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PATHOGENETIC FEATURES OF MORPHODENSITOMETRIC CHARACTERISTICS OF CARDIOMYOCYTES AND MARKER PROFILE OF THE LEFT VENTRICULAR REMODELING IN RATS WITH EXPERIMENTAL INTERMITTENT HYPOXIA OF DIFFERENT DURATION

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Abstract

Myocardial remodeling is considered as a three-component complex process, including cardiomyocyte hypertrophy, their apoptosis, interstitial and perivascular fibrosis. At the same time, the nature of remodeling and the direction of restructuring in the myocardium depend on the prevalence of one of these processes, the degree of their severity and the impact duration. Therefore, to understand the effect of short- and long-term intermittent hypoxia on the direction of myocardial remodeling and its type, **the aim** of this study was to determine the pathogenetic features of the morphodensitometric characteristics of cardiomyocytes and

the marker profile of left ventricular myocardial remodeling in the rats with experimental intermittent hypoxia durated 15 and 60 days.

Material and methods. A total of 30 male Wistar rats aging 6-10 months were used for the experiment and assigned to 3 experimental groups of 10 animals each: control; rats exposed to 15-day hypoxia; rats exposed to 60-day hypoxia. The object of the study was the left ventricular myocardium. The number of nuclei in an image, their average linear size and density (the ratio of the total area of nuclei to the area of the cytoplasm), and the concentration of RNA in the nucleus and cytoplasm were measured in the sections stained by Einarson via morphodensitometric method. An immunofluorescence method was used to study the content of immunoreactive material to markers of remodeling (cardiotrophin-1, type I collagen, titin, annexin V). As **a result** of the study, it was found that intermittent hypoxia led to a decrease in the number of nuclei in cardiomyocytes in both experimental groups compared to the control. Such changes in the number of nuclei were accompanied by significantly larger nuclear size compared to the control (in rats with 15-day hypoxia by 52.9 %, in rats with 60-day hypoxia - by 153.4 %) and a decrease in the density of nuclei in relation to the cytoplasm by 19.5 % and 21.2 %, respectively. In both groups, a significantly lower control concentration of RNA in the nuclei of cardiomyocytes was found alongside with an increase in their area. Such changes were accompanied by a significantly higher concentration of RNA by 23.2 % in the cytoplasm in case of long-term intermittent hypoxia. The marker profile of remodeling parameters in rats exposed to 15-day hypoxia was characterized by a higher content of cardiotrophin-1 and titin by 11.5 % and 23.1 %, respectively, as compared to the control. The content of type I collagen was 19.9 % higher, while the content of annexin V did not change significantly. In rats with long-term 60-day hypoxia, the content of cardiotrophin-1 was higher by 73.6 %, titin - by 124.9 %, type I collagen - by 41.9 %, and annexin V - by 95.9 % in comparison to the control. When calculating the titin / collagen ratio in rats based on their content, a significant increase in myocardial stiffness was determined in the 60-day hypoxia group which was 1.44, while it was 0.91 in the control, and in rats with 15-day short-term hypoxia – 0.93.

Conclusions. Intermittent hypoxic effects, regardless of duration, result in morpho-structural rearrangements of the myocardium, are characterized by an increase in viscoelastic mechanical properties and the development of hypertrophy, but with an increase in the duration of exposure (60 days) – by an additional formation of interstitial fibrosis and apoptosis of cardiomyocytes. Short-term hypoxia forms a hypertrophic type of myocardial remodeling with a decrease in the number of nuclei alongside with an increase in their size

and a moderate decrease in RNA concentration. The marker profile of remodeling changes in a physiological manner and is characterized by an increase in the content of cardiotrophin-1, type I collagen and titin, while maintaining the titin / collagen ratio and normal apoptotic rate. Long-term hypoxia causes a fibro-apoptotic type of pathological myocardial remodeling including an almost two-fold decrease in the number of nuclei with an increase in their size and a decrease in RNA concentration. The marker profile of remodeling is characterized by an increase in all 4 components, a significant increase in the titin / collagen ratio and high apoptotic rate of cardiomyocytes.

Key words: titin, annexin V; cardiotrophin-1; type I collagen; myocardium; left ventricle of the heart; intermittent hypoxia; Wistar rats.

Introduction

Analysis of the results of large-scale studies has shown that various impacts on the cardiovascular system (CCC) result in myocardium remodeling, the component ratio of which depends on the severity and duration of driving factors. The left ventricular myocardium is sensitive to pressure overload and oxygenation levels, so it is the first to adapt through physiological myocardial remodeling (PhMR) [1]. In a case of overstimulation, its long-lasting effect or ineffective adaptation, the physiological effect becomes pathological, and remodeling turns pathological (PMR). A demonstrative example of this action is hypoxia. Short-term intermittent stimuli have a therapeutic effect, causing PhMR, while long-term influence and overload tend to PMR. Most often, researchers believe that intermittent hypoxia at last causes physiological changes in the myocardium, but all of these studies were short-term estimating a limited number of parameters without a comprehensive description of morpho-structural changes in the heart "geometry" [2].

Today, myocardial remodeling is considered as a three-component complex process, including hypertrophy of cardiomyocytes, their apoptosis, interstitial and perivascular fibrosis. The nature of remodeling and the direction of restructuring in the heart muscle depend on the prevalence of one of these processes and their severity [3].

In this regard, in recent years, in accordance with cardiological and pathoanatomical guidelines for the purpose of determining the component ratio of myocardial remodeling and evaluating morpho-structural changes in it, it is recommended to study a panel of biomarkers consisting of cardiotrophin-1 – a marker of hypertrophy [4], titin – a protein that is a part of the sarcomere for modeling the myocardial passive elasticity [5], type I collagen – a key component of the myocardial interstitial skeleton, which in case of excessive biosynthesis and

impaired degradation leads to the development of fibrosis [6], and annexin V – as a marker of cardiomyocyte apoptosis [7].

Therefore, to understand the influence of short- and long-term intermittent hypoxia (IH) on the direction of myocardial remodeling and to establish its type, **the aim** of the study was to determine the pathogenetic features of morphodensitometric characteristics of cardiomyocytes and marker profile of the left ventricular remodeling in rats with experimental intermittent hypoxia of different duration (15 and 60 days).

Materials and methods. The experiment was conducted on the basis of the Training Medical Laboratory Center of the Zaporizhzhia State Medical University (Certificate of technical qualification 033/18 of 12.12.2018, valid until 25.12 2023). All devices used for study were certified and underwent annual metrological control (Laboratory of Experimental Pathophysiology, License 2CK2 YMK2 T6PB SG5N SJLS4).

The experimental part of the study was carried out exactly in accordance with the National “Common Ethical Principles of Animal Experiments” (Ukraine, 2001), in agreement with the Directive 2010/63EU of the European Parliament and of the Council of 22 September 2010 on the protection of animals used for scientific purposes [8].

To detect the pathogenetic features of the marker profile in left ventricular remodeling, a total of 30 male rats, weighing 220-290 g, 6-10-month-old were divided into 3 experimental groups: the 1st – the control group (10 intact male Wistar rats); the 2nd – 10 male Wistar rats exposed to 15-day intermittent hypoxia (IH 15), the 3rd – 10 male Wistar rats exposed to 60-day intermittent hypoxia (IH 60).

Hypoxic training was performed in a ventilated pressure chamber with a volume of 1.0 cubic meter (m³) at an altitude of 6000 m (pO₂ = 9.8 %) using a model widely used for the studies at the Department of Pathophysiology of ZSMU [9]. The animals were housed in the pressure chamber daily from 10 am to 4 pm. The training was conducted in the following mode: an oxygen tension in the pressure chamber corresponded to the altitude of 1 km on the 1st day, 2 km - on the 2nd day, 3 km – on the 3rd, 4 km - on the 4th, 5 km - on the 5th, 6 km - on the 6th day and in the days that followed. Hypoxic training at the altitude of 6 km was performed for 10 days in 15-day hypoxia and for 55 days in 60-day hypoxia. [10].

The animals were euthanized via rapid decapitation after thiopental anesthesia (45 mg/kg body weight, intraperitoneally). The study objects in the experimental animals was fragments of the left ventricle, which were embedded in paraplast blocks after standard histological preparation and then sectioned into 5 µm-thick slices using a rotary microtome Microm-325 (MicromCorp, Germany).

The images of sections stained by Einarson via morphodensitometric method were taken. AxioScope (Carl Zeiss, Germany) microscope images were analyzed in an interactive mode in AxioVision 40 V 4.8.2.0 software program (License No. 3005339) using ImageJ software (National Institutes of Health, USA); the number of cardiomyocyte nuclei, the average nuclear linear size (μm), the nuclear density (a ratio of the total nuclear area to the cytoplasmic area), the concentration of RNA in the nucleus and cytoplasm (OD) were calculated.

The study of the immunoreactive material (IRM) to remodeling markers content (IF) was carried out using the immunofluorescence technique in accordance with the protocol of the immunohistochemical study [11]. Goat polyclonal antibodies Cardiotrophin-1 were used to detect cardiotrophin-1, (N-20): sc-20867; primary goat polyclonal antibodies COL1A1 (C-18): sc-8784 - type I collagen; goat polyclonal antibodies Annexin V (R-20): sc-1929 - annexin V. FITC-conjugated murine anti-goat IgG: sc-2356 were used as secondary antibodies to the above mentioned markers. Primary murine monoclonal antibodies Titin (E-2): sc-271946 and secondary FITC-conjugated murine anti-rabbit IgG: sc-2359 were used to detect titin. All antibodies, produced by Santa Cruz Biotechnology, Inc., were applied at a dilution of 1:200.

To understand the degree of fibrosis development in the experimental rats, the titin/collagen ratio was calculated according to their content.

All statistical computations were performed in the Microsoft Excel 2016 table processor (Microsoft Corp., USA). For all parameters, the arithmetic mean (M), its dispersion and mean error (m) were calculated. To determine the significance of differences between the study results among the experimental and control groups of rats, the Student's coefficient (t) was calculated, followed by a verification of significant differences between the samples (p) and the confidence interval of the mean according to the Student's distribution tables. A P_{St} value < 0.05 were considered statistically significant [12].

Results. As a study result, it was found that intermittent hypoxia led to a decrease in the number of nuclei in cardiomyocytes in both experimental groups compared to the control. In IH60 rats, this indicator was significantly lower than that in the control by 39% (Table 1).

Such changes in the number of nuclei were accompanied by a significant increase in their size as compared to the control (in IH15 rats by 52.9%, in IH60 rats – by 153.4%) and a decrease in the nuclear density compared to the cytoplasm by 19.5% in IH15 and 21.2% in IH60 rats (Table 1).

Table 1 – Morphodensitometric characteristics of the myocardium in rats with experimental hypoxia, (M±m)

Parameters	Control	IH15	IH60
Number of the nuclei	1069,1±24,2	1013,8±23,1	651,8±15,3*
The nuclear size, μm	6,1±0,2	9,3±0,2*	15,5±0,3*
The nuclear density, μm	21,9±0,5	17,6±0,5*	17,2±0,5*
RNA concentration in the nucleus, OD	0,241±0,007	0,157±0,002*	0,140±0,003*
RNA concentration in the cytoplasm, OD	0,052±0,001	0,053±0,000	0,064±*0,001

Note. * – significance of the differences in comparison with the control group, P<0.05.

An interesting pattern was found in both groups as a significantly lower RNA concentration in the nuclei of cardiomyocytes with an increase in their area. Thus, this indicator was 34.9% lower in animals with IH15 than the control one, while in IH60 rats – by 41.9% (Table 1). Such changes were accompanied by 23.2% higher concentration of RNA in the cytoplasm in IH60, and this indicator was characterized only by a tendency to increase in IH15 rats (Table 1).

The marker profile of remodeling parameters in rats of the experimental groups changed in a way different from the morphodensitometric parameters. The content of cardiotrophin-1 and titin in IH15 rats was significantly higher by 11.5% and by 23.1% than that in the control. The content of collagen type I showed significant changes in the direction of increase by 19.9% in comparison with the control, while the content of annexin V did not change significantly compared to the control values (Table 2).

Table 2 – The content of immunoreactive material to markers of the left ventricular myocardium remodeling in the rats with experimental hypoxia, (M±m)

Parameters	Control	IH15	IH60
Cardiotrophin-1	620,8±21,6	692,3±19,7*	1077,4±38,0*
Collagen type I	664,6±22,1	796,9±18,5*	943,2±36,8*
Titin	602,2±18,2	741,1±21,3*	1354,5±27,2*
Annexin V	594,1±23,7	598,8±19,2	1163,8±50,9*

Note. * – significance of the differences in comparison with the control group P<0.05.

In rats with long-term hypoxia IH60, the content of cardiotrophin-1 was higher by 73.6% than that in the control, titin – by 124.9%, the value of collagen type I exceeded the

control index by 41.9%, and annexin V – by 95.9% (Table 2). Calculated the titin/collagen ratio revealed a significant increase in myocardial stiffness in the group of rats with IH60, which amounted to 1.44, while it was 0.91 in the control, and 0.93 – in IH15.

Discussion. The experimental study showed that the hypoxic effect, regardless of its duration, was accompanied by changes in the left ventricle myocardium and a decrease in the number of nuclei with a compensatory increase in their size, especially in IH60 rats (2.5 times larger than that in the control). However, the number of nuclei in IH60 rats was less by 35.7% than that in IH15. Due to changes in the total area of the nuclei and cytoplasm of cardiomyocytes, the nuclear density showed the downward trend. Such morpho-structural changes of nuclei and their ratio to the cytoplasm are a sign of the hypertrophy development as a response to hypoxic effects. It was expected to identify major changes in the myocardium of rats with long-term hypoxia – IH60 (Table 1 and 2). Similar facts have been proven by Antonio F. Corno et al. in the study confirming the formation of myocardial hypertrophy in response to chronic and intermittent hypoxia [13].

The identified lower RNA concentration in the nuclei of cardiomyocytes in the rats of both experimental groups should be considered as a result of the increase in their area while maintaining the total RNA content in the first case of IH15, and as a possible nuclear dysfunction - in the second case of IH60 [14].

The determined nature of the marker profile has enabled to assess the type of remodeling and its direction towards physiological or pathological, as well as to identify the dependence on the hypoxic exposure duration. Thus, under hypoxic effects in rats, an increase in the marker of myocardial hypertrophy cardiotrophin-1 was observed. Its content was quantitatively dependent on the exposure duration and IH60 index exceeded IH15 by 2 times. According to Mirtschink P. and others, this increase could be initiated by HIF-factor, which is the main regulator of key indicators in response mediated by gene transcription at low oxygen levels [15].

At the present stage, it is impossible to characterize the morpho-structural rearrangements in the myocardium and describe the nature of fibrotic changes without taking into account the content of titin and collagen type I, the increase in expression of which was reliably found in rats of both experimental groups. However, it is important to note that in rats with IH15, the ratio remained as close as possible to the control values (0.93:0.91) amid the moderate increase in their content. Probably, the 15-day hypoxic effect helps to increase the elasticity of the matrix skeleton as an element of adaptation to new functioning conditions, but is insufficient for the formation of fibrosis. In contrast to our results, Sataeva T. P. and

Zadniptyany I. V. obtained significant manifestations of myocardial remodeling with the prevalence of fibrosis in 15-day exposure at 3.5 km that developed due to the activation of fibroblasts and reduced collagen degradation. But in their study, the authors did not differentiate the type of collagen, which provides no confidence that its accumulation is pathological [16].

Prolonged 60-day hypoxic effect resulted in a significant increase in the content of titin and collagen with the almost two-fold increase in their ratio compared to the control (1.44:0.91) demonstrating the development of fibrosis amid the compensatory increase in elastic properties of the heart muscle. The researchers suggest that such changes in titin content were associated with oxidative modification of all three filamentous systems of the sarcomere and titin isoforms imbalance towards increasing a more severe form of N2B. These changes were especially pronounced in oxidative stress, which have been developed in prolonged hypoxia [17].

Determination of the annexin V content as the marker of cardiomyocyte apoptosis showed that there were no significant changes in 15-day hypoxic exposure. This fact suggested the physiological mode of cardiac muscle remodeling in rats of this group. In the case of long-term hypoxia, the content of annexin V was found to be 124.8 % higher than that in the control, which was a sign of significant cell death processes with the development of so-called myogenic dilation of the heart [18].

Conclusions

1. Intermittent hypoxic effects, regardless of duration, result in morpho-structural rearrangements of the myocardium, are characterized by an increase in viscoelastic mechanical properties and the development of hypertrophy, but with an increase in the duration of exposure (60 days) – by an additional formation of interstitial fibrosis and apoptosis of cardiomyocytes.

2. Short-term hypoxia forms a hypertrophic type of myocardial remodeling with a decrease in the number of nuclei alongside with an increase in their size and a moderate decrease in RNA concentration. The marker profile of remodeling changes in a physiological manner and is characterized by an increase in the content of cardiotrophin-1, type I collagen and titin, while maintaining the titin / collagen ratio and normal apoptotic rate.

3. Long-term hypoxia causes a fibro-apoptotic type of pathological myocardial remodeling including an almost two-fold decrease in the number of nuclei with an increase in their size and a decrease in RNA concentration. The marker profile of remodeling is

characterized by an increase in all 4 components, a significant increase in the titin / collagen ratio and high apoptotic rate of cardiomyocytes.

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