilogenesis of collagen (Brown & Vogel, 1989; Scott, 1996; Iozzo, 1997).

The analysis of the differentiated electrophoretic behavior of proteins with 68 and 75 kDa in the presence of β-Mercaptoethanol made it clear that those are not oligomeric proteins but probably proteins with intrachain disulfides bonds which, being ruptured change their conformation and/or dissociate from some other component. Considering the protein with kDa 75, some other protein seems to have dissociated in the presence of the reducing agent. The component of 68 kDa appears more polydisperse in the presence of β-Mercaptoethanol, probably due to the change in its conformation.

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5. REFERENCES

AULT, N.K. & HOFFMAN, A.H. (1992) A composite micromechanical model for connective tissues: Part II – Aplication to rat tail tendon and joint capsule. *J. Biomech. Eng.* 114: 137-141.

BARROS, P.S.M.; SAFATLE, A.M.V. & RIGUEIRO, M. (1999) Experimental lamellar corneal graft in dogs using preserved equine pericardium. *Braz. J. Vet. Res. Anim. Sci.*, 36: 00-00.

BASHEY, R.I.; BASHEY H.M. & JIMENEZ, S.A. (1978) Characterization of pepsin-solubilized bovine heart-valve collagen. *Biochem. J.*, 173: 885-894.

BEELEY, J.G. (1985). Glycoprotein and proteoglycan techniques. Elsevier. New York; 16: 268-270.

BLUM, B., BEIER, H., GROSS, H. J. (1987) Improved silver staining of plant proteins RNA and DNA in polyacrylamide gels. *Electrophoresis.* 8: 93-99.

BRADFORD, M.M. (1976) A rapid sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein dye binding. *Ann. Biochem.,* 72: 248-54.

BROWN, D. C., VOGEL, K. G. (1989) Characteristics of the *In* Vitro interaction of a small proteoglycan (PGII) of bovine tendon type I collagen. *Matrix,* 9: 468 – 79.

CHANDA, J.; KURIBAYASHI, R. & ABE, T. (1997). Use of glutaraldehyde-chitosan treated porcine pericardium as a pericardial substitute. *Biomaterials,* 17: 1087-1091.

DIETRICH, C.P. & DIETRICH, S.M. (1976) Electrophoretic behaviour of acidic mucopolysaccharides in diamine buffers. *Anal Biochem*, 70: 645-647.

FARNDALE, R.W.; BUTTLE, D.J. & BARRET, A.J. (1986) Improved quantitation and discrimination of sulphated glycosaminoglycans by use of dimethylmethylene blue. Biochem. *Biophys. Acta*, 883: 173-177.

FENTIE, I.H.; ALLEN, D.J.; SCHENCK, M.H & DIDIO, L.J.A. (1986) Comparative electron microscopic study of bovine, porcine and human parietal pericardium, as materials for cardiac valve bioprostheses. *J. Submicrosc. Cytol.* 18: 53-65.

FISHER, L. W. Decorin. *In:* Extracellular matrix, Anchor and Adhesion Proteins. (Kreis T., Vale R.). San Francisco, 358p. p. 241 – 254, 1999.

GATHERCOLE, L.J. & KELLER, 1991) A. Crimp Morphology in Fibre-Forming Collagens. *Matrix*, 11v, p214-234.

GRODZINSKY, A.J. (1983) Electrochemical and physicochemical properties of connective tissue. *CRC Crit. Rev Biomed Eng.*, 9: 133-199.

HEINEGÅRD, D., PIMENTEL E. R. *Cartilage matrix proteins. In:* Articular cartilage and Osteoarthritis. (Kuttner K. E., Scheleyerbach R., Peyron J. G., Hascall V. C., eds). New York, p95 – 111, 1992.

HEINEGÅRD, D. & SOMMARIN, Y. Methods in Enzimology. 144v, p319-373, 1987.

HOLLINSHEAD, W. H. O sistema circulatório. In: HOLLISHEAD, W.H. Livro texto de anatomia Humana. São Paulo : ed. Harper & Row do Brasil,. 971p. p.82-83, 1980.

IOZZO, R.V. (1997) The family of the small leucine-rich proteoglycans: key regulators of matrix assemblay and cellular growth. *Crit. Rev. in Biochem. and Mol. Biol.* .32: 141-174.

JORGE-HERRERO, E.; FERNANDEZ, P.; TURNAY, J.; OLMO, N.; CALERO, P.; GARCIA, R.; FREILE, I. & CASTILLO-OLIVARES, J.L. (1999) Influence of different chemical cross-linking treatment on the properties of bovine pericardium and collagen. *Biomaterials,* 20: 539-545.

KHOR, E. (1997) Methods for the treatment of collagenous tissues for bioprostheses. *Biomaterials,* 18: 95-105.

KLAUS, W. & OSBORN, M. (1969) The reliability of molecular weight determinations by dodecil sulfate- polyacrylamide gel electrophoresis. *J. Biol. Chem.* 244: 4406-4412.

KOOB, T.J. & VOGEL, K.G. (1987) Site-Related Variations in Glycosaminoglycan Content and Swelling Properties of Bovine Flexor Tendon. *J. Orthop. Res.* 5: 414-424.

KUC, I. M., SCOTT, P.G. (1997) Increased diameters of collagen fibrils precipitated *in vitro* in the presence of decorin from various connective tissues. *Connective Tissue Research.,* 36:287-296.

LAEMMLI, V.K. (1970) Cleavage of structural proteins during the assembly of the head of bacteriofhage Ty. *Nature*, 227:680-685.

LANGDON, S.E.; CHERNECKY, R.; PEREIRA, D.A. & LEE, J.M. (1999) Biaxial mechanical/structural effects of biaxial strain during crosslinking of bovine pericardial xenograft materials. *Biomaterials*, 20: 137-153.

LOKE, W.K.; KHOR, E.; WEE, A.; TEOH, S.H. & CHIAN, K.S. (1996) Hybrid biomaterials based on the interaction of polyurethane oligomers with porcine pericardium. *Biomaterials*, 17: 2163-2172.

MICHELACCI, YM, HORTON, DSPQ (1989). Proteoglycans from the cartilage of young hammerhead shark Sphyrna lewini. Comp. Biochem. Physiol. 92: 651-658.

MOORE, K.L. & DALLEY, A.F. Torax. In: MOORE, K.L. & DALLEY, A.F *Anatomia orientada para a clínica.* Rio de Janeiro, Guanabara Koogan, 4ª ed. 1023p.p.52-151, 2001.

OLMOS, Z.R.; JASSO,V.R.; SOTRES, V.A.; CEDILLO, L.I.; ARREOLA, R.J.L. & GAXIOLA, G.M., (1997). Utilidad del pericardio bovino tratado con glutaraldeído en las resecciones pulmonares no anatómicas en perros. *Rev. Inst. Nal. Enf. Resp. Mex.* 10:155-159.

PETITE, H.; DUVAL, J.; FREI, V.; ABDUL-MALAK, N.; SIGOT-LUIZARD, M. & HERBAGE, D. (1995) Cytocompatibility of calf pericardium treated by glutaraldehyde and by the acyl azide methods in na organotypic culture model. *Biomaterials*, 16: 1003-1008.

PIRES, A.C.; SAPORITO, W.F. & LEAO, L.E.V. (1997) Pericárdio bovino utilizado como remendo no sistema cardiovascular. *Ver. Brás. Cir. Cardivasc.* 12: 176-187.

REDDY G.K.; GUM, S.; STEHNO-BITTEL, L. & ENWEMEKA, C.S. (1998) Biochemistry and biomechanics of healing tendon : Part I. effects of rigid plaster casts and functional casts. *Medicine & Science in Sports & Exercises.* .794-800.

RILEY, G.P.; HARRALL, R.L.; CONSTANT, C.R.; CHARD, M.D.; CAWSTON, T.E. & HAZLEMAN, B.L. (1994) Tendon degeneration and chronic shoulder pain: changes in the collagen composition of the human rotator cuff tendons in rotator cuff tendinitis. *Annals of the Rheumatic Diseases*. 53: 359-366.

SACKS, M.S.; CHUONG, C.J.C. & MORE, R. (1994) Collagen Fiber Architeture of Bovine Pericardium. *ASAIO Journal*, M632-M637.

SCOTT, J.E., (1996) Proteodermatan and Proteokeratan Sulfate (Decorin, Lumican/ Fibromodulin). Proteins Are Horse Shoe Shaped. Implications for Their Interactions With Collagen. *Biochemistry*. 35: 8795-8799.

SIMIONESCU, D.; IOZZO, R. & KEFALIDES, N.A. (1989) Bovine Pericardial Proteoglycan: Biochemical, Immunochemical and Ultrastructural Studies. *Matrix* 9: 301-310.

SLACK, C.; FLINT, M.H. & THOMPSON, B.M. (1984) The effect of tensional load on isolated embryonic chick tendons in organ culture. *Conn. Tiss. Res.*12: 229-247.

STEGEMANN, H. & STALDER, K. (1967) Determination of hydroxyproline. *Clinica Chimica Acta*, 18: 267-273.

STEHNO-BITTEL, L.; REDDY G.K.; GUM, S. & ENWEMEKA, C.S. (1998) Biochemistry and biomechanics of healing tendon : Part I. effects of rigid plaster casts and functional casts. *Medicine & Science in Sports & Exercises*. .788-793.

URBAN, J.P.G.; MAROUDAS, A.; BAYLISS,M.T. & DILLON, M.T. (1979) Swelling pressures of proteoglycans at concentrations found in cartilaginous tissues. *Biorheology*, 16:447-464.

VIDAL, B.C. (1966) Macromolecular Desorientation in Detached Tendons. *Protoplasma Bd* LXII/2-3: 121-131.

VIDAL, B.C. (1980) The part played by proteoglycans and structural glycoproteins in the macromolecular orientation of collagen bundles. *Cell. Mol. Biol.* 26: 415-421.

VIDAL, B.C. (1984) Ordem molecular y haces de colageno. *Trab. Inst. Cajal.* 75v, p19-27.

VIDAL, B.C. Métodos em Biologia Celular. In: VIDAL, B.C. & MELLO, M.L.S. *Biologia Celular*. Rio de Janeiro. São Paulo : Atheneu, 348p. p05-34, 1987.

VIDAL, B.C. (1995) From collagen type I solution to fibers with a helical pattern: a self-assembly phenomenon. *C.R. Acad. Sci. Paris*, 318:831–836.

VIDAL, B. C. (2003) Image analysis of tendon helical superstructure using interference and polarized light microscopy. *Micron,* 34:423-432.

VIDAL, B.C. & MELLO, M.L.S. (1972) Anisotropic properties of Toluidine blue-stained collagen. *Ann. Histochim.* , 18: 106-122.

VISWANADHAM, R.K.; AGRAWD, D.C. & KRAMER, E. (1976) Environmental effects on the mechanical properties of reconstituted collagen hollow fiber membranes. *J. Poly. Sci. Phys.*, 14: 2195-2209.

WHITTAKER, P.; BOUGHNER, D.R.; PERKINS, D.G. & CANHAM, P.B. (1987) Quantitative structural analysis of collagen in chordae tendineae and its relation to floppy mitral valves and proteoglycan infiltration. *Br Heart*, 57v, p264-269.

YANNAS I.V. & GRODZINSKY A.J. (1973) Electromechanical energy conversion with collagen fibers in an aqueous medium. *J. Mechanochem Cell Motility*, 2: 113-125.

ZINGALES, B. (1984) Analysis of protein sodium dodecyl sulphate-polyacrilamide gel electrophoresis. In: Genes and antigens of parasites. Rio de Janeiro, Fiocruz, 357-363,

FIGURE LEGENDS

Figure 1. In totum preparation of the ULL region of pericardium stained with Toluidin Blue pH 4,0 and observed under polarizing microscope (Bar 30μm).

Figure 2. In totum preparation of UC region of pericardium. Notice vessel cells nuclei (black arrows) and fibroblasts (white arrows) stained with Toluidin Blue pH 4,0. (Bar: 26μm).

Figure 3. In totum preparation of the LC region of pericardium stained with Toluidin Blue pH 4,0 and observed under polarizing microscope. Notice birefringence of collagen fibers (CF) and the great ramification of vascular tree (arrows). (Bar: 130μm).

Figure 4. In totum preparation of LC region of pericardium stained with Toluidin Blue pH 4,0 and observed under polarizing microscope. Collagen fibers (CF) and vessels (arrows) (Bar: 550μm).

Figure 5. In totum preparation of LLL region of pericardium seen under polarizing microscope. Observe the multidirectionality (arrows) of the collagen fibers birefringent in white (Bar: 77μm)

Figure 6. Unstained in totum preparation of LC region of pericardium. The collagen fibers show typical brilliancy of their birefringence, from first-class white to grayish, observed under crossed polarizers. Observe their preferential direction (arrows) (details in the text). (Bar: 65μm).

Figure 7. Micrography of tangential section of pericardium stained with Toluidin Blue pH 4,0 and observed with polarizers in relative angle smaller than 90°. Observe crimp (C) and direction of collagen fibers (arrow). (Bar:60μm).

Figure 8. Same area as figure 7 observed with crossed polarizer and analyzer. Notice greenish birefringence of collagen fibers (Bar: 115μm).

Figure 9. SDS-PAGE of extracts obtained from pericardium.

A- SDS-PAGE of proteins extracted with GuHCl in conditions of 3M (slots 2 and 6), 4M (slots 3 and 7), 5M (slots 4 and 8) and 6M (slots 5 and 9).-SH = without β-mercaptoethanol; +SH = with β-mercaptoethanol.

B- SDS-PAGE of proteins extracted with 4MGuHCl from the right and left parts of fresh and frozen tissues. Observe that the band pattern was the same in every case. MM= molecular mass markers; col = type Icollagen, α1 and α2= α chains; β= band resulting from two α chains.

Figure 10. Chromatography in DEAE-Sephacel and SDS-PAGE

A- Chromatography of material extracted from whole pericardium in GuHCl 4M. G indicates the beginning of the gradient 0,1-1,0M NaCl.

B- SDS-PAGE in the presence of β-mercaptoethanol of fractions eluted from the column. The apparent molecular mass of proteins eluted from the columm are pointed by arrows. Arrowsheads= polydisperse bands; MM = molecular mass markers and col = Type I collagen.

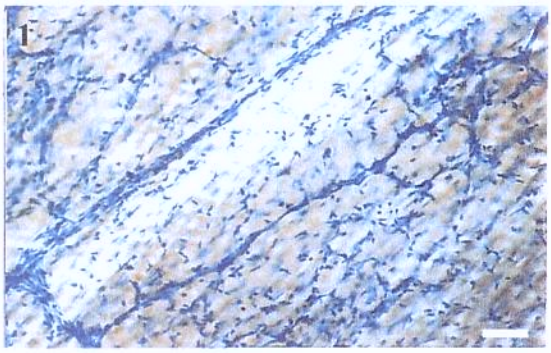
Figure 11. Electrophoresis in Agarose-PDA of GAGs extracted from pericardium with papain and treated with chondroitinase AC (2) and ABC (3). The material not treated with chondroitinase appears as extense polydisperse band (1). On the left are the GAG standards CS (chondroitin sulfate), DS (derrmatan sulfate) and HS (heparan sulfate). The arrow indicates the direction of the running.

Figure 12. Electrophoresis in agarose-PDA gel of GAGs obtained by β-elimination. Observe the control (1) and the samples treated with chondroitinases AC (2) and ABC (3). The GAG standards are shown on the left. The arrow indicates the direction of the running.

Figure 13. SDS-PAGE of the fractions 19 and 20 of DEAE-Sephacel

A- Running in the presence (+SH) and the absence (-SH) of β-Mercaptoethanol. MM= molecular mass markers. The arrows indicate components with 75 (fraction 19) and 68 kDa (fraction 20). Staining with CBB.

B- SDS-PAGE of proteins with 75 and 68 kDa purified by elution from the gel. The arrow indicates the band corresponding to the protein, which dissociated from the component with 75 kDa in the presence of β-mercaptoethanol (+SH). Notice the more disperse aspect of the component with 68 kDa in the presence of β-mercaptoethanol. MM= molecular mass markers. Staining with silver.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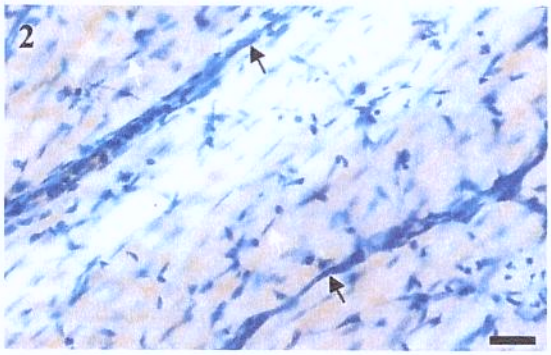
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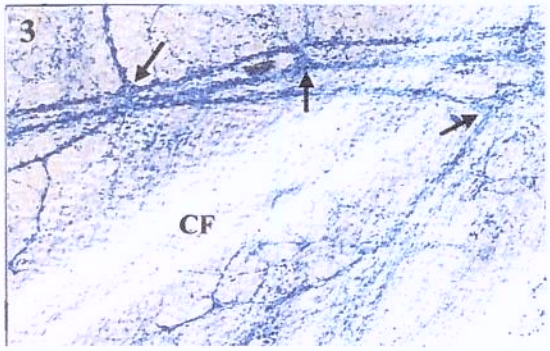
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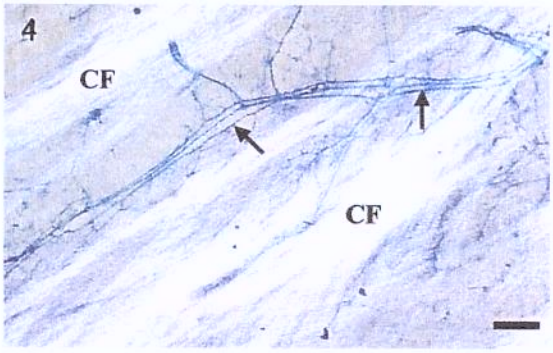
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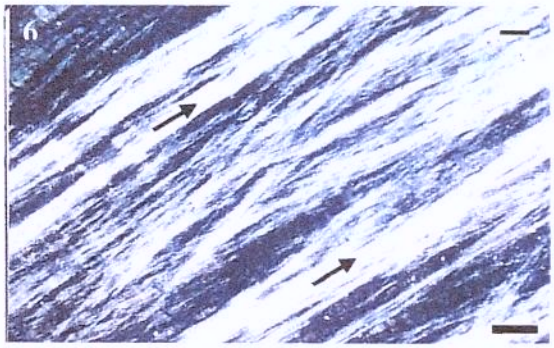
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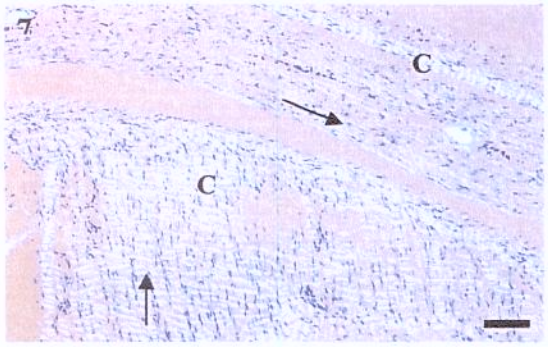
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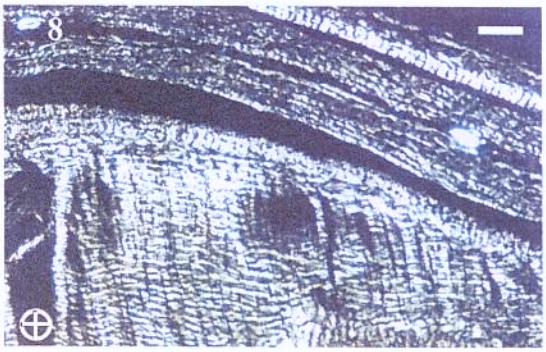
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Figure 9

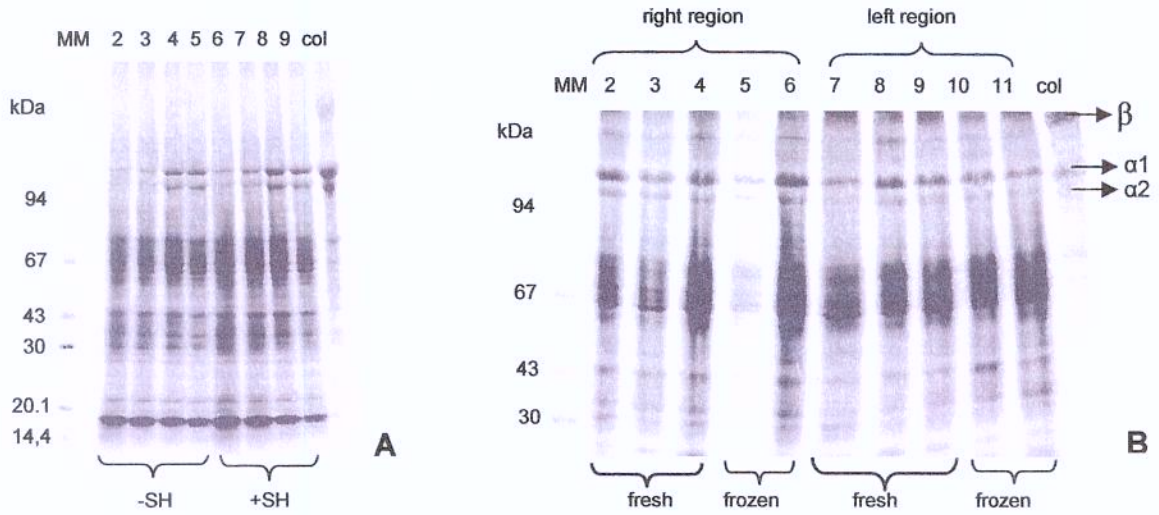


Figure 10

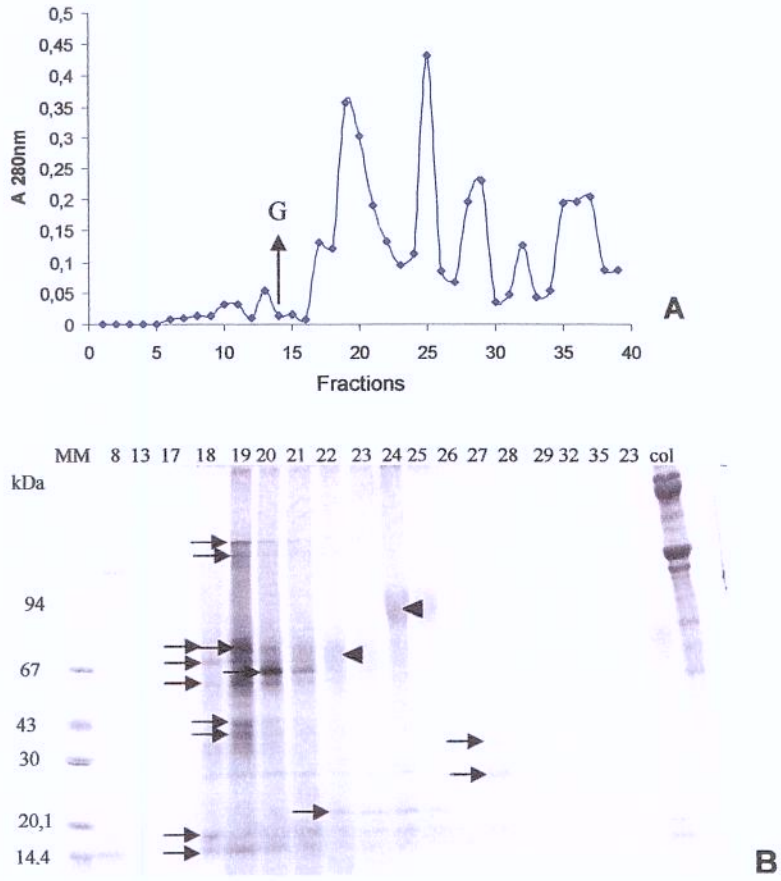


Figure 11

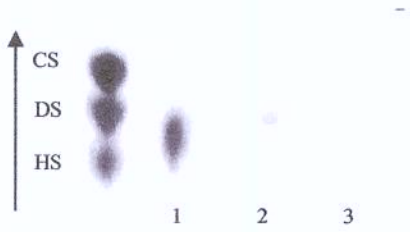


Figure 12

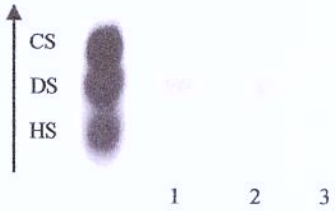


Figure 13

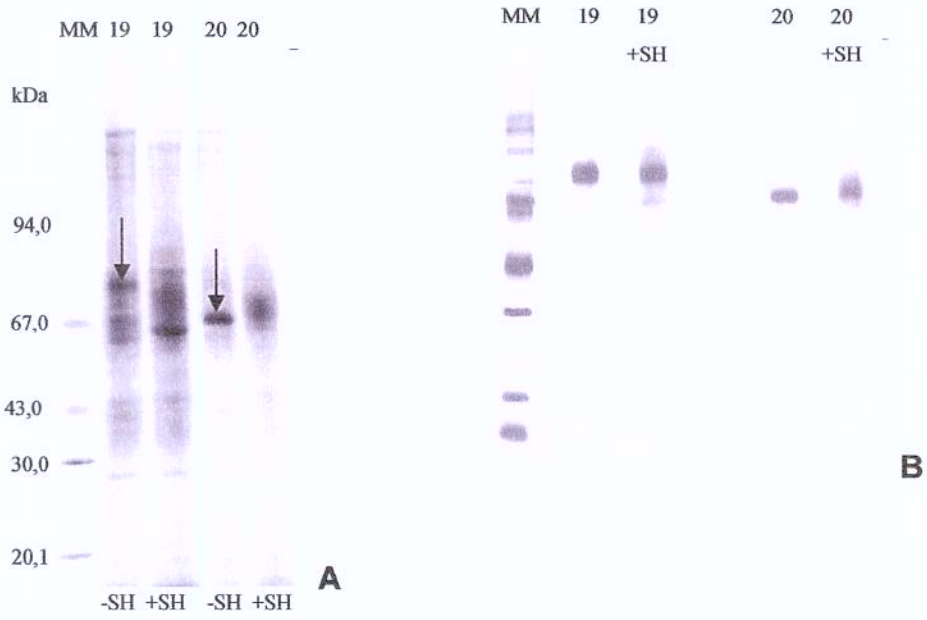
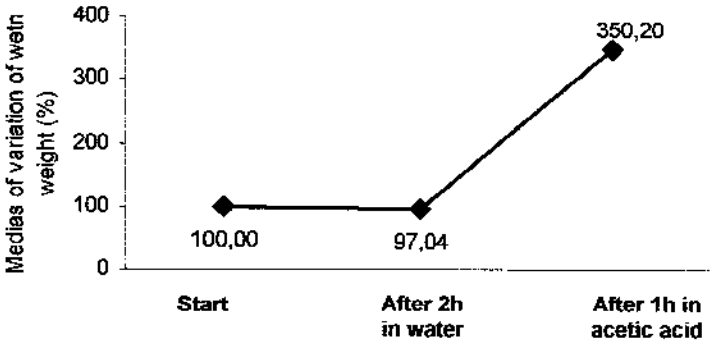


Table 1. Amino acid composition of soluble collagen of porcine pericardium. Presented values in residue of amino acid per 1.000 total residues

Amino acid	N° of residues	Percentage
Aspartic acid	53	5,3%
Glutamic acid	85	8,5%
Serine	39	3,9%
Glycine	313	31,3%
Histidine	3	0,3%
Arginine	65	6,5%
Threonine	18	1,8%
Alanine	109	10,9%
Proline	118	11,8%
Tyrosine	1	0,1%
Valine	33	3,3%
Methionine	6	0,6%
Isoleucine	14	1,4%
Leucine	31	3,1%
Phenylalanine	19	1,9%
Lysine	33	3,3%
Hydroxiproline	90	9,0%
Cysteine	0	0

Ratio Hydroxiproline/Proline : 0,76



Graphic 1. Media values of swelling of 5 different pericardia

Capítulo 2

Esse capítulo contém artigo intitulado **"IDENTIFICATION OF ELASTIC FIBERS AND LAMELLAE IN PORCINE PERICARDIUM AND AORTA BY CONFOCAL, FLUORESCENCE AND POLARIZED LIGHT MICROSCOPY"**, publicado na revista Acta Histochemica, 108 (2); 125-132.

IDENTIFICATION OF ELASTIC FIBERS AND LAMELLAE IN PORCINE PERICARDIUM AND AORTA BY CONFOCAL, FLUORESCENCE AND POLARIZED LIGHT MICROSCOPY

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Short title: Elastic fibers of porcine pericardium and aorta

SUMMARY

Pericardial connective tissue has been used to construct bioprostheses to repair various types of injuries including aortic wall repairs. The arrangement and the distribution of elastic and collagen staple fibers are related directly to the tissue's biomechanical properties which are determinant in the choice tissue for the construction of bioprostheses. Although elastic fibers can be visualized with several histochemical methods, the specificity and mechanism of binding involved remain to be clarified. In this work, we compared the elastic net of the porcine pericardial matrix with that of the aortic wall using 1-anilino-8-naphtalene sulfonate and dansyl chloride as fluorescent probes and permanganate-bisulfite-toluidine method. Polarized light and fluorescence microscopy were simultaneously used to analyze the tissues. Some samples also were examined with confocal microscopy. Aorta and pericardium treated with 1-anilino-8-naphtalene sulfonate and dansyl chloride showed elastic fibers and lamellae with an intense blue fluorescence. Stained with the permanganate-bisulfite-toluidine method, the aortic elastic lamellae were clearly metachromatic and, under polarized light, they showed a greenish birefringence. Dansyl chloride provided the better fluorescence of elastic fibers in the confocal microscopy, even though it has not proven advantageous in this work.

Key words: elastic fibers, pericardium, aorta, fluorescence, polarized light, confocal microscopy.

1. INTRODUCTION

Fluorescent probes, such as 1-anilino-8-naphtalene sulfonate (ANS) and 1-dimethylamino-naphthalene-5-sulfochloride or dansyl chloride (DCl) have been used to study the polarity, hydrophobicity, reactivity and molecular conformation of proteins (Stryer, 1965; Beyer et al., 1973; Radda, 1973; Yguerabide, 1973). Anilinonaphthalene sulfonates and related dyes are suitable probes for identifying non-polar molecular sites since the non-polar regions are sterically accessible and sufficiently large to bind the dye. There is a highly efficient transfer of electronic excitation energy from aromatic amino acid residues to bound ANS that is seen as fluorescence (Stryer, 1965). ANS has been used to study the degree of histone aggregation (Laurence, 1966; Penzer, 1972), to assess structural alterations in the elastin network (Gosline et al., 1975; Gosline, 1976), and in the histochemistry of collagen and elastin (Vidal, 1978, 1980).

DCl, introduced by Weber in 1952 for the preparation of conjugated proteins, is widely used in structural studies of complex molecules and in quantitative analyses of primary, secondary and tertiary amines (Bartzatt, 2001, 2003). According to Rosselet & Ruch (1968), the specificity of DCl in acetone can be used for the microfluorometric detection of α-NH_2 and ε-NH_2. With protein-bound amino acids, the reaction is specific for lysine so that DCl can been used as a fluorescent label for lysine residues in studies of elastic and collagen fibers (Vidal, 1980; Timberlake et al., 2002).

The permanganate-bisulfite-toluidine (PBT) method developed by Fischer (1979), which uses potassium permanganate as the oxidizing agent and toluidine blue as the dye, results in the metachromasy and birefringence of elastic fibers that allows their ready identification *in situ* and permits an assessment of their association with collagen fibers.

Elastic fibers and lamellae interact closely with several extracellular matrix components, especially collagen fibers. The arrangement and the distribution of elastic and collagen fibers contribute significantly to the biomechanical behavior of connective tissues which constitutes a determinant factor in the choice of the tissue to be used in the construction of bioprostheses.

Pericardial connective tissue has been used to construct bioprostheses to repair various types of injuries, especially cardiac injury (Olmos et al., 1997; Barros et al., 1999). Ventricular wall, cardiac valve and aortic wall repairs have been successfully done using bioprostheses made from bovine pericardial tissue (Pires et al., 1997). However, damage and limited durability are often problems in cardiac bioprostheses, although the precise mechanisms involved are unknown (Chanda et al., 1997; Páez et al., 2001). Therefore, alternative biomaterials for bioprostheses must be searched. In this work, as a part of a comprehensive study and in order to increase our knowledge about porcine pericardium, we compared the elastic net of the porcine pericardial matrix with that of the aortic wall, since there is not much available data in pertinent literature.

The collagen of the tissues was extracted to allow better visualization of the elastic fibers and to simultaneously provide information about the physicochemical

stability of the tissues. The methods mentioned above were used to study the identification, arrangement and distribution of the elastic fibers. Polarized light and fluorescence microscopy were simultaneously used to analyze the tissues. In order to compare results of the several microscopies, some samples also were analyzed with confocal microscopy.

2. MATERIAL AND METHODS

Six-month-old pigs of Large White lineage with 92Kg of weight were used. Fragments of aorta and pericardium collected immediately after the pigs (n=10) had been killed in an abattoir were washed in phosphate-buffered saline (PBS) at 4°C and fixed in 4% paraformaldehyde (in phosphate buffer, pH 7.4, containing 0.15 M NaCl) for 24 h at 4°C (control group, n=5). In some cases, before fixation, the collagen of the aortic fragments was extracted using one of several procedures (treated group, n=5) in order to assess the physicochemical stability of the elastic fibers and lamellae and to facilitate their visualization in situ. The procedures used were:

1) Boiling in water for 1 h
2) Autoclaving in water at 120°C for 2 h
3) Extraction in acetic acid (5% or 10%) for 24 h, with or without subsequent autoclaving for 1 h
4) Extraction in NaOH (0.5%,1% or 5%) overnight, followed by autoclaving for 2 h at 120°C

5) Boiling in 4% trichloracetic acid (TCA) for 30 min

The fragments were subsequently dehydrated and embedded in paraffin, and longitudinal or transversal sections 7μm thick were obtained. The sections were mounted on slides and deparaffinized in xylol and chloroform/methanol. For comparative purposes, the same procedures were applied to porcine pericardium, which was also prepared *in totum*. The latter preparations, as well as sections of tissues that had or had not been extracted, were subjected to the following reactions:

Dansyl chloride (DCl)

The sections were washed in 95% ethanol and stained with 0.1% dansyl chloride in 95% ethanol saturated with sodium bicarbonate for 6 h in the dark at room temperature. The slides were then rinsed in the ethanol-sodium bicarbonate solution above prior to dehydrating in absolute ethanol and clearing in xylol. The slides were subsequently mounted with a coverslip in Nujol oil or Eukite, both of which are non-fluorescent media (Vidal, 1980).

Anilino-naftalene sulphonate (ANS)

The sections were stained with 0.1% ANS in butanol for 30 min in the dark at room temperature and then rinsed in butanol, cleared rapidly in xylol and mounted with a coverslip in Nujol oil or Eukite (Vidal, 1978).

Permanganate-bisulfite-toluidine (PBT) method

The sections were oxidized with 0.02-0.5% potassium permanganate in 0.1 N HCl (pH 1) for 15 min and then distained in 1% oxalic acid, followed by sulfatation with potassium bisulfite saturated solution for 30 min and staining with 0.05% toluidine blue in 0.1 N HCl (pH 1) for 15 min. The sections were subsequently mounted in natural Canada balsam with a coverslip (Fischer, 1979).

All of the slides, control and treated, were examined with a Zeiss Axiophot microscope equipped for epifluorescence with an HBO-100W stabilized mercury lamp as the light source. The filters used for fluorescence were: G 365 (BP exciting filter transmitting a λ = 546/547 nm), an FT 395 chromatic splitter, an LP 420 barrier filter (filter set 2) and a G 485/20 - 578/14 (BP exciting filter transmitting a □ = 485 and 578 nm), as well as an FT 500/600 chromatic splitter, BP 515-540 and LP 610 (filter set 24). The preparations were observed simultaneously between crossed analyzer and polarizer in order to detect birefringence. Control slides stained with DCI and ANS also were examined with a laser scanning microscope (LSM-510 META – Carl Zeiss,).

3. RESULTS

Observed by usual fluorescence microscopy, aortic elastic fibers and lamellae, stained with the ANS-butanol solution, showed a strong blue fluorescence (Fig. 1). Collagen that was stained with ANS under these conditions

showed less fluorescence than elastin. When observed under fluorescence and polarized light simultaneously, the elastic fibers and lamellae were easily distinguished from the intensely birefringent collagen fibrils (Figs. 2 and 3). In the media tunicae of the aorta, the collagen fibrils were arranged obliquely relative to the elastic lamellae that connected them (Fig. 3). In contrast, in the adventitia tunicae, these fibrils were arranged in essentially the same orientation as the elastic lamellae (Fig.2). As with ANS, aorta treated with DCI showed elastic fibers and lamellae with an intense blue fluorescence (data not shown). After staining with the PBT method, the aortic elastic lamellae were clearly metachromatic (Fig. 4) and, under polarized light, they showed a greenish birefringence (Fig. 5).

Prior treatment of the aortic wall with mild extraction procedures resulted in a significant reduction in interlamellar collagen that enhanced the visualization of the elastic fibrils and lamellae (Figs. 6-8). After harsh extraction procedures, such as with NaOH, the aortic elastic fibers and lamellae showed marked structural modifications (data not shown).

Fine elastic fibrils closely associated with collagen fibers were observed in the pericardium stained with ANS (Fig. 9). When observed simultaneously in fluorescence and polarized light, the elastic fibrils were easily distinguished from collagen fibrils that appeared strongly birefringent (Fig. 10). In previously autoclaved pericardium from which part of the collagen had been extracted, staining with ANS, revealed the presence of fine elastic fibrils. In certain regions, these fibrils were parallel to each other (Fig. 11). In pericardium previously treated with 5% acetic acid and then autoclaved, staining with DCI revealed fine, interlaced

elastic fibers running in different directions (Fig. 12). Extraction procedures involving the use of NaOH resulted in marked disorganization of the pericardial fiber matrix, regardless of the NaOH concentration used (data not shown). The pericardium showed poor reactivity staining with the PBT method (data not shown).

The best results in confocal microscopy were obtained with DCI which showed elastic lamellae of the aorta wall strongly fluorescent (Fig. 13). The study of the pericardial elastic fibers by this method was not satisfactory. Thus, the confocal microscopy has been used simply to analyze some sample. Although the ANS and DCI dyes were found to be suitable for confocal microscopy, in this work no significant advantages were found with the use of the mentioned technique as compared to fluorescence microscopy for identification and distribution of elastic and collagen fibers.

4. DISCUSSION

Anilinonaphthalene sulfonates and their dye correlates have been used as probes to identify non-polar molecular sites to which these compounds can bind by hydrophobic forces. The resulting highly efficient transfer of electronic excitation energy from aromatic amino acid residues to the bound ANS leads to fluorescence (Stryer, 1965). ANS has been used to study structural alterations in the elastin network (Gosline et al., 1975; Gosline, 1976) and in the histochemistry of collagen and elastin by fluorometric measurements (Vidal, 1978, 1980). It has been verified that the fluorescence detected after ANS staining of the human skin is deeper in

the elastic fiber as compared to that of the collagen fibers, although the emission peaks for both fibers was positioned at λ = 470nm (Vidal, 1978). The weak greenish fluorescence shown by pericardial and aortic fibrillar collagen when stained with ANS in butanol very probably reflected the lower number of hydrophobic residues in this protein as compared to the elastic fibers and lamellae. These findings agree with those of Vidal (1978).

DCI has been used in structural studies and in quantitative analyses of complex molecules that contain primary, secondary and tertiary amino and carboxylated aromatic groups (Bartzatt, 2001, 2003). According to Rosselet & Ruch (1968), DCI in acetone is sufficiently specific for the microfluorometric detection of α-NH_2 and -εNH_2 groups, and also for lysine when used to detect amino acid residues bound to proteins. As shown here, collagen treated with DCI was less fluorescent than elastic lamellae, indicating that there was less amount of available N-terminal amino groups in the collagen. These results are also in accordance with those reported by Vidal (1978).

The simultaneous use of polarized light and fluorescence microscopy to analyze sections treated with DCI and ANS made it possible to distinguish between elastic and collagen fibrils. Although both collagen and elastic fibrils are fluorescent, the characteristically strong natural birefringence of fibrillar collagen is a decisive factor in the identification of this macromolecule.

There is evidence that the positive metachromatic reaction seen with the PBT method in the elastic lamellae of the aortic wall results from the release of

aldehyde groups following the cleavage of desmosine and isodesmosine cross-linkages during oxidation with potassium permanganate. These groups, which are transformed into hydroxysulfonic acid after the addition of bisulfite, can be detected by toluidine blue at pH 1.0, as demonstrated by Romhányi et al. (1975) and Fischer and Romhányi (1977) in elastic fibers of bovine ligamentum nuchae. In the conditions used here, aortic elastic lamellae treated by the PBT method showed metachromasy and, when observed in polarized light, had a relatively strong birefringence. This birefringence shown by elastic lamellae may indicate a linear arrangement of the dye molecules and a helical organization of these structures, contrary to established knowledge that elastic fibers are not birefringent. Our results agree with those of Fischer (1979) for aortic wall. The treatment to achieve PBT reaction is hard enough to support the hypothesis that some material was removed disclosing oriented elastin microfibrillar structure.

In contrast, it was not possible to detect pericardial elastic fibers by this method. In the latter case, the elastic fibers were closely interlaced with collagen fibers and possibly masked the reaction through steric hindrance or by blocking the production of free aldehyde by permanganate. Another explanation for this phenomenon could be the presence of only a limited number of reducible cross-linkages in the pericardial matrix elastin opening the question to the possibility of variations in elastin types.

Although the confocal microscopy use has not been advantageous in this work, the ANS and DCI have proved to be suitable dyes for such type of microscopy and it may prove important for future studies of different tissues.

Despite the fact that the methods used are not unpublished, in the present study they were concatenated to get complementary information in a new way. On the other hand, they provide new data about the part played by elastic fibers in pericardium which are relevant in the preparation of bioprostheses.

ACKNOWLEDGMENTS

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5. REFERENCES

BARROS PSM, SAFATLE AMV, RIGUEIRO M. Experimental lamellar corneal graft in dogs using preserved equine pericardium. Braz J Vet Res Anim Sci 1999; 36:00-00.

BARTZATT R Fluorescent labeling of drugs and simple organic compounds containing amine functional groups, utilizing dansyl chloride in Na_2CO_3 buffer. J Pharmacol and Toxicol Meth 2001; 45:247-253.

BARTZATT R. Dansylation of aromatic, aliphatic, and medicinal carboxylic acid compounds in 1M Na_2CO_3 buffer. Analytica Chimica Acta 2003; 488:203-209.

BEYER CF, CRAIG LC, GIBBONS WA. Structural requirements for binding of the fluorescent probe TNS with peptides. Nature New Biol 1973; 241:78-79.

CHANDA J, KURIBAYASHI R, ABE T. Use of glutaraldehyde-chitosan treated porcine pericardium as a pericardial substitute. Biomaterials 1997; 17:1087-1091.

FISCHER J, ROMHÁNYI GY. Optical studies on the molecular-sterical mechanism of metachromasia. Acta Histochem 1977; 59:29-39.

FISCHER J. Ultrastructure of elastic fibers as shown by polarization optics after the topo-optical permanganate-bissulfite-toluidine blue (PBT) reaction. Acta Histochem 1979; 65:87-98.

GOSLINE JM, YEW FF, WEISS-FOCH T. Reversible structural changes in a hydrophobic protein, elastin as indicated by fluorescence probe analysis. Biopolymers 1975; 14:1811-1826.

GOSLINE JM. The physical properties of elastic tissue. Int Rev Connect Tissue Res 1976; 7:211-249.

LAURENCE DJR. Interaction of calf thymus histones fractions in aqueous solution with 8-anilino-naphthalene-1-sulphonic acid. Biochem J 1966; 99:419-422.

OLMOS ZR, JASSO VR, SOTRES VA, CEDILLO LI, ARREOLA RJL, GAXIOLA GM. Utilidad del pericardio bovino tratado con glutaraldeído en las resecciones pulmonares no anatómicas en perros. Rev Inst Nal Enf Resp Mex 1997; 10:155-159.

PAEZ JMG, JORGE-HERRERO E, CARRERA A, MILLÁN I, ROCHA A, CALERO P, CORDÓN A, SALVADOR J, SAINZ N, MÉNDEZ J, CASTILLO-OLIVARES JL. Chemical treatment and tissue selection: factors that influence the mechanical behaviour of porcine pericardium. Biomaterials 2001; 22:2759-2767.

PENZER GR. 1-Anilinonaphtalene-8-sulphonate. The dependence of emission spectra on molecular conformation studied by florescence and proton-magnetic resonance. Eur J Biochem 1972; 25:218-228.

PIRES AC, SAPORITO WF, LEAO LEV. Pericárdio bovino utilizado como remendo no sistema cardiovascular. Ver Brás. Cir Cardiovasc 1997; 12:176-187.

RADDA GK. Probes for enzyme conformation. In Thaer AA and Sernetz M, ed. Fluorescence Techniques in Cell Biology. Berlin, Heidelberg, New York, Springer, 1973. p. 261-272.

ROMHÁNYI G, DEÁK GY, FISCHER J. Aldehyde-bisulfite-toluidine blue (PBT) staining as a topo-optical reaction for demonstration of linear order of vicinal OH groups in biological structures. Histochemistry 1975; 43:333-348.

ROSSELET A, RUCH F. Cytofluorometric determination of lysine with dansylchloride. J Histochem Cytochem 1968; 16:459-466.

STRYER L. The interaction of naphtalene dye with apomyoglobin. A fluorescent probe of non-polar binding sites. J Molec. Biol 1965; 13:482-495.

TIMBERLAKE GT, REDDY GK, STEHNO-BITTEL L, WEBER JF, AMSLINGER S, GIVENS RS. Photoactivated Coumaryl-diazopyruvate Fluorescent Label for Amine Functional Groups of Tissues Containing Type-I Collagen. Photochemistry and Photobiology 2002; 76:473-479.

VIDAL BC. The use of the fluorescent probe 8-anilinonaphtalene sulfate (ANS) for collagen and elastin histochemistry. J Histochem Cytochem 1978; 26:196-201.

VIDAL BC. Aorta Elasticae and Tendon Collagen Reativity to 8-Anilino-1-Naphtalene Sulphate (ANS) and Dansylchloride. Cell Mol Biol 1980; 26:583-588.

YGUERABIDE J. Nanosecond fluorescence spectroscopy of biological macromolecules and membranes. In Thaer AA and Sernetz M, ed. Fluorescence Techniques in Cell Biology. Berlin, Heidelberg, New York, Springer, 1973. p. 261-272.

WEBER, G., 1952. Polarization of the fluorescence of macromolecules. 2.Fluorescent conjugates of ovalbumin and bovine serum albumin. Biochem. J. 51:155-61.

FIGURES LEGENDS

Figure 1. Porcine aorta stained with ANS and examined using fluorescence microscopy. The elastic lamellae are shown fluorescents in blue (details in the text).

Figures 2 and 3. Porcine aortic adventitia tunicae and media tunicae, respectively, stained with dansyl chloride and simultaneously examined using fluorescence and polarized light microscopy. Black arrows indicate birefringent collagen fibrils. Red arrows show fluorescent elastic fibrils and lamellae.

Figure 4. Porcine aorta stained by the PBT method and observed under non-crossed polarizers. Note the metachromatic elastic fibrils and lamellae (arrow).

Figure 5. Same region as in figure 4 but seen under polarized light. The elastic fibers (arrow) show an unexpected greenish birefringence.

Figure 6. Previously boiled porcine aorta stained with ANS and examined using fluorescence microscopy.

Figure 7. Autoclaved porcine aorta stained with dansyl chloride and examined using fluorescence microscopy. Note that elastic lamellae are better visualized after partial extraction of interlamellar collagen.

Figure 8. Fluorescence microscopy of porcine aorta treated with 5% acetic acid prior to staining with dansyl chloride. Note slight disarrangement of the elastic lamellae as compared with figure 7.

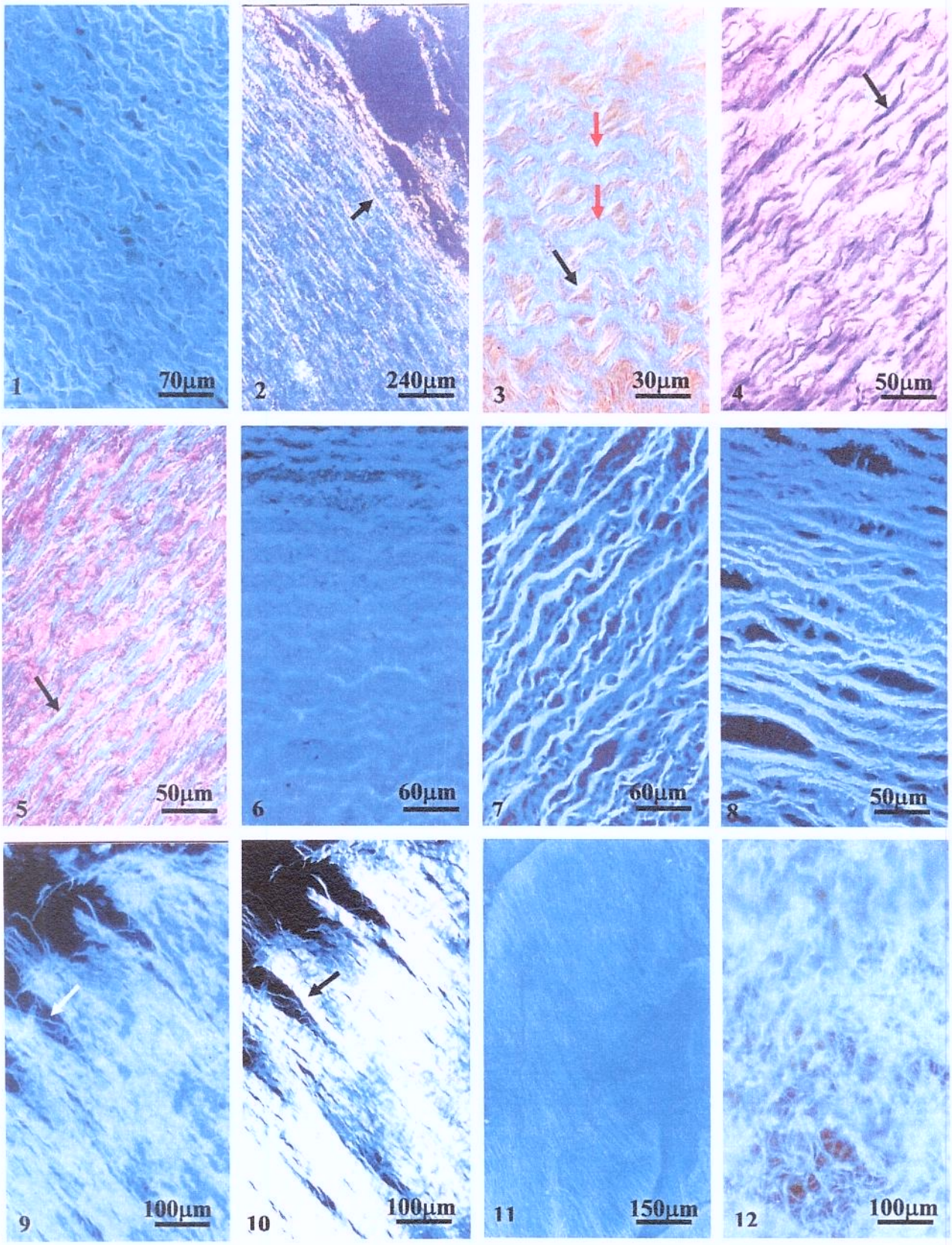
Figure 9. Fluorescence microscopy of a tangential section of porcine pericardium stained with ANS. Fluorescent elastic fibers are blue (white arrow) and are closely associated with collagen fibers which are less fluorescent.

Figure 10. Same region as in figure 9 observed simultaneously under polarized light and fluorescence microscopy. Note the birefringent collagen fibers (arrow) and fluorescent elastic fibers in blue. Both staple fibers can be identified simultaneously under such microscopy.

Figure 11. An *in totum* preparation of autoclaved porcine pericardium stained with ANS. Note the parallel arrangement of elastic fibers in this region.

Figure 12. An in totum preparation of autoclaved porcine pericardium in 5% acetic acid and stained with dansyl chloride. Note arrangement in network of elastic fibers in this region which is different that shown in the previous figure.

Figure 13 a and b. Confocal microscopy of non treated porcine aorta wall fragment stained with dansyl chloride. The central picture of figure 13a has been enlarged in figure 13b. Note the strong fluorescence of the elastic lamellae stained with DCl. Bars: 200µm.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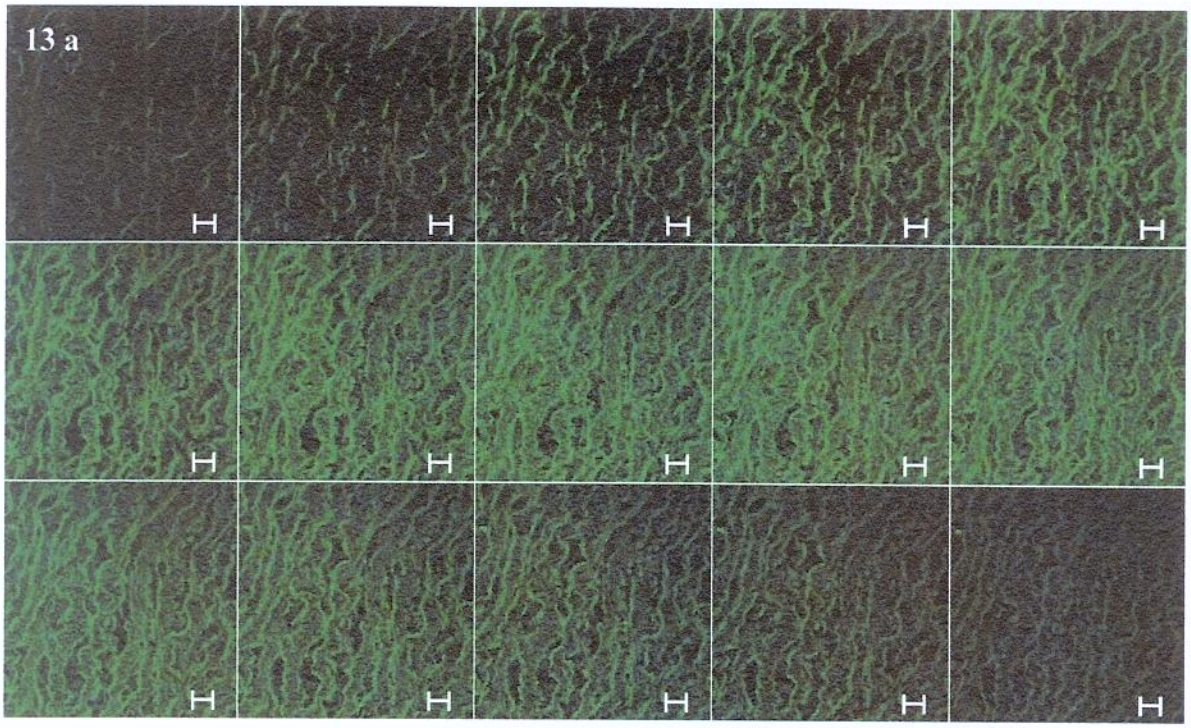
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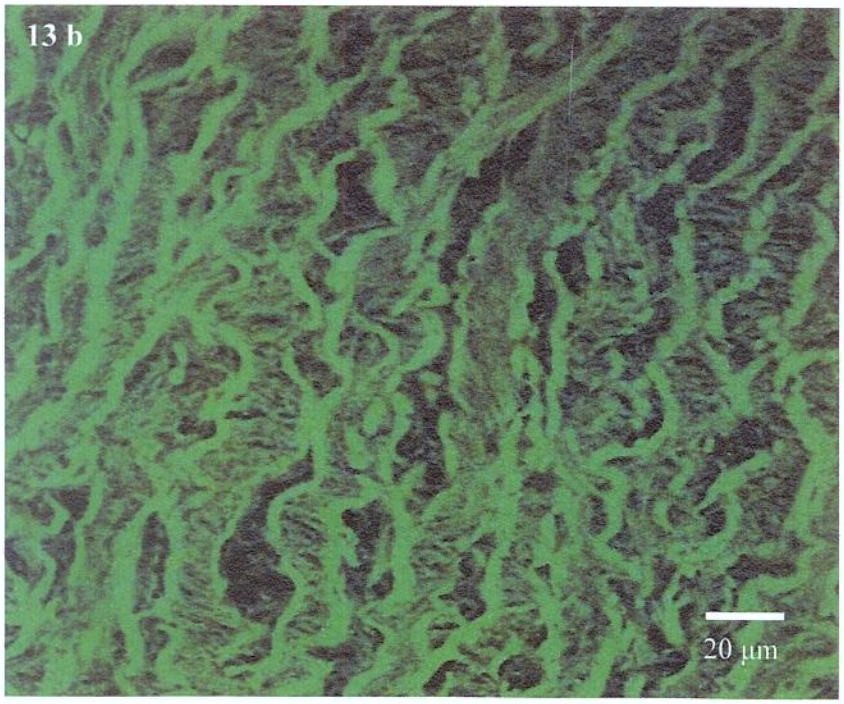
13 b
20 µm

Considerações finais

CONSIDERAÇÕES FINAIS

► O pericárdio porcino apresentou expressiva celularidade, representada especialmente por fibroblastos, característica de tecidos ainda jovens. Apesar da sua alta celularidade comportou-se como tecido maduro, com alta estabilidade colagênica.

► A matriz fibrosa apresentou-se constituída de uma rede de fibras colágenas relativamente finas dispostas em camadas multidirecionalmente orientadas. Em toda a extensão da matriz fibrosa observaram-se finas fibras elásticas fortemente entrelaçadas às fibras colágenas.

► Observou-se discreta predominância circunferencial de fibras colágenas na região anterior esquerda do pericárdio enquanto que na região anterior direita predominou a direção base-ápice com relação ao coração.

► A quantificação de aminoácidos mostrou que a hidroxiprolina corresponde a 9% do total de aminoácidos do colágeno pericardial.

► O pericárdio apresentou-se constituído por 47% de colágeno, calculado a partir da dosagem de hidroxiprolina no tecido.

► A matriz do pericárdio porcino apresentou-se constituída de pequenas quantidades de GAGs.

► Análise da matriz pericardial em SDS-PAGE revelou dezesseis proteínas aniônicas apresentando massa molecular aparente entre 11 e 109kDa e uma banda polidispersa de 85kDa com um comportamento eletroforético muito semelhante ao do decorim. O tratamento por condroitinase AC e ABC identificou o GAG dermatam sulfato.

Referências Gerais

REFERÊNCIAS GERAIS

AULT, N.K. & HOFFMAN, A.H., 1992. A composite micromechanical model for connective tissues: Part II – Aplication to rat tail tendon and joint capsule. J. Biomech. Eng., 114: 137-141.

BARROS, P.S.M.; SAFATLE, A.M.V. & RIGUEIRO, M., 1999. Experimental lamellar corneal graft in dogs using preserved equine pericardium. Braz. J. Vet. Res. Anim. Sci., 36: 00-00.

BARTZATT, R., 2001. Fluorescent labeling of drugs and simple organic compounds containing amine functional groups, utilizing dansyl chloride in Na_2CO_3 buffer. J Pharmacol and Toxicol Meth., 45:247-253.

BARTZATT, R., 2003. Dansylation of aromatic, aliphatic, and medicinal carboxylic acid compounds in 1M Na_2CO_3 buffer. Analytica Chimica Acta, 488:203-209.

BASHEY, R.I.; BASHEY H.M. & JIMENEZ, S.A., 1978. Characterization of pepsin-solubilized bovine heart-valve collagen. Biochem. J., 173: 885-894.

BEELEY, J.G. (1985). Glycoprotein and proteoglycan techniques. Elsevier. New York; 16: 268-270.

BERTIPAGLIA, B; ORTOLANI, F.; PETRELLI, L.; GEROSA, G.; SPINA, M.; PAULETTO, P.; CASAROTTO, D.; MARCHINI, M. & SARTORE, S., 2003. Cell Characterization of Porcine Aortic Valve and Decellularized Leaflets Repopulated with Aortic Valve Interstitial Cells : The VESALIO Project (Vitalitate Exornatum Succedaneum Aorticum Labore Ingenioso Obtenibitur). *Ann Thorac Surg*, 75:1274-1282.

BEYER, C.F., CRAIG, L.C. & GIBBONS, W,A., 1973. Structural requirements for binding of the fluorescent probe TNS with peptides. Nature New Biol., 241:78-79.

BLUM, B., BEIER, H. & GROSS, H. J., 1987. Improved silver staining of plant proteins RNA and DNA in polyacrylamide gels. Electrophoresis., 8: 93-99.

BRADFORD, M.M., 1976. A rapid sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein dye binding. Ann. Biochem., 72: 248-54.

BROWN, D. C. & VOGEL, K. G., 1989. Characteristics of the In Vitro interaction of a small proteoglycan (PGII) of bovine tendon type I collagen. Matrix, 9: 468 – 79.

CARRINO, D. A., SORREL, J. M., CAPLAN, A. I., 2000 Age-related changes in the proteoglycans of human skin. *Archives of Biochemistry and Biophysics*, 373:91–101.

CARVALHO, H.F. Matriz extracelular – Sistema Elástico. In: CARVALHO,H.F. & RECCO-PIMENTEL, S.M. *A célula 2001*. São Paulo, Ed. Manole, 2001. 287p. p.228-234.

CARVALHO, H.F. & VIIDAL, B.C., 1995. The Elastic System of a pressure-bearing tendon of bullfrog Rana catesbeiana. *Annals of Anatomy*, 177:1-8.

CHAKRAVARTI, S.; MAGNUSON, T.; LASS, J.H.; JEPSEN, K.J.; LaMANTIA, C. & CARROLL, H., 1998. Lumican regulates collagen fibril assembly: skin fragility and corneal opacity in the absence of lumican. The Journal of Cell Biology, 14:1277-1286.

CHANDA, J.; KURIBAYASHI, R. & ABE, T., 1997. Use of glutaraldehyde-chitosan treated porcine pericardium as a pericardial substitute. *Biomaterials,* 17:1087-1091.

CHANG, Y., TSAI, C.C.; LIANG, H.C. & SUNG, H.W., 2002. In vivo evaluation of cellular and acellular bovine pericardia fixed with a naturally occurring crosslinking agent (genipin). *Biomaterials,* 23:2447-2457.

COMPER, W.D. Water, 1996. Dynamic Aspects. In: *Extracelular Matrix : Molecular components and interactions* vol. 2. Amsterdan , Harwood Academic Publishers, 1-21.

COTTA-PEREIRA, G., RODRIGO, F.G., BITTENCOURT-SAMPAIO, S., 1976. Oxytalan, elaunin and elastic fibers in the human skin. *J. Invest. Dermatol.*, 66:146-148.

COTTA-PEREIRA, G., DEL-CARO, L.M., MONTES, G.S.,1984 Distribution of elastic system fibers in hyaline and fibrous cartilages of rat. *Acta anat.*, 119:80-85.

DIETRICH, C.P. & DIETRICH, S.M., 1976. Electrophoretic behaviour of acidic mucopolysaccharides in diamine buffers. Anal Biochem, 70: 645-647.

DUNCAN, A.C. & BOUGHNER, D., 1998. Effect of dynamic glutaraldeyde fixation on the viscoelastic properties of bovine pericardial tissue. *Biomaterials*, 19:777-783.

FARNDALE, R.W.; BUTTLE, D.J. & BARRET, A.J., 1986. Improved quantitation and discrimination of sulphated glycosaminoglycans by use of dimethylmethylene blue. Biochem. Biophys. Acta, 883: 173-177.

FENTIE, I.H.; ALLEN, D.J.; SCHENCK, M.H & DIDIO, L.J.A., 1986. Comparative electron microscopic study of bovine, porcine and human parietal pericardium, as materials for cardiac valve bioprostheses. *J. Submicrosc. Cytol.* 18: 53-65.

FISCHER J., 1979. Ultrastructure of elastic fibers as shown by polarization optics after the topo-optical permanganate-bissulfite-toluidine blue (PBT) reaction. Acta Histochem., 65:87-98.

FISCHER, J. & ROMHÁNYI, G.Y., 1977. Optical studies on the molecular-sterical mechanism of metachromasia. Acta Histochem., 59:29-39.

FISHBEIN, M. C., LEVY, R. J., FERRANS, V. J., 1982. Calcification of cardiac valve bioprostheses. Biochemical, histologic, and ultrastructural observations in a subcutaneous implantation model system. *J. Thorac. Cardiovasc. Surg.* 83:602-609.

FISHER, L. W., 1999. Decorin. In: Extracellular matrix, Anchor and Adhesion Proteins. (Kreis T., Vale R.). San Francisco, 358p. p. 241 – 254.

GARDNER, E.,1978. O coração e o pericárdio. In: GARDNER, E.; GRAY, D.J. & O'RAHILLY, R. *Anatomia Regional do Corpo Humano.* Rio de Janeiro : Guanabara Koogan, 815p.299-317.

GATHERCOLE, L.J. & KELLER, 1991. A. Crimp Morphology in Fibre-Forming Collagens. Matrix, 11v, p214-234.

GOSLINE JM, YEW F.F. & WEISS-FOCH, T., 1975. Reversible structural changes in a hydrophobic protein, elastin as indicated by fluorescence probe analysis. Biopolymers, 14:1811-1826.

GOSLINE JM., 1976. The physical properties of elastic tissue. Int Rev Connect Tissue Res., 7:211-249.

GRAY, H. & GOSS, C.M. 1988. O coração. In: GRAY, H. & GOSS, C.M. *Anatomia.* Rio de Janeiro: Guanabara Koogan, 29ªed. 1147p.433-461.

GRODZINSKY, A.J., 1983. Electrochemical and physicochemical properties of connective tissue. CRC Crit. Rev Biomed Eng., 9: 133-199.

HASCALL, V.C. & HASCALL, K.G., 1985. Proteoglycans. In: HAY, E.D. Cell biology of extracellular matrix. New York : Plenum Press, 417p. 39-63.

HAY, E. D., 1991. *Cell biology of extracellular matrix.* New York: Plenum Press, 417p.

HEDBOM, E., ANTONSSON, P., HJERPE, A., AESCHLIMANN, D., PAULSSON, M., PIMENTEL, E. R., WENDEL, M., OLDBERG, A., HEINEGARD, D., 1992. Cartilage matrix proteins. An acidic oligomeric protein (COMP) detected only in cartilage. *J. Biol. Chem.* 267;6132-6136.

HEINEGÅRD, D. & PIMENTEL E. R., 1992. Cartilage matrix proteins. In: Articular cartilage and Osteoarthritis. (Kuttner K. E., Scheleyerbach R., Peyron J. G., Hascall V. C., eds). New York, p95 – 111.

HEINEGÅRD, D. & SOMMARIN, Y., 1987. Methods in Enzimology. 144v, p319-373.

HOLLINSHEAD, W. H., 1980. O sistema circulatório. In: HOLLISHEAD, W.H. Livro texto de anatomia Humana. São Paulo : ed. Harper & Row do Brasil,. 971p. p.82-83.

IOZZO, R.V., 1997. The family of the small leucine-rich proteoglycans: key regulators of matrix assemblay and cellular growth. Crit. Rev. In: Biochem. and Mol. Biol., 32: 141-174.

ISHIHARA,T.; FERRANS, V.; JONES, M.; BOYCE, S.W. & ROBERTS, W.C., 1981. Structure of bovine parietal pericardium and of unimplanted Ionescu-Shiley pericardial valvular bioprotheses. *J. Thorac. Cardiovasc. Surg.* 81:747-757.

JORGE-HERRERO, E.; FERNANDEZ, P.; TURNAY, J.; OLMO, N.; CALERO, P.; GARCIA, R.; FREILE, I. & CASTILLO-OLIVARES, J.L., 1999. Influence of different chemical cross-linking treatment on the properties of bovine pericardium and collagen. Biomaterials, 20: 539-545.

KHOR, E., 1997. Methods for the treatment of collagenous tissues for bioprostheses. Biomaterials, 18: 95-105.

KLAUS, W. & OSBORN, M., 1969. The reliability of molecular weight determinations by dodecil sulfate-polyacrylamide gel electrophoresis. J. Biol. Chem., 244: 4406-4412.

KOOB, T.J. & VOGEL, K.G., 1987. Site-Related Variations in Glycosaminoglycan Content and Swelling Properties of Bovine Flexor Tendon. J. Orthop. Res., 5: 414-424.

KUC, I. M. & SCOTT, P.G., 1997. Increased diameters of collagen fibrils precipitated in vitro in the presence of decorin from various connective tissues. Connective Tissue Research., 36:287-296.

LAEMMLI, V.K., 1970. Cleavage of structural proteins during the assembly of the head of bacteriofhage Ty. Nature, 227:680-685.

LANGDON, S.E.; CHERNECKY, R.; PEREIRA, D.A. & LEE, J.M., 1999. Biaxial mechanical/structural effects of biaxial strain during crosslinking of bovine pericardial xenograft materials. Biomaterials, 20: 137-153.

LAURENCE, D.J.R., 1966. Interaction of calf thymus histones fractions in aqueous solution with 8-anilino-naphthalene-1-sulphonic acid. *Biochem. J.*, 99:419-422.

LINSENMAYER, T.F., 1985. Collagen. In: HAY, E.D. *Cell biology of extracellular matrix*. New York: Plenum Press, 417p. p.5-37.

LOKE, W.K.; KHOR, E.; WEE, A.; TEOH, S.H. & CHIAN, K.S., 1996. Hybrid biomaterials based on the interaction of polyurethane oligomers with porcine pericardium. Biomaterials, 17: 2163-2172.

MARIA, S.S., 1998. Estudo citoquímico, imunocitoquímico e de análise de imagem de células fibroblásticas em proliferação e apoptose na ausência e presença de colágeno tipo I hiperpolimerizado. Tese de doutorado. IB UNICAMP.

MELLO, M.L.S & VIDAL,B.C., 1973. Linear dichroism and anomalous dispersion of the birefringence on sperm heads. *Acta histochem.*, 45:109-114.

MELLO, M.L.S. & VIDAL, B.C., 2003. Experimental tendon repair: glycosaminoglycan arrangement in newly synthesized collagen fibers. *Cellular and Molecular Biology*, 49:579-585.

MICHELACCI, Y.M. & HORTON, D.S.P.Q., 1989. Proteoglycans from the cartilage of young hammerhead shark Sphyrna lewini. Comp. Biochem. Physiol., 92: 651-658.

MOORE, K.L. & DALLEY, A.F., 2001. Torax. In: MOORE, K.L. & DALLEY, A.F *Anatomia orientada para a clínica.* Rio de Janeiro, Guanabara Koogan, 4ª ed. 1023p.52-151.

NEAME, P.J.; CHOI, H.U.; ROSEMBERG, L.C., 1989. The primary structure of the core protein of the small leucine-rich proteoglycan (PGI) from bovine articular cartilage. *J. Biol. Chem*, 264:653-661.

NELSON, D.L. & COX, M.M., 2002. Carboidratos e glicoconjugados e aminoácidos, peptídeos e proteínas. In: NELSON, D.L. & COX, M.M. Lehninger – *Princípios de Bioquímica.* São Paulo: Savier, 975p. 94-240.

OLMOS, Z.R.; JASSO,V.R.; SOTRES, V.A.; CEDILLO, L.I.; ARREOLA, R.J.L. & GAXIOLA, G.M., (1997). Utilidad del pericardio bovino tratado con glutaraldeído en las resecciones pulmonares no anatómicas en perros. *Rev. Inst. Nal. Enf. Resp. Mex*. 10:155-159.

PAEZ, J.M.G.; JORGE-HERRERO, E.; CARRERA, A.; MILLÁN, I.; ROCHA, A.; CALERO, P.; CORDÓN, A.; SALVADOR, J.; SAINZ, N.; MÉNDEZ, J. & CASTILLO-OLIVARES, J.L., 2001. Chemical treatment and tissue selection: factors that influence the mechanical behaviour of porcine pericardium. Biomaterials, 22:2759-2767.

PALMA, P.C.R.; VIDAL, B.C.; RICETTO, C.L.Z.; HERRMAN, V. & NETTO JR, N.R., 1996. A propósito de um novo sling pubovaginal absorvível. Experiência inicial. *J. Bras Ginec.*, 106:335-339.

PENZER, G.R., 1972. 1-Anilinonaphtalene-8-sulphonate. The dependence of emission spectra on molecular conformation studied by florescence and proton-magnetic resonance. Eur J Biochem., 25:218-228.

PETITE, H.; DUVAL, J.; FREI, V.; ABDUL-MALAK, N.; SIGOT-LUIZARD, M. & HERBAGE, D., 1995. Cytocompatibility of calf pericardium treated by glutaraldehyde and by the acyl azide methods in na organotypic culture model. Biomaterials, 16: 1003-1008.

PIEZ, K. A., REDDI, A. H., 1984. Fibronectin, Laminin, and other Extracellular glycoproteins. *In:* HAKAMORI, S., FUKUDA, M., SEKIGUCHI, K., CARTER, W. G. *Extracellular Matriz Biochemistry,* New York: Elservier Science. 452p. 288 – 305.

PIRES, A.C.; SAPORITO, W.F. & LEAO, L.E.V., 1997. Pericárdio bovino utilizado como remendo no sistema cardiovascular. *Ver. Brás. Cir. Cardivasc.* 12:176-187.

RADDA, G.K., 1973. Probes for enzyme conformation. In Thaer AA and Sernetz M, ed. Fluorescence Techniques in Cell Biology. Berlin, Heidelberg, New York, Springer, p. 261-272.

REDDY G.K.; GUM, S.; STEHNO-BITTEL, L. & ENWEMEKA, C.S., 1998. Biochemistry and biomechanics of healing tendon : Part I. effects of rigid plaster casts and functional casts. Medicine & Science in Sports & Exercises, 794-800.

RICETTO, C.L.Z., 1997. Estudo anátomo-patológico de utilização experimental de colágeno bovino purificado na embolização da artéria renal. Tese de mestrado. FCM UNICAMP.

RILEY, G.P.; HARRALL, R.L.; CONSTANT, C.R.; CHARD, M.D.; CAWSTON, T.E. & HAZLEMAN, B.L., 1994. Tendon degeneration and chronic shoulder pain: changes in the collagen composition of the human rotator cuff tendons in rotator cuff tendinitis. Annals of the Rheumatic Diseases, 53: 359-366.

ROMHÁNYI, G.; DEÁK, G.Y. & FISCHER, J., 1975. Aldehyde-bisulfite-toluidine blue (PBT) staining as a topo-optical reaction for demonstration of linear order of vicinal OH groups in biological structures. Histochemistry , 43:333-348.

ROSSELET, A. & RUCH, F., 1968. Cytofluorometric determination of lysine with dansylchloride. J Histochem Cytochem., 16:459-466.

SACKS, M.S.; CHUONG, C.J.C. & MORE, R., 1994. Collagen Fiber Architeture of Bovine Pericardium. ASAIO Journal, M632-M637.

SCHMIDT, C.E. & BAIER, J.M., 2000. Acellular vascular tissues: natural biomaterials for tissue repair and tissue engineering. *Biomaterials*, 21:2215-2231.

SCOTT, J.E., 1996. Proteodermatan and Proteokeratan Sulfate (Decorin, Lumican/ Fibromodulin). Proteins Are Horse Shoe Shaped. Implications for Their Interactions With Collagen. Biochemistry, 35: 8795-8799.

SIMIONESCU, D.T.; LOVEKAMP, J.J. & VYAVAHARE, N.R., 2003. Degeneration of bioprosthetic heart valve cusp and wall tissues is initiated during tissue preparation: An ultrastructural study. *J. Heart Valve Disease.*, 12:226-234.

SIMIONESCU, D.; IOZZO, R. & KEFALIDES, N.A., 1989. Bovine Pericardial Proteoglycan: Biochemical, Immunochemical and Ultrastructural Studies. Matrix, 9: 301-310.

SLACK, C.; FLINT, M.H. & THOMPSON, B.M., 1984. The effect of tensional load on isolated embryonic chick tendons in organ culture. Conn. Tiss. Res.,12: 229-247.

STEGEMANN, H. & STALDER, K., 1967. Determination of hydroxyproline. Clinica Chimica Acta, 18: 267-273.

STEHNO-BITTEL, L.; REDDY G.K.; GUM, S. & ENWEMEKA, C.S., 1998. Biochemistry and biomechanics of healing tendon : Part I. effects of rigid plaster casts and functional casts. Medicine & Science in Sports & Exercises, 788-793.

STOCK, U.A. & VACANTI J.P., 2001. Tissue engeneering: current state and prospects. *Annu Rev Med.*, 52:443-451.

STRYER L., 1965. The interaction of naphtalene dye with apomyoglobin. A fluorescent probe of non-polar binding sites. J Molec. Biol., 13:482-495.

SUNG, H.W.; CHANG, Y.; CHIU, C.T; CHEN, C.N. & LIANG, H.C., 1999. Mechanical properties of a porcine aortic valve fixed with a naturally occuring crosslinking agent. *Biomaterials,* 20:1759-1772.

TIMBERLAKE, G.T.; REDDY, G.K.; STEHNO-BITTEL, L.; WEBER, J.F.; AMSLINGER, S. & GIVENS, R.S., 2002. Photoactivated Coumaryl-diazopyruvate Fluorescent Label for Amine Functional Groups of Tissues Containing Type-I Collagen. Photochemistry and Photobiology, 76:473-479.

URBAN, J.P.G.; MAROUDAS, A.; BAYLISS,M.T. & DILLON, M.T., 1979. Swelling pressures of proteoglycans at concentrations found in cartilaginous tissues. Biorheology, 16:447-464.

van der REST, M., GARRONE, R., 1991. Collagen family of proteins. *Faseb J.*, 5:2814 – 2823.

VIDAL, B.C.,1963. Pleochroism in Tendon and its bearing to acid mucopolysaccharides. *Protoplasma*, 56:529-536.

VIDAL, B.C.,1964. The part played by the mucopolysaccharides in the form birefringence of collagen. *Protoplasma*, 59:472–479.

VIDAL, B.C., 1966. Macromolecular Desorientation in Detached Tendons. *Protoplasma Bd* LXII/2-3:121-131.

VIDAL, B.C., 1972. Toluidine blue: Cotton effect-like phenomena produced by crystal aggregates obtained by drying on microscopic slides. *Ann. Histochim.* , 17:151-157.

VIDAL, B.C., 1978. The use of the fluorescent probe 8-anilinonaphtalene sulfate (ANS) for collagen and elastin histochemistry. *The Journal of Histochemistry and Cytochemistry*, 26:196-201.

VIDAL, B.C., 1980a. The part played by proteoglycans and structural glycoproteins in the macromolecular orientation of collagen bundles. *Cell. Mol. Biol.* 26:415-421.

VIDAL, B.C., 1980b. Aorta Elasticae and Tendon Collagen Reativity to 8-Anilino-1-Naphtalene Sulphate (ANS) and Dansylchloride, *Cellular & Molecular Biology,* 26:583-588.

VIDAL, B.C., 1984. Ordem molecular y haces de colageno. *Trab. Inst. Cajal.* 75:19-27.

VIDAL, B.C., 1987. Métodos em Biologia Celular. In: VIDAL, B.C. & MELLO, M.L.S. *Biologia Celular.* Rio de Janeiro. São Paulo : Atheneu, 348p. p05-34.

VIDAL, B.C., 1995. From collagen type I solution to fibers with a helical pattern: a self-assembly phenomenon. C.R. Acad. Sci. Paris, 318:831–836.

VIDAL, B.C., 2003. Image analysis of tendon helical superstructure using interference and polarized light microscopy. Micron, 34:423-432.

VIDAL, B.C. & MELLO, M.L.S., 1972. Anisotropic properties of Toluidine blue-stained collagen. Ann. Histochim. , 18: 106-122.

VISWANADHAM, R.K.; AGRAWD, D.C. & KRAMER, E., 1976. Environmental effects on the mechanical properties of reconstituted collagen hollow fiber membranes. J. Poly. Sci. Phys., 14: 2195-2209.

VOGEL, K.G. & HEINEGÅRD, D., 1985. Characterization of proteoglycans from adult bovine tendon. *J. Biol. Chem.*, 260:9298-9306.

WEBER, G., 1952. Polarization of the fluorescence of macromolecules. 2.Fluorescent conjugates of ovalbumin and bovine serum albumin. Biochem. J. 51:155-61.

WHITTAKER, P.; BOUGHNER, D.R.; PERKINS, D.G. & CANHAM, P.B., 1987. Quantitative structural analysis of collagen in chordae tendineae and its relation to floppy mitral valves and proteoglycan infiltration. Br Heart, 57: 264-269.

YANNAS I.V. & GRODZINSKY A.J., 1973. Electromechanical energy conversion with collagen fibers in an aqueous medium. J. Mechanochem Cell Motility, 2: 113-125.

YGUERABIDE J., 1973. Nanosecond fluorescence spectroscopy of biological macromolecules and membranes. In Thaer AA and Sernetz M, ed. Fluorescence Techniques in Cell Biology. Berlin, Heidelberg, New York, Springer, 261-272.

ZINGALES, B., 1984. Analysis of protein sodium dodecyl sulfate-polyacrilamide gel electrophoresis. In: Genes and antigens of parasites. Rio de Janeiro, Fiocruz, 357-363.