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THE USE OF LUMINESCENCE MICROSCOPY FOR DIAGNOSIS OF SUDDEN CARDIAC DEATH DUE TO ISCHEMIC HEART DISEASE IN THE FORENSIC PRACTICE

Khachatryan P.S.[2,3*], Zilfyan A.V.[1], Bisharyan M.S.[2],, Avagyan S.A.[1]

[1] Scientific-Research Center, Yerevan State Medical University, Yerevan, Armenia
[2] Republican Scientific-Practical Center of Forensic Medicine, Ministry of Health, Yerevan, Armenia
[3] Department of Pathological Anatomy and Clinical Morphology, Yerevan State Medical University, Yerevan, Armenia

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Abstract

Sudden cardiac death remains a major health issue accounting for a significant percentage of all cardiac deaths. The majority of deaths investigated by forensic pathologists is certified as natural. The use of appropriate microscopy in these cases will assist the pathologist in identifying the disease process and any other underlying medical conditions that may exist.

Microscopy in daily expert practice requires the use of hematoxylin and eosin, which are mandatory for each forensic examination. Histological sampling of the myocardium may reveal an acute infarct or cardiosclerosis, but sometimes there were no significant changes while using routine hematoxylin-eosin staining. In these cases it is important to use different techniques. Over the years histological techniques use a distinguished method of luminescent microscopy by acridine orange staining to assess the morphofunctional condition of the myocardium.

We examined three groups of deceased from sudden death: the first group included 15 cases of blunt head trauma without damaging of organs in thoracic cavity, the second group with 15 cases of sudden coronary death without macroscopic changes in the heart. The final third group included 15 cases of myocardial infarction with the presence of necrotic changes visible by naked eye. Tinctorial features of cardiomyocytes in the processing of microscope slides in all three groups /death from casual trauma without damaged myocardium, and sudden cardiac death cases in the presence or absence of macroscopically visible changes/ treated with acridine orange show green fluorescence localized within structurally intact cells, green and yellow – in cardiomyocytes at the early stages of dystrophic processes, orange-red – in the muscle cells in advanced dystrophic processes in the myocardium.

Thus, the method of myocardial samples staining with acridine orange including subsequent luminescence microscopy examination is very informative in determining the severity of the damage of cardiomyocytes and the spreading pattern of a regional pathological process.

Keywords: *sudden cardiac death, histological examination, acridine orange, luminescent microscopy.*

Introduction

Sudden cardiac death is currently described as natural, unexpected death occurring within one hour of the onset of final symptoms. The term "sudden death" has been used in literature for over 250 years, but no single definition has been identified yet. Sudden death is assumed to be either instantaneous or the one that occurs during several minutes, 1 or 6 or up to 24 hours after the onset of continual symptoms of a mortal disease. However, such ways of determining it are often useless because sudden death is unwitnessed in about 40% cases, for example when it occurs during sleep. Most often sudden deaths have a cardiovascular cause when this time lapse is short [*de la Grandmaison GL, 2006*].

According to the World Health Organization (WHO) every year an estimated 20 million people

Address for Correspondence:
*****Department of Pathological Anatomy and Clinical Morphology, Yerevan State Medical University after M. Heratsi, 2 Koryun Street, 0025, Yerevan, Armenia
Tel.: (+374 010) 56 52 47
E-mail: parik82@gmail.com

die of cardiovascular diseases globally, particularly more than half from different forms of ischemic heart disease [*WHO, 2012*]. According to the Ministry of Health of Republic Armenia more than 6000 people die from CVD, including 55% from heart attack. Specific feature of sudden death due to ischemic heart disease is the death of 30-40% of victims in the first 10 minutes, and approximately the same in the next 2 hours. Two thirds of lethal outcomes occur at the prehospital stage, even in the presence of well-administered emergency medical care. [*Kirichenko AA,2002; Chugh SS et al., 2008; Bokeria LA, Revishvili AS, 2011*].

Diagnosis of ischemic heart disease in forensic autopsies practice is hindered by the lack of medical documentation with clinical records. However, diagnosis of chronic forms of ischemic heart disease usually does not present great difficulties, since at the time of post-mortem examination it is already possible to reveal significant pathological changes of the heart [*Milroy CM, 2012; Chang J et al., 2013*]. The greatest difficulties arise in the diagnosis of acute forms of ischemic heart disease, especially during sudden death of relatively young people in the presence of seeming well-being. It is known that the focus of necrosis in patients with acute myocardial infarction is formed only by the end of the first day after the onset of ischemic attack [*Boyko YA, 2000; Goldstein JA et al., 2000; Basso C et al., 2008*]. In these situations, when sudden death occurs at a very early stage of myocardial infarction, the myocardial changes are nonspecific and cannot easily be detected by traditional macroscopic examination, or histological staining.

The medical literature contains information about the possibility of using luminescence method in postmortem diagnosis of early ischemic damage of myocardial tissue. Evaluation of structural changes in areas of myocardial ischemia was evaluated by the authors based on the spontaneous luminescence of the myocardial tissue, i.e. without the use of fluorescent probes. Meanwhile, over the years histological techniques use distinguished method of luminescent microscopy by acridine orange (AO) staining to assess the morphofunctional condition of the myocardium [*Ranji M et al., 2009; Gohel H et al., 2012*]. Possibilities of luminescent microscopy using usual staining of samples with hematoxylin-eosin and those embedded in polystyrene were studied [*Khromova* AM, *Valiullina DN, 2001*]. Fluorescent staining should be used when available in all forensic cases of sudden death by heart attack to ensure its highly probable determination, which is otherwise not visible grossly and on routine histopathology examination by hematoxylin and eosin stains. However, this very informative method has not found a proper application in specialized forensic laboratories. Unavailability of the UV fluorescent microscope and the old technique of the procedure with cryostat sections might become a limiting factor. The fact that the study can be conducted on paraffin embedded sections eliminates this hurdle, as the tissue blocks can be mailed to such "referral centers", if the equipment is not available locally.

MATERIALS AND METHODS

Myocardial samples taken from 45 dead bodies of persons aged 30-60 were examined to reveal the possibilities of luminescence microscopy method in the diagnosis of sudden cardiac death from ischemic heart disease in forensic practice. In all cases there were no comorbidities and no traces of alcohol or drugs in the blood or other fluids of the body. The material was divided into three groups: the first group included 15 cases of blunt head trauma without damaged organs in thoracic cavity. In second group – 15 cases of sudden coronary death without macroscopic changes in the heart. The final third group included 15 cases of myocardial infarction with the presence of necrotic changes visible by naked eye.

In order to determine the severity of the structural changes in the myocardium in all groups studied, we have used acridine orange staining method with subsequent examination of slides under luminescence microscope by Bertalanffy Bikis [*Pirs E, 1960*]. This method is very informative because it allows in each case (even on the same slide) to determine foci of structurally intact cardiomyocytes, area of myocardium with apparent damage, including the evaluation of the prevalence of regional pathological process and profundity of dystrophic changes in parenchyma and stromal structures. Luminescence method of dyeing with AO is based on the dynamic of transformation of the cardiomyocytes' tinctorial properties

depending on the prevalence and severity of regional disease process in a wide variety of cardiovascular diseases.

For staining procedure fresh 1% AO solution was prepared (pH 7.2). Paraffin embedded sections were treated with AO solution for 3-5 seconds and repeatedly rinsed in phosphate buffer for 10-15 minutes changing the buffer solution 2-3 times to wash away non-specific deposits of AO. The slides were immediately viewed under UV fluorescent microscope ("Boeco", Germany).

The entire gamma of tinctorial properties was observed most prominently in the longitudinal sections of the myocardium. Thus, "structurally intact" cardiomyocytes during the process of staining with AO gain intense green color. The nucleus, nucleoli and cytoplasm of cardiomyocytes, including intracellular longitudinally oriented myofibrils are also endowed with intense green fluorescence. Initial stages of dystrophy in myocardial tissue, as a rule, manifested a marked decrease in the green fluorescence, which is replaced with the yellow-orange fluorescence in compound components of cardiomyocytes. During deeper destructive injury of myocardial structures (dyscomplexation of adjacent sarcomeres, foci of myofibrils destruction, myocytolysis, karyopyknosis and karyolysis) areas of myocardium with prevalence of catabolic processes begin to acquire new tinctorial properties. Thus, as a result of depolymerization and decay of nucleus, nucleolus and structural components of cytoplasm all of the aforementioned intracellular components of cardiomyocytes stain in orange, orange-red and red colors, depending on the severity of damage.

Results

In the first group cytoangioarchitectonics of the myocardium was intact in all parts. Longitudinally and transversely oriented sarcomeres manifested loose orientation to each other, resulting from moderate edema of the interstitium. Cardiomyocytes showed moderately compact distribution of myofibrils in the cytoplasm and the presence of intensively basophilic oval shaped nuclei. Capillaries were clearly seen in intermuscular connective tissue along longitudinally oriented cardiomyocytes. In general, the structure of the organization of the myocardium in persons who died from an accidental injury (I study group) did not differ from that of a typical myocardium of practically healthy population of the same age. However, in some areas there were signs of focal activation of fibroblastic processes (Fig. 1).

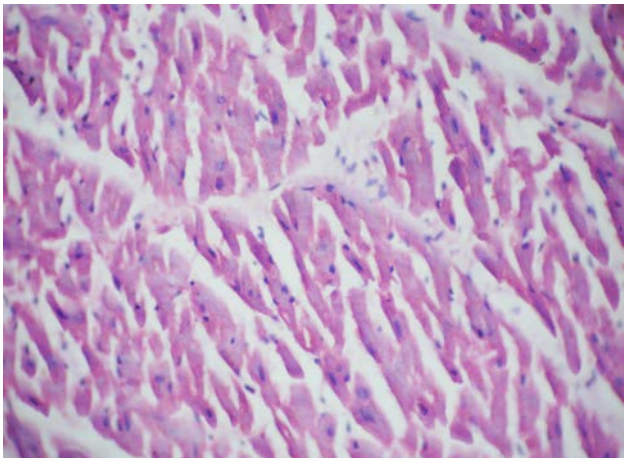

Figure 1. *Structural changes in the myocardium of study group I. H&E ob. 20, oc. 10. Cytoangioarchitectonics of the myocardium is intact. Moderate swelling of the stroma.*

In the second study group the cytoangioarchitectonics of myocardium was changed. Everywhere there were signs of dyscomplexation of myofibrils, pyknosis or, on the contrary, swelling of nucleus, lumpy destruction (Fig. 2a). Microcirculatory disturbances with swelling of endotheliocytes and perivascular edema were detected in the areas with dystrophic changes of cardiomyocytes. Erythrodiapedesis indicated high degree of vascular permeability. In this condition, some areas showed signs of activation of the fibroblastic cells developing looseness in connective tissue, which was revealed in the foci of dystrophy and destruction of cardiomyocytes. Microvessels walls (venules, capillaries, arterioles) were in the state of fibrosis, localized especially in the intramuscular connective tissue (Fig. 2b).

The polymorphism of structural changes in myocardium that we have found indicates that regional pathological process had prolonged progressive nature. Thus, relatively fresh areas with prevalence of alterative processes were found due to activation of focal reparative-proliferative reactions (fibrosis and sclerosis in intramuscular connective tissue, including walls of vessels).

During the examination of the slides prepared from the samples of the third group under the light

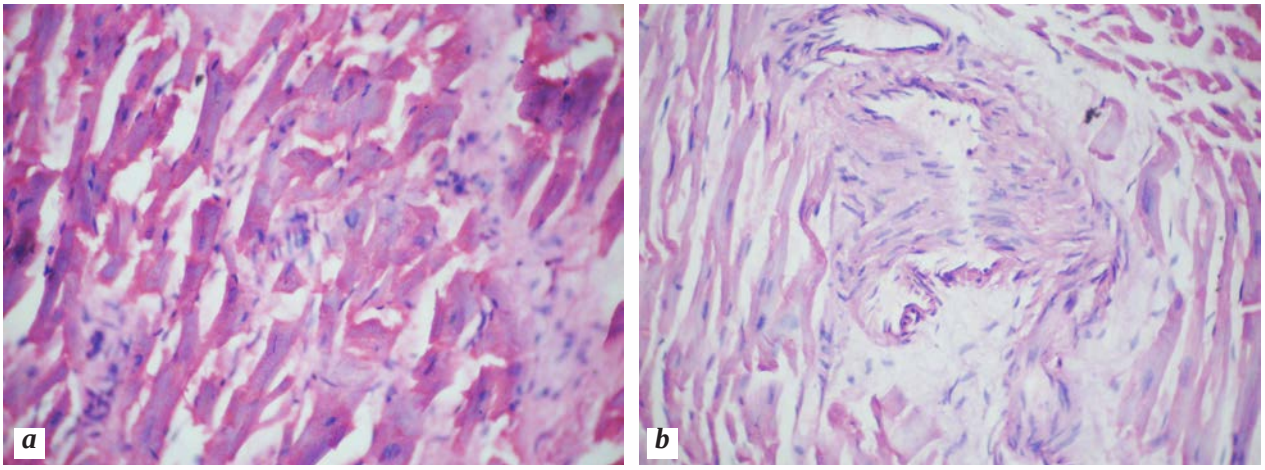

FIGURE 2. *Structural changes in the myocardium of study group II. hemotoxylin-eosin, ob. 40, oc. 10.* ***a)*** *Diffuse changing of cytoangioarchitectonics of myocardium. Signs of lumpy destruction of cardiomyocytes in the presence of moderately edematous stroma and focal activation of fibroblastic processes;* ***b)*** *Pronounced fibroblastic processes in the walls of adjacent microvessels in the presence of proliferation of endothelial and smooth muscle cells of arterioles. Signs of focal dyscomplexation and cardiomyocytes decay, activation of fibroblastic cells.*

microscope the structure of myocardium was normal in some areas. In these areas cardiomyocytes had a strict linear orientation. Intermuscular arteriolar and venular microvessels were also detected very clearly. In most cases examined in this study group were found micronecrotic foci of myocardium. In these areas the boundary between adjacent groups of sarcomeres was not contoured. They were presented by structureless (lumpy or even homogenous) eosinophilic masses quite often showing the signs of karyorrhexis (hematoxylin bodies) (Fig. 3a).

Exudative processes prevailed in the perifocal area, presented by signs of increased vascular permeability (plasmorrhagia, perivascular swelling, erythrodiapedesis, and moderate swelling in connective tissue) (Fig. 3b).

In this condition, signs of activation of fibroblastic processes were detected in some areas. As a result, areas of fibrosis were found at the place of previous localization of cardiomyocytes, sclerotic processes were revealed in the thickness of some vessel's walls, including capillaries.

The results of our fluorescence-microscopic analysis have demonstrated that transversely oriented muscle cells with intense green luminescence of their constituent components were observed in the myocardial samples of the first study group throughout the entire myocardial areas (Fig. 4a). Besides cardiomyocytes, stromal cells – endothe-

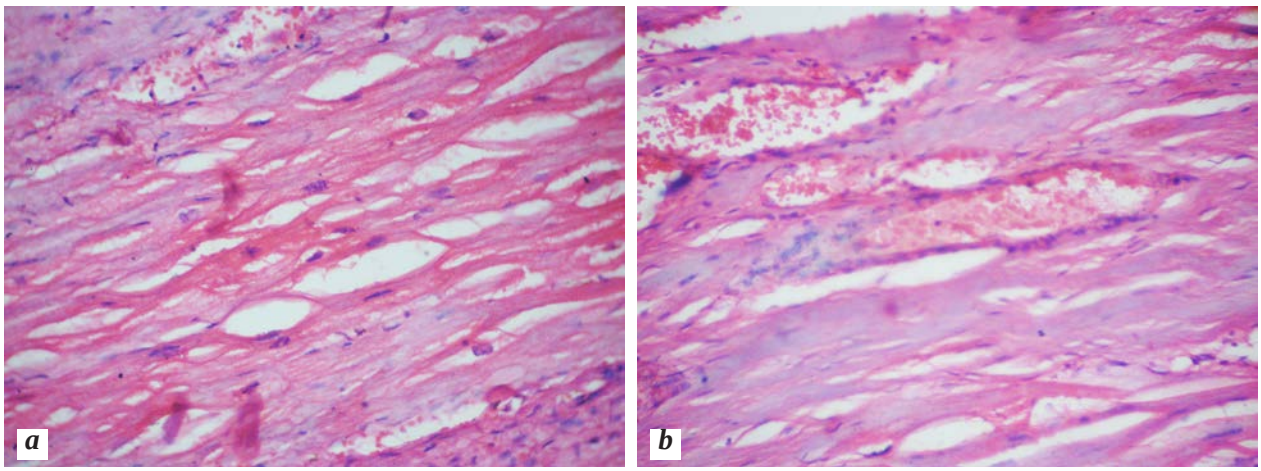

FIGURE 3. *Structural changes in the myocardium of study group III. hemotoxylin-eosin, ob. 40, oc. 10.* ***a)*** *Signs of focal droplet-lumpy decay and cardiomyocytolysis of adjacent sarcomeres with the presence of ‹hematoxylin bodies›;* ***b)*** *Destruction area's perifocal zone. Pronounced dilation and hyperemia of intermuscular microvessels.*

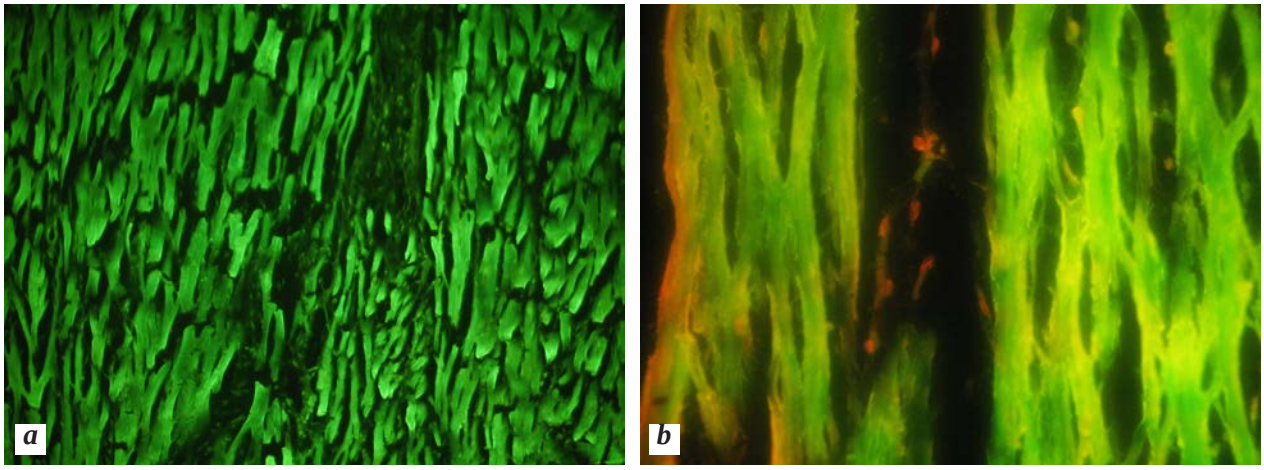

Figure 4. *Fluorescent-microscopic characteristics of first study group. Staining with acridil-orange.* ***a)*** *Longitudinally oriented cardiomyocytes endow green fluorescence of their cytoplasm ob. 10, oc. 7;* ***b)*** *Changing of tinctorial features of muscle cells on the background of green glow of longitudinally oriented cardiomyocytes. A focus of yellow and orange fluorescence along some sarcomeres was revealed ob.40 oc. 10.*

liocytes, smooth muscle cells of vascular wall, fibroblasts, and adventitial cells – also displayed intense green fluorescence. Green glow was replaced by yellow-green only in a few visual fields on a very limited area over the adjacent cardiomyocytes, (Fig. 4b).

In the second study group, AO staining of slides revealed mosaic pattern. Groups of sarcomeres with green and green-yellow fluorescence alternated with adjacent sarcomeres of cardiomyocytes whose cytoplasm and nuclear material displayed intense red-orange or red fluorescence (Fig. 5 a, b).

It is noteworthy that the orange-red fluorescent foci localized in the cardiomyocytes were observed not only in the areas of their decay, but also in those where integrity of the muscle cells was relatively intact.

In the third study staining of the slides with AO revealed relatively severe changes, strictly localized in the foci of micronecrosis and in the perifocal area. Thus, areas of necrotic myocardial tissue had an intense homogeneous orange-red fluorescence (Fig. 6a). In some areas free of infarction zones, in the presence of diffuse green fluorescence in most groups of the sarcomeres, only in some areas the sarcomeres changed their tinctorial properties, i.e. the glow gave way to orange-red (Fig. 6b).

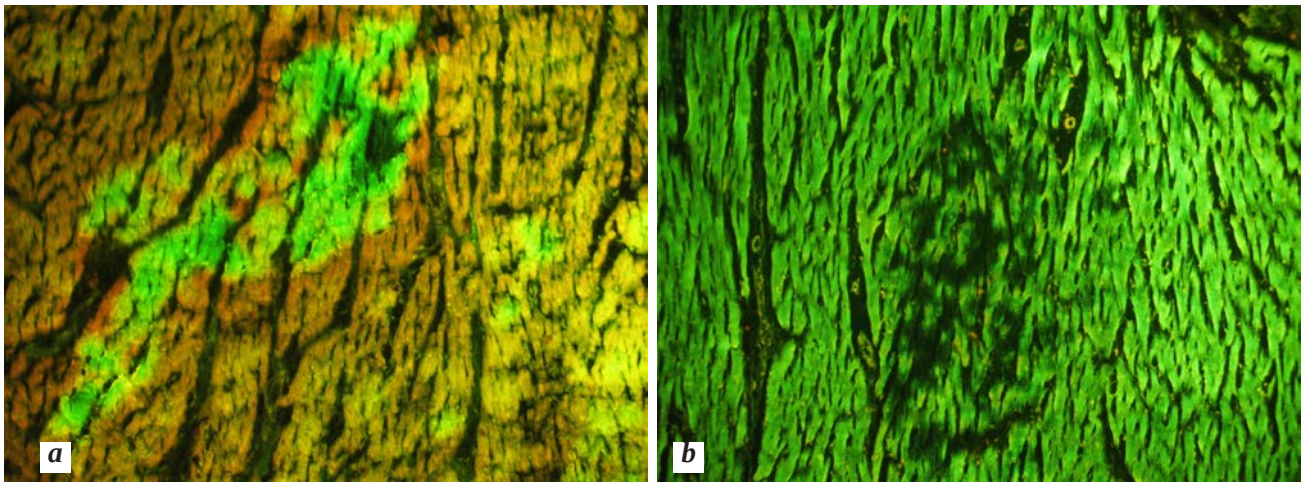

Figure 5. *Fluorescent-microscopic characteristic of II study group. Staining with acridil-orange.. ob.10 oc. 7.* ***a)*** *Diffuse intense and moderate green fluorescence of cardiomyocytes' cytoplasm;* ***b)*** *Focal green fluorescence of muscle cells alternating with areas in which cardiomyocytes have a yellow-orange fluorescence.*

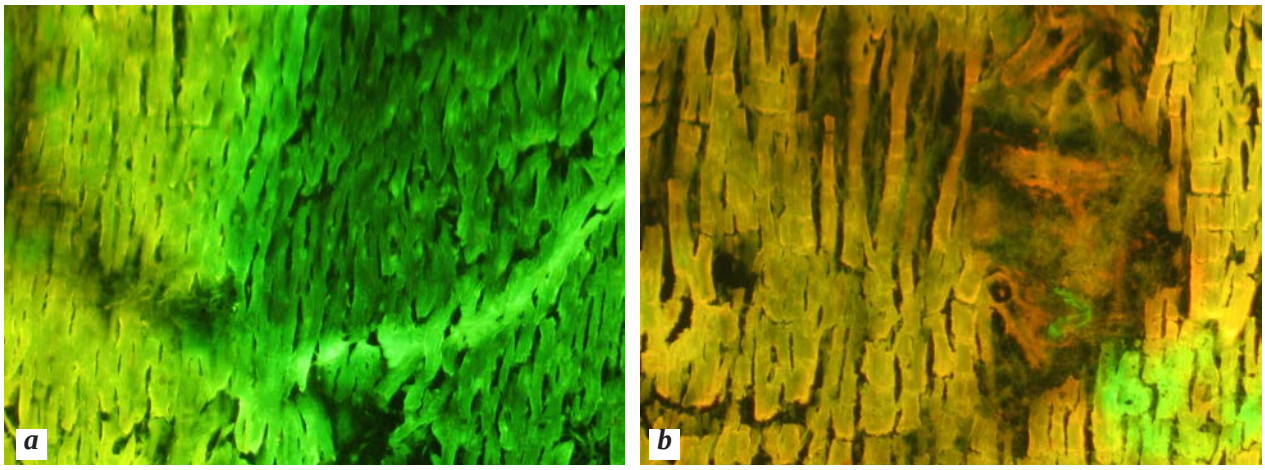

FIGURE 6. *Fluorescent-microscopic characteristics of study group III. Staining withacridil-orange.. **a)** Focal orange-red glow in the field of dystrophy and cardiomyocytes decay ob.40, oc. 10. Mainly yellow-orange fluorescence, including few cells with green glow in the cytoplasm in the perifocal area of adjacent sarcomeres. **b)** In areas remote from necrosis mosaic pattern is observed: fields with green glow of the cytoplasm of cardiomyocytes are interspersed with patches of yellow fluorescence of adjacent sarcomeres ob. 10, oc. 10.*

Discussion

Thanks to the research findings, in our case, that is, in all the three study groups, the method of myocardial samples staining with AO followed by luminescence microscopy examination was very informative in determining the severity of the damage of cardiomyocytes and the spreading pattern of the regional pathological process. Tinctorial features of cardiomyocytes in the treatment of microscope slides with AO in all the three groups were manifested as green fluorescence localized within structurally intact cells: green and yellow – in cardiomyocytes in the early stages of dystrophic processes, orange-red – in the muscle cells, in far advanced dystrophic processes in the myocardium.

The fluorescent method we used – AO staining of slides – also in conjunction with other informative morphological methods, allows for differential diagnosis of pathological processes occurring in the myocardium in the groups we studied.

The method is based on the unique tinctorial properties of AO, which makes it possible to determine the severity of the regional pathological process based on identifiable transformation of cardiomyocytes' color range of fluorescence from green to orange-red.

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