J Membrane Biol (2008) 221:15–25
DOI 10.1007/s00232-007-9081-5

Extracellular Matrix of Porcine Pericardium: Biochemistry and Collagen Architecture

Antonella Sachsida Braga-Vilela · Edson Rosa Pimentel · Sergio Marangoni · Marcos Hikari Toyama · Benedicto de Campos Vidal

Received: 4 July 2007 / Accepted: 9 October 2007 / Published online: 3 December 2007

Abstract Pericardial tissue has been used to construct bioprostheses employed in the repair of different kinds of injuries, mostly cardiac. However, calcification and mechanical failure have been the main causes of the limited durability of cardiac bioprostheses constructed with bovine pericardium. In the course of this work, a study was conducted on porcine fibrous pericardium, its microscopic structure and biochemical nature. The general morphology and architecture of collagen were studied under conventional light and polarized light microscopy. The biochemical study of the pericardial matrix was conducted according to the following procedures: swelling test, hydroxyproline and collagen dosage, quantification of amino acids in soluble collagen, component extraction of the extracellular matrix of the right and left ventral regions of pericardium with different molarities of guanidine chloride, protein and glycosaminoglycan (GAG) dosage, sodium dodecyl sulfate-polyacrylamide gel electrophoresis and total GAG analysis. Microscopic analysis showed collagen fibers arranged in multidirectionally oriented layers forming a closely knit web, with a larger number of fibers obliquely oriented, initiating at the lower central region toward the upper left lateral relative to the heart. No qualitative differences were found between proteins extracted from the right and left regions. Likewise, no differences were found between fresh and frozen material. Protein dosages from left frontal and right frontal pericardium regions showed no significant differences. The quantities of extracted GAGs were too small for detection by the method used. Enzymatic digestion and electrophoretic analysis showed that the GAG found is possibly dermatan sulfate. The proteoglycan showed a running standard very similar to the small proteoglycan decorin.

Keywords Extracellular matrix · Porcine pericardium · Collagen · Proteoglycan glycosaminoglycan · Bioprosthesis

A. S. Braga-Vilela (✉)
Department of Biologic Sciences, UNIFAL, Rua Gabriel Monteiro Silva 714, 37 130 000 Alfenas, Minas Gerais, Brazil
e-mail: antonella@unifal-mg.edu.br

E. R. Pimentel · B. de Campos Vidal
Department of Cell Biology, UNICAMP, São Paulo, Brazil

S. Marangoni
Department of Biochemistry, UNICAMP, São Paulo, Brazil

M. H. Toyama
Department of Biochemistry, UNESP-CLP-SV, São Paulo, Brazil

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). Pericardial tissue has been used for bioprosthesis construction for decades, especially for the repair of cardiac injuries (Olmos et al., 1997; Barros, Safatle & Rigueiro, 1999). Repair of the ventricular wall, cardiac valves and aortic wall for the correction of aneurysms has been successfully performed using pericardium bioprostheses (Pires, Saporito & Leao, 1997). Pericardium from different animals has been tested for the construction of bioprostheses, and 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 that of human and bovine pericardium. Thus, its use in the

construction of bioprostheses appears to be a distinct possibility (Fentie et al., 1986; Chanda, Kuribayashi & Abe, 1997).

The biomechanical properties of pericardium, as well as other collagenous tissues, are directly related to the distribution and orientation of the collagen fiber bundles (Sacks, Cuhong & More, 1994) and to the wave-like structures (WLSs) or crimp of such fibers (Loke et al., 1996; Langdon et al., 1999). Analysis of the WLS shows the arrangement of type I collagen molecules in the collagen bundle and possible direction of changes of these fibrillar elements (Ault & Hoffman, 1992). The morphological variability of the collagenous bundle probably reflects functional differentiation resulting from different biomechanical properties of the tissues. Alterations in the collagenous 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 WLSs (Vidal, 2003; Gathercole & Keller, 1991).

The anisotropic optical properties, birefringence and dichroism 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 dichroism, using toluidine blue (pH 3.5–4.0), reveals the molecular arrangement of acid glycosaminoglycans (GAGs), birefringence predominantly reveals the crystalline structure of collagen polypeptide chains. Both phenomena are intimately related, due to the fact that about 13% of the form birefringence of collagen bundles is due to the GAGs associated with them, as observed in young rat tendon (Vidal, 1966, 1980).

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

Besides the fibrous components, other typical components of the extracellular matrix (ECM), such as proteoglycans, structural glycoproteins and collagenous proteins, are part of the fibrous layer of the pericardium. A small proteoglycan containing a single chain of dermatan sulfate-type GAG was found in bovine pericardium (Simionescu, Iozzo & Kefalides, 1989). However, the literature is still scarce concerning data related to porcine pericardium. Proteoglycans, although comprising a small fraction of the tissue mass (<1% of bovine flexor tendon net weight), contribute significantly to the physicochemical properties of connective tissues, such as the phenomena of swelling and osmotic resistance to compression forces.

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

The purpose of this work was to study the microscopic structure and biochemical nature of porcine pericardium. The surveyed data and the additional knowledge regarding its molecular supraorganization will be useful for reviewing pericardium preparation methods in order to obtain membranes which are better prepared and free from antigenic matter.

Materials and Methods

Animals

Six-month-old pigs of the Large White lineage, with an average weight of 95 kg, were used. The pericardia were collected immediately after slaughter at the abattoir, fixed kept fresh or frozen (3 days at −20°C) for the following experimental procedures.

Morphology

The ventral surface of the pericardium (n = 10) was used, with the regions identified as follows: 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 4% paraformaldehyde (in phosphate-buffered saline [pH 7.4] and 0.15 M NaCl). Part of the fragments was processed according to the routine histological procedure for embedding in Histosec/Paraplast Plus (Merck, Darmstadt, Germany) and microtomy with a thickness of 7 μm, and the remaining part was submitted for in totum preparations. Part of the in totum preparations and sections were stained with toluidine blue (Merck) in McIlvaine buffer (pH 4.0), and the remainder was kept without staining. The slides were analyzed under polarized light microscopy, using a Zeiss Polarizing Microscope, equipped with Planachromatic objective, and a Zeiss Axiophot 2 microscope, equipped with Pol-Neofluar infinitive objectives/infinitive focus (Zeiss, Oberkochen, Germany).

Analysis of Collagen Amino Acids

Pericardial collagen (n = 5) was obtained by extraction in 5% acetic acid for 72 h at 4°C, precipitation in 2.5 M NaCl for 24 h at 4°C and dialysis against water for 96 h at 4°C. The amino acid analysis was realized by reverse-phase high-performance liquid chromatography in order to separate phenylthiocarbamoyl derived from amino acids. The amino acids were obtained from collagen samples submitted to hydrolysis with 6N HCl in the presence of 0.1% phenol in vapor phase at 106°C for 24 h. The amino acid content was evaluated by a PICO-TAG (Waters, Eschborn, Germany) amino acid analyzer.

Swelling Test

Pericardium fragments (n = 5) were washed in water, compression-dried between sheets of filter paper and weighed. They were then immersed in water for 2 h and again dried and weighed. They were subsequently immersed in 3% acetic acid for 1 h 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, 1987).

Hydroxyproline Dosage

In order to quantify hydroxyproline, the fragments from five pericardia were weighed and submitted to hydrolysis in 6N HCl (1 ml/10 mg tissue) for 24 h at 106°C. The hydrolysate was then treated with a chloramine-T solution for 20 min at 20°C; perchloric/aldehyde acid was then added, and the mixture was left in a water bath at 60°C for 15 min according to the method described by Stegemann & Stalder (1967). Absorbance was read at 550 nm in a Hewlett-Packard (Palo Alto, CA) 845A spectrophotometer. Different concentrations of hydroxyproline (Sigma, St. Louis, MO) were used for the standard curve.

Extraction of ECM Components

The samples of the left frontal and right frontal regions of both fresh and frozen pericardia (n = 5) were cut into fragments of approximately 1 x 1 mm and submitted to extraction with 15 volumes of 3, 4, 5 or 6 M guanidine chloride (GuHCl) containing 1 mM phenylmethylsulfonyl fluoride (PMSF), 20 mM ethylenediaminetetraacetic acid (EDTA) in 50 mM sodium acetate buffer (pH 5.8) (Heinegård & 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.

Protein and Sulfated GAG Dosages

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

Ion Exchange Chromatography

The total extracts in GuHCl were dialyzed against 20 volumes of 7 M urea, 0.05 M Tris-acetate (pH 8.0) buffer. After four changes of dialysis, 3 ml of dialyzed material was applied to a diethylaminoethyl-Sephacel (DEAE-Sephacel 1.5 x 2.7 cm) ion exchange column equilibrated with 7 M urea, 0.05 M Tris-acetate (pH 8.0) buffer. The fractions were eluted at a flow rate of 1.5 ml/min, using a gradient of 0.1–1 M NaCl in the same buffer with 7 M urea. Fractions of 2.8 ml were collected, and protein elution was monitored by absorbance at 230 and 280 nm in a Hewlett-Packard 8452 A spectrophotometer.

Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was performed according to Zingales (1984), using a gradient of 4–16% of acrylamide in the presence of SDS and stacking gel with 3.5% acrylamide and buffer system according to Laemmli (1970). The proteins were precipitated in a mixture of acetate-ethanol after 12 h at a temperature of −4°C. The precipitate was suspended in a sample buffer containing 0.05 M Tris-HCl (pH 6.8), 2% SDS, 10% glycerol and 0.002% bromophenol blue. β-Mercaptoethanol (5%) was used under reducing conditions. Gel staining was realized with Coomassie brilliant blue or by silver impregnation (Blum, Beier & Gross, 1987). The relative molecular masses were deduced from the retenction factor (Rf) of molecular mass markers (Klaus & Osborn, 1969).

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 h in 0.5 M NaOH at 4°C, followed by precipitation with ethanol and washing with acetone (Michelacci & Horton, 1989). The GAGs were analyzed in agarose-propylene diamine (PDA) gel.

Enzymatic Treatment

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 h and treated with papain (40 mg/g of tissue) in 0.03 M sodium citrate buffer (pH 5.5) containing 0.04 M EDTA and 0.08 M β-mercaptoethanol (80 ul/1 ml) and incubated at 50°C for 24 h (Michelacci & Horton, 1989). The GAGs obtained after precipitation with ethanol were analyzed in agarose-PDA gel.

Digestion with chondroitinases ABC/AC

Samples containing GAGs obtained from β-elimination and digestion with papain were treated with chondroitinase ABC and AC (Seikagaku, Tokyo, Japan). For chondroitinase ABC (0.04 U), the sample was suspended in 10 ml of 50 mM sodium acetate buffer, 10 mM EDTA and 50 mM Tris (pH 6.0). For chondroitinase AC (0.08 U) the buffer was the same but with pH 8.0 (Beeley, 1985). Digestion lasted for 20 h at 37°C. After ethanolic precipitation, the GAGs were analyzed in gel.

Electrophoresis in Agarose-Propylene Diamine Gel

The GAGs obtained by enzymatic digestion were analyzed by electrophoresis in agarose gel in 50 mM acetate-propylene diamine buffer (pH 9.0) as described by Dietrich & Dietrich (1976), using CS, dermatan sulfate (DS) and heparan sulfate (HS) as standard.

Protein Elution from SDS-PAGE Gel

Bands corresponding to 75- and 68-kDa proteins in SDS-PAGE gel were trimmed, chopped and placed in 0.5% SDS with 50 mM Tris-HCl buffer (pH 7.4) for elution at ambient temperature under constant agitation for 24 h. The supernatant containing the eluted protein was then precipitated in ethanol-acetate for 12 h 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 by SDS-PAGE.

Results

Morphological Analysis

The in totum preparations stained with toluidine blue at pH 4.0 revealed staining only in their polyanionic components. Examining these preparations by polarized light microscopy enabled detection of collagen fibers due to their birefringence, as well as their distribution, orientation and aggregation.

The morphological aspects of porcine pericardium can be seen in Figures 1–3. A highly cellularized tissue can be seen, whose cells are mainly fibroblasts, with some 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 between arterioles, capillaries and small veins (Fig. 3).

ECM showing little or no metachromasia can be seen in total preparations stained with toluidine blue at pH 4.0 (Figs. 3 and 4), revealing small amounts of acid GAGs.

A network with a closed 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, permitted the observation of multidirectionally oriented fibers (Fig. 5 and 6), frequently

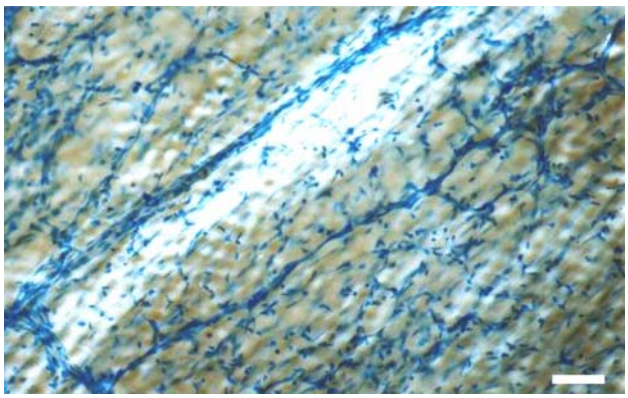


Fig. 1 In totum preparation of the ULL region of pericardium stained with toluidine blue (pH 4.0) and observed using polarized light microscopy. Bar = 30 μm

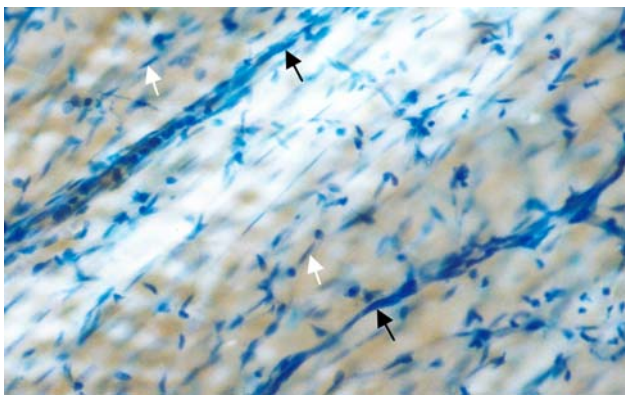


Fig. 2 In totum preparation of UC region of pericardium. Note the vessel cell nuclei (*black arrows*) and fibroblasts (*white arrows*) stained with toluidine blue (pH 4.0). Bar = 26 μm

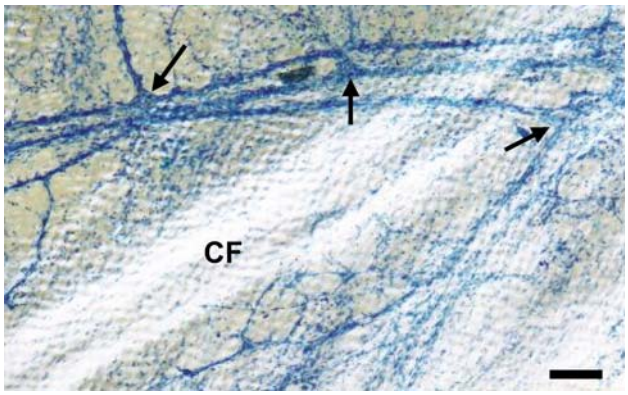


Fig. 3 In totum preparation of the LC region of pericardium stained with toluidine blue (pH 4.0) and observed using polarized light microscopy. Note the birefringence of collagen fibers (*CF*) and the great ramification of the vascular tree (*arrows*). Bar = 130 μm

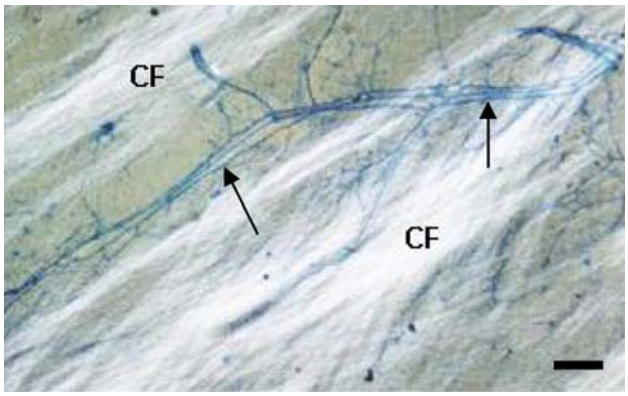


Fig. 4 In totum preparation of LC region of pericardium stained with toluidine blue (pH 4.0) and observed using polarized light microscopy. Collagen fibers (*CF*) and vessels (*arrows*). Bar = 550 μm

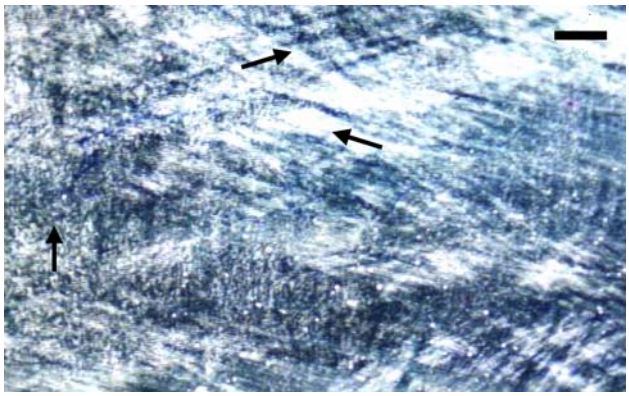


Fig. 5 In totum preparation of LLL region of pericardium seen using polarized light microscopy. Observe the multidirectionality (*arrows*) of the collagen fibers birefringent in white. Bar = 77 μm

crossing each other at acute angles. It was also possible to observe a predominance in direction, with a larger number of fibers obliquely oriented, from the LC region toward the ULL (Fig. 6). A discreet predominance of fibers in the

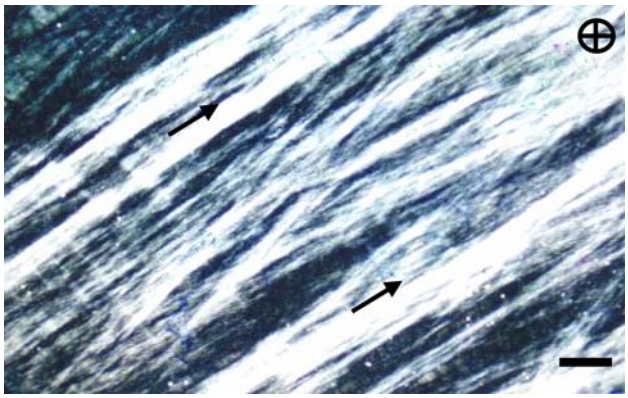


Fig. 6 Unstained in totum preparation of LC region of pericardium. The collagen fibers show the typical brilliance of their birefringence, from first-class white to grayish, observed under crossed polarizers. Observe their preferential direction (*arrows*) (details in the text). Bar = 65 μm

apex-base direction of the heart was verified in the pericardium right frontal region (*data not shown*). Collagen fibers, with an apparently rectilinear course and showing a typically undulant pattern (crimp), were observed in diverse pericardium regions (Fig. 7 and 8).

Analysis of Amino Acids

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

Swelling Test

Fragments of pericardium ($n = 5$) submitted to the swelling test showed a small weight decrease after 2 h immersion in

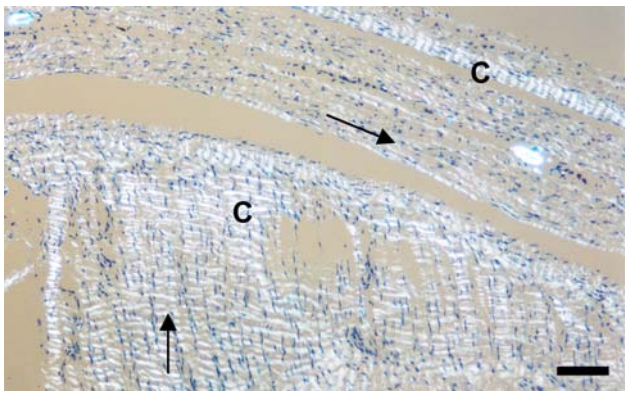


Fig. 7 Micrography of tangential section of pericardium stained with toluidine blue (pH 4.0) and observed with polarizers at a relative angle smaller than 90°. Observe crimp (*C*) and direction of collagen fibers (*arrow*). Bar = 60 μm

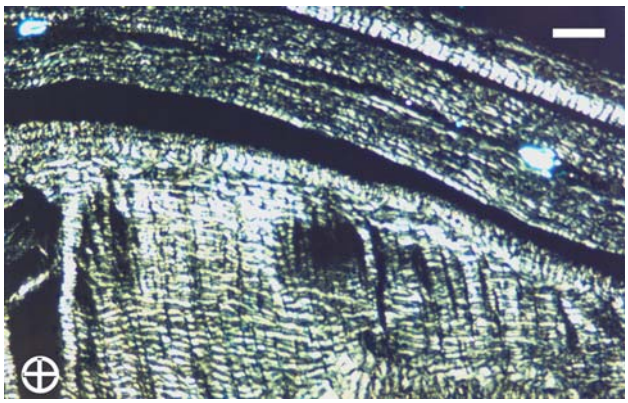
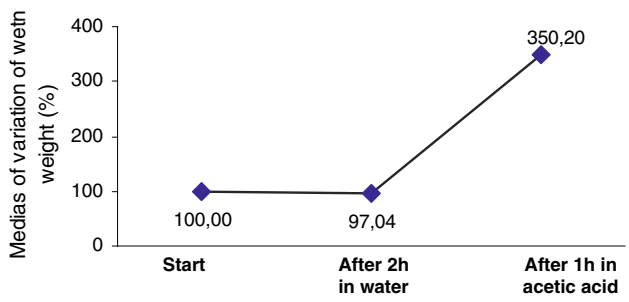


Fig. 8 Same area as Figure 7 observed with crossed polarizer and analyzer. Note the greenish birefringence of collagen fibers. Bar = 115 μm

Table 1 Amino acid composition of soluble collagen of porcine pericardium: values in residue of amino acid per 1,000 total residues

Amino acid	Residues (*n*)	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%
Hydroxyproline	90	9.0%
Cysteine	0	0
Hydroxyproline/Proline ratio	0.76	



Graph 1 Media values of swelling of 5 different pericardia

water. A significant increase was observed after 1 h immersion in 3% acetic acid, as shown by the analysis of Graph 1.

Hydroxyproline Dosage

The average hydroxyproline dosage and its conversion into an estimated quantity of collagen were 42.21 (standard deviation [SD] ±2.01) and 469.0 μg/mg of tissue, respectively, considering that hydroxyproline corresponds to 9% of the total amount of amino acids of pericardial collagen.

Extraction of ECM Components

The electrophoretic analysis of ECM proteins of the pericardium, obtained after extraction with 3, 4, 5 and 6 M GuHCl, showed no apparent differences (Fig. 9a). Although no differences were found in the extraction with different concentrations of GuHCl, extraction with 4 M GuHCl was chosen as the standard procedure as it is the most widely used concentration in extraction procedures of ECM components, principally to avoid any kind of molecular interactions. Analysis of electrophoresis in reducing (with β-mercaptoethanol) and nonreducing conditions (in the absence of β-mercaptoethanol) showed the same band pattern in both conditions. Additionally, no differences were found in extracts obtained from the right and left regions of the pericardium (Fig. 9b). Likewise, no differences were detected between fresh and frozen tissue (Fig. 9b).

Protein and Sulfated GAG Dosage

Analyses of the protein dosages in the extracts obtained from the left and right pericardium regions showed no significant differences (α = 0.05). For each gram of pericardium 20.94 mg (SD ±2.7) of proteins for the right region and 21.08 mg (SD ±3.4) for the left region were found. The dosage of sulfated GAGs showed no such polysaccharides. It is possible that the extracted amounts were too small for detection by the method used.

Ion Exchange Chromatography and SDS-PAGE

The extracted material was fractionated in a DEAE-Sephacel column, and the components bound to DEAE were eluted with a gradient of 0.1–1 M NaCl (Fig. 10a). Samples of each fraction were analyzed in SDS-PAGE

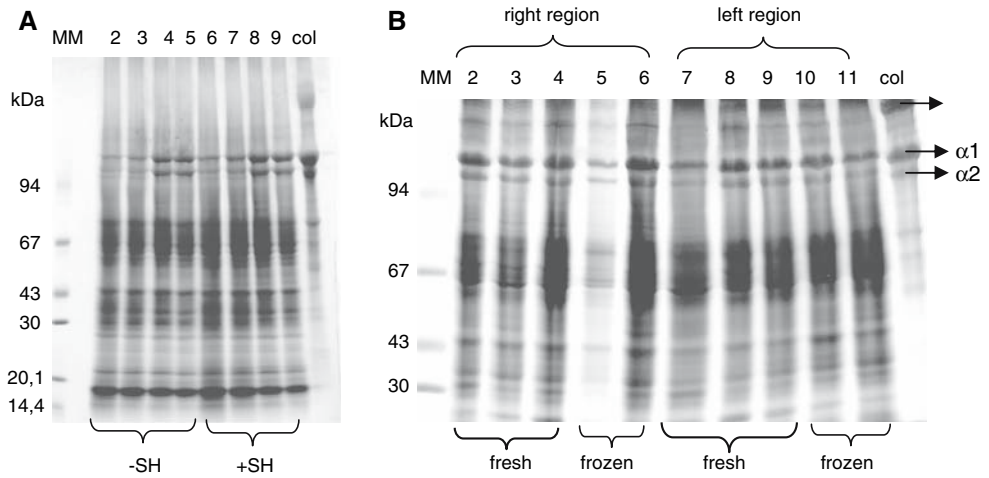


Fig. 9 SDS-PAGE of extracts obtained from pericardium. **a** SDS-PAGE of proteins extracted with GuHCl in conditions of 3 M (lanes 2 and 6), 4 M (lanes 3 and 7), 5 M (lanes 4 and 8) and 6 M (lanes 5 and 9). *–SH*, without *β*-mercaptoethanol; *+SH*, with *β*-mercaptoethanol. **b** SDS-PAGE of proteins extracted with 4 M GuHCl 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 I collagen; *α1* and *α2*, α chains; *β*, band resulting from two α chains

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 collagen chains were found only in the material not bound to DEAE-Sephacel.

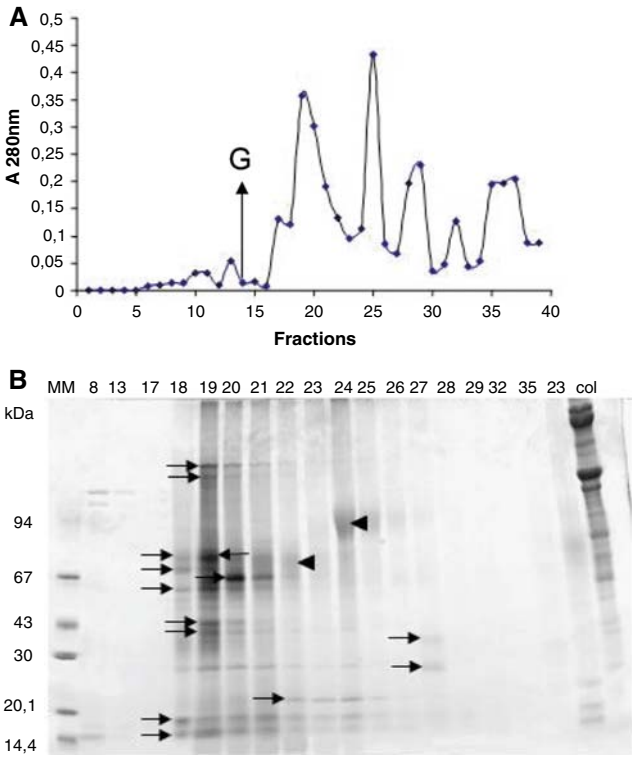


Fig. 10 Chromatography in DEAE-Sephacel and SDS-PAGE. **a** Chromatography of material extracted from whole pericardium in 4 M GuHCl. G indicates the beginning of the gradient 0.1–1.0 M NaCl. **b** SDS-PAGE in the presence of *β*-mercaptoethanol of fractions eluted from the column. The apparent molecular masses of proteins eluted from the column are indicated by *arrows*. *Arrowsheads*, polydisperse bands; *MM*, molecular mass markers; *col*, type I collagen

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 an intermediate position between HS and DS. After treatment with chondroitinases AC and ABC, observation revealed that the GAG was completely digested by chondroitinase ABC but not by chondroitinase AC, indicating it was DS (Fig. 11). The extensive polydisperse band seen in the control probably contained some contaminant nucleic acid.

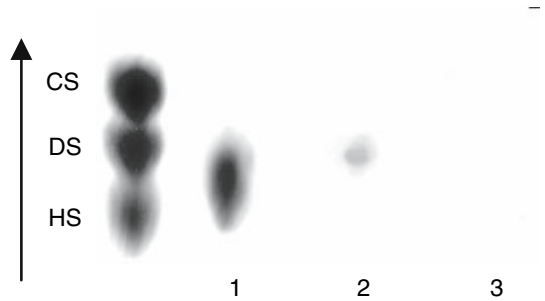


Fig. 11 Electrophoresis in agarose-PDA of GAGs extracted from pericardium with papain and treated with chondroitinases AC (lane 2) and ABC (lane 3). The material not treated with chondroitinase appears as an extensive polydisperse band (lane 1). On the left are the GAG standards CS, DS and HS. *Arrow* indicates the direction of the running

Electrophoresis in Agarose-PDA Gel of GAGs Obtained by β-Elimination of Chromatography Fractions

The polydisperse component with an apparent molecular mass of 80–100 kDa, probably a small PG, eluted from DEAE-Sephacel was submitted to β-elimination; and the liberated GAG was analyzed in agarose-PDA gel (Fig. 12). The band aligned in the same direction as the standard DS and was completely digested by chondroitinase ABC but not by chondroitinase AC, indicating that the polydisperse component is a small PG containing DS.

Analysis of Proteins with 75 and 68 kDa

Fractions 19 and 20 of DEAE-Sephacel showed two proteins with an apparent mass of 75 and 68 kDa, whose electrophoretic behavior was different in the presence or absence of β-mercaptoethanol (Fig. 13a). For individual analysis of the two proteins, the bands were trimmed from the gel, eluted and submitted to a new electrophoresis in the presence and absence of β-mercaptoethanol (Fig. 13b). Considering the protein with 75 kDa, in the presence of β-mercaptoethanol, another band could be seen, besides that 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 of the sharp band observed in the absence of the reducing agent.

Discussion

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

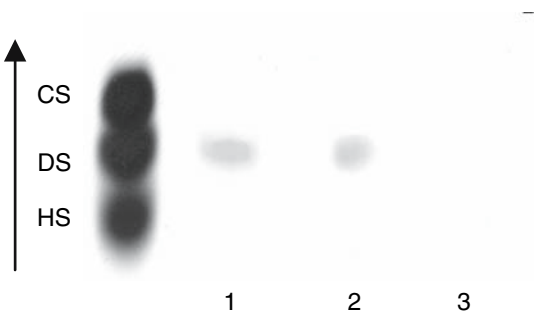


Fig. 12 Electrophoresis in agarose-PDA gel of GAGs obtained by β-elimination. Observe the control (lane 1) and the samples treated with chondroitinases AC (lane 2) and ABC (lane 3). The GAG standards are shown on the *left*. *Arrow* indicates the direction of the running

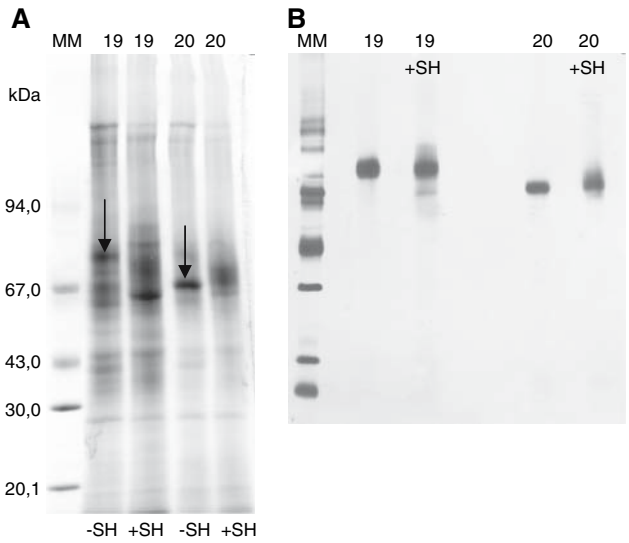


Fig. 13 SDS-PAGE of fractions 19 and 20 of DEAE-Sephacel. **a** Running in the presence (+*SH*) and absence (−*SH*) of β-mercaptoethanol. *MM*, molecular mass markers. *Arrows* indicate components of 75 (fraction 19) and 68 (fraction 20) kDa. Staining realized with Coomassie brilliant blue. **b** SDS-PAGE of proteins of 75 and 68 kDa purified by elution from the gel. *Arrow* indicates the band corresponding to the protein, which dissociated from the component of 75 kDa in the presence of β-mercaptoethanol (+*SH*). Note the more disperse aspect of the component of 68 kDa in the presence of β-mercaptoethanol. *MM*, molecular mass markers. Staining realized with silver

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 with pericardial function. As the pericardium suffers distension during diastolic movements, the multidirectional arrangement of the collagen fibers guarantees the restraint of this movement, helping to stabilize the pericardium and preventing superdilation.

The observation in this work of a discreet predominance of direction, with a higher number of fibers obliquely oriented, from the LC region toward the ULL could reflect the continuity of pericardium fixation fibers, through the pericardiacophrenic ligament to the tendinous center of the diaphragm. These predominant fibers probably take a helical course, from the pericardiacophrenic ligament toward the region of posterior fixation of the pericardium. Sacks et al. (1994) noted a circumferential predominance of fibers in the left frontal region of bovine pericardium, while in the right frontal region, the apex-base direction was predominant, in agreement with the present findings. Concurring with the reports of Langdon et al. (1999), the observation of WLS in the collagenous pericardial bundles was also frequent in the present study. However, what Langdon et al. called "crimps" (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 the present work, the occurrence of WLS is due to a change in the molecular orientation in the collagen bundles, even though they may appear rectilinear. These structures are detected by changes in birefringence of the fibers under polarized light microscopy. Even when tension forces are applied to the tissue, changes in 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 toluidine blue at pH 4.0 showed a pale matrix with little or no metachromasia, reflecting very small quantities of available anionic groups. At pH 4.0, toluidine blue bound with carboxyl radicals and sulfate of acid GAGs, as well as phosphate radicals, DNA and RNA, as indicated by the staining observed in the nuclei of nucleic acid phosphate groups. Staining with toluidine blue enables the analysis and quantification of available anionic radicals and of electrostatic combinations, which are important for supramolecular organization and self-assembling (Vidal, 1995).

The toluidine blue molecule, which in solution behaves like liquid crystal, is small, has a planar geometry and possesses excellent optical anisotropic properties (Vidal, 1987). In the ECM, its molecules combine with GAGs, creating orderly piles and showing metachromasia (Vidal, 1984). The present topochemical findings, which suggest a low content of available anionic radicals that are important in the production of metachromasia, are in agreement with the behavior of pericardium swelling conditions. The PGs contribute significantly to the physicochemical properties of the ECM. This is especially due to the large number of negatively charged groups, carboxyl and sulfate, which form their chains of GAGs. Thus, the PGs in the cartilage, e.g., exert swelling pressure on the collagenous matrix, promoting osmotic resistance to compression loads (Urban et al., 1979). A hypothesis that should be considered is the possibility that water fixed by the acid radicals could concomitantly create hydrostatic pressure. Swelling tests performed with bovine flexor tendon showed that regions with relatively low levels of GAGs lose weight in water and swell in acetic acid (Koob & Vogel, 1987), a fact that has also been verified with pericardium. In contrast, tendon regions which swell more in water and less in acetic acid have relatively high GAG levels. Low levels of PGs are typical in tendons predominantly subjected to tension forces (Koob & Vogel, 1987). However, the content of negative charges is but one of the factors that contribute to the swelling properties of the tissue. Therefore, the type and ratio of collagen, size and organization of fibrils, amount of intermolecular crosslinking and interfibrillary interactions must still be considered, 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, Agrawd & Kramer (1976) and Yannas & Grodzinsky (1973). The topochemical and physicochemical findings presented here enable us to state that the pericardial ECM shows small quantities of PGs.

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 crosslinks deriving from hydroxylysine are more stable than those deriving from lysine and that the great amount of these crosslinks might be responsible for collagen's high insolubility, which also occurs in cardiac valves (Bashey, Bashey & Jimenez, 1978). These authors demonstrated that collagen in the cardiac valves possesses higher quantities of hydroxylysine compared to skin collagen, suggesting that the increase in numbers and in the extension of crosslinks might represent an evolutionary adaptation of collagen, making it capable of withstanding the constant mechanical stress to which it is submitted in the cardiac valves. This fact 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, estimated by converting the amounts of hydroxyproline obtained into amounts of sample collagen. Variations concerning the percentage corresponding to hydroxyproline in the molecule of type I collagen can be found in the literature. Analysis of amino acids in collagen I of porcine pericardium showed that around 9% correspond to hydroxyproline, differing from the reports of Bashey et al. (1978), Slack, Flint & Thompson (1984), Riley et al. (1994), Reddy et al. (1998) and Stehno-Bittel et al. (1998) concerning collagen I of other tissues. Such findings may suggest a functional adaptation of the collagen molecule, possibly with a consequent change in its biomechanical properties, adapting them to the mechano-physiological requirements of the pericardium. The percentages of the remaining amino acids as well as the hydroxyproline/proline ratio of 0.76 verified in the soluble pericardial collagen are compatible with those of type I collagen (Bashey et al., 1978).

The dosage of proteins and GAGs in the total extracts of pericardium showed large amounts of proteins and only traces of GAGs, in total agreement with the topochemical and physicochemical findings that were obtained.

SDS-PAGE of the material eluted from DEAE-Sephacel revealed 16 anionic proteins present in the pericardial matrix, showing apparent molecular masses between 11 and 109 kDa. These proteins perform structural functions and probably regulatory functions as they interact with the various matrix components.

The polydisperse band of 85 kDa found in the pericardium exhibited 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 PG decorin is strengthened by the result of electrophoresis in agarose-PDA gel after β-elimination of the chromatography fractions containing the 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 by chondroitinase AC. It is known that decorin in soft tissues possesses DS and not CS, such as found in bone decorin (Fisher, 1999). The presence of small PGs like decorin should be expected in the pericardial matrix since it probably takes part in the regulation of collagen fibrillogenesis (Brown & Vogel, 1989; Scott, 1996; Iozzo, 1997).

Analysis of the differentiated electrophoretic behavior of proteins of 68 and 75 kDa in the presence of β-mercaptoethanol made it clear that these are not oligomeric proteins but probably proteins with intrachain disulfide bonds that, upon being ruptured, change their conformation and/or dissociate from some other component. Considering the protein of 75 kDa, 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.

Acknowledgement This study was financially supported by Coordenação de Aperfeiçoamento de Pessoal do Ensino Superior, Fundação de Amparo à Pesquisa de São Paulo and Fundação de Apoio à Pesquisa de Minas Gerais, Brazil.

References

Ault NK, Hoffman AH (1992) A composite micromechanical model for connective tissues: Part II. Application to rat tail tendon and joint capsule. J Biomech Eng 114:137–141

Barros PSM, Safatle AMV, Rigueiro M (1999) Experimental lamellar corneal graft in dogs using preserved equine pericardium. Braz J Vet Res Anim Sci 36:71–76

Bashey RI, Bashey HM, Jimenez SA (1978) Characterization of pepsin-solubilized bovine heart-valve collagen. Biochem J 173:885–894

Beeley JG (1985) Glycoprotein and proteoglycan techniques, vol 16. Elsevier, New York, pp 268–270

Blum B, Beier H, Gross HJ (1987) Improved silver staining of plant proteins RNA and DNA in polyacrylamide gels. Electrophoresis 8:93–99

Bradford MM (1976) A rapid sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein dye binding. Ann Biochem 72:248–254

Brown DC, Vogel KG (1989) Characteristics of the in vitro interaction of a small proteoglycan (PGII) of bovine tendon type I collagen. Matrix 9:468–479

Chanda J, Kuribayashi R, Abe T (1997) Use of glutaraldehyde-chitosan treated porcine pericardium as a pericardial substitute. Biomaterials 17:1087–1091

Dietrich CP, Dietrich SM (1976) Electrophoretic behaviour of acidic mucopolysaccharides in diamine buffers. Anal Biochem 70:645–647

Farndale RW, Buttle DJ, Barret AJ (1986) Improved quantitation and discrimination of sulphated glycosaminoglycans by use of dimethylmethylene blue. Biochem Biophys Acta 883:173–177

Fentie IH, Allen DJ, Schenck MH, Didio LJA (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 LW (1999) Decorin. In: Kreis T, Vale R (eds) Extracellular matrix, anchor and adhesion proteins. Oxford University Press, New York, pp 241–254

Gathercole LJ, Keller A (1991) Crimp morphology in fibre-forming collagens. Matrix 11:214–234

Grodzinsky AJ (1983) Electrochemical and physicochemical properties of connective tissue. CRC Crit Rev Biomed Eng 9:133–199

Heinegård D, Pimentel ER (1992) Cartilage matrix proteins. In: Kuttner KE, Scheleyerbach R, Peyron JG, Hascall VC (eds) Articular cartilage and osteoarthritis. Raven Press, New York, pp 95–111

Heinegård D, Sommarin Y (1987) Proteoglycan: an overview. Methods Enzymol 144:319–373

Hollinshead WH (1980) O sistema circulatório. In: Hollishead WH (ed) Livro texto de anatomia humana. Harper & Row do Brasil, São Paulo, pp 82–83

Iozzo RV (1997) The family of the small leucine-rich proteoglycans: key regulators of matrix assembly and cellular growth. Crit Rev Biochem Mol Biol 32:141–174

Jorge-Herrero E, Fernandez P, Turnay J, Olmo N, Calero P, Garcia R, Freile I, Castillo-Olivares JL (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 dodecyl sulfate-polyacrylamide gel electrophoresis. J Biol Chem 244:4406–4412

Koob TJ, Vogel KG (1987) Site-related variations in glycosaminoglycan content and swelling properties of bovine flexor tendon. J Orthop Res 5:414–424

Kuc IM, Scott PG (1997) Increased diameters of collagen fibrils precipitated in vitro in the presence of decorin from various connective tissues. Connect Tissue Res 36:287–296

Laemmli VK (1970) Cleavage of structural proteins during the assembly of the head of bacteriophage Ty. Nature 227:680–685

Langdon SE, Chernecky R, Pereira DA, Lee JM (1999) Biaxial mechanical/structural effects of biaxial strain during crosslinking of bovine pericardial xenograft materials. Biomaterials 20:137–153

Loke WK, Khor E, Wee A, Teoh SH, Chian KS (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 KL, Dalley AF (2001) Torax. In: Moore KL, Dalley AF (eds) Anatomia orientada para a clínica, 4th ed. Rio de Janeiro, Guanabara Koogan, pp 52–151

Olmos ZR, Jasso VR, Sotres VA, Cedillo LI, Arreola RJL, Gaxiola GM (1997) Utilidad del pericárdio bovino tratado com glutaraldeido em lãs resecciones pulmonares no anatômicas em perros. Ver 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 an organotypic culture model. Biomaterials 16:1003–1008

Pires AC, Saporito WF, Leao LEV (1997) Pericárdio bovino utilizado como remendo no sistema cardiovascular. Ver Brás Cir Cardivasc 12:176–187

Reddy GK, Gum S, Stehno-Bittel L, Enwemeka CS (1998) Biochemistry and biomechanics of healing tendon: Part I. Effects of rigid plaster casts and functional casts. Med Sci Sports Exerc 30:794–800

Riley GP, Harrall RL, Constant CR, Chard MD, Cawston TE, Hazleman BL (1994) Tendon degeneration and chronic shoulder pain: changes in the collagen composition of the human rotator cuff tendons in rotator cuff tendinitis. Ann Rheum Dis 53:359–366

Sacks MS, Chuong CJC, More R (1994) Collagen fiber architeture of bovine pericardium. ASAIO J 40:M632–M637

Scott JE (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 NA (1989) Bovine pericardial proteoglycan: biochemical, immunochemical and ultrastructural studies. Matrix 9:301–310

Slack C, Flint MH, Thompson BM (1984) The effect of tensional load on isolated embryonic chick tendons in organ culture. Connect Tissue Res 12:229–247

Stegemann H, Stalder K (1967) Determination of hydroxyproline. Clin Chim Acta 18:267–273

Stehno-Bittel L, Reddy GK, Gum S, Enwemeka CS (1998) Biochemistry and biomechanics of healing tendon: Part I. Effects of rigid plaster casts and functional casts. Med Sci Sports Exerc 30:788–793

Urban JPG, Maroudas A, Bayliss MT, Dillon MT (1979) Swelling pressures of proteoglycans at concentrations found in cartilaginous tissues. Biorheology 16:447–464

Vidal BC (1966) Macromolecular disorientation in detached tendons. Protoplasma Bd 62:121–131

Vidal BC (1980) The part played by proteoglycans and structural glycoproteins in the macromolecular orientation of collagen bundles. Cell Mol Biol 26:415–421

Vidal BC (1984) Ordem molecular y haces de colageno. Trab Inst Cajal 75:19–27

Vidal BC (1987) Métodos em Biologia Celular. In: Vidal BC, Mello MLS (eds) Biologia Celular. São Paulo, Atheneu, pp 5–34

Vidal BC (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 BC (2003) Image analysis of tendon helical superstructure using interference and polarized light microscopy. Micron 34:423–432

Vidal BC, Mello MLS (1972) Anisotropic properties of toluidine blue-stained collagen. Ann Histochim 18:106–122

Viswanadham RK, Agrawd DC, 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 DR, Perkins DG, Canham PB (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 IV, Grodzinsky AJ (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-polyacrylamide gel electrophoresis. In: Genes and antigens of parasites. Rio de Janeiro, Fiocruz, pp 357–363