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THE INFLUENCE OF GRAPE POLYPHENOLS OF THE MYOCARDIUM IN YOUNG RATS UNDER CONDITIONS OF HYPOBARIC COLD HYPOXIA

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Abstract

The aim of this research was to reveal the cardioprotective properties of food concentrate polyphenols grapes "Fenokor" in experimental hypobaric hypoxia. The study was conducted on mature male Wistar rats (n = 25) aged 1-1.5 months divided into three experimental groups. In the I (control) group, an isotonic NaCl solution was administered intragastrically to intact animals. Hypobaric cold hypoxia was modeled for rats of groups II and III once a day for 30 days. Animals III series 30 minutes before hypoxia intragastrically through a tube were injected an aqueous solution "Fenokor" containing grape polyphenols.

In rats of II group without correction there was an unreliable decrease in heart mass by 2.7%, a decrease in the diameter of cardiomyocytes by 24.3% was accompanied by a decrease in their concentration in 1 mg of tissue by 16%. Accelerated compensatory physiological growth of cardiomyocytes in animals of group III caused a slight decrease in the diameter of a number of cardiomyocytes by 3.7% with a decrease in their concentration by 11.4%, and as a consequence, that mostly preserved of the total number of cardiomyocytes in the heart by the end of the experiment. Hypertrophic growth of the number of cardiomyocytes is caused by the end of the experiment statistically significant compensatory increase in the mass of the heart 9.7%.

Thus, the main morphological signs of hypoxic myocardial damage in young rats were presented by the phenomena of mixed dystrophy, edema and destruction of contractile cardiomyocytes, lysis of myofibrils and mitochondrial cristae, contracture changes. The administration of "Fenokor" demonstrated its cytoprotective properties, contributed to the preservation of myocardial structure of rats in conditions of histotoxic hypoxia.

Keywords: *myocardium, hypoxia, cardiomyocytes, grape polyphenols.*

Introduction

Understanding the fundamental role of hypoxia and peroxidation in the pathogenesis of many cardiovascular pathologies stimulated the development of tools that can enhance the energy functions of cells. Considering the interaction of the two leading processes responsible for the development and course of most pathologies, many authors see the following sequence of events: during hypoxia and ischemia, inhibition of energy production in cells and the development of acidosis is observed, which, in turn, activates the processes of free radicals and inhibits antioxidant protection [*Czibik G, 2010*]. Lipid peroxidation products (POL) by damaging cell membranes (including mitochondrial) exacerbate the disturbances of energy metabolism, creating a so-called "vicious circle" [*Holt EM et al, 2009*]. In this regard, the application of remedies that contribute to the regulation of energy metabolism may break that vicious circle on its early stages due to the effect of antioxidants which decrease the initial level of re-

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active oxygen species (ROS), which have an indirect effect on cellular energy [*Kjaergaard K, 2006*].

Nowadays a whole arsenal of lowering blood pressure drugs combined with antianginal and antihypoxic properties that affect cell metabolism is currently applied in the treatment of cardiovascular pathologies [*Ravens U et al, 2013*]. In fact, most of these are symptomatic drugs that do not eliminate the main cause of the disease — damage to the cardiovascular system at the subcellular level [*Rowe CA et al, 2011*]. Therefore, many researchers suggest the consideration of ultramicroscopic cadiomyocyte studies as primary markers (criteria) for calculating the risk of cardiovascular diseases, as well as for evaluating the effectiveness of preventive or therapeutic interventions, including the administration of polyphenolic grape compounds [*Chou EJ et al, 2001*].

Deep research of the mechanisms of polyphenolic effects on human health began with the discovery of the so-called "French paradox" [*Li H et al, 2012*]. The "French paradox" was proclaimed during a large-scale international epidemiological study. At the same time it was shown that among the French people the prevalence of atherosclerosis and mortality from cardiovascular diseases were significantly lower than in Canada, Great Britain, Italy, Scotland, and the USA [*Mink PJ et al, 2007*]. And this fact controversies the abundant French diet consisting of meat and fatty foods enriched by saturated fats and cholesterol. This phenomenon was associated with the widespread consumption of red wine in France and was first associated with the "wine alcohol" [*O'Byrne DJ et al, 2002*]. However, M.G. Hertog and co-authors revealed that positive influence of grape wines are possible due to the presence of large quantities of various flavonoids [*Hertog MGL, 1993*]. At the same time, they provided primary data on the inverse relationship between the consumption of flavonoids (including wine flavonoids) and mortality from cardiovascular diseases, which was revealed during long-term (5-25 years) observations involving large groups of people. Later it was shown that not only red wines with a high content of flavonoids, but also white wines with their moderate polyphenolic content have a pronounced protective effect on the development of atherosclerosis in contrast to alcoholic beverages based on pure ethyl alcohol [*Rowe CA et al, 2011*]. The redox properties of different polyphenolic and flavonoid classes are provided by various compositions of functional chemical groups. Their variety leads to a diversity in the biochemical and pharmacological properties of different polyphenols and, consequently, their clinical application stategies [*Wightman, JD, Heuberger RA, 2015*].

Grape wines' effects have not been fully studied yet in regard to their promising effects in sanatorium-and-spa treatment of cardiopathies, that is mainly restricted due to the negative effects of relatively large doses of alcohol, which are present in the wine [*Mink PJ, 2007*]. However "Cabernet Sauvignon" grapes is used as a component of spa treatment in health resorts of the southern coast of the Crimea in the form of non-alcoholic food concentrate "Fenokor", which contains the whole spectra of polyphenols of grapes present in red wine but at the same time it is not containing any alcohol. Grape polyphenols are represented in it mainly by anthocyanins, leucoanthocyanins, catechins. The total content of total grape polyphenols in the concentrate is 20–28 g / dm^3, whereas in red wine it is only 0.2 g / dm^3. The flavonoid polyphenols in the "Fenokor" composition are represented mainly by anthocyanins in the form of glycosides of delphinidin, malvidin, cyanidin, petunidin, peonidin, and also quercetin and its glycoside, (+) - B-catechin, (-) - epicatechin [*Kubyshkin AV, 2017*].

The purpose of this study was to reveal the cardioprotective properties of the "Fenokor" concentrate of grape polyphenols in young rats exposed to hypobaric hypoxia.

Material and methods

The experiment was carried out on mature male Wistar rats (n = 25) aged 1–1.5 months, divided into three experimental groups. The I (control) group consisted of the intact animals (n = 5) which intragastrically received 15 *ml* of isotonic solution of sodium chloride by the probe. For rats of II (n = 10) and III (n = 10) experimental groups hypobaric cold hypoxia was simulated once a day for 30 days. Modeling of acute hypobaric hypoxia was carried out in a hypobaric chamber for laboratory animals placed in a refrigeration unit at a temperature of 0 ° C. Conditions simulating a stay at the "height" were provided by identifying the threshold resolu-

tion of the atmosphere. The "elevation" of animals was carried out to a height of 6000 *m*. The exposition at the "height" was 30 minutes. In 30 *min* before placement in the hypobaric chamber the III group of animals additionally intragastrically received an aqueous solution of Fenokor (LLC RESSFOOD, Yalta, patent No RU 150139 U1) by catheter at a dose of 2.5 *ml/kg* equivalent to 87.5 *mg* of polyphenolic compounds mixed with 0.5 ml of water [*Zadnipryany IV et al, 2017*].

Animals were housed in vivarium according to the rules and International recommendations of the European Convention for the Protection of Animals (1997). Experimental studies were carried out in accordance with the ethical requirements for working with experimental animals, Order of the USSR Ministry of Health No. 755 of August 12, 1987 "Rules for Working with Experimental Animals", Federal Law "On the Protection of Animals Against Cruel Treatment" of January 1, 1997, Order of the Ministry of Health Of the Russian Federation No. 267 dated June 19, 2003 "On the Approval of Laboratory Practice Rules" and approved by the local ethical committee of the S. I. Georgievsky Medical Academy of V.I. Vernadsky Crimea Federal University.

After sacrificing the animals under ether anesthesia left ventricular myocardium fragments were fixed for 24 hours in 10% formalin solution, then the material was degreased and dehydrated in increasing alcohols, embedded in paraffin, and histological preparations were made with sections about 5 microns. For these purposes, standard equipment and reagents for paraffin wiring were used, the preparations were stained with iron hematoxylin (Rego modification) for review staining, and also with azocarmine to detect newly formed collagen. The obtained microslides were investigated using an Olympus CX-31 microscope (Japan). Morphometric measurements were made at a magnification of 400 using the licensed J Image program.

For electron microscopic examination, myocardial samples with a size of no more than 1 mm were fixed in a 2.5% glutaraldehyde solution in 0.1 M phosphate buffer with a pH of 7.2–7.4. After fixation, the myocardial samples were washed in 3 portions of Milligig's buffer for 20 minutes at a temperature of 4 ° C. The material was postfixed in a 1% solution of osmium tetroxide in the same buffer for 2 h in the cold, again washed in Milligig's buffer three times for 10 minutes. Dehydration of the tissue in a series of alcohols of increasing concentration was carried out according to the following scheme: alcohol 30% - 15 minutes, alcohol 50% - 15 minutes, alcohol 70% - 15 minutes, alcohol 96% - 15 minutes, two portions of alcohol 100% - 10 minutes each, alcohol 100%: acetone (1: 1) - 10 min, acetone - 15 min. Then the tissue was left overnight for impregnation in a mixture of epoch and araldite, and finally the tissue samples were also poured into the mixture of epon and araldite. Polymerization was carried out at 60°*C* for 24 *hours*. Semithin and ultrathin sections were obtained on an LKBIII ultratome (Sweden). The sections were viewed in a Selmi-125 electron microscope (Ukraine) at an accelerating voltage of 125 *kV* [*Kuo J, 2007*].

Statistical data processing was calculated on the licensed software Statistica 10.0. When analyzing the results of histomorphometric research methods, we estimated the arithmetic average for the whole group, the standard deviation, the average error, the coefficient of variation, the deviation of the value in the experiment from the value in the control in percent. The obtained quantitative data of morphometry (no less than 100 cardiomyocytes were studied in 10–15 fields of view for each animal) were subjected to a preliminary analysis for compliance of the distribution of characters with the normal law. Comparisons between groups were determined by a one-way ANOVA with a post-hoc Student t-test (SPSS Inc., Chicago, IL). Probabilities of $p<0.05$ were considered statistically significant.

RESULTS

The development of adaptive-compensatory processes in the myocardium occurs at its various structural levels. For each level there are basic structural criteria characterizing the state of the myocardium: at the tissue level - parenchyma and stroma, at the cellular level - the nucleus and cytoplasm, at the subcellular level - organelles. In control animals, the sarcoplasm of cardiomyocytes was uniformly stained with eosin, azure, and other acidic dyes. Cardiac myocytes had cylindrical shape, were connected to each other by intercalated disks and found transverse striation. In the light karyoplasm of the nuclei, small lumps of heterochromatin and one or two nucleoli were distinguished.

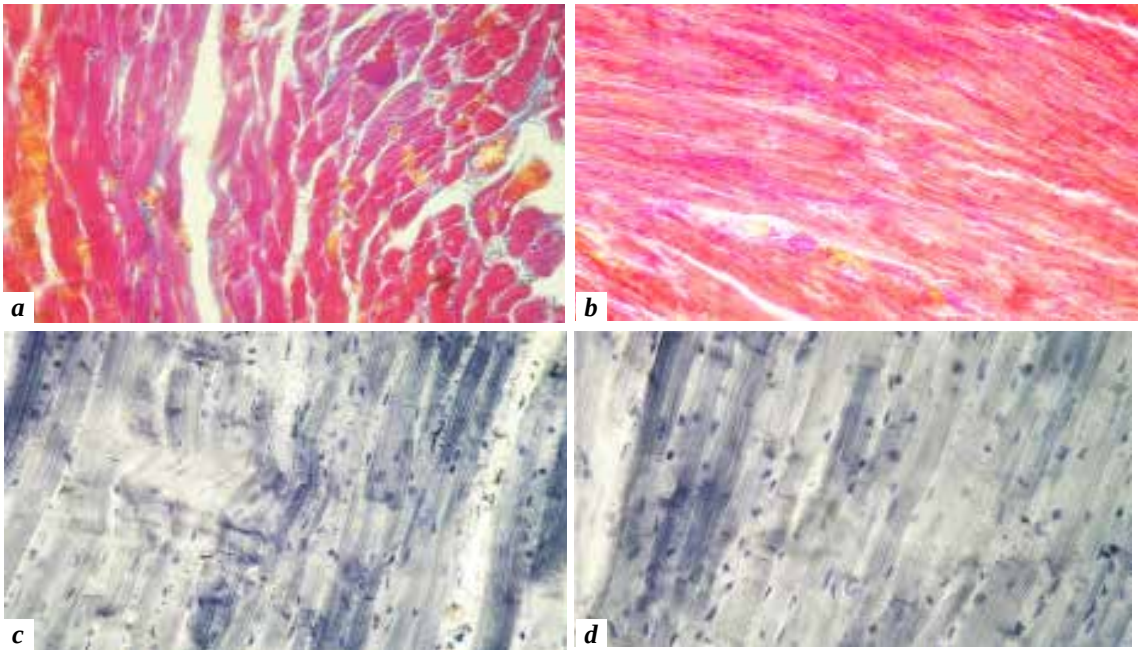

Figure 1. *Light microscopy of rat myocardium after exposure to hypobaric hypoxia.*
a *- thickened collagen fibers in the interstitium, vivid stromal edema. Rat myocardium after hypobaric hypoxia. Staining by azocarmine, x 400.*
b *- dark ischemic myofibrils with undulating deformity. Plethora of vessels. Myocardium rats after hypobaric hypoxia. Staining by Rego, x 400.*
c *- thin collagen layers in the interstitium, moderate edema of the perivascular space. Rat myocardium after hypobaric hypoxia with correction. Staining by azocarmine, x 400.*
d - mosaic color and heterogeneity of cardiomyocytes. Rat myocardium after hypobaric hypoxia with correction. Staining by Rego, x 400.

In all layers of the myocardium of group II, without correction of hypoxia, diffuse mononuclear infiltration and swelling of the stroma were recorded, numerous collagen fibers were detected in the perivascular space, which together provoked muscle fibers loosening (Fig. 1a). The combination of the above changes in the stroma aggravated the proper architectonics of the cardiac myofibrils. Contractile cardiomyocytes demonstrated the mosaic staining. Cardiomyocytes with lytic changes accounted for approximately 15% of the total. The remaining ones was characterized by a darker staining of sarcoplasm. The pronounced phenotypic heterogeneity of cardiomyocytes within a single muscle fiber attracted attention: along with hypertrophic cells the thinned ones with dystrophic changes were observed (Fig. 1b). Hemodynamic disorders were detected: venous and capillary plethora, spasm of intramural arteries, foci of hemorrhages. Foci of infiltration in the stroma were small, met most often perivascular and in places of atrophy and death of cardiomyocytes.

In group III, against the background of correction by polyphenol concentrate the connective tissue stroma of the myocardium contained few fibroblasts located between the muscle fibers combined with the gentle bundles of collagen fibers (Fig. 1c). Other mononuclear cells were observed in the perivascular spaces, sometimes forming small clusters. Mosaic staining of cardiomyocytes appeared, due to the presence of cells with both unchanged tinctorial properties, and cells with pronounced lytic changes of sarcoplasm (Fig. 1d). Cardiomyocytes with signs of lysis of the sarcoplasm accounted for about 5% of all cardiomyocytes, in rare cases there were ruptures of muscle fibers along the intercalted discs. Hemodynamic disorders showed compensatory reactive responses: the venules were enlarged, full-blooded, some arterioles were spasmodic, the capillaries were dilated, filled with red blood cells (Fig. 1b). As a result of hemodynamic disorders, moderate interstitial edema developed. In general overview the preservation of the structure of myocardial tissue was observed.

Evaluation of the regularities of cardiac remod-

eling in hypobaric hypoxia as well as other basic pathological processes is impossible without elucidation of the dynamical changes in the total number of cardiomyocytes in the ventricles of the heart.

In rats of group II without correction, there was an non-significant decrease in heart mass by 2.7%, a decrease in the diameter of cardiomyocytes by 24.3% was accompanied by a decrease in their concentration in 1 mg of tissue by 16%, probably due to pronounced intermuscular edema (Table.).

Accelerated compensatory physiological growth of cardiomyocytes in young animals caused a slight decrease in the diameter of a number of cardiomyocytes by 3.7% combined with a decrease in their concentration by 11.4%, and that resulted in the maintainance of the total number of cardiomyocytes in the myocardium by the end of the experiment. Hypertrophic growth of a number of cardiomyocytes caused statistically unreliable compensatory increase of heart mass in those young animal (Table).

According to electron microscopy data in young animals of the II group, chromatin condensation was observed in individual nuclei of cardiomyocytes, as well as the segregation of the fibrillar and granular components by the nucleolon part of nucleolus. In many cells a moderate expansion of the perinuclear intermembrane space was recorded. In general, the compact arrangement of ultrastructures in cardiomyocytes and the preservation of their intracellular architectonics should be noted. However, a significant number of cardiomyocytes showed diffuse lysis of the myofibrillary apparatus (Fig. 2a). In some cells, zones were present in which the structure of the myofibrillary beams was disturbed for a considerable length. In such areas, a chaotic arrangement of myofilaments was noted, there were no regular sarcomeres.

Mitochondria were distinguished by polymorphism; this was especially true for their size. The proportion of small organelles was quite significant. Lytic changes in the matrix: they were minimal, but there was a decomposition of the cristae, their uneven expansions. Medium-mitochondria were dominated by forms with vesicular cristae, but organelles with lysed matrix and few ruptured cristae also appeared. The vesicles of the agranular sarcoplasmic reticulum were most commonly extended, sometimes quite significantly in the subarcolemmal zones. In the endothelium of capillaries signs of active regeneration were observed: the number of comppartments of the granular endoplasmic reticulum increased, polisomes and the plasmalemma formed outgrowths into the lumen of microvessels for the increased pinocytotic activity.

In rats aged 1 month (III group) ultrastructural pathological changes in most cardiomyocytes were expressed rather moderately. The nuclei contained mostly euchromatin, heterochromatin in the form of small clumps was located marginally. In the nuclei there often were 1-2 nucleolus of the looped form, sometimes fragmentation of the nucleolus was noted. In some cardiomyocytes an expansion of the intermembrane perinuclear space was noted. In the same animals a more pronounced segregation of the fibrillar and granular components of the nucleolonemes was also noted. Lytic changes of myofibrillary fibers were slight and did not lead to a violation of their integrity. At the same time, in some cardiomyocytes was determined significant

TABLE1.

Quantitative assessment of rat cardiomyocyte population after hypoxic exposure (M±m)

Index	Control n=5	Hypobaric hypoxia	
		Untreated n=10	Treated by Fenocor n=10
Fixed heart weight (*mg)*	458.2±5.9	446.3±30.4*	507.3±2.8
Diameter of cardiomyocytes (*μm)*	14.76±0.43	11.87±0.33*	14.22±0.68
Density of cardiomyocytes in 1 *mg* of tissue x10^3	8.5±0.54	7.14±0.79*	7.51±0.19
NOTE: **- $p < 0.05$ compared to control.*			

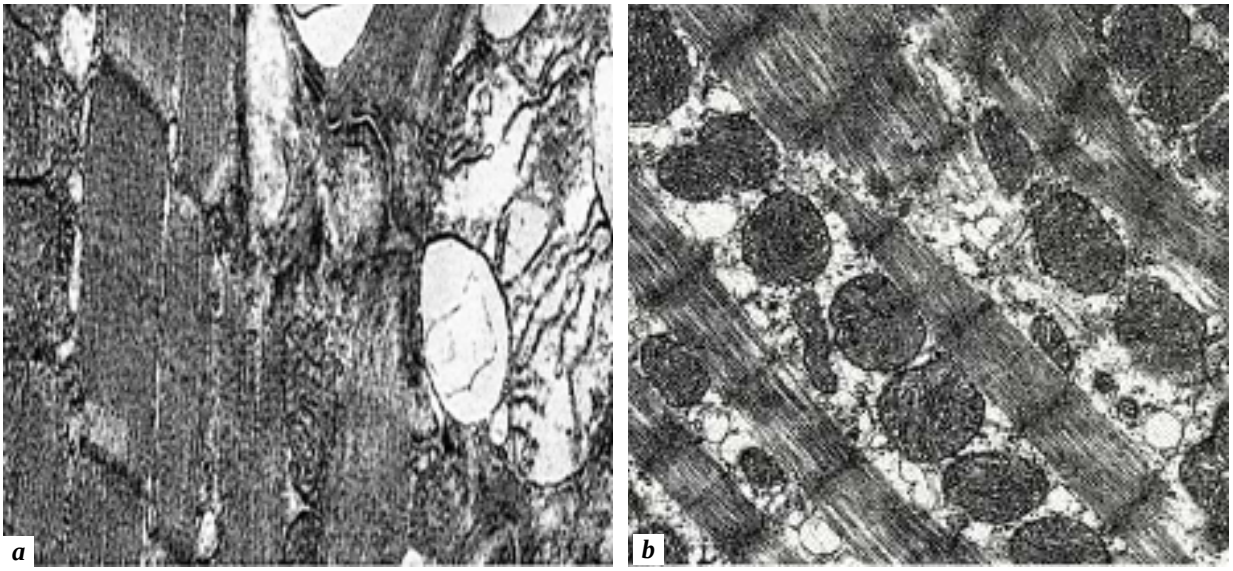

Figure 2. *Notation: 1- mitochondria, 2 - myofibrils.*
a *- diffuse lysis of myofibrillary apparatus. Lysis of the mitochondrial matrix. Fragment of the contractile cardiomyocyte of rat group II. TEM x 20,000.*
b *- A large number of heterogeneous mitochondria with electron-dense cristae. Fragment of the contractile cardiomyocyte of rat group III. TEM x18,000.*

lysis of the sarcoplasmic matrix and the almost complete disappearance of glycogen granules. A large number of polysomes and thin newly formed filaments differed between some chaotically located myofilaments, which indicated the preservation of the intracellular regenerative potential of cardiomyocytes. Small myelin-like structures appeared in the perinuclear space, sub-caparolemmally and close to the intercalated discs, reflecting an increase in autophagocytosis. The fine structure of mitochondria did not change significantly. Only in individual cardiomyocytes, there was a decrease in the matrix and a decrease in the number of cristae in mitochondria (Fig. 2b). Massive residual bodies were recorded in the capillary lumens, and in the interstitial space - collagen secreting fibroblasts and leukocytes.

Discussion

As is known, the characteristic features of the hypoxic syndrome are the excessive accumulation of intermediate products of glycolysis, lipolysis, proteolysis, the development of metabolic acidosis with subsequent secondary nonspecific metabolic and functional shifts, aggravating the course of the underlying disease. The cause of excessive formation of free radicals during hypoxia is blockade of the end link of the respiratory chain in mitochondria, electron leakage along the path to cytochrome oxidase, which leads to one-electron oxygen reduction with the formation of its active forms [*Parildar H et al, 2008*].

A comprehensive morphological study allowed us to identify the main compensatory regenerative strategies developing at different levels of structural organization in the myocardium of experimental animals against the background of cold hypobaric hypoxia. More pronounced structural changes in the myocardium and ultrastructural changes in cardiomyocytes were found in animals without prior correction with polyphenol concentrate. Cardiotoxic effects of hypoxia manifested in changes in heart mass, total number and diameter of cardiomyocytes, structural reorganization of the myocardium and intracellular reorganization of cardiomyocytes and endothelial cells, which was accompanied by a decrease in the diameter of cardiomyocytes, their total number in the heart, as well as a decrease in heart mass. These are due to significant damage to the structures of cardiomyocytes, impaired biosynthetic processes (processes of intracellular regeneration) and cell death (numerical deficiency). Circulatory disorders in the form of significant venous plethora and the development of pronounced interfibrillar and interstitial edema contributed to myocardial fibrillation. And finally, under ischemic conditions, against the background of antigenic stimulation by leuko-

cytes, the leukocyte mechanism of activation of lipid peroxidation (POL) is invariably activated [*Williams RJ et al, 2004*].

The above indicates that the efferent element of systemic functional and metabolic disorders in hypoxia of various origins is the activation of free-radical oxidation, in particular, lipid peroxidation. The oxidation of functional groups of biologically active substances may result in the degradation of structural proteins and cell membrane lipids, in particular, the observed lysis of mitochondria and myofibrils, as well as modification of nucleic acids. In this connection, experimental studies aimed at studying the metabolic effects of various types of antioxidants due to that antihypoxants are evidenced membrane protectors under conditions of acute hypoxia [*Vaisman N, Niv E, 2015*].

Heart failure, which develops as a result of inhibition of intracellular regeneration processes (plastic processes) and progressive numerical deficiency of cardiomyocytes, is designated as plastic heart failure, in contrast to the alternative, in which irreversible metabolic or ischemic damage of cardiomyocytes occurs. Damage and death of cardiomyocytes are diffuse and with constant hypoxic exposure may be accompanied by the development of diffuse or small-focal cardiosclerosis. Remodeling of the heart in chronic regenerative-plastic insufficiency is, as a rule, in the dilated version.

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The antioxidant activity of polyphenolic grape concentrates is due to the presence of flavonoid (anthocyanins, quercetin, epicatechin, tannins) and non-flavonoid (gallic and syringic acid, resveratrol) components [*Baczkó I, Light PE, 2015*]. The mechanism of the antioxidant activity of plant polyphenols is associated with their ability to chelate metal ions of variable valence, at least this mechanism occurs in the case of induced lipid peroxidation [*Sano A et al, 2007*]. In our research after the introduction of the polyphenol complex in group III rats the preservation of the intracellular regenerative potential of cardiomyocytes was established, which contributed to an increase in their diameter in the dynamics of the experiment. In young animals, the preservation of the proliferative activity of cardiomyocytes and the ability for hypertrophic growth ensured the restoration and increase in heart mass by the end of the experiment. However, the cytoprotective effect of the above antioxidant still could not fully compensate for the destructive effect of potent hypoxic stress. This is evidenced by the detection of cardiomyocytes, in the cytoplasm of which edema developed and areas of lysis of the components of myofibrils were noted. It should be noted that even in such cells fairly large mitochondria were encountered, which did not have damage to the structure. This, apparently, is also explained by the positive effect of antioxidant correction.

Conclusion

Thus, the main morphological signs of hypoxic myocardial damage in young rats were represented by phenomena of mixed dystrophy, edema and destruction of contractile cardiomyocytes, lysis of myofibrils and mitochondrial cristae, and contractural changes. However, the above ischemic and hypoxic changes were more pronounced in the myocardium in rats that did not receive correction. Apparently, the depopulation of cardiomyocytes and the reduction of regenerative-plastic reactions in them constitute the main mechanisms for the development of heart failure during hypoxic exposure and determine the nature of cardiac remodeling according to the dilation variant.

The use of “Fenokor”, which demonstrated cytoprotective properties, contributed to the preservation of the structure of the myocardium of rats under conditions of histotoxic hypoxia. The structure of the myocardium observed in young rats on the background of the introduction of the concentrate of grape polyphenols, generally reflected the tendency to minimize damage volumes, manifested in the form of morphological preservation of organelle cardiomyomas. and myofibrils.

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