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Estimation of connexin 43 in myocard by experimental modeling of acute ischemia

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ABSTRACT

We induced acute ischemia by ligation of the left coronary artery in the area between left atrial appendage and pulmonary trunk. Animals were sacrificed after 1 hour, 3 hours, 12 and 24 hours. Using immunohistochemical methods to determine the intensity of connexin 43 in cardiomyocytes allows to identify sites of acute ischemic myocardial damage. We estimated the distribution of connexin in the area of intercalated discs and Z-bands in the myocardium by experimental modeling of acute ischemia in laboratory male rats of "Wistar" line. Immunohistochemical research of myocardium carried out with the purpose to estimate expression severity of connexin 43 can be recommended for use in cases of morphological examination of dead after sudden cardiac death for diagnostic of myocardial ischemia.

Introduction

Connexin 43 (Cx43) from connexinformed is an important cell protein composing the intercellular gap junctions of cardiac myocytes. Cx43 takes part in functioning of intercellular canals, that allows to provide diffusion of low-molecular compositions between nearby cardiac myocytes. In view of abovementioned the role of Cx43 in providing cardiac myocytes metabolism becomes clear. It takes part in providing intercellular interactions realizing by means of gap junctions [1, 2]. Estimation of cell protein distribution being part of intercellular junctions of cardiac myocytes allows to extend the understanding of mechanisms being the base of injury of cardiac myocytes structure response to acute ischemia [3, 4].

Aim of the Research

Revealing of Cx43 expression in intercellular junctions of cardiac myocytes by experimental modeling of acute myocardial ischemia.

Materials and Methods

White rats of "Wistar" line were used as experimental animals. Choice of rats as laboratory animals is connected with the fact that pathology of acute site injuries of myocardium in these animals is investigated enough detailed and described in the literature that is convenient to carry out the experiment [5–7]. In medicine and biology the opportunities of modern informative immunohisto-

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chemical and immunocytochemical methods of organs and tissues examination have been using during the last decades very intensively [8, 9]. Antibodies to Cx43/GJA1 (rabbit polyclonal, ABCAM, USA) were used for estimation of Cx43 expression in intercellular junctions of cardiac myocytes.

Experiments in modeling of acute myocardial ischemia were carried out in 43 male rats, weight of the animals was 180–200 g. Control group consisted of 3 animals.

Laboratory animals were kept in standard conditions of vivarium, in one cage 5 individuals, which had free access to water by strong keeping international rules of bioethics suggested by international organizations and associations including the World Medical Association's Declaration of Helsinki (2013).

At the first stage of experiment the weight of animal was determined for the following calculation of chloral hydrate doze, which with the purpose of general anesthesia was inserted intraperitoneally 300 mg/kg of body weight [6]. After general anesthesia the front area of the animal chest was shaven, animals were located on the back on the device for feet fixation. By animals of experimental groups acute myocardial ischemia was carried out after pleuracotomy suturing area of myocardium in projection of left coronary artery in the area between left atrial appendage and pulmonary trunk. After occlusion of left coronary artery the chest wall was sutured immediately.

Animals were sacrificed in 1 hour, 3 hours, 12 and 24 hours (10 animals in each time period and 3 animals from control group). By sacrificed animals the chest and abdominal cavity were lanced, working heart was cut off and put into Petri dish with ice for prevention of fibrillation before the moment of last activity. The hearts were fixed in 10% buffered formaline (Biovitrum, Russia) during 24 hours. After fixation the organs were incised in frontal plane into two parts, then standard entry of material in histoprocessor (STP-200, Leica) was carried out. Slices of about 5 μm thick were prepared on the rotary microtome from enclosed in paraffin samples, slices were stained by hematoxylin and eosin.

Histological and immunohistochemical investigations were carried out on the received materials of experimental animals, hearts were fixed in 10% buffered formaline (Biovitrum, Russia) during 24 hours. After fixation process the hearts were incised in frontal plane into four parts, fragments 3–5 mm thick were cut off. Then the standard entry of material in histoprocessor (STP-200, Leica) was carried out. Slices about 5 μm thick were prepared on the rotary microtome from the placed in paraffin samples, the slices were stained by hematoxylin and eosin.

The procedure of immunohistochemical staining was carried out according to recommendations of manufacturing company of antibodies, and according to recommendations of immunohistochemical researches guidelines [8]. Before the carrying out of immunohistochemical staining the prepared slices were dewaxed and tissue antigens were unmasked in PT Link module (Dako, Denmark) in citrate buffer (pH 6.0) at the temperature of 95° during 60 min. Then endogenous peroxidase was blocked by 3% H_2O_2 solution, protein blocking was carried out by serum. The received slices were incubated with antibodies to Cx43/GJA1 (rabbit polyclonal, ABCAM, USA). For immuno-staining the polymer system of detection was used with peroxidase mark (EnVision FLEX, DAKO, Denmark). At the last stage of the experiment the cell nucleuses were stained by hematoxylin.

Square of DAB-positive products of immunohistochemical reaction was estimated as a percent of picture square by microscope Axio Scope.A1 with the camera AxioCam MRc5 and software ZEN blue (C. Zeiss). 35 pictures with zoom 40×12 were estimated for each parameter.

Results and Discussion

By examination of animals sacrificed after 1 and 3 hours we have seen the staining changes of heart muscle in the area of front wall of the left ventricle in the form of edematic, a little bit pale site with indistinct boundaries. After 12 hours the edematic pale site on the front wall of the left ventricle was seen clear. After 24 hours on the front wall of left ventricle we could see the yellow-brown site with the distinct boundaries.

By microscopy of slices stained by hematoxylin and eosin of the animal of the control group the myocardium kept its distinctive histological structure, the cardiomyocytes had uniform staining of cytoplasm and nucleuses, there were also some symptoms of the beginning fragmentation of separated groups of myocytes and non-uniform blood filling of vessels.

At the next stage of microscopic investigation myocardium slices of animals from the control group stained immunohistochemical with Cx43 were estimated. By immunohistochemical staining allowing to detect Cx43 the cardiomyocytes had pale basophilic staining with seeable cross striation, cell nucleuses had distinct boundaries, oblong oval shape and basophilic staining. Selectively stained Cx43 was good seen in intercellular gap junctions. Cx43 was represented as the brown strips in microscopy with low zoom. By microscopy with high zoom Cx43 was represented as accumulations of the brown grains in the area of intercellular junctions of cardiac myocyte (Figure 1).

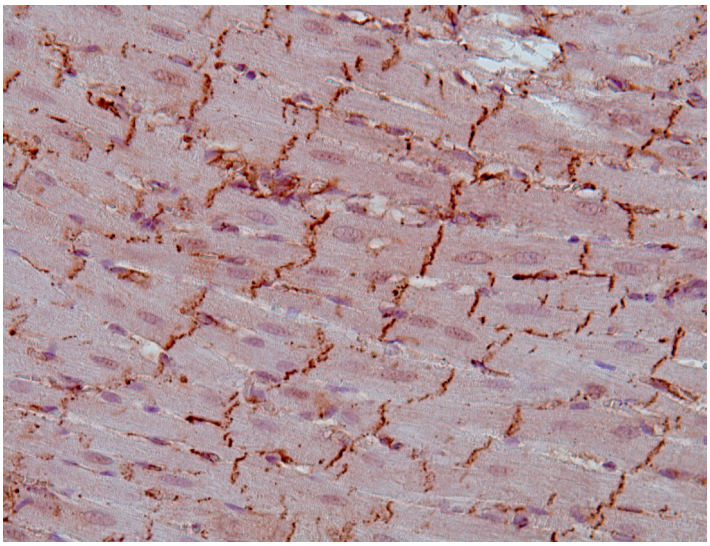

Figure 1. Connexin 43 in the area of intercalated disks of cardiac myocytes in the control animal group. Zoom 400

Estimation of myocardium slices stained by hematoxylin and eosin 1 hour after occlusion of left coronary artery allowed to detect the starting intermuscular edema combined with acute blood circulation disorder. Small arteries were in the condition of spasm, blue vessels were extended and fully blooded. In a number of vessels we observed dowel extension of endotheliocytes. In capillaries the sludging of erythrocytes was pointed out (Figure 2). The main part of cardiac myocytes kept normal structure, in some view fields indications of metachromasy were expressed enough.

By immunochemical staining of slices allowing to determine Cx43 the changes of intercalated disks were pointed out in the area of myocardial ischemia. By microscopy of the big zoom Cx43 was seen clear not in all intercellular areas in a form of strip-like located compact accumulations of brown grains. In some places the brown grains filled in intercellular gaps, sometimes partly.

Morphology of myocardium slices stained by hematoxylin and eosin in 3 hours form the occlusion moment was characterized by intensification of indications of blood circulation disorder; sludging process were seen clear in vessels of microcirculatory bloodstream as well as in small arteries and veins. In arteries on the background of sludging the separation of plasma was pointed out. While augmentation of myocardial ischemia after occlusion of the left coronary artery during 3 hours we fixed appearance of expressed perivascular edema. Statistical changes of arterial bed vessels in 3 hours after ischemia changed

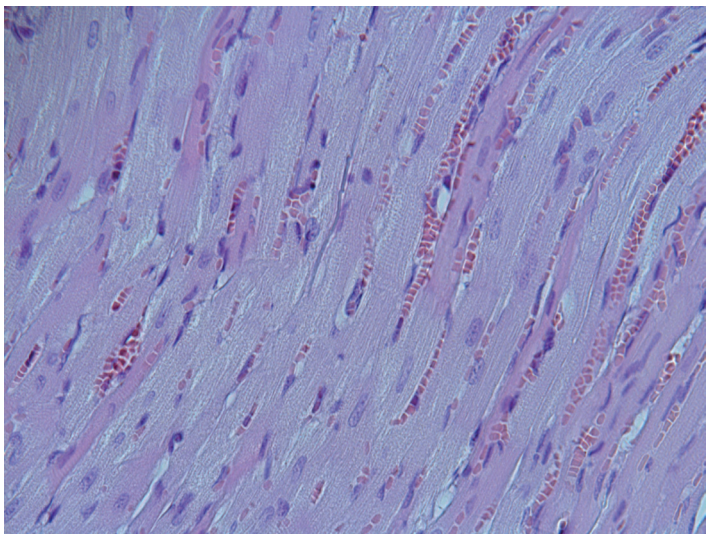

Figure 2. Acute blood circulation disorders in the area of ischemia in 1 hour after occlusion. Zoom 400

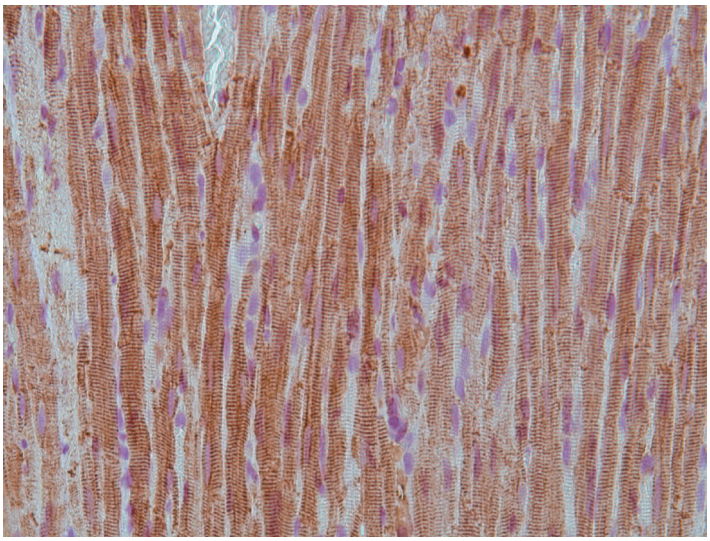

Figure 3. Reduction of Cx43 expression in the area of ischemia after 12 hours of occlusion. Zoom 200

by paresis, at the same time vascular congestion of veins was kept. More expressed wave-shaped deformation of muscle fibers was pointed out.

The above data showed that the carried out microscopy investigation of slices histochemically modified for identification of Cx43 allowed to identify significant reduction of Cx43 density in intercellular junctions, which in the low zoom looked like reduction, shortening and incomplete staining of intercellular zones of cardiac myocytes junctions. Together with above described changes we revealed the areas of connexin distribution on the side surfaces of cells. Also we pointed out the staining of myocytes cytoplasm in pale brown. Such changes in cells had a nature of pussles.

Acute myocardial ischemia was accompanied by even more blood circulation disorders in 12 hours, increased edema of stroma and perivascular spaces coupled with site hemorrhage and responsive changes as a polymorphcellular infiltration by polymorphonuclear leukocytes and lymphocytes.

By microscopy of the stained slides allowing to estimate expression of Cx43 in the area of ischemia it was pointed out that Cx43 fast fully disappeared from the area of intercellular cardiac myocytes junctions, we observed non-uniform staining of cardiac myocytes cytoplasm in brown (Figure 3). At the same time by low zoom the process of metachromasy was good seen both in the area of ischemia and on the periphery of the focus.

In 24 hours of acute ischemia in the area of myocardial necrosis perivascular and stroma edema was clearly expressed, there were site hemorrhages. Expressed responsive changes were represented by polymorphcellular infiltration of polymorphonuclear leukocytes and lymphocytes. By estimation of myocardium structure we pointed out different-sized cardiac myocytes, having among them thickened swollen cells with homogenized cytoplasm without nucleuses. By microscopy of myocardium slices with immunohistochemical staining we can not distinguish cross striation in many cardiac myocytes, this striation was kept as separate foci only. Cardiac myocytes cytoplasm was of intensive brown color.

Comparative estimation of ischemia zone morphology allowed to identify that by staining of slides stained immunohistochemic square of myocardium injury exceeded the same one in slices stained by hematoxylin and eosin.

As seen above, morphological investigation of peculiarities of Cx43 distribution under acute myocardial ischemia the following revealed the following: protein of intercellular junctions of cardiac myocytes (Cx43) taking part in providing of intercellular interaction was represented by areas of brown color in the zone of intercalated disks (in control group). While intensification of myocardial ischemia we noticed reduction of Cx43 expression in intercellular junctions of cardiac myocytes and its appearance on the side surfaces of muscle cells. At the same time staining of cellular cytoplasm in brown was observed that was connected with metabolic disturbance of Cx43.

Conclusion

By estimation of myocardial ischemia intensity the reduction of Cx43 expression was pointed out in intercellular junctions of cardiac myocytes. Taking part in providing of intercellular interaction protein of intercellular junctions of cardiac myocytes (Cx43) is a cell structure element, which sensitively responded

to ischemic impact. Reduction of the revealed (Cx43) in intercellular junctions of cardiac myocytes in the left heart ventricle correlates with duration of left coronary artery occlusion and can be considered as one of the indications of acute myocardial ischemia.

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