Int. J. Morphol.,
29(3):907-913, 2011.

The Microfibril-Elastin Fiber System Distribution in Left Atrioventricular Valve of the Rat

Distribución del Sistema de Microfibrillas y fibras de Elastina en la Valva Atrioventricular Izquierda de la Rata

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ROCHA, V. N.; NETO-FERREIRA, R.; MANDARIM-DE-LACERDA & CARVALHO, J. J. The microfibril-elastin fiber system distribuition in left atrioventricular valve of the rat. ***Int. J. Morphol., 29(3)***:907-913, 2011.

SUMMARY: The microfibril-elastin fiber system, an important constituent of the extracellular matrix, was studied in the rat left atrioventricular valve to investigate the interrelationship of oxytalan, elaunin and elastic fibers in left atrioventricular valve morphology. The elastin fibers forms continuous bundles observed along the length of the valve in atrial and ventricular layers and oriented parallel to endothelium. The elaunin and oxytalan fibers are distributed in the thickest fiber bundles along the length of the valve. The thinner fibers which radiated towards both the atrial and spongiosa layers, either as isolated or arborescent fiber bundles were identified as oxytalan fibers. With transmission electron microscopy elastic fibers were seen mainly in the atrial layer. The spongiosa layer was composed of elaunin and oxytalan fibers and ventricular layer showed elaunin fibers arranged in continuous bundles parallel to the endothelium. Both fibrillin and elastin were seen and identified by immunocytochemistry with colloidal gold in the left atrioventricular valve spongiosa and atrial layers. These observations allow us to suggest that the microfibril-elastin fiber system plays a role in the mechanical protection and maintenance of the integrity of the rat left atrioventricular valve.

KEY WORDS: Elastic fibers; Elaunin fibers; Oxytalan fibers; Left atrioventricular valve.

INTRODUCTION

Healthy heart valves maintain unidirectional blood flow and the anisotropic matrix architecture assures sustained and adequate function under high-pressure conditions that remodel in response to changes in local mechanical forces (Mendelson & Schoen, 2006; Balguid *et al*., 2007; Butcher *et al.*, 2011).

In humans, the left atrioventricular valve has four distinct layers: a) atrial - situated deep to the subendocardium of the atrial surface and is rich in elastic fibers, b) spongiosa - composed of proteoglycans and glycosaminoglycans, c) fibrosa - composed of densely packed collagen, and d) ventricular - situated deep to the subendocardium of the ventricular surface and has elastic fibers (more delicate and shorter than atrial layer) (Mendelson & Schoen).

However, information is scarce about the microfibril-elastin fiber system in the left atrioventricular valve. The microfibril-elastin fiber system is composed of two main components: fibrillin and elastin. The proportion and distribution of these components differentiates three types of fibers, the oxytalan, elaunin and elastic fibers, which together form the microfibril-elastin fiber system. The oxytalan fibers are constituted only by microfibrils, the elaunin fibers are constituted of microfibrils and small quantities of elastin dispersed between microfibrils, and the elastic fibers have great quantities of elastin between microfibrils. The elastic, elaunin and oxytalan fibers, maintain the resilience of local tissue requirements (Montes, 1996; Ramirez, 2000; Ushiki, 2002).

Elastic fibers play a role in connective tissues that are normally subject to stretch and expansible forces, because they provide the mechanical basis of numerous body functions, such as respiration (Ramirez & Sakai, 2010), phonation (Riede *et al.*, 2010), and maintenance of vascular

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tone (Ramirez & Sakai). Elastic fibers are made of fibrillin microfibrils, which surround and are embedded in an amorphous core of elastin (Ramirez & Dietz, 2009). The microfibrils are composed of several distinct glycoproteins including fibrillin (Hubmacher *et al*., 2008), which occur as isolated bundles or randomly intermingled with variable amounts of elastin (Alexander & Garner, 1983). Among the microfibrillar molecules, fibrillin-1 and fibrillin-2 are the best characterized (Kielty, 2006) but other members of the fibrillin family play important roles in the elastic fiber development (Chapman *et al.*, 2010; Ramirez & Sakai). Mature elastic and elaunin fibers have microfibrillar and amorphous matrix components (elastin), while oxytalan fibers contain only microfibrils (Prost-Squarcioni *et al.*, 2008).

The microfibril-elastin fiber system was investigated to determine the interrelationship of the oxytalan, eulanin and elastic fibers in the rat left atrioventricular valve. These results should help investigations of tissue-engineered heart valves and valve disease.

MATERIAL AND METHOD

Sample and Procedures. Ten male Wistar rats (weight between 300-350 g), obtained from colonies of the State University of Rio de Janeiro, were kept in standard housing conditions (21±2ºC, humidity 60±10%, 12:12h dark-light cycle) and received water and standard rodent chow (Nuvilab, Parana, Brazil) *ad libitum.* All procedures were approved and carried out in accordance with conventional guidelines of "Care and Use of Laboratory Animals" (US National Institutes of Health, revised 1996).

Rats were anesthetized with intraperitoneal injection of sodium pentobarbital (50 mg/kg) and subsequently euthanized by exsanguination. The thorax was opened and the heart excised and washed in sodium chloride solution (0.9 %). The heart was opened and the parietal leaflet was identified. One fragment of approximately 2 mm thick of the central part of the parietal leaflet of each animal was used. The section was performed to atrio-ventricle being removed part of the atrial and ventricular myocardium including the endocardium and every valvar segment, from insertion until the free edge. Each fragment was processed for light (n = 6) or electron (n = 4) microscopy.

Light Microscopy. Six parietal leaflet fragments were fixed in 4% paraformaldehyde in 5mM $CaCl_2$ and 0.1% glutaraldehyde and processed following routine histological procedures and embedded in paraplast plus (Sigma-Aldrich, St. Louis, USA) and sectioned (3 μm). The sections were stained with orcinol-new fuchsin for identification of elastic fibers. Another sections were stained with Weigert's resorcin-fuchsin, with or without pre-oxidation with potassium peroxymonosulfate (oxone) to identify oxytalan and elaunin fibers, respectively (Fullmer, 1960).

Electron Microscopy. Four parietal leaflet fragments were fixed in 2.5 % glutaraldehyde (Riedel-de-Haen, Germany) in 0.1 M cacodylate buffer (pH 7.2) and 0,25% tannic acid (Merck, Germany) (Cotta-Pereira *et al*., 1976a). After fixation (at least 12 hours) the samples were rinsed three times (15 min each) in 0.1 M cacodylate buffer and pos-fixed in 1% osmium tetroxide (Sigma-Aldrich, St. Louis, USA) with 0.8% potassium ferricyanide in 0.1 M cacodylate buffer for one hour, then rinsed three times (15 min each) in 0.1 M cacodylate buffer. After rinsing, dehydration was through a graded series of acetone (30%, 50%, 70%, 90% and twice in 100%), samples were embedded in Epon (Embed-812). Semi-thin sections (1 μm) were cut and stained with toluidine blue (Vetec, Rio de Janeiro, Brazil) and observed with a light microscope (Carl Zeiss, Oberköchen, Germany). Ultrathin sections were obtained from selected areas (central region) with ultramicrotome (Leica Ultracut-UCT, Leica Mikrosysteme Gmbh, Austria), counterstained with uranyl acetate and lead citrate, and examined with a Zeiss EM 906 transmission electron microscope (Carl Zeiss, Oberköchen, Germany) at 80 Kv.

Immunostaining techniques. Samples of 1 mm thickness from the central part of the parietal valve fixed in 4% paraformaldehyde in 5mM CaCl2 and 0.1% glutaraldehyde were washed three times (15 min each) in 0.1 M phosphate buffer and dehydrated into 30%, 50%, 70%, 90% ethanol and twice in absolute ethanol (1 hour in each). Samples were gradually infiltrated with LR White resin (Acrylic Resin, London Resin Company, England) and finally polymerized at 37°C for 48 hours. Ultra-thin sections were obtained from selected areas with a diamond knife and collected on 300-mesh nickel grids and reacted with antibodies as described below.

Sections were washed by floating first on 50 mM NH4Cl in 0.1 M phosphate-buffer pH 7.2 (PBS) for 15 min, second on phosphate-buffer saline pH 7.2 containing 3% bovine serum (PBS/BSA 3%) for 20 min and finally on phosphate-buffer saline containing 1% bovine serum (PBS/BSA 1%) for 10 min. Next, sections were incubated for 2 hours in primary antibody to show fibrillin-1 (Santa Cruz Biotechnol., code sc-20084, rabbit polyclonal antibody, CA, USA) or elastin (Santa Cruz Biotechnology, code sc-17580, goat polyclonal antibody, CA, USA) both diluted 1:50 in PBS/BSA 1%. To remove unbound antibody, the grids were

washed sequentially in PBS/BSA 1%, PBS/BSA 3% and PBS (two washes of 5 minutes each). The grids were incubated in secondary antibody protein-A 10 nm colloidal gold labeled (Sigma-Aldrich, St. Louis, USA, code P-1039) diluted 1:50 in PBS for 1 hour. The unbound secondary antibody was removed by washing in PBS and after in distilled water. Finally, the grids were counterstained with uranyl acetate and lead citrate, and examined using a Zeiss EM 906 transmission electron microscope at 80 Kv. The following controls were employed: (a) for negative controls, treatment with primary antibody was omitted, and (b) for positive controls Wistar rat aortic sections were used.

RESULTS

Fibers of varying diameters were observed with light microscopy. With orcinol new-fuchsin stain, continuous bundles of fibers were observed along the length of the valve in atrial and ventricular layers oriented parallel to endothelium (Fig. 1 a). Sections without oxidation and stained by Weigert's resorcin fuchsin showed the thickest fiber bundles along the length of the valve (Fig. 1 b). After oxidation, the thicker bundles show thin oxytalan fibers in a fan-like radiation towards to both the atrial and spongiosa layers, either isolated or as arborescent fiber bundles (Fig. 2a and b).

Under transmission electron microscopy, elastic fibers predominated in the mitral atrial layer, characterized by large amounts of elastin (Fig. 3a). In addition, the spongiosa layer was composed mainly by elaunin fibers formed by microfibrils interspersed with elongated patches

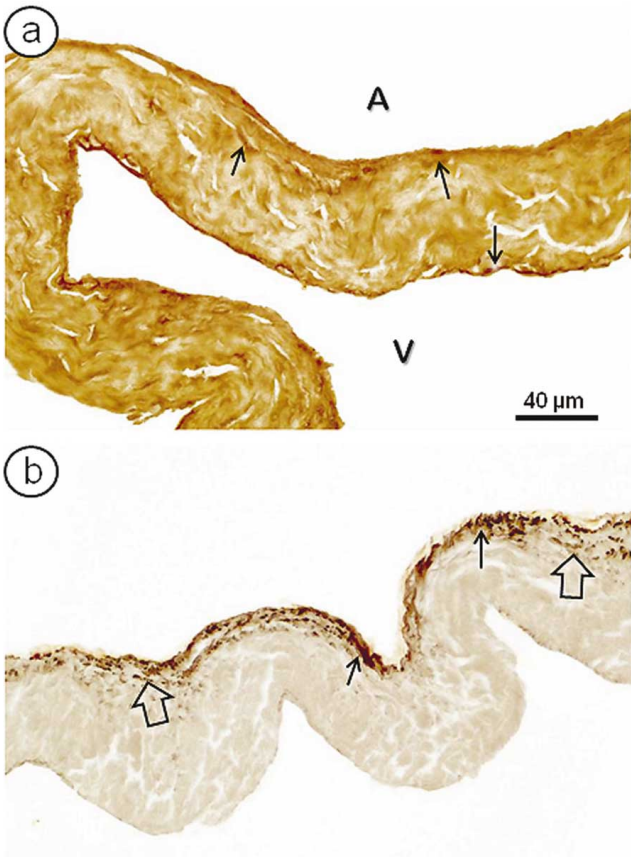

Fig. 1. Photomicrograph, parietal leaflet longitudinal section: (a) elastic fibers (arrows) deep to endocardium of the atrial (A) and ventricular (V) surfaces and dispersing in the espongiosa layer. Orcinol new-fuchsin stain, Magnification 600X, bar 40 µm. (b) elastic fibers (arrows) deep to atrial endocardium (A) and elaunin fibers (open arrows) the spongiosa layer. Weigert's resorcin-fuchsin stain without prior oxidation, bar 40 µm.

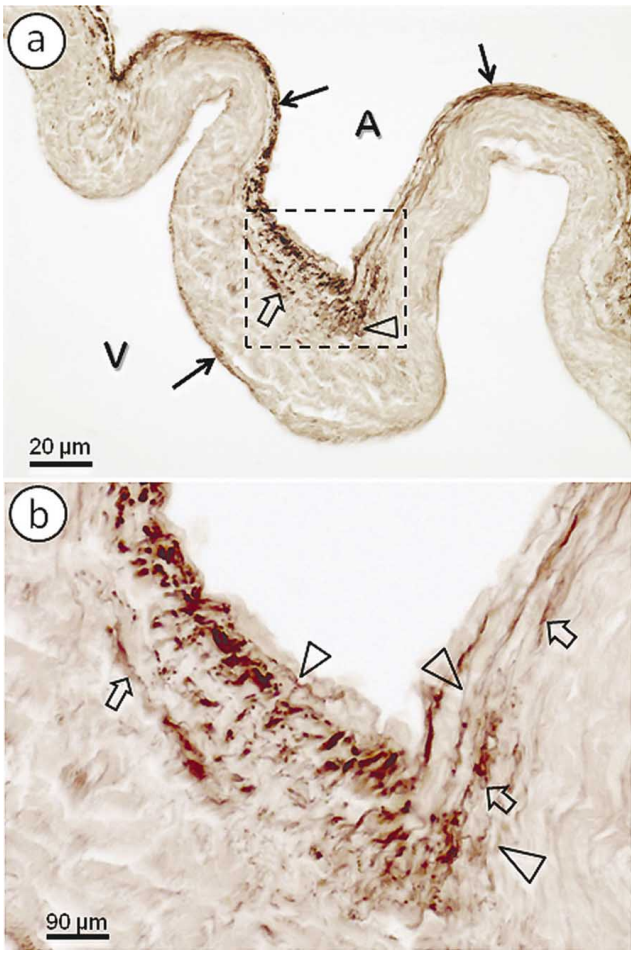

Fig. 2. Photomicrograph, parietal leaflet longitudinal section: (a) elastic fibers (thin arrows) beneath endothelial cells of the atrial (A) and ventricular (V) layers, elaunin (open arrow) and oxytalan (head arrow) fibers are seen in the spongiosa layer; (b) enlargement of rectangular area of the previous figure showing elaunin (open arrows) and oxytalan (head arrows) fibers in the spongiosa and atrial layers. Weigert's resorcin-fuchsin with prior oxidation with oxone stain, bar 40 µm.

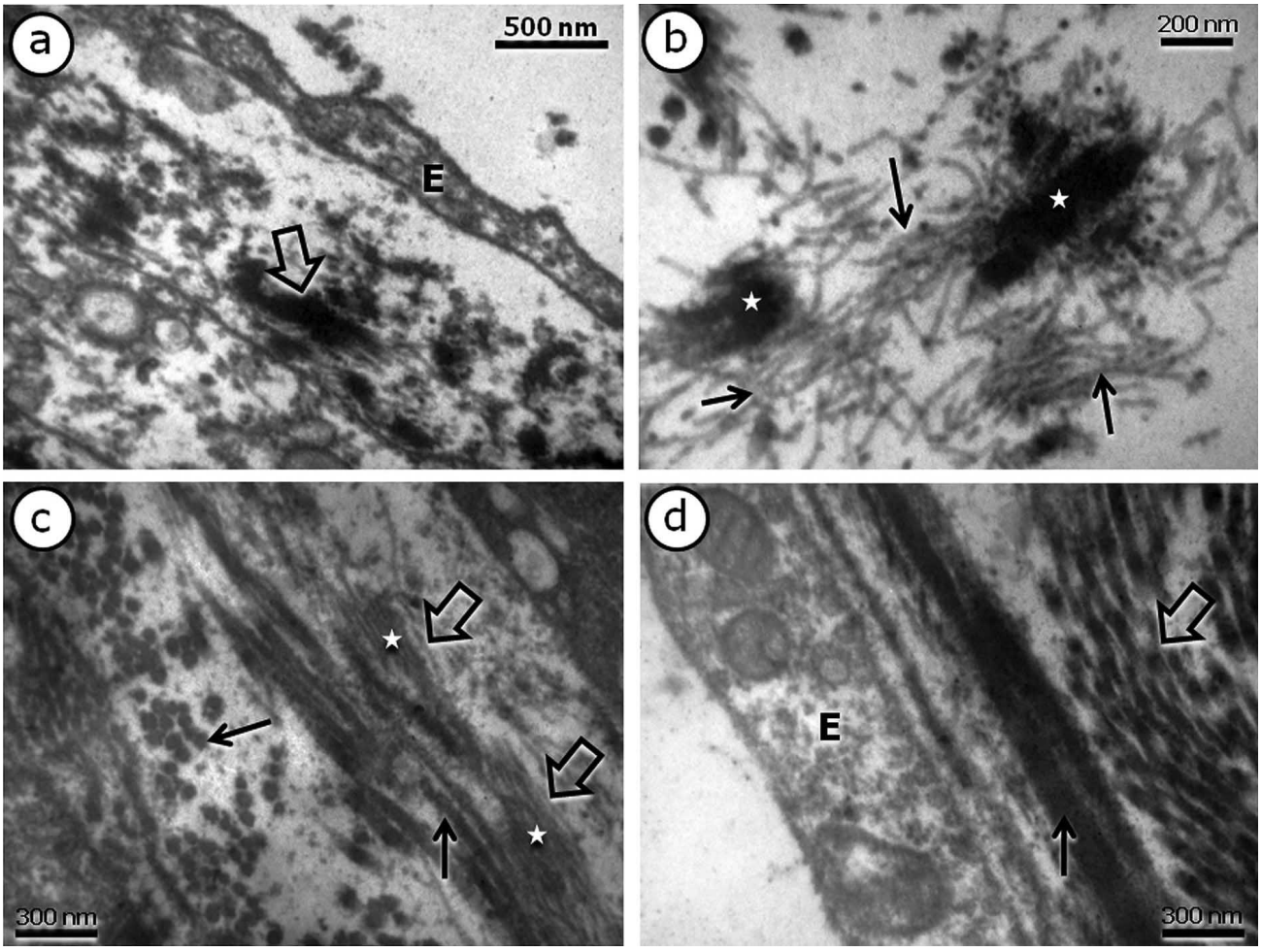

Fig. 3. Electron micrographs of the rat parietal leaflet: (a) atrial and part of spongiosa layers showing the endothelium (E) and elastic fibers (open arrows); (b) spongiosa layer with microfibrils (arrows) intermingled with variable amounts of elastin (stars) of the elaunin fibers; (c) extracellular matrix with microfibrils (open arrows) of the oxytalan fibers (stars) near to collagen fibers (arrows); (d) continuous elaunin fibers (arrow) bundles parallel to the endothelial cells (E) of the ventricular layer. The collagen fibrils (open arrow) are localized beneath the elaunin fibers.

of amorphous material (Fig. 3b), and oxytalan fibers characterized by large bundles of microfibrils without elastin (Fig. 3c). The rat left atrioventricular valve ventricular layer showed elaunin fibers arranged in continuous bundles with parallel direction to the endothelium (Fig. 3d). Both fibrillin (Fig. 4a) and elastin (Fig. 4b) were seen in the left atrioventricular valve spongiosa and atrial layers. They were identified by the colloidal gold particles of the immunocytochemistry technique.

DISCUSSION

It is known that the essential extracellular matrix components of the left atrioventricular valve include glycoproteins, collagen and elastin (Tamura *et al.*, 1995; Vesely, 1998). The glycoproteins serve predominantly as a shock and shear absorber, while the collagen fibers comprise the strongest portion of the leaflet (Mendelson & Schoen; Vesely, 2005). The elastin maintains a specific collagen fiber configuration and returns the fibers to this state, once external forces have been released (Vesely, 1998; Mendelson & Schoen; Patel *et al*., 2006).

These components of the extracellular matrix have different roles in the valve microstructure. The glycoproteins act to resist compressive forces and dissipate shocks during valve closure. The collagen components of the left atrioventricular valve provide the mechanical strength required to maintain functionality under rigorous conditions of the cardiac cycle (Mendelson & Schoen). The elastin

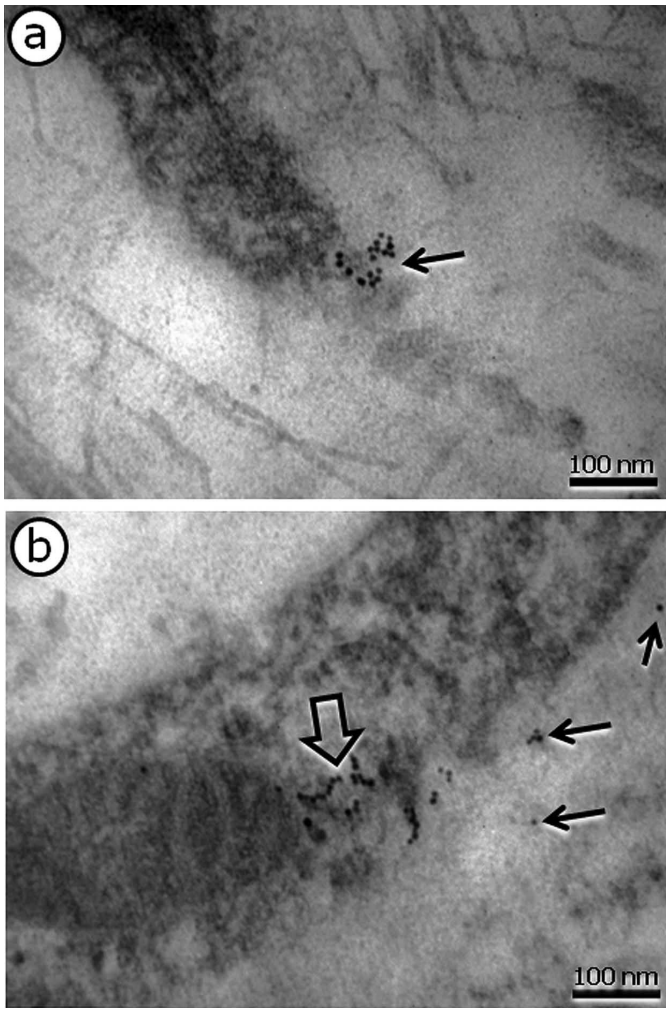

Fig. 4. Immuno-electron micrographs with antibody-coated gold particles (arrows): (a) localization of fibrillin in the extracellular matrix; (b) localization of elastin in both cell (open arrow) and extracellular matrix (arrow).

provides resilience to the valve during opening and closure returning the collagen structures back to their resting states between loading cycles (Vesely, 2005; Flanagan *et al.*, 2006). Besides the essential components of the extracellular matrix in parietal valve, we showed in this work the existence of oxytalan and elaunin fibers, which constitute the microfibril-elastin system, suggesting that microfibril-elastin system is important in valve architecture maintenance.

In the present study, a heterogeneous distribution of the microfibril-elastin system was observed in the rat left atrioventricular valve. Distinct morphological networks of the elastic system are usually present in the extracellular matrix of most organ systems, being particularly abundant in tissues that are subjected to mechanical stress such as the skin, mainly at the dermoepidermal junction, where the elastic system is involved in anchoring the epidermis to the dermis (Cotta-Pereira *et al*., 1976b). In parietal valve, we found a higher amount of oxytalan and elaunin fibers in the ventricular region, possibly because to be a region subject to a constant mechanical stress caused by blood pressure.

The microfibril-elastin system thus plays a role in distributing stress forces uniformly in tissues (Montes). The oxytalan fibers composed of microfibrils devoid of elastin are widely distributed in the connective tissue where they interconnect elastic fibers to other components of the extracellular matrix and surrounding cells (Ramirez; Ushiki). We also observed, oxytalan and elaunin fibers between the atrial and fibrous layers of the mitral spongiosa layer, possibly providing a link between the elastic fibers of the atrial layer and the collagen fibers of the fibrous layer. Such pattern of distribution is indicative that collagen, in coevolution with the elastic system (and other macromolecules of the extracellular matrix), largely contribute to accommodate functional diversity (Montes).

Stretching increased the levels of fibrillin-1 and fibrillin-2, but did not affect the gene expression level of either type of fibrillin. Bundles of oxytalan fibers became thicker under stretching conditions. Therefore, tension strain functionally regulates the microfibril assembly in periodontal ligament fibroblasts and thus may contribute to the homeostasis of oxytalan fibers in periodontal ligaments (Tsuruga *et al*., 2009). Likewise, increased neutrophil elastase observed in periodontal disease degrades the oxytalan fibers and interfibrillar substances in the periodontal ligament to decrease its mechanical strength (Ujiie *et al*., 2008).

In the present study, oxytalan and elaunin fibers were observed in the region under great mechanical stress (left atrioventricular valve ventricular layer). These fibers were also found in the mitral spongiosa layer, between different components of the atrial (elastic fibers) and fibrous (collagen fibers) layers. These observations allow us to suggest that the microfibril-elastin fiber system plays a role in the mechanical protection and maintenance of the integrity of the rat left atrioventricular valve.

ACKNOWLEDGEMENTS

The authors would like to thank Ana Lucia R. Nascimento and Alan C. N. de Moraes for their technical assistance. This work was sponsored by the Brazilian agencies CAPES, CNPq, and FAPERJ.

ROCHA, V. N.; NETO-FERREIRA, R.; MANDARIM-DE-LACERDA & CARVALHO, J. J. Distribución del sistema de microfibrillas y fibras de elastina en la valva atrioventricular izquierda de la rata. ***Int. J. Morphol., 29(3)***:907-913, 2011.

RESUMEN: Fue estudiado el sistema de fibras microfibrillas-elastina, un componente importante de la matriz extracelular, en la valva atrioventricular izquierda de rata, con la finalidad de investigar la interrelación de oxitalán, elaunin y fibras elásticas en la morfología de dicha valva. Las fibras de elastina forman paquetes continuos a lo largo de la valva en las capas atriales y ventriculares, orientadas paralelamente al endotelio. Las fibras de elaunin y oxitalán se distribuyen en haces de fibras más gruesas a lo largo de la valva. Las fibras más delgadas, las cuales se irradiaban hacia las capas atrial y esponjosa, ya sea como haces de fibras aisladas o arborescentes, fueron identificadas como fibras oxitalán. En la capa atrial a través de microscopía electrónica de transmisión se observaron principalmente fibras elásticas. La capa esponjosa estaba compuesta por fibras de elaunin y oxitalán; la capa ventricular mostró fibras de elaunin dispuestas en haces continuos paralelos al endotelio. Tanto fibrilina y elastina se observaron e identificaron por inmunocitoquímica con oro coloidal en las capas esponjosa y atrial de la valva atrioventricular izquierda. Estas observaciones nos permiten sugerir que el sistema de fibras de elastina-microfibrillas tienen participación en la protección mecánica y la mantención de la integridad de la valva atrioventricular izquierda en la rata.

PALABRAS CLAVE: Fibras elásticas; Fibras de elaunin; Fibras de oxitalán; Valva atrioventricular izquierda.

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Received: 31-03-2011
Accepted: 14-06-2011