Quantitative analysis of types I and III collagens in the anterior and posterior papillary muscles of the left ventricle of infarcted rats

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Abstract

The objective of this study is quantitatively analyze type I and III collagens in the papillary muscles of infarcted rats comparing them with a control group and carry out a immunohistochemical approach to assess the presence of types I, II, III, IV and VII collagens in the infarction scar region and in the rest of the myocardium. This study used 24 rats divided into two groups: infarcted and non-infarcted. After six weeks, the rats of both groups were killed in order to remove the anterior and posterior papillary muscles of the left ventricle. The material was observed under a light microscope, with polarization, to quantify, in percentage, types I and III collagens; one of the infarcted hearts was also randomly selected to undergo an immunohistochemical analysis to identify the presence of types I, II, III, IV and VII collagens in the post-infarction scar region and in the remaining myocardium. The anterior papillary muscles of the infarcted group presented a higher collagen concentration when compared with the control group; the means were found to be between 6.4 and 0.6% respectively. In posterior papillary muscle analysis, the mean for the infarcted group was 3.2 and 0.5% for the control group. Myocardial infarction leads to an increase in the concentrations of types I and III collagens in the anterior and posterior papillary muscles. Immunohistochemistry showed the presence of types I, II, III, IV and VII collagens in the infarction scar region and a lower amount of these same collagens in the rest of the myocardium.

Keywords: myocardial infarction, collagen, immunohistochemistry, papillary muscle, polarization.

1 Introduction

Myocardial collagen concentrations change in heart diseases, especially in some of them: myocardial infarction, essential hypertension, aortic stenosis, renovascular hypertension. Myocardial infarction is followed by remodeling in the infarcted area and the remaining myocardium (ANVERSA, OLIVETTI and MELISSARI, 1979; BRILLA, JANICKI and WEBER, 1991; JANICKI, 1992; WEBER, JANICKI, PICK et al., 1987).Ventricular remodeling is an adaptive response of the heart to hemodynamic and neurohormonal stimuli and genetic factors, associated with a change in heart shape, size, composition and function. Among the etiologies, acute myocardial infarction is currently one of the most important causes of heart remodeling and it contributes significantly to ventricular dilation, myocardial fibrosis, ventricular dysfunction and onset of congestive heart failure (EPIFANIO, ZORNOFF, MATSUBARA et al., 2005).

Post-infarction remodeling is a phenomenon that begins right after coronary occlusion. Myocytic necrosis occurs during this phase and is followed by stretching of the infarcted area, that is, an infarction expansion that eventually results in muscle rupture or formation of a ventricular aneurysm. Approximately 72 hours after the acute event, remodeling encompasses the entire heart. During that period, ventricular dilation, change in geometry, and hypertrophy of the remaining musculature is evident. The inability of the heart to normalize wall stress results in progressive cardiac dilation, recruitment of the myocardium around the scar, and deterioration of the contractile function (SUTTON and SHARPE, 2000).

During the first 24 hours, most humans (94%) present wavy fibers which indicate intracellular edema and 90% present characterized necrosis. When the necrosis phase ends, a deposition of new components in the extracellular matrix begins, forming a "scaffold" for new collagen deposition. Fibronectin, laminin and type IV collagen appear between the third and fourth days of infarction healing in rats

(MORISHITA, KUSACHI, YAMASAKI et al., 1996), approximately at the same time that messenger RNA for type III (first) and type I (a little later) is detected (CLEUTJENS, VERLUYTEN, SMITHS et al., 1995).

A study shows that collagen content increases steadily from the first to the sixth week after an experimental infarction in some mammals. Necropsy observations indicate the same amount of time for human myocardium. In rats, collagen begins to increase around the fourth or fifth day after infarction and continues to increase for approximately three weeks. The post-infarction healing process includes a mixture of types I and III collagens and other minor subtypes. Apparently, an initial network of type III collagen forms a basic structure for a subsequent deposition of type I collagen fibers (HOLMES, BORG and COVELL, 2005). Collagen deposition in the myocardium has two effects on the structure and function of the heart; increased collagen deposition is a prerequisite to prevent dilation of the infarcted area, yet its excessive accumulation in the infarcted and non-infarcted areas of the myocardium leads to tissue stiffness, increasing the incidence of arrhythmias and adverse effects on the elasticity of the myocardium which results in ventricular systolic and diastolic dysfunction. This abnormal increase in collagen concentration is called fibrosis (JACK, CLEUTJENS and CREEMERS, 2002).

Post-infarction myocardial remodeling is a complex, dynamic and time-dependent process. This process involves different changes between the infarcted and non-infarcted regions, especially in the extracellular matrix Types I, II, III, IV, V, VII and XI collagens are involved in heart remodeling (JUGDUTT, 2003).

Other factors are essential for the good functioning of the heart. The system consisting of four valves that control the blood flow within the heart cavities is one of the most important. Two of these valves called right (tricuspid) and left (mitral) atrioventricular valves are found in the annulus fibrosus of the heart, between the atriums and the ventricles. They are connected to the papillary muscles by the chordae tendineae. These muscles are formed by myocardial projections; therefore, they exert a contractile function and are important structures for controlling these valves (MOORE and DALLEY, 2007).

Given the structural characteristic of the papillary muscles (the cardiomyocytes are aligned parallel to each other and oriented towards the source of the forces that act upon the muscle) they assume particularly promising peculiarities for functional studies of contraction and relaxation (COOPER and TOMANEK, 1982; THOMPSOM, MARINO, UBOH et al., 1984).

Despite the various studies on this muscle, little importance has been given to the collagen structure when heart dilation occurs after a myocardial infarction. This situation, which is followed by increased diastolic pressure, changes the structure of the entire wall of the ventricle and probably of the papillary muscle. Such changes impair the contractile function of the cardiomyocytes; therefore, it is important to know them in order to better understand the functional part of the papillary muscles (ICARDO and COLVEE, 1998).

Icardo and Colvee (1998) demonstrated that collagen concentration increases in the papillary muscle of hypertensive individuals and that this increase could impair the contractile capacity of the papillary muscle and also its electrical conductivity.

Hoang et al. (1999) studied 10 infarcted rats treated with medication and compared them with placebo-treated controls. The results showed that collagen accumulated in the papillary muscle of the placebo-treated control group.

2 Material and methods

This study was analyzed and approved by the Research Ethics Committee (REC) of the Federal University of São Paulo – *Escola Paulista de Medicina/Hospital São Paulo* (Ref. REC 1354/04).

Twenty-four adult female Wistar-EPM rats divided into two groups were used in this study: a control group submitted to simulated surgery (SS) and a group of rats submitted to myocardial infarction (MI), each group consisting of 12 rats.

In the MI group, infarction was induced by the already established technique of our laboratory. The rats were anesthetized (2-(2-chlorophenyl)-2-methylamino-cyclohexan-1-one [Ketamine] 50 $mg.kg^{-1}$ + Xylazine 10 $mg.kg^{-1}$, intraperitoneal), intubated, ventilated and after thoracotomy in the left hemithorax, at the level of the *ictus cordis*, the heart was exposed and the anterior interventricular branch of the left coronary artery was occluded with a stitch (Prolene 6-0 suture), causing definitive and total ischemia of the myocardium. The heart was quickly placed in its natural position and the thorax was closed with a purse-string suture prepared beforehand.

The rats in the SS group were anesthetized (2-(2-chlorophenyl)-2-methylamino-cyclohexan-1-one [Ketamine] 50 $mg.kg^{-1}$ + Xylazine 10 $mg.kg^{-1}$, intraperitoneal) and submitted to the same procedures that the MI group experienced but without occlusion of the anterior interventricular branch. The Prolene 6-0 suture passed deeply around the artery and was removed right away. Again the heart was rapidly placed in its natural position.

After a period of 6 weeks, the rats in both groups were anesthetized and sacrificed with a lethal dose of anesthetics. The hearts were removed and placed in a fixating solution of 10% paraformaldehyde in distilled water. After a period of three hours, the hearts were cut at their equator and divided into two halves: an upper half containing the atriums, part of the ventricles and the vessels in the base of the heart and a lower half containing the ventricles and the apex of the heart. Both halves were again placed in a 10% paraformaldehyde solution for another 21 hours for fixation of the material.

The upper halves were used to determine if post-infarction healing occurred. They were placed in paraffin, cut into 5 μm slices and stained with picrosirius red. One of these upper halves was randomly selected for immunohistochemical analysis of types I, II, III, IV, V and VII collagens.

The myocardial infarction size (MIS) was estimated in the cross-sectional cut of the left ventricle (LV) of these upper halves by measuring the perimeter of the ventricular cavity (PVC) and the length of the arch formed by the MI scar (MISc). The infarction size, in percentage, was calculated with the Equation 1:

$$MIS = MISc/PVC \times 100 \quad (1)$$

Animals whose scars occupied more than 30% of the left ventricular muscle mass (N = 12) were included in the study. Twenty animals did not have scars more than 30% and were exclused.

The lower halves of these hearts were dissected and the anterior and posterior papillary muscles were removed. The remaining portion of the lower halves were discarded. These muscles were again placed in a 10% paraformaldehyde solution for 21 hours for fixation.

The papillary muscles were positioned in the paraffin blocks in such a way that the histological cuts followed their longitudinal length. This allows the longitudinal collagen fibers that are aligned with the muscle fibers in this region of the heart to be seen. The 5 μm slices obtained from these blocks were stained with picrosirius red and analyzed with a polarization microscope.

The streptavidin-biotin technique was used to analyze the immunohistochemical reactions of types I and III collagen.

The slides were treated with '20 volume' hydrogen peroxide to block endogenous peroxidase. The samples were then rinsed with tap water and with a phosphate buffer solution (PBS), pH 7.4.

The cuts were incubated for 16 to 18 hours in PBS, pH 7.4, with 1% bovine albumin (BSA) and the following antibodies: rabbit anti-human type I collagen monoclonal antibody (Novatec™-USA) and rabbit anti-human type III collagen monoclonal antibody (Novatec™-USA).

After rinsing with PBS, pH 7.4, the signal was amplified with biotinylated antibodies from the Dako LSAB-HPR (Dako A/S™) Kit in a moist chamber at room temperature.

The samples were developed and counterstained with Harris hematoxylin. Next, the samples were dehydrated with an increasing ethanol series and made transparent in xylol baths. The slides were mounted with coverslips in Etellan resin (Sigma™), analyzed and photographed with a light microscope (Olympus BX50™).

3 Results

Immunohistochemical analysis revealed that this infarcted heart presented higher concentrations of types I (Figure 1e and f), II (Figure 1d), III (Figure 1b), IV (Figure 1c) and VII (Figure 1a) collagens in the post-infarction scar region when compared with the remaining portion of the left ventricular wall. Type I collagen prevailed, followed by type III; the remaining collagens in were present in lower amounts.

Immunohistochemical analysis revealed that this infarcted heart presented higher concentrations of types I, II, III, IV and VII collagens (Figure 1a-i) in the post-infarction scar region when compared with the remaining portion of the left ventricular wall. Type I collagen prevailed, followed by type III; the remaining collagens in were present in lower amounts.

The slides with the papillary muscles stained with picrosirius red were analyzed under a polarization microscope, thus it was possible to evidence the collagen and differentiate between types I and III.

With polarization, type I collagen colors vary from orange to red (Figure 2) while type III has shades of blue or green.

The posterior papillary muscles of the MI group (Figure 2) present a concentration of type I collagen ranging from 0.2% (sample E2) to 11.3% (sample O2) in the entire muscle and the mean type I collagen concentration of the twelve samples was 3.2%.

Meanwhile, type I collagen concentration in the anterior papillary muscles of this group ranged from 0.7% (sample E1) to 39% (sample G1) (Figure 2) and the mean concentration of the twelve samples was 6.4%.

In the SS group, both the posterior and anterior papillary muscles (Figure 2) present a lower collagen concentration than the MI group. The mean concentration of the twelve samples was 0.5 and 0.6% respectively.

When type III collagen was quantified in the MI samples, the posterior papillary muscles presented values ranging from 0.1 to 0.4% and four samples did not present significant values (<0.1%). The mean collagen concentration of these samples was 0.3%.

Likewise, three samples of the anterior papillary muscles (Figure 2) of the MI group did not have significant values. The values ranged from 0.1 to 0.8% and the mean concentration was 0.3%.

In the SS group, the mean concentrations of type III collagen in the posterior and anterior papillary muscles were 0.2 and 0.1% respectively.

4 Conclusion

According to our results, the collagen matrix concentration of the infarcted hearts was higher when compared with that of the control group. These data are in agreement with those reported by (ANVERSA, OLIVETTI and MELISSARI, 1979; BRILLA, JANICKI and WEBER, 1991; JANICKI, 1992) Weber et al. (1987) who report that myocardial infarction is the main heart remodeling factor and consequently an important predisposing factor for a greater accumulation of the collagen matrix.

Our samples were collected six weeks after occlusion of the anterior interventricular branch of the coronary artery. Holmes et al. (2005) studied chronic infarction in dogs and sheep and noticed that collagen accumulation in these animals lasted approximately 6 weeks. On the other hand, in rats, there is a collagen increase between the fourth and fifth days and it continues for three weeks after artery occlusion.

Immunohistochemistry for type I collagen demonstrated that there was a greater accumulation of collagen matrix in the post-infarction scar region than in the remaining portions of the myocardium in the ventricles. Similar results were obtained by Holmes et al. (2005) and Omens et al. (1997), who studied type I collagen in infarcted rat hearts and stated that the healing process involves an increase of this collagen matrix. The same was found by Holmes et al. (1996) when they studied infarcted pig hearts.

An increase in the concentration of type I collagen is understandable since this is the main type of collagen found in vertebrates and its function is associated with resistance to tension (JUNQUEIRA and MONTES, 1983). Thus, given the fragility presented by the cardiac muscle after an infarction, collagen assumes the vital role in maintaining tissue architecture.

Our results also point toward a high concentration of type III collagen in the post-infarction scar region and a reasonably smaller accumulation was found in the remaining myocardium. Morishita et al. (1996) and Holmes et al. (2005)

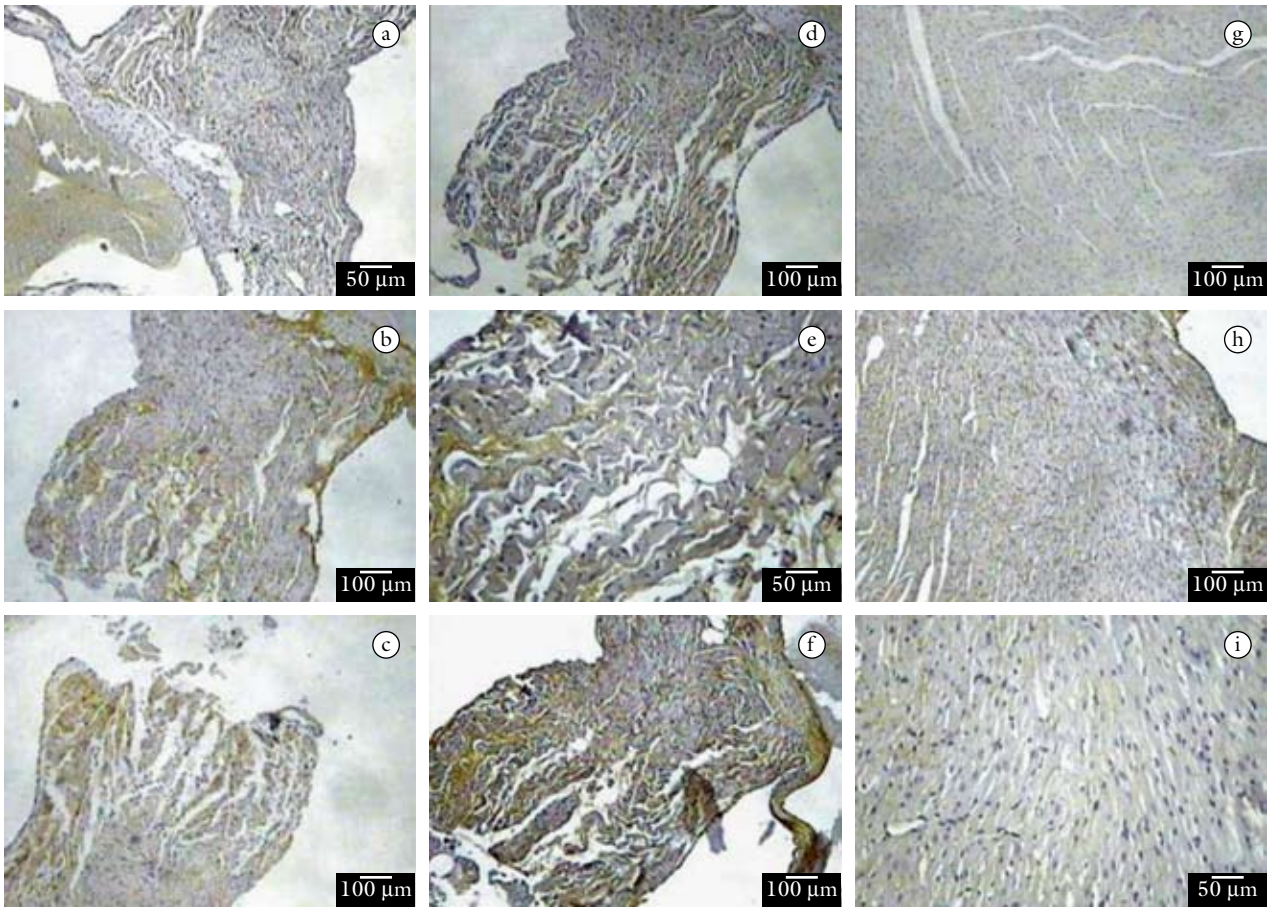

Figure 1. Immunohistochemistry of the region close to the infarction scar (a to f) and of the non-infarcted region (g to i). a) type VII collagen; b) type III collagen; c) type IV collagen; d) type II collagen; e) and f) type I collagen. A higher concentration of all types of collagen is seen in the region close to the infarction scar when compared with the remaining myocardial regions (g, h and i).

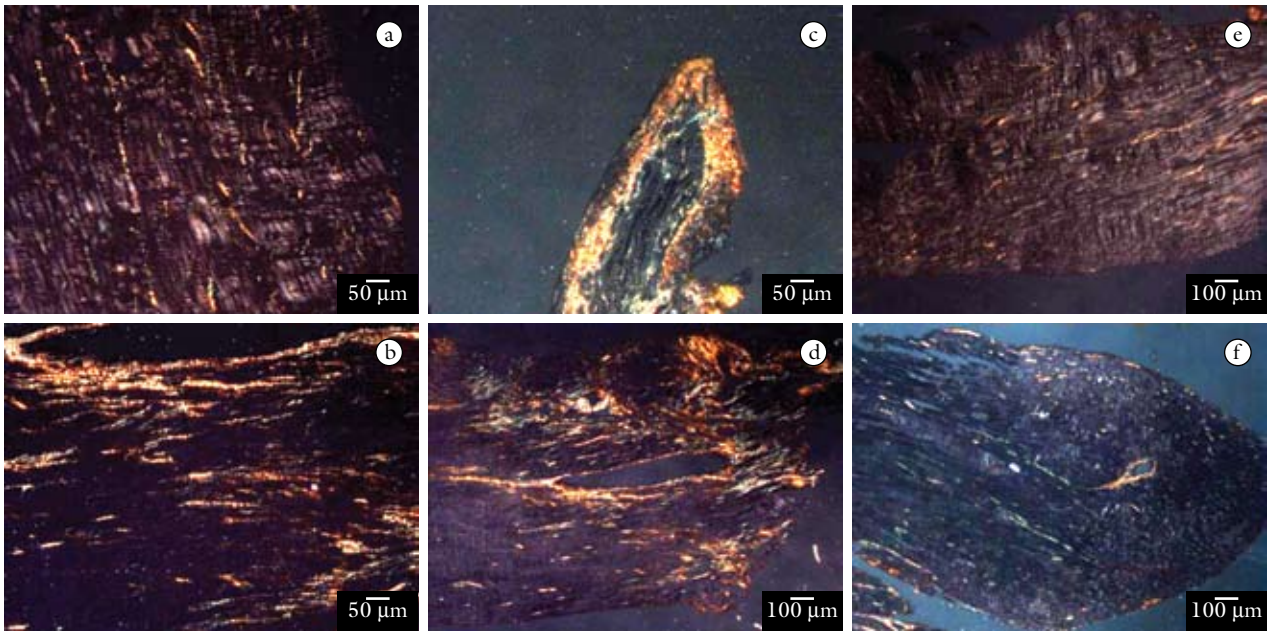

Figure 2. Polarization microscopy. Type I collagen appears in shades of red and type III collagen in shades of blue and green. a) posterior papillary muscle, control group; b) and d) posterior papillary muscle of an infarcted heart; c) anterior papillary muscle, infarcted heart; e) and f) anterior papillary muscle, control group.

describe that, once the necrosis phase of infarction ends, type III collagen fibers start to form a net for the deposition of type I collagen. Messenger RNA for type III collagen is detected between the third and fourth days and later for type I (CLEUTJENS, VERLUYTEN, SMITHS et al., 1995).

Regarding the other collagen types used in this study (types II, IV and VII), literature is relatively scarce when it comes to cardiac muscle and its changes in dilated cardiomyopathies and myocardial infarction. The infarcted hearts we studied contained types II and VII collagens both in the infarction scar region and in the non-infarcted region, but their concentrations were lower than those of types I, III and IV.

Jugdutt (2003) demonstrated that the same types of collagen that we studied, plus types V and XI, are involved in the post-infarction remodeling process of the myocardium. However, he does not mention if this occurred in the same regions that we studied.

Collagen concentration increased in the anterior and posterior papillary muscles of the rats in the MI group when compared with the rats in the SS group.

The collagen percentages in the anterior papillary muscle of the MI group ranged from 0.7 to 39% (mean = 6.4%). The posterior papillary muscle of the MI group also presented higher type I collagen concentration than that of the SS group. Our results showed that this collagen type ranged from 0.2 to 11.3% (mean = 3.2%) in this muscle. In the SS group, the mean percentages of type I collagen in the anterior and posterior papillary muscles are 0.6 and 0.5% respectively.

The size of the post-infarction scars found in the hearts of the MI group did not differ significantly. Yet, the significantly different type I collagen concentrations in the anterior papillary muscle E1 (0.7%) and G1 (39%) and in the posterior papillary muscle E2 (0.2%) and O2 (11.3%) can be explained since the amount of blood irrigated by the coronary arteries varies greatly, and that includes irrigation to the papillary muscles. This allows us to understand why some hearts with the same signs of heart failure (post-infarction scar occupying more than 30% of the total area of the left ventricle) did not have a high accumulation of type I collagen in the anterior and posterior papillary muscles; additionally, the G1 sample had much more collagen than the remaining samples.

Regarding type III collagen, present in lower amounts in the myocardium, the mean percentage in the anterior papillary muscles of the MI group was 0.3% and of the SS group was 0.1%. The same was observed for the posterior papillary muscles, where the mean percentage for the MI group was 0.3% and for the SS group was 0.2%. Even though the concentration percentages of collagen type III were lower than those of type I, they were still higher in the anterior and posterior papillary muscles of the MI group than in the SS group.

Few studies were found in the literature regarding specific quantification of types I and III collagen in the papillary muscles of hearts with post-infarction dilated cardiomyopathies.

Icardo et al. (1998) demonstrated an increased collagen concentration in the papillary muscles of hypertensive individuals yet they did not specify the type of collagen they analyzed nor did they compare the values obtained from the various papillary muscles.

In another study, Hoang et al. (1999) studied infarcted rats treated with an anti-hypertensive drug that blocked the activation of angiotensin II AT1 receptor. The placebo-treated control group presented a higher concentration of types I and III collagen in the papillary muscles of the left ventricle than the treated group.

According to Fishbein et al. (1978), myocardial infarction may bring about many complications for the papillary muscles. The main complication is the rupture of this muscle, which may lead to severe mitral failure and even death in some cases. On the other hand, infarction of the papillary muscle may progress to fibrosis of this muscle in the long run, making it more curved, and sometimes accompanied by papillary muscle-related mitral valve prolapse and, consequently, a secondary mitral valve failure.

Zalaquett et al. (1995) studied nine cases of papillary muscle rupture, seven of them compromising the posterior papillary muscle and only two compromising the anterior papillary muscle; this difference may be explained by the fact that the posterior papillary muscle is only irrigated by the posterior interventricular branch, generally branching from the right coronary artery, while the anterior papillary muscle receives more irrigation from branches of the anterior interventricular artery and from marginal branches of the circumflex artery. In rats, our results do not corroborate Zalaquett et al. (1995) if we take into account the fact that collagen accumulation is associated with an irrigation deficit since the occluded artery was the anterior interventricular artery while the posterior interventricular artery remained intact. Still, our results show that the posterior papillary muscles were also affected by the infarction. On the other hand, some authors, such as Cleutjens et al. (1995), state that the collagen matrix increases not only in the infarction scar region but also in the remaining myocardium. Thus, we can also propose that, in our study, collagen accumulation in both papillary muscles may be associated not only with the absence of blood supply, but also with the remodeling of the entire heart after an infarction.

Heart remodeling after an infarction or even after other pathological situations is a mechanism that is not yet entirely known. We already know that collagen accumulation in the infarcted region is an attempt to keep the structural integrity of the ventricular wall and that this remodeling ends up affecting the entire heart, mainly increasing the collagen matrix of types I and III collagen in many regions of the ventricle.

The main focus of our study was to quantitatively analyze these collagens in the anterior and posterior papillary muscles of the left ventricle of infarcted rats in order to enrich the knowledge of this remodeling mechanism that also involves this specific part of the heart.

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