ORIGINAL ARTICLE

QUANTIFICATION OF LEFT VENTRICULAR MYOCARDIAL COLLAGEN SYSTEM IN CHILDREN, YOUNG ADULTS, AND THE ELDERLY

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Abstract Studies on the collagen system of the human myocardium are still limited compared to those on small laboratory animals. The aim of this work was to observe the collagen tissue of the myocardium of the human heart as a function of age. The types of collagen, as well as the density of collagen tissue and the diameter of collagen fibrils, were examined. Fragments of the left ventricular wall from 15 hearts, 5 from children, 5 from young adults, and 5 from elderly individuals, were analyzed by using the Picrosirius-polarization method and by transmission electron microscopy (TEM). The results showed the presence of collagen type III and collagen type I, both in the endomysium and perimysium of the 3 groups studied. Measurements of collagen content in myocardial tissue displayed that both endomysial and perimysial collagen increase in number and thickness in the adult and elderly. These histochemical results coincided with the observations obtained with the electron microscope in showing an increase in the number of collagen fibrils with a large diameter in the adult and elderly hearts. The present results on cardiac collagen may be important for assessing the pathogenesis of several cardiopathies in the hearts of children, young adults, and the elderly.

Key words: collagen fibrils, morphometry, human heart, children, young adult, elderly

Resumen ***Cuantificación del sistema de colágeno del ventrículo izquierdo del miocardio en niños, adultos jóvenes y ancianos***. Los estudios sobre el colágeno del miocardio humano son aún escasos en comparación con los hechos en pequeños animales de laboratorio. El objetivo de este trabajo fue cuantificar el tejido colágeno del miocardio del corazón humano en función de la edad. Se estudiaron los tipos de colágeno, su densidad y el diámetro de las fibrillas de colágeno. Para esto se utilizaron fragmentos de la pared del ventrículo izquierdo de 15 corazones, cinco de niños, cinco de adultos jóvenes y 5 de personas de edad avanzada. Las muestras se analizaron mediante el método de Picrosirius-polarización y por microscopía electrónica de transmisión (MET). Los resultados mostraron la presencia de colágeno tipo III y de tipo I, tanto en el endomisio como en el perimisio de los tres grupos estudiados. Además, aumenta el colágeno tanto en el endomisio como en el perimísio, así como su número y grosor a medida que aumenta la edad. Los resultados histoquímicos coincidieron con las observaciones obtenidas con el microscopio electrónico, en las que se observa un aumento en el número de fibrillas de colágeno de gran diámetro en los corazones de los adultos y los ancianos. Estos resultados podrían ser importantes para la evaluación de la patogénesis de varias cardiopatías en los corazones de niños, jóvenes y ancianos.

Palabras clave: fibrillas de colágeno, morfometría, corazón humano, niños, jóvenes adultos, ancianos

Collagen is an important component of the interstitial matrix of the myocardium. Collagen fibers surround and support cardiomyocytes and coronary microcirculation[1, 2]. Therefore, the distribution of the collagen tissue of the myocardium is such that it plays an important role in the elastic properties of the left ventricle. The major types of collagen present in the myocardium of the left ventricle are I and III, with type I predominating[3].

The total collagen content of some tissues has been found to increase with aging[4-6]. However, quantitative studies on the collagen fibrils of the human myocardium as a function of age are scarce[4, 7]. Our group has previously demonstrated that in the hearts obtained from old subjects, there were no significant differences in the arrangement of the collagen fibers in relation to the hearts obtained from young adult subjects. Furthermore, measurements of collagen content in myocardial tissue suggest that both perimysial and endomysial collagen type I fibers increase in number and thickness in the old individuals[8].

However, the pool of comparison between collagen deposition and organization in children, young adults and elderly has been poorly investigated, and its detailed

structure in the normal state in these different age groups is required. In this sense, the aim of the present work was to determine the types of collagen, to measure the collagen content, and the collagen fibril diameters of the left ventricle of the human heart and to observe any differences among children, young adults, and the elderly.

Materials and Methods

Small fragments of myocardial tissue were dissected from the anterior wall of the LV of 5 children aged 1 year old (G1), 5 adults aged 28 years old (G2), and 5 elderly subjects aged 80 years old (G3) at postmortem examination (12 hours after death). The precise age of all the subjects was known. In each case, the immediate cause of death was unrelated to specific chronic heart conditions such as hypertension. All of the samples were from male individuals. Hearts with apparent signs of diseases were excluded.

Collagen content was determined by light microscopy in samples that were fixed in 10% Bouin's solution for 24 h, included in paraffin and used to make tangential histological sections of 5 μm. The sections were stained with the Picrosirius technique[9] and studied with polarized light. The best results were obtained when high light intensity was used. When studied by this method, the tissues containing collagen type I showed thick fibers, intensely birefringent, of yellow or red colors. Collagen type III appears as thin, pale (weakly birefringent), greenish fibers[10].

To determine the range of collagen content in the 3 groups, histological sections were analyzed with the KS-400 digital analyzing computer program (Zeiss), capable of quantifying, in percentage/area (of 12 000 μm^2) green, red, or yellow collagen fibers. Three sections per heart were analyzed. For that, the microscope was dotted with appropriate polarization lenses. In each section, 5 randomized fields were used, and the percentage of collagen fibers was quantified in each field. The whole collagen fibers of 225 fields were quantified in the child, young adult, and elderly left ventricles. Histograms were obtained, the means calculated, and the data tested for significance ($P < 0.05$).

To determine the diameter of collagen fibrils, the samples were fixed for about 12 hours in 2.5% glutaraldehyde in 0.1 M phosphate buffer, post fixed in 1% osmium tetroxide in phosphate buffer, dehydrated through a graded series of ethanols and propylene oxide, and embedded in Epon resin. Ultrathin sections of the samples were stained with uranyl acetate[11], and lead citrate[12], and viewed in an electron microscope. Ten micrographs for each of the hearts were obtained from regions where the fibers were transversely sectioned. The final magnification was x 130 000. The minimum diameter of each fibril was measured with the Kontron S-400 image analyzer program (Zeiss). Commencing at one corner of each field and radiating out in an arc, the diameter of the fibrils present in the field was determined. A total of 9 000 fibrils were measured in the 3 groups.

Statistical significance was evaluated by ANOVA and *post hoc* Scheffe's test, and *P* values of less than 0.05 were considered significant.

Results

In the child hearts, the sections stained with Picrosirius and examined with polarization microscopy showed, in the epimysium, yellow or red, thick strongly birefringent collagen fibers characteristic of collagen type I and also thin fibers of green color, typical of collagen type III (Fig. 1A, 1A'). Between the cardiac myocytes, the endomysium from the child group had the same composition but with thin green fibers predominating. The same results were obtained for the hearts of the adult group, but thick, red strongly birefringent fibers predominate, both in the endomysium and perimysium (Figs. 1B, 1B'). In the elderly group, thin, pale fibers greenish in color were scarce, and red strongly birefringent collagen fibers were predominant, both in the endomysium and perimysium (Fig. 1C, 1C').

Statistical analysis comparing the quantitative results obtained for the 3 groups showed that there was a significant difference ($P < 0.05$) in collagen content (2.1 ± 0.1%) in child hearts compared with that obtained in adult hearts (3.2 ± 0.2%) and elderly hearts (5.6 ± 0.3%). These results show a progressive increase of collagen content in the left ventricle with growth and aging.
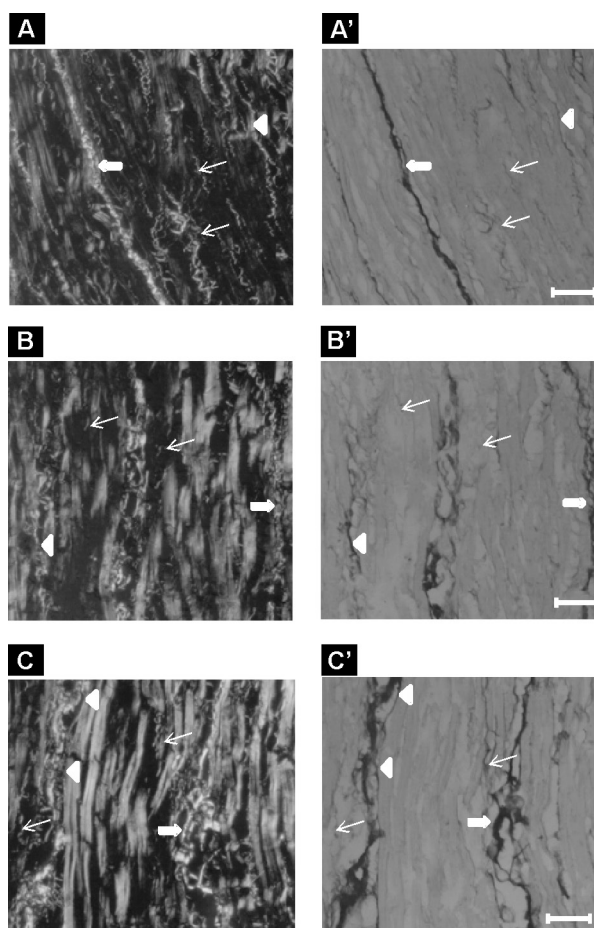

Fig. 1.– Histological sections of the human myocardium stained with Picrosirius and observed with (A, B, and C) and without polarized light (A', B' and C') from child (A, A'), adults (B, B') and elderly (C, C'), showing thin collagen fibers of a green color (collagen type I of the endomysium (thin arrows) and perimysium (arrowheads) and collagen fibers of red color (collagen type I) of the perimysium (thick arrows). Scale bar: 100 μm.
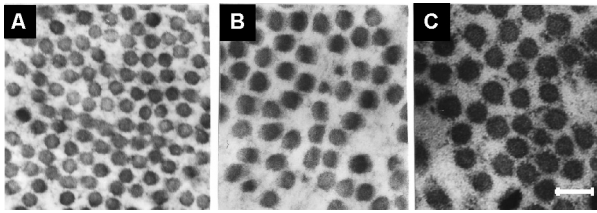
Fig. 2.– Electron micrographs of human myocardial interstitium to show collagen fibrils sectioned transversely, from a child heart (A), adult heart (B) and old heart (C). The fibrils show good preservation. The majority of collagen fibrils from the adult and old hearts (B, C) are larger in diameter than the fibrils from the child heart (A) at the same magnification. Scale bar: 0.1 µm.
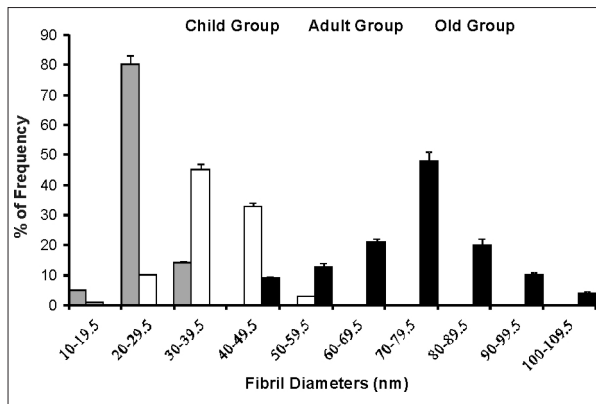

Fig. 3 – Histograms of the distribution of fibril diameters for most of the collagen fibers in the human myocardium from child, adults and old. On the abscissa, the diameters of the fibrils in nanometers, on the ordinate the percentage. Fifteen individuals and 9 000 fibrils.

Electron microscopic studies have shown collagen fibers composed of closely packed fibrils. In the child hearts, collagen fibers were composed mainly of fibrils of small diameter (Fig. 2A). In the adult group, the collagen fibers were composed of fibrils of small and large diameter (Fig. 2B), and in the elderly group, the collagen fibers were formed mainly by fibrils of a large diameter (Fig. 2C).

Histograms showing collagen fibril diameter distribution in child, young adult, and elderly hearts appear in Fig. 3. Statistical analysis showed that collagen fibril diameters are greater in the elderly heart compared to the child and young adult hearts and that collagen fibril diameter is greater in the young adult heart in relation to the child heart ($P < 0.05$).

Discussion

There are 3 major findings in this study. First, it was demonstrated that polarized light facilitates quantitative image analysis of collagen from child, adult, and elderly hearts collected in autopsies. Second, there are differences in the amount of collagen fibers in child, young adult, and elderly hearts. Third, there is an apparent increase in collagen type I and a decrease in collagen type III from childhood to old age.

The present study showed that collagen types I and III could be easily quantified in the human cardiac muscle by polarized light in specimens obtained during autopsies. Though the material studied was fixed for some hours after death, the appearance of the fibers suggested good preservation. This method was used previously with the same results[13]. Collagen fiber types were identified by their different color[3]. When examined in a bright field, cardiac collagen has the same characteristics as other tissues: it appears red and cardiac muscle yellow after Picrosirius red staining. This bright-field contrast between collagen and cardiomyocytes has been used, in conjunction with digital image analysis, to assess myocardial collagen content[14]. However, it is the combination of Picrosirius red and polarized light microscopy that provides a more powerful method of identifying collagen fibers[9]. When this technique is used, it provides a convenient and quantitative histological approach that can be used to quantify collagen content[15]. Actually, this method has been successfully used by others to quantify the collagen content in several organs and tissues[16, 17]. The present results confirm that the combination of Picrosirius red and polarized light microscopy provides a powerful method for identifying collagen fibers in the heart[8] and of quantifying the collagen content[14, 15, 18].

The present work revealed the quantitative differences in the amount of collagen fibers in child, young adult, and elderly hearts. The collagen content increases from childhood to adulthood and to old age. Studies in rats also provide consistent evidence of an increase in myocardial collagen associated with growth and aging[19, 20]. Furthermore, measurements of collagen content in myocardial tissue suggest that it is the type I collagen fibers that increase in number and thickness in the aged. At the same time, electron microscopic observations have shown an increase in the number of collagen fibrils with a large diameter in the aging heart[21]. These and the present results suggest a continuous formation of new collagen fibers in the ventricular wall, after birth. However, the exact mechanism responsible for the myocardial fibrosis in the senescent myocardium is unclear

Fibroblasts are the cardiac cells responsible for fibrillar collagen biosynthesis in the heart[20-24]. Synthesis and organization of collagen increases during neonatal development as the heart adapts to increased hemodynamic load. The regulation of fibrillar collagen mRNA levels following pressure overload may result from hemodynamic changes and their impact on cardiac fibroblasts[25]. How fibroblasts organize collagen within the heart is unknown[24]. In this context, an experiment performed by Carver et al.[26] demonstrated that mechanical stretch stimulates collagen

protein and mRNA synthetic rates in cyclically stretched fibroblast.

It is well established that the aging process of the heart is characterized by a loss of myocytes[27]. This reduction occurs because myocytes are postmitotic cells and are not replaced as they die. Thus, the loss of myocytes with age could be one cause of the accumulation of collagen in the wall of the left ventricle. Another possible mechanism for collagen accumulation with age could be an inhibition of collagen degradation[25].

In the present study, we have shown that there is an increase in collagen type I and a decrease in collagen type III with age. As mentioned above, it is possible that it is related to cardiac pressure overload. Then, the increase in cardiac collagen type I may represent a response to increased loading conditions imposed by age-related changes in the blood vessels. The regulation of mRNA levels for fibrillar collagen following pressure overload may result from hemodynamic changes and their impact on cardiac fibroblast[25]. Alternatively, increase in collagen synthesis could be caused by the interaction of released elements, such as hormones, neurotransmitters, and growth factors, that interfere either directly or through a second messenger with the biosynthetic pathway of collagens in cardiac fibroblasts[25, 28-34]. In fact, it has been shown that the renin-angiotensin system and transforming growth factor-β1 (TGF-β1) plays a critical role in the development of cardiac fibrosis[34].

Preliminary studies indicate that TGF-β1 mRNA increases with pressure overload[35]. TGF-β1 is a biologically active peptide present in normal cells, including fibroblasts. The presence of TGF-β1 in normal rat myocardium has been demonstrated[19, 36]. It has been shown that TGF-β1 and elevated glucose levels stimulate the expression of collagen, which shows up in the extracellular matrix[16, 37]. TGF-β_1 stimulates the deposition of collagen, leading to an increased myocardial collagen content[33, 38]. Thus, TGF-β1 possibly increases with pressure overload, which could explain the increase in collagen transcription in adult and elderly hearts.

It is well known that the tensile strength of type I is compared to that of steel, whereas collagen type III has more distensible properties than collagen type I[39]. Because these 2 types of collagen vary with respect to their physical and mechanical properties, a balance between the 2 types of collagen is of utmost importance in maintaining the normal contractility of the myocardium. The collagen network of myocardium is associated with the elastic modulus[40]. This arrangement permits some slippage of the myocytes as diastolic volume increases during cardiac function. However, as the collagen bundles straighten, an upper limit is placed on the distensibility of the ventricle[41]. Thus, the increase in myocardial collagen and type I collagen linkage with aging may contribute to the decrease in the ventricular elasticity with age. In conclusion, there are differences in the amount of collagen fibers in child, young adult, and elderly hearts, with an apparent increase in collagen type I and a decrease in collagen type III from childhood to old age. This study also highlights the importance of the screening in the changes of heart structures involved in aging process, in order to determine the main points of therapeutics interventions to minimize the cardiac dysfunction in elderly.

Conflict of interest: The authors declare that they do not have any conflict of interest.

References

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[…] "Clinical judgment," for instance, is a phrase that has fallen into disgrace, replaced by "evidence-based practice", the practice of medicine based on scientific data. But evidence is not new; throughout our medical education beginning more than three decades ago, we regularly examined the scientific evidences for our clinical practices.[…].

[…]. "Juicio clínico", por ejemplo, es una frase que ha caído en desgracia, reemplazada por "medicina basada en la evidencia", la práctica de la medicina basada en datos científicos. Pero la evidencia no es nueva, a lo largo de nuestra educación médica, comenzada más de tres décadas atrás, regularmente examinamos las evidencias científicas para nuestras prácticas clínicas.

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