

## Histological and immunohistochemical changes of the myocardium in dilated cardiomyopathy

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### Abstract

Dilated cardiomyopathy is a major cause of heart failure and a major cause of morbidity and mortality. It is a multifactorial disease that includes both hereditary and acquired forms. It is estimated that around 20–35% of patients with dilated cardiomyopathy have hereditary forms. It is the third most common cause of heart failure and the most common cause of heart transplant. Dilated cardiomyopathy can be a secondary condition of many diseases such as coronary heart disease, diabetes, pheochromocytoma, infections, malnutrition, ingestion of toxic substances (alcohol, cocaine), ingestion of chemotherapeutic drugs, autoimmune diseases. In our study, we aimed to describe the changes of myocardial cells and interstitial connective tissue in patients clinically diagnosed with alcoholic dilated cardiomyopathy. The material studied consisted of heart fragments sampled from the left ventricle (LV) during necropsy from a total of 28 patients, aged between 58 and 73 years, with a clinical and laboratory diagnosis of dilated cardiomyopathy, hospitalized in the Cardiology Center of the Emergency County Hospital of Craiova in 2009 and 2010. In dilated cardiomyopathy, myocardial muscle fibers appeared slightly elongated or wavy, with hypochromatic, heterogeneous, vacuolar sarcoplasm, by a decrease of myofibril numbers. Lipofuscin granules were frequently seen in the sarcoplasm. Nuclear changes were consistent with sarcoplasmic alterations. Changes of the interstitial connective tissue were sometimes extensive and sometimes barely noticeable. The most common alteration of this structure was the onset and development of a mainly perivascular collagen fibrillogenetic process.

**Keywords:** dilated cardiomyopathy, myocardium, immunohistochemistry, alcohol consumption.

### ■ Introduction

Dilated cardiomyopathy is defined by the presence of left ventricular dilation associated with left ventricular systolic dysfunction, which causes global systolic impairment in the absence of abnormal ventricular loading conditions (hypertension, valvular disease) or coronary insufficiency. Right ventricular dilation and dysfunction may be present, but are not required for the diagnosis [1]. Dilated cardiomyopathy is a major cause of heart failure and a major cause of morbidity and mortality in both developing and industrialized countries [2]. It is a multifactorial disease that includes both hereditary and acquired forms. It is estimated that around 20–35% of patients with dilated cardiomyopathy have hereditary forms [2], and genetic studies in recent years revealed several genetic mutations in some patients with this disease [3]. Familial disease should be suspected when there is a family history of premature death by heart disease or disease of the electrical conduction system of the heart, skeletal disease or myopathy [2]. It seems that familial disease has mostly an autosomal dominant transmission [4].

The prevalence of dilated cardiomyopathy in the general population is unknown, but clearly it varies by age and geographical area. Recent studies have noted that dilated cardiomyopathy is one of the most common forms of myocardial disease, largely irreversible, with an estimated prevalence of 1:2500; it is the third most common cause of heart failure and the most common cause of heart transplant [5].

According to some authors, idiopathic dilated cardiomyopathy is the most common cause of congestive heart failure in young people, with an estimated prevalence in the USA of at least 36.5 to 100 000 [6].

Dilated cardiomyopathy can be a secondary condition of many diseases such as coronary heart disease, diabetes, pheochromocytoma, infections (viral, bacterial, fungal or parasitic), malnutrition, ingestion of toxic substances (alcohol, cocaine), ingestion of chemotherapeutic drugs, autoimmune diseases, etc. [5]. In most cases of dilated cardiomyopathy, no identifiable cause is defined.

Histopathological aspects of the myocardium in dilated cardiomyopathy are less described as cardiac biopsy was practiced only in well-equipped cardiology clinics. Knowledge of structural changes in myocardial

cells and the myocardium as a whole could explain the pathophysiology of the disease and allow a correct therapeutic approach.

In our study, we aimed to describe the changes of myocardial cells and interstitial connective tissue in patients clinically diagnosed with alcoholic dilated cardiomyopathy.

## Materials and Methods

The material studied consisted of heart fragments sampled from the left ventricle (LV) during necropsy from a total of 28 patients, aged between 58 and 73 years, with a clinical and laboratory diagnosis of dilated cardiomyopathy, hospitalized in the Cardiology Center of the Emergency County Hospital of Craiova in 2009

and 2010. After sampling, the biological material was immediately placed in 4% formaldehyde solution buffered to a pH of 7.2–7.4 with monosodium phosphate and processed through the usual technique for paraffin inclusion. Four  $\mu\text{m}$  thick serial sections were cut using a rotary microtome (Microm HM350) equipped with a waterfall based section transfer system (STS, Microm). Sections were stained with Hematoxylin–Eosin or Masson's trichrome. For the immunohistochemical study, sections were cut using the same equipment, but with a thickness of 3  $\mu\text{m}$ . Sections were collected on poly-L-lysine coated slides, dried in a thermostat at 37°C for 24 hours in order to obtain a perfect adhesion of the biological material to the surface of the histological slide, and then stained using different antibodies (Table 1).

**Table 1 – Antibodies used for the immunohistochemical study**

Antibody	Manufacturer	Clone	Host/Target/Clonality	Antigen retrieval	Dilution
Anti-CD34	Dako	QBEnd 10	Ms/Hu/Monoclonal	Sodium citrate, pH 6	1:50
Anti-vimentin	Dako	V9	Ms/Hu/Monoclonal	Sodium citrate, pH 6	1:100
Anti-desmin	Dako	D33	Ms/Hu/Monoclonal	Sodium citrate, pH 6	1:50
Anti-collagen IV	Dako	CIV 22	Ms/Hu/Monoclonal	Sodium citrate, pH 6	1:25

For single immunohistochemistry, after antigen retrieval, sections were cooled down to room temperature and were incubated for 30 minutes in a 1% hydrogen peroxide solution.

The sections were next washed in PBS, followed by a blocking step of 30 minutes in 2% skim milk. Next, the slides were incubated with the primary antibodies overnight at 4°C, and the next day, the signal was amplified for 30 minutes using a peroxidase polymer-based secondary detection system (EnVision, Dako). The signal was detected with 3,3'-diaminobenzidine (DAB) (Dako) and the slides were coverslipped in DPX (Fluka) after Hematoxylin counterstaining.

The sections were imaged with a Nikon Eclipse 55i microscope (Nikon, Apidrag, Romania) equipped with a 5-megapixel cooled CCD camera. Images were captured and archived using a Nikon frame grabber and the Image ProPlus 7 AMS software (Media Cybernetics Inc, Buckinghamshire, UK).

## Results

Systematic analysis of myocardial tissue from the left ventricle by conventional histological staining, allowed us to observe that dilated cardiomyopathy is characterized by significant changes within both myocardiocytes as well as the interstitial connective tissue. Most often, myocardial fibers were more elongated than hypertrophied, which means that in dilated cardiomyopathy systolic function is impaired.

Myocardial cells showed variable staining patterns because of changes in intracytoplasmic structures. An attenuation of the myofibrils was the most common observation. In some myocardial cells the reduction of myofibrils appeared relatively homogenous, translated into a reduction of staining intensity and attenuation of myofibrils, while in other cells the reduction of myofibrils was heterogeneous which resulted in a vacuolar sarcoplasmic pattern, particularly on cross-sections of myocardial cells (Figures 1 and 2). Most often myofibrils

in the central area of the myocardial cells were affected and to a lesser extent those in the periphery, highlighting that the main cause of myofibril reduction could be a lower oxygen intake in the perinuclear area. The reduction of the contractile support of myocardial cells explains the systolic myocardial dysfunction, dilation of cardiac cavities and chronic heart failure.

Another sarcoplasmic change present in many cases was the accumulation of 1–2  $\mu\text{m}$  yellow granules on the Hematoxylin–Eosin staining, mostly in a perinuclear pattern. From the histological point of view, these granules could be lipofuscin granules, a wear pigment that accumulates in suffering or aging cells (Figure 3).

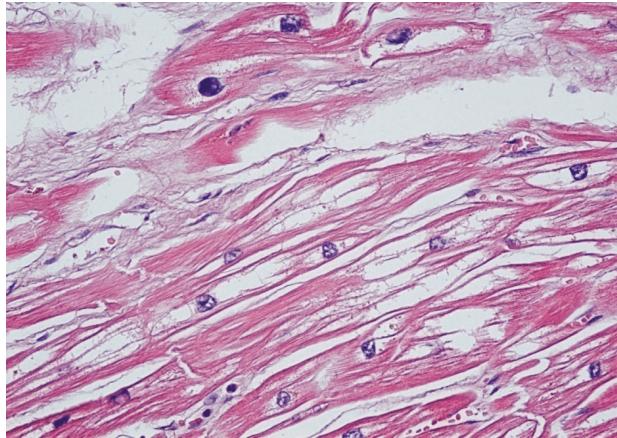
Nuclear changes were also quite common. Most often we found deformed, twisted, hyperchromatic nuclei, with chromatin arranged in an uneven pattern; sometimes pyknotic nuclei with chromatin organized in homogeneous disintegrating blocks were seen. Nuclear changes were consistent with sarcoplasmic changes in the sense that if sarcoplasmic changes were significant, so were nuclear ones.

We believe that nuclear changes reveal an irreversible important cell suffering.

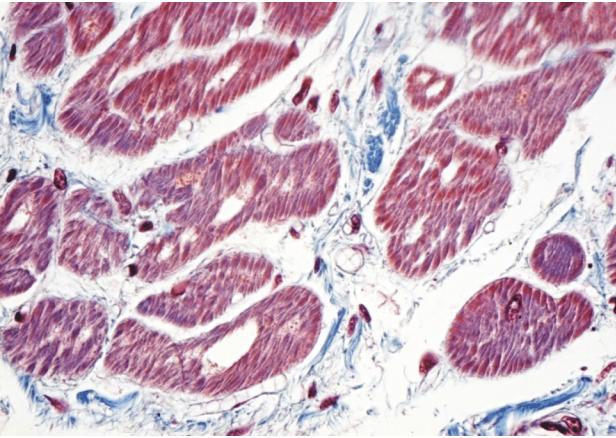
Another microscopic aspect observed in patients with dilated cardiomyopathy was the rupture of muscle bundles occurring within the intercalary disk (Figure 4). Intercalary disks are very strong junction areas between muscle fibers. Their rupture shows the existence of powerful mechanical forces or serious metabolic conditions that allowed the rupture of desmosomal junctions. Intercalary disk rupture leads to diminished myocardial contraction force and disruption of depolarization wave transmission through the ventricular myocardium.

Myocardial connective tissue changes were sometimes significant and sometimes barely noticeable. The most frequent and constant change in this structure was the emergence and development of collagen fibrillogenesis process. In the early stages, this process was reported to

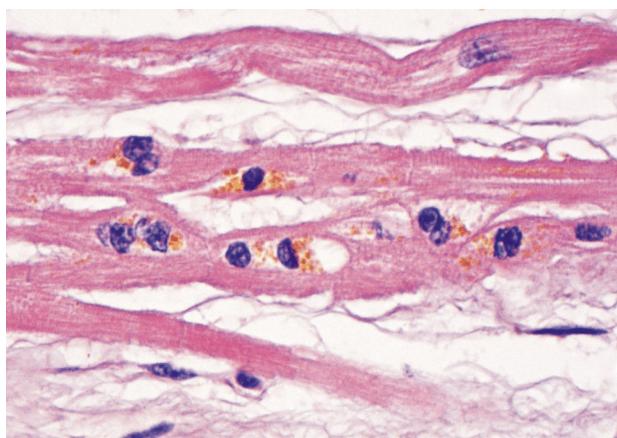
be more intense in the perivascular areas, which could be explained by the increasing synthesis activity of fibroblasts present in this space. In the advanced stages of dilated cardiomyopathy, this fibrillogenesis was also present in the spaces between muscle bundles as well as the interfibrillar interstitium (Figure 5).



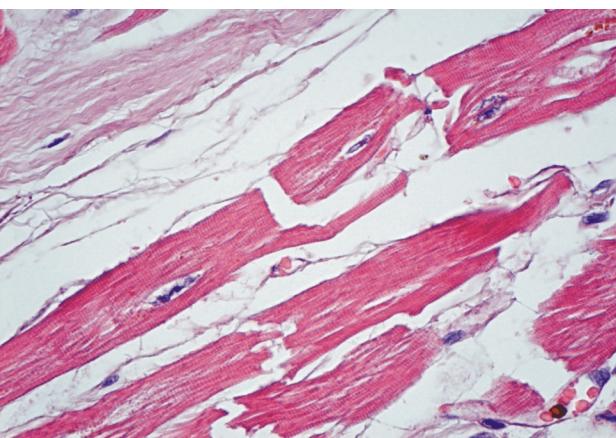
**Figure 1 – Mycardiocytes with marked and uneven reduction of myofibrils and uneven appearance of the sarcoplasm (HE stain,  $\times 100$ ).**



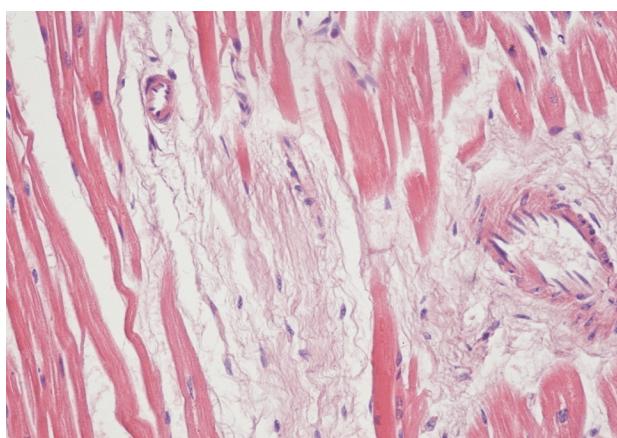
**Figure 2 – Mycardiocytes with heterogeneous sarcoplasm and vacuoles (Masson's trichrome stain,  $\times 100$ ).**



**Figure 3 – Mycardiocytes with lipofuscin granules (HE stain,  $\times 400$ ).**



**Figure 4 – Ruptured myocardial fibers (HE stain,  $\times 400$ ).**

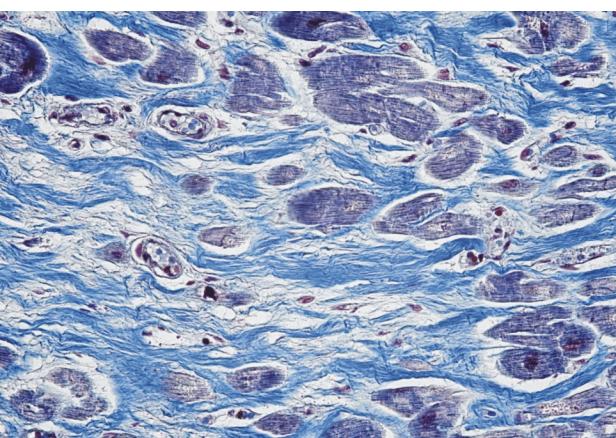


**Figure 5 – Myocardial fibrillogenesis (HE stain,  $\times 200$ ).**

The microscopic examination lead to the assumption that collagen fibers are composed mainly of collagen types I and III.

To assess changes in the contractile apparatus of

At this level, the collagen fibrillogenesis expanded progressively, dissociating heart muscle fibers, isolating them from the neighboring ones and finally destroying them. Thus, myocardiocytes were replaced by extensive areas of collagen fibers (true myocardiosclerosis) (Figure 6).



**Figure 6 – Advanced myocardiosclerosis (Masson's trichrome stain,  $\times 200$ ).**

myocardial cells, we used the anti-desmin antibody, knowing that desmin is the main intermediate filament protein that interacts with other proteins to form a continuous cytoskeletal network, which preserves the

spatial relationship of the myocardiocytic contractile apparatus.

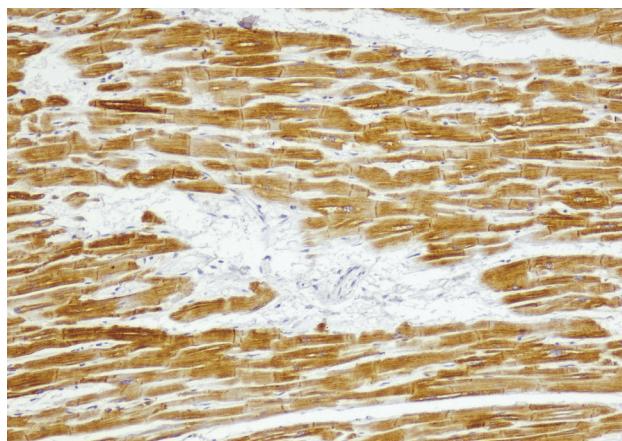
Thus, desmin ensures cellular integrity, transport and mechanical and chemical signaling within myocardiocytes. The evaluation of the immunohistochemical reaction to desmin allowed us to note that dilated cardiomyopathy is characterized by a heterogeneous reaction, which means that myocardial cells contain varying amounts of desmin. In areas with an early interstitial collagen fibrosis, the reaction to desmin was reduced, which denotes a drastic reduction in cytoskeletal intermediate filaments and destruction of the contractile apparatus. It is possible that interstitial fibrillogenesis processes begin because of a chronic myocardial ischemia, which would explain myocardiocyte suffering manifested by a reduction of intermediate filaments, cytoplasmic organelles and myofibrils (Figure 7). The presence of vacuolar non-reactive areas within myocardiocytes (Figure 8) proves that in dilated cardiomyopathy etiopathogenic factors can partially destroy the cytoskeletal intermediate filaments and with them, the contractile apparatus represented by myofibrils.

For the evaluation of myocardial interstitial connective tissue reaction in dilated cardiomyopathy,

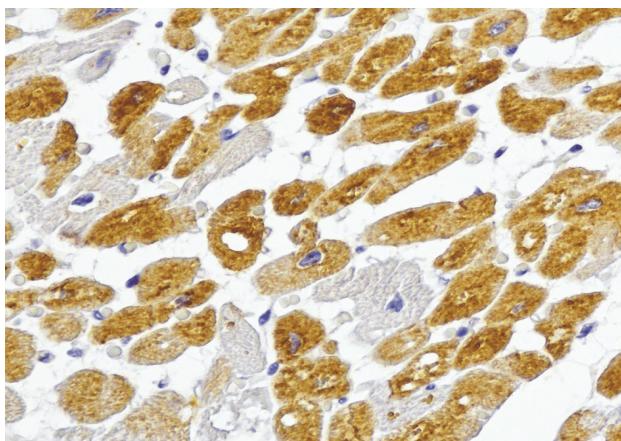
we investigated the vimentin immunohistochemical reaction knowing that vimentin is a major intermediate filament protein present in mesenchymal-derived cells, and the collagen IV immunohistochemical reaction. In our study, we found that the reaction to vimentin was intense in the interstitial tissue of the ventricular myocardium, where proliferative and fibrilllogenetic processes were more intense (Figure 9). We believe that vimentin specifically marked the fibroblast-type connective cells, responsible for collagen fibrillogenesis. In other words, the reaction to vimentin can be used for early diagnosis of myocardiosclerosis.

With regard to collagen IV, we found an increased expression in myocardial vessels with intense arteriolosclerosis (Figure 10).

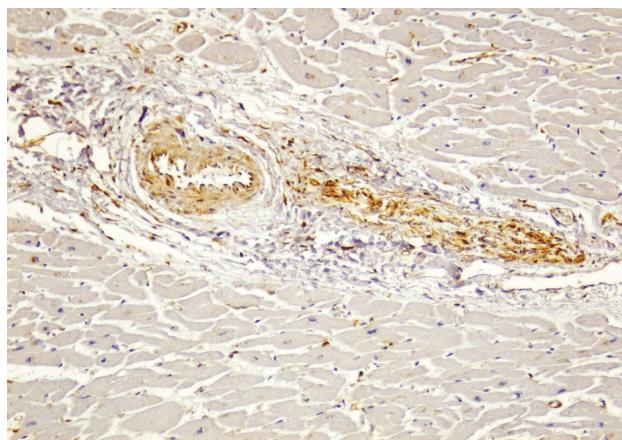
Changes in myocardial microcirculation were investigated using the anti-CD34 antibody, which marks endothelial cells. We noted that in areas less affected by pathological processes induced by dilated cardiomyopathy, myocardial microcirculation was well expressed, rich anastomotic blood capillaries, mostly around myocardiocytes (Figure 11). However, in areas with myocardiosclerosis, the vascular structures were completely remodeled, with decreased number and size of blood vessels (Figure 12).



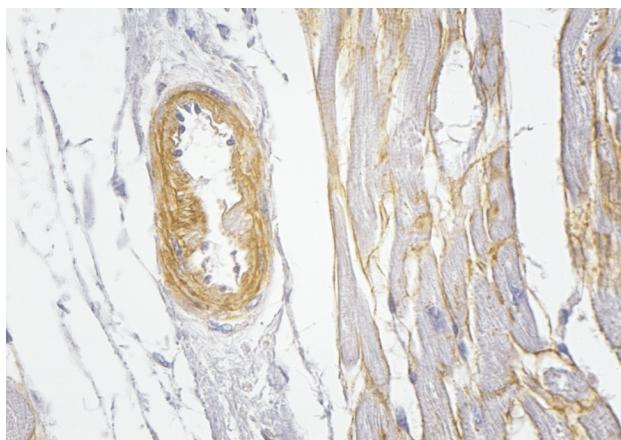
**Figure 7 – Myocardium with collagen fibrosis (immunohistochemical staining for desmin,  $\times 100$ ).**



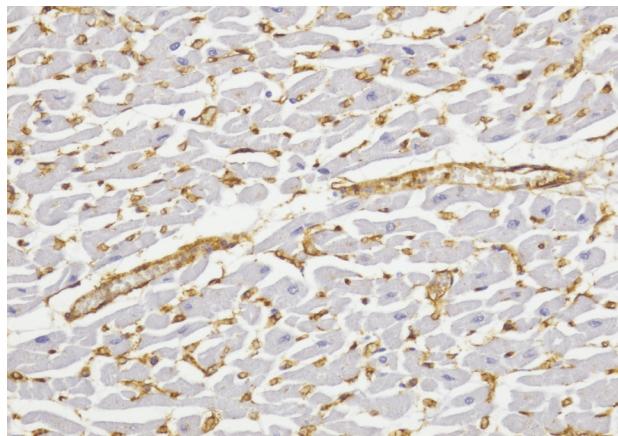
**Figure 8 – Cross-section myocardiocytes with very variable immunohistochemical reaction to desmin, associated with areas with intracytoplasmic vacuolization, with no reaction to desmin (immunohistochemical staining for desmin,  $\times 200$ ).**



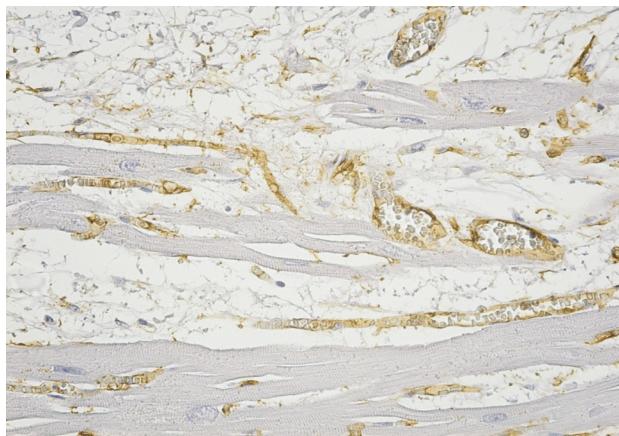
**Figure 9 – Intense reaction to vimentin (immunohistochemical staining for vimentin,  $\times 100$ ).**



**Figure 10 – Myocardial arteriole with increased amount of collagen IV (immunohistochemical staining for collagen IV,  $\times 200$ ).**



**Figure 11 – Relatively normal myocardium (immuno-histochemical staining for CD34,  $\times 100$ ).**



**Figure 12 – Marked reduction of vascularization (immuno-histochemical staining for CD34,  $\times 100$ ).**

## Discussion

Cardiomyopathies are a major cause of morbidity and mortality in both children and adults. Some cardiomyopathies are diseases that require a strong financial support and are a frequent indication for heart transplantation due to the severe heart failure that they generate. In 1995, the *World Health Organization (WHO)/International Society and Federation of Cardiology (ISFC) Task Force* recommended that cardiomyopathies be classified into two main groups: specific cardiomyopathies and primary cardiomyopathies [6].

The pathophysiology of dilated cardiomyopathy is heterogeneous both in terms of its pathogenesis and pathology. The common features for all dilated cardiomyopathies are represented by a weak contraction, decrease of left ventricular wall thickness followed by a progressive expansion of the ventricular cavity [7].

Although the etiology of this disease is largely unknown, numerous studies of medical genetics in recent years have shown that patients with dilated cardiomyopathy have single or multiple mutations involving structural proteins of the myocardocyte cytoskeleton or sarcolemma [8, 9]. Familial dilated cardiomyopathy was proposed to be considered as a form of "cytoskelopathy" [8]. Secondary causes of dilated cardiomyopathy include coronary heart disease, cardiomyopathy, myocarditis, nutritional deficiency, some systemic diseases, viral infections, cardiotoxins, alcohol abuse, muscle dystrophic diseases etc. In most cases of dilated cardiomyopathy no identifiable cause is defined. According to some authors, the most common cause of dilated cardiomyopathy is alcohol consumption. A wide range of structural abnormalities were observed in the myocardium, associated with high alcohol consumption, but is difficult to define precisely the point at which these abnormalities can be considered as being significant for dilated cardiomyopathy [5].

In our study, we sought to identify changes of left ventricular myocardium in order to highlight lesions leading to heart failure and subsequent death in individuals with high alcohol intake. It was difficult to ascertain by history whether individuals taken in the

study had a history of viral or bacterial infection that triggered the dilated cardiomyopathy.

Histological changes identified by us, namely the reduction of the contractile apparatus, the presence of lipofuscin granules in large quantities, nuclear changes, rupture of intercalary disks, explain the morphological basis of clinical signs in patients with dilated cardiomyopathy. The intensity of these microscopic changes was variable from one patient to another and even from one area to another within the same myocardium. Other authors [10] have also shown that histological changes associated with dilated cardiomyopathy non-specific and not all may be present simultaneously. Consistent with other observations [10], we also found that myocardocytes experience an extensive loss of myofibrils, giving an empty or vacuolated appearance to the sarcoplasm.

The changes of the myocardial interstitium identified by us are common and are mentioned by other researchers. The dominant lesion found in our cases was myocardial fibrosis. The collagen fibrillogenesis process may be due on one hand to myocardial hypoxia and on the other hand to the presence of large numbers of fibroblasts. It is known that, under hypoxic conditions, fibrocytic-type connective tissue cells have the ability to transform into fibroblasts, young connective cells that synthesize and excrete large amounts of connective matrix. Marijanowski MM *et al.* [11] demonstrated in patients with dilated cardiomyopathy the presence of increased amounts of collagen I and III, as well as an increase in the collagen type I/type III ratio.

The presence of an increased fibrotic process involves a possible role of TGF- $\beta$ 1 in the pathology of dilated cardiomyopathy. Sanderson JE *et al.* [12] found that TGF- $\beta$ 1 plasma levels were two times higher in patients who developed idiopathic dilated cardiomyopathy, compared with control groups, and Wenner CE and Yan S [13] showed that TGF- $\beta$ 1 is involved in the proliferation of mesenchymal cells such as fibroblasts, necessary for stroma formation in many organs.

In our study, we sometimes found large amounts of fibrillar collagen, arranged in coarse bundles within the myocardial interstitium. Sometimes, myocardiosclerosis

development was centrifugal, with myocardiocyte fascicle dissection, isolation and destruction of myocardial fibers. We believe that this accumulation of fibrillar collagen in the interstitial space of myocardium reduces cardiac performance in both the systole and diastole. Most researchers agree that cardiac interstitial fibrosis may contribute to ventricular dysfunction and affects prognosis in patients with dilated cardiomyopathy [14]. Disproportionate accumulation of fibrillar collagen is responsible for the reduction of the pumping capacity of the heart and the stiffness of the cardiac wall [15]. In addition, by modifying local vasculature, interstitial fibrosis reduces the O<sub>2</sub> supply and hastens the death of myocardiocytes.

As far as the changes in myofibrils and myocardiocyte cytoskeleton are concerned, we found that in areas with advanced interstitial fibrosis, desmin response was low, uneven, with desmin-negative vacuolar spaces within the myocardiocyte sarcoplasm. We believe that desmin-negative areas represent areas where the cytoskeleton is destroyed. In this respect, several hypotheses have been issued on the molecular mechanisms of heart failure onset. One of these states that desmin changes could disrupt the interaction between sarcomeres and the Z-band, which reduces the force transmitted by sarcomeres to the myocardiocyte membrane [16]. In addition, the increasing number of apoptotic cardiomyocytes partly explains cardiac dysfunction [17].

Some studies consider that coronary microcirculation can be directly affected by cardiomyopathy [18]. Thus, according to some authors [19], in end stage dilated cardiomyopathy myocardial blood flow is severely reduced. Studies using positron emission tomography showed that 82% of patients with dilated cardiomyopathy have reduced myocardial flow due to a dysfunction of coronary microcirculation [20]. We believe that it is currently difficult to determine whether coronary microcirculation impairment causes dilated cardiomyopathy phenomena, or they are secondary to the evolution of dilated cardiomyopathy.

## Conclusions

In dilated cardiomyopathy myocardial muscle fibers appeared slightly elongated or wavy, with hypochromatic, heterogeneous, vacuolar sarcoplasm, by a decrease of myofibril numbers. Lipofuscin granules were frequently seen in the sarcoplasm, mainly around the nucleus, or diffusely scattered.

Nuclear changes were consistent with sarcoplasmic alterations, our study showing deformed, twisted, hyperchromatic nuclei with heterogeneous chromatin and even disintegrating nuclei.

Changes of the interstitial connective tissue were sometimes extensive and sometimes barely noticeable. The most common alteration of this structure was the onset and development of a mainly perivascular collagen fibrillogenetic process.

Ventricular blood supply appeared to be reduced and arterioles shown signs of intense arteriolosclerosis.

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