

Morpho-functional indicators changes of rats' myocardium in experimental doxorubicin-induced chronic heart failure and its pharmacological modulation with new 4-amino-1,2,4-triazole derivative

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Received 15 September 2021 ♦ Accepted 11 November 2021 ♦ Published 10 December 2021

Citation: Bak PG, Belenichev IF, Kucherenko LI, Abramov AV, Khromylova OV (2021) Morpho-functional indicators changes of rats' myocardium in experimental doxorubicin-induced chronic heart failure and its pharmacological modulation with new 4-amino-1,2,4-triazole derivative. *Pharmacia* 68(4): 919–925. <https://doi.org/10.3897/pharmacia.68.e75298>

Abstract

Bromide 1 - (β -phenylethyl)-4-amino-1,2,4-triazolium (Hypertril) has the properties of a beta-blocker and of NO-mimetic, is assigned to the IV class of toxicity. All these effects make Hypertril a promising drug for the treatment of cardiovascular diseases. The aim of this paper was to study the cardioprotective action of Hypertril in terms of the effect on the morpho-functional parameters of the myocardium in rats with experimental chronic heart failure (CHF). CHF was modeled on 80 white outbred rats weighing 190–220g by administering doxorubicin at a cumulative dose of 15 mg/kg. Hypertril and the reference drug metoprolol succinate were administered within 30 days after CHF modeling, intragastrically at doses of 3.5 mg/kg and 15 mg/kg. Morphometric analysis of the cellular structure of the myocardium was carried out on an Axioskop microscope (Zeiss, Germany), in an automatic mode using a macro program developed in a specialized programming environment VIDAS-2.5 (Kontron Elektronik, Germany). The administration of Hypertril to animals with CHF led to an increase in the density of nuclei of cardiomyocytes, the area of myocardocyte nuclei, an increase in the nuclear cytoplasmic ratio and an increase in the concentration of RNA in the nuclei and cytoplasm of cardiomyocytes compared with the group of untreated animals, which indicated the presence of a pronounced cardioprotective effect in the drug candidate. In terms of such indicators as the density of surviving cardiomyocytes and the content of RNA in them, the nuclear-cytoplasmic ratio of Hypertril is significantly ($p < 0.05$) superior to metoprolol.

Keywords

chronic heart failure, Hypertril, endothelial dysfunction, β -blocker, metoprolol, cardioprotection

Introduction

The beginning of the current millennium was marked by a significant spread of cardiovascular diseases, which ranked 2–3 in the structure of mortality in industrialized

countries. One of the formidable complications, mortality from which ranges from 10% to 50% in patients with cardiovascular pathology, is chronic heart failure (CHF). Therefore, the development of remedies for the treatment of these pathologies of the cardiovascular system is an ur-

gent task of modern medicine. According to the recommendations of the European Community of Cardiology, diuretics, ACE inhibitors and β -blockers are important components of the complex therapy of heart failure, especially after myocardial infarction. However, modern β -adrenergic blockers do not show adequate efficiency and require additional combination (ACE inhibitors, diuretics, thrombolytics), exhibit side reactions (Bozkurt et al. 2021). Unavoidable sign of CHF is the cardiac remodeling (CR) which is regarded as the pathogenesis component and progressing factor. CR includes the whole complex of adaptive-restructuring processes related to the geometry, morpho-functional and molecular biochemical levels of heart organization (Urbich et al. 2020). The search for optimal remedies for treating CHF, as well as methods and ways of inhibiting CR, is constantly ongoing. The foregoing served as a rationale for the creation of a new drug of the original structure (bromide 1 - (β -phenylethyl) -4-amino-1,2,4-triazolium, working title Hypertril) which has NO-mimetic, β 1-adrenergic blocking, antihypertensive, anti-ischemic action and belongs to the IV class of toxicity (LD_{50} is 683.4 mg/kg with intragastric administration to rats) (Mazur et al. 2010,2019; Chekman et al. 2013). SPA "Farmatron" together with the scientific and technological complex "Institute of Single Crystals" of the NAS of Ukraine developed a laboratory methods and technological formulas for the synthesis of the substance and the production of ampoule solutions Hypertril substance, for which standardization was carried out (certificate №2, series020213). According to the decision of the State Expert Center of the Ministry of Health of Ukraine, Phase 1 of clinical trials of Hypertril was permitted, and successfully completed. Hypertril is currently undergoing Phase 2 of the clinical trials as an antihypertensive and antianginal drug. The aim of this paper was to study the cardioprotective action of Hypertril in terms of the effect on the morpho-functional parameters of the myocardium in rats with experimental CHF.

Materials and methods

Ethics statement

All animal experiments were conducted in compliance with the guidelines for experimental animal care and use, ARRIVE guidelines and the guidelines of the International Association for the Study of Pain, and were approved by the Animal Care and Use Committee of Zaporozhye State Medical University. Adequate measures were taken to minimize animal suffering. All procedures were strictly implemented by the code of ethics.

Establishment and grouping of rats

The studies were carried out on 80 white outbred rats weighing 190–220 g, of both sexes, obtained from the breeding station of the Institute of Physiology named after

A.A. Bogomolets of the Academy of Medical Sciences of Ukraine. To reproduce CHF, the doxorubicin model was used (Khloponin 2009), which can be considered as the most effective, leading to the development of severe and progressive pathology in most animals. The use of doxorubicin (intraperitoneally at a cumulative dose of 15 mg/kg, divided into 6 injections for 14 days) leads to a decrease in the contractility of the left ventricular myocardium, its eccentric remodeling and the formation of progressive CHF in rats. The study used Doxorubicin "Ebewe" 50mg/25ml (EBEWE Pharma GmbH Nfg.KG, Austria). Doxorubicin was diluted with physiological solution according to the instructions to 25 ml and injected at a dose of 2.5 mg/kg (0.125 ml/100g of weight) intraperitoneally 1 time in two days for 14 days. Hypertril was administered intragastrically once a day at a dose of 3.5 mg/kg (Mazur et al. 2010) in the form of a suspension of 1% starch mucilage for 30 days after 14-day administration of doxorubicin; metoprolol succinate - according to the same scheme at a dose of 15 mg/kg (Khloponin 2009). In the experiment, there were four groups of 20 animals each - intact received 1% solution of starch mucilage; control - untreated with chronic heart failure (CHF) - received 1% solution of starch mucilage; animals with CHF receiving Hypertril; animals with CHF receiving Metoprolol succinate. We used: Hypertril (substance and metoprolol succinate (Betaloc ZOK) - in tablets of 47.5 mg manufactured by Astra Zeneca UK Ltd. (Sweden).

Morphometric analysis

At the end of the experiment, hearts were removed from rats under anesthesia (sodium thiopental, 40 mg/kg). The hearts of the animals were removed, the apical part was isolated from them, which was placed in a Carnoy fixator for 24 hours. After the standard procedure of tissue dehydration and its impregnation with chloroform and paraffin, the myocardium was embedded in paraplast (MkCormick, USA). Using a Microm-325 rotary microscope (Microm Corp., Germany), serial histological sections with a thickness of 5 μ m were prepared, which were then dewaxed in xylene, rehydrated in descending ethanol concentrations (100%, 96%, 70%), and washed in saline. For specific detection of RNA, histological sections were stained for 24 hours with gallocyanine-chromium alum according to Einarson and embedded in a polymer medium EUKITT (O.Kindler GmbH, Germany) for subsequent microscopy. The myocardium was studied using an Axioskop microscope (Zeiss, Germany) in transmitted light. Using an 8-bit CCD camera COHU-4922 (COHU Inc., USA) the images of myocardial areas were entered into the VIDAS-386 computer image analysis system (Kontron Elektronik, Germany) and digitized using a densitometric scale with 256 gray gradations. In each series, about 500 sites from different parts of the myocardium were examined. The study of morphometric and densitometric characteristics was carried out on a computer system for digital image analysis VIDAS-386

(“Kontron Elektronik”, Germany). The image obtained with the AXIOSKOP microscope was entered into a computer system for digital image analysis using a highly sensitive video camera COHU-4922 (COHU Inc., USA) and digitized on a densitometric scale with 256 gray gradations. Morphometric analysis of the cellular structure of the myocardium was carried out automatically using a macro program developed in a specialized programming environment VIDAS-2.5 (Kontron Elektronik, Germany). The following indicators were determined: the area of the nuclei of cardiomyocytes (μm^2); the concentration of RNA in the nuclei of cardiomyocytes in units of optical density (U_{od}), which were calculated as the logarithm of the ratio of the optical density of the cell nucleus to the optical density of the intercellular substance; the concentration of RNA in the cytoplasm of cardiomyocytes in optical density units (U_{od}), which were calculated as the logarithm of the ratio of the optical density of the cell cytoplasm to the optical density of the intercellular substance; the density of cardiomyocyte nuclei as an indicator of the number of cell nuclei per 1 mm^2 of the area of myocardial tissue; nuclear-cytoplasmic coefficient as an indicator of the total area of cardiomyocyte nuclei per 1 mm^2 of the area of myocardial tissue. The nuclear-cytoplasmic coefficient was determined automatically using the VIDAS-2.5 software package (Kontron Elektronik, Germany) as the ratio of the total area of cardiomyocyte nuclei per 1 mm^2 of the area of myocardial tissue.

Statistical analysis

The results of the study were processed using the statistical software package SPSS 16, Microsoft Excel 2003, STATISTICA for Windows 7.0 (StatSoft Inc. № AXXR712D-833214FAN5). Data were presented as the mean \pm standard deviation (SD). Analysis of variance (ANOVA) for normal distribution or Kruskal-Wallis test for non-normal distribution was used to compare independent variables in more than two samples. To analyze the regularities of the relationship between individual indicators, a correlation analysis was carried out using the Pearson or Spearman correlation coefficient. A value of $P < 0.05$ was considered statistically significant.

Results

Morphological changes in the myocardium after 14-day administration of doxorubicin and the absence of experimental therapy (45-day follow-up) manifested themselves in pronounced circulatory disorders and significant lytic changes in some cardiomyocytes and contracture lesions of others. Morphological equivalents of lytic damage to cardiomyocytes at the light-optical level are the violation of compact packing and rarefaction of myofibrils, pronounced clearing of the cytoplasm, the formation of foci of “devastation”, mainly near the nuclei. Necrosis and apoptosis of individual cells with accumulations of mononuclear cells in these areas was

observed. Circulatory disorders in the form of significant venous plethora and the development of pronounced inter-fibre and interstitial edema contributed to myocardial dissociation. Our description of the morphological picture of the myocardium in experimental animals does not contradict the results of other researchers working with this model. Our description of the morphological picture of the myocardium in experimental animals does not contradict the results of other researchers working with this CHF model (Khloponin 2009). Analysis of the density of cardiomyocyte nuclei in the heart revealed a decrease in this parameter ($p < 0.05$). A decrease in the density of cardiomyocyte nuclei, indicating cell death, is due to the cardiotoxic effect of this doxorubicin and cell death mainly by apoptosis (Muller et al. 2000). We assume that it is confirmed by studies by other scientists that a decrease in the density of cardiomyocytes may indicate apoptosis as a result of the toxic effects of Doxorubicin. As a result of the death of cardiomyocytes, small foci of fibrosis were formed (Mukhopadhyay et al. 2009b; Gilleron et al. 2009c). According to the data and results of other researchers (Khloponin 2009; Mazur et al. 2010), mononuclear cardiomyocytes retain the ability to enter the cell cycle, which ends with karyokinesis, which leads to a decrease in their number and, accordingly, to an increase in the pool of binuclear cells. A decrease in the proportion of mononuclear cardiomyocytes may indicate depletion of the regenerative reserves of the myocardium. We also found a decrease in the concentration of RNA in the nuclei and cytoplasm of cardiomyocytes, which indicated an imbalance in the biosynthesis processes in the myocardium. The main role in the morphological rearrangement of cardiac muscle tissue belongs to the almost complete suppression of intracellular biosynthetic processes as the basis for intracellular regeneration and renewal of cardiomyocyte ultrastructures. At the same time, in some (few) cardiomyocytes, the manifestations of inhibition of RNA and protein synthesis are insignificant, in others, its prolonged blockade and low level lead to hypoplasia of intracellular structures, and in others, the synthesis of RNA and protein is completely suppressed, which is manifested by their progressive atrophy. In the control group, the most pronounced phenomena of interstitial edema were recorded. In untreated animals with CHF, there was a decrease in the area of cardiomyocyte nuclei compared with the intact group. A decrease in the area (atrophic changes) is associated with a violation of the reproduction of intracellular structures, i.e. plastic provision of the functions of muscle cells of the heart as a result of a decrease in protein synthesis under the action of doxorubicin. These changes against the background of an increase in the mass of the heart indicated the replacement of the muscle tissue of the heart with fibrous tissue. Heart failure, which develops in such cases, can be regarded as regenerative-plastic failure (Chekman et al. 2013). The main types of damage to cardiomyocytes in regenerative-plastic insufficiency include lytic changes with reduction of myofibrillar bundles; the main type of cell death is apoptosis. Damage and death of cardiomyocytes are diffuse and in chronic course are accompanied by the development of diffuse

or small focal fibrosclerosis. The remodeling of the heart in chronic regenerative plastic insufficiency takes place, as a rule, according to the dilatation variant, which is the reason for the development of congestive heart failure (Khloponin 2009; Bozkurt et al. 2021). Modeling of CHF led to a violation of the myocardial histology - a decrease in the density by 21.2% and an area by 23% of the nuclei of myocardiocytes, a decrease in RNA concentration in them by 4.5% with a decrease in the RNA concentration in the cytoplasm of myocardiocytes by 14.6% compared to intact animals and a decrease in the nuclear-cytoplasmic index of the myocardium by 81%, indicating myocardial hypertrophy (Table 1).

Table 1. Influence of Hypertril and the reference drug on the morphofunctional characteristics of cardiomyocytes in experimental CHF.

Test item	Intact	CHF (control)	CHF+ Hypertril, 3,5 mg/kg	CHF+Metoprolol, 15 mg/kg
area of myocardiocyte nuclei, μm^2	12,6 \pm 0,21	9,7 \pm 0,38	13,0 \pm 0,22 *	8,4 \pm 0,31
density of nuclei per 1 mm ² of myocardial area	9788 \pm 208	7711 \pm 215	9326 \pm 296 * ¹	7911 \pm 166*
Nuclear-cytoplasmic ratio	0,64 \pm 0,003	0,12 \pm 0,004	0,37 \pm 0,003* ¹	0,11 \pm 0,002
RNA concentration in the nuclei of myocardiocytes, E _{OH}	0,23 \pm 0,001	0,21 \pm 0,001	0,25 \pm 0,002* ¹	0,22 \pm 0,001
RNA concentration in the cytoplasm of myocardiocytes, E _{OH}	0,082 \pm 0,001	0,070 \pm 0,001	0,103 \pm 0,001* ¹	0,065 \pm 0,002 *

* - changes are significant in relation to animals of the control group ($p < 0,05$); ¹ - changes are significant in relation to the group of animals treated with metoprolol ($p < 0,05$)

These changes indicated ischemic disturbances in the metabolism of cardiomyocytes, in particular, an imbalance in the processes of biosynthesis in the myocardium, cell destruction, and increased necrosis / apoptosis. The course administration of Hypertril to animals with CHF at a dose of 3.5 mg / kg had a significant cardioprotective effect, as evidenced by an increase in the density of cardiomyocyte nuclei by 21%.

In parallel, in the myocardium of rats with CHF treated with Hypertril, there was an increase in the area by 34% compared with the control group ($p < 0.05$). The introduction of Hypertril also led to an increase in the nuclear-cytoplasmic index by 2 times ($p < 0.05$) compared to the same indicators in the control group, which indicated a decrease in myocardial hypertrophy in rats with CHF receiving Hypertril. Experimental therapy with Hypertril led to an increase in the concentration of RNA in the nucleus by 19% and in the cytoplasm of myocardiocytes by 47% compared to untreated animals, which indicates the stimulation of transcription processes and the anti-ischemic and reparative properties of Hypertril (Chekman et al. 2013; Mazur et al. 2019).

Discussions

Such kind of positive effect of Hypertril on myocardium morpho-functional parameters in CHF rats is logically explained by our previous studies. This is due to its antioxidant and NO-mimetic effects. The mechanism of the antioxidant action of Hypertril is most likely associated with

the inhibition of ROS formation by the transmitter systems - adrenaline and nitroxydergic. Taking into account the results obtained, it can be assumed that Hypertril not only inhibits the formation of superoxide radical in the reaction pair-adrenaline-adrenochrome, but also reduces the formation of cytotoxic forms of NO, normalizing the eNOS / iNOS ratio (Mazur et al. 2010,2019; Chekman et al. 2013) and, interacting with NO (spin trap) with the formation of a more stable radical complex. Numerous studies have shown that CHF is accompanied by significant violations of the nitroxydergic system of the myocardium. The dynamics of the final NO metabolites in CHF shows that a decrease in the level of nitrites in the blood and urine is typical for endothelial dysfunction in cardiovascular pathology, indicating a depression of NO generalization, which is associated with inhibition of the endothelial NO- synthase gene, a lack of cofactors for NO synthesis a decrease in the amount of L-arginine and the main cofactor NOS-tetrahydropterin, oxidation of very low density lipoproteins, an increase in the local concentration of peroxynitrite in the vascular wall, decrease in antioxidant protection, increase in endogenous NO inhibitors (Muller et al. 2000). Since NO is an important mediator of vasodilation, its deficiency plays a key role in the development of arterial hypertension, impaired arterial tone, decreased coronary reserve, left ventricular hypertrophy and the formation of left ventricular diastolic dysfunction. In patients with CHF, changes in the endogenous nitric oxide system were revealed. Cardiomyocytes express two types of nitric oxide synthases (NOS): eNOS и iNOS. ENOS activity is regulated by the contractile state of the myocardium, while iNOS is induced by cytokines. In CHF, under the influence of ROS, the expression of eNOS decreases and the concentration of the necessary cofactors of NO-synthase decreases, and NO deficiency is observed (Cosentino et al. 2002; Belenichev et al. 2021b). The results of several studies have shown that IL-1b, TNF-a, INF stimulate NO synthesis in cardiomyocytes by inducing iNOS. However, upon suppression of eNOS expression and activation of oxidative stress, NO degradation in the myocardium and in the vessel wall increases due to its conversion into peroxynitrite under the action of ROS, as evidenced by an increase in nitrotyrosine (Trujillo et al. 2000a; Octavia et al. 2012). Cytotoxic forms of NO have a direct toxic effect on the myocardium, activates the processes of interstitial growth and fibrosis, which enhances the negative inotropic effect of NO on the myocardium and causes geometric remodeling of the heart. In the case of a further increase in iNOS expression, cytokine-dependent NO production is enhanced, leading to a decrease in contractility. The switching of the phenotype of fibroblasts to myofibroblasts is due to transforming growth factor beta-1 (TGF-b1), whose expression is regulated by ROS and NO, in particular by peroxynitrite, and is associated with the onset of their expression of smooth muscle alpha-actinin (a-SMA) and desmin (Liu et al. 2006; Mukhopadhyay et al. 2009b; Gilleron et al. 2009c; Octavia et al. 2012). Data on the connection between the expression of TGF-b1 and the activity of iNOS and NADPH oxidase were obtained. (Muller et al. 2000; Mihm et al. 2002a Gilleron et al. 2009c).

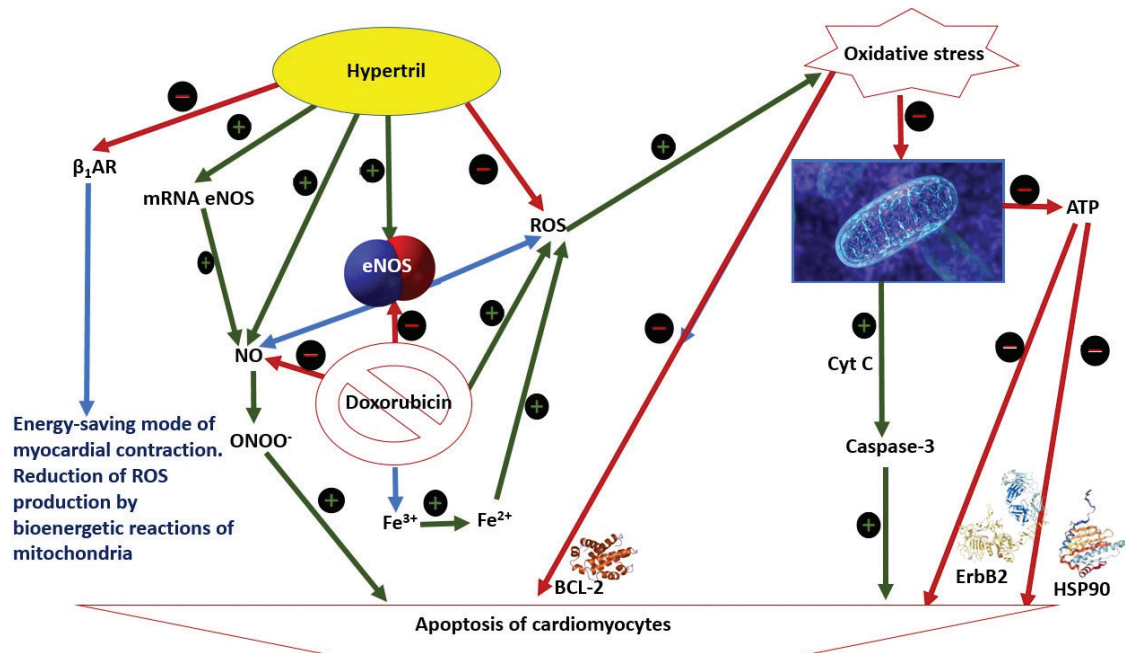


Figure 1. Hypothetical mechanism of the compound “Hypertril” positive effect on the morpho-functional parameters of rat cardiomyocytes in experimental doxorubicin-induced CHF.

Increase in the number of myofibroblasts in various fibrotic and sclerotic processes in the heart (Mihm et al. 2002a; Youn et al. 2005; Bien et al. 2007; Tassigny et al. 2008). ROS and NO in CHF are involved in the mechanisms of initiation of apoptosis, both through the caspase mechanism and through damage to mitochondrial membranes (Youn et al. 2005; Liu et al. 2006; Bien et al. 2007; Tassigny et al. 2008; Khloponin 2009; Octavia et al. 2012; Belenichev et al. 2021b). In the clinic and in experimental models of CHF, an increase in iNOS activity and an increase in the level of nitrites and nitrotyrosine are observed with decompensated CHF (Cosentino et al. 2002; Khloponin 2009; Mukhopadhyay et al. 2009b; Gilleron et al. 2009c; Belenichev et al. 2021b). Therefore, the use of drugs that have a stimulating effect on NO synthesis in patients with CHF is sufficiently justified. Some studies in recent years have shown that, on the one hand, β -blockers have a beneficial effect on the incidence of serious complications (including general and cardiovascular mortality) in patients with CHF, on the other hand, there is convincing evidence of the positive effect of certain β -blockers on the synthesis NO in patients with cardiovascular disease (Cosentino et al. 2002; Khloponin 2009; Konyakhin 2009a; Mazur et al. 2010; Chekman et al. 2013; Kolesnik et al. 2014; Ryzhov et al. 2017; Mazur et al. 2019). Hypertril, being a B1-blocker, has a pronounced negative chronotropic effect and normalizes heart rate, the amplitude of the ventricular R-wave and the amplitude of the T-wave of repolarization in rats with CHF. Also, Hypertril reduces the amplitude of the ST segment in rats with CHF. The normalization (bringing to the value of intact animals) the duration of the depolarization phase (QRS complex) and ventricular repolarization (T wave) as well as electrical diastole (TP interval) should be considered as significant effect of Hypertril from the point of view of CHF therapy (Belenichev et al. 2021a). This indicates that

Hypertril application prevents the formation of diastolic dysfunction. Hypertril reduces disturbances in the myocardial L-arginine-NO-NOS system during myocardial ischemia and arterial hypertension. This preparation shows the unique NO-mimetic properties, increases the expression of mRNAeNOS and eNOS in the myocardium and vascular endothelium, thereby compensate NO deficiency, which explains the mechanism of its cardioprotective properties. Hypertril enhances the protective effects of NO, increasing cardiomyocyte resistance to adverse effects by reducing its conversion to peroxynitrite (Chekman et al. 2013; Mazur et al. 2019). Hypertril also inhibits doxorubicin-induced oxidative damage of cardiomyocyte mitochondria (Mazur et al. 2010; Chekman et al. 2013; Mazur et al. 2019). Thus, Hypertril is able to inhibit the apoptosis of cardiomyocytes.

Course administration of metoprolol led to less pronounced cardioprotective and anti-ischemic effect compared to Hypertril. Such indicators as the area of the nuclei of cardiomyocytes and the nuclear-cytoplasmic ratio in the myocardium of rats with CHF receiving metoprolol were lower than in the group of untreated animals. In terms of such indicators as the density of surviving cardiomyocytes and the content of RNA in them, the nuclear-cytoplasmic ratio of Hypertril is significantly ($p < 0.05$) superior to metoprolol.

Conclusion

The administration of Hypertril to animals with CHF led to an increase in the density of nuclei of cardiomyocytes, the area of cardiomyocyte nuclei, an increase in the nuclear-cytoplasmic index and an increase in the concentration of RNA in the nuclei and cytoplasm of cardiomyocytes compared with the group of untreated animals, which indicated the presence of a pronounced cardioprotective

effect in the potential drug. In terms of efficiency, Hypertril reliably surpasses metoprol succinate.

Ethics statement

All animal experiments were carried out on the basis of the guidelines for experimental animal care and use, and were approved by the Animal Care and Use Committee of the Zaporozhye State Medical University.

Author contributions

Belenichev I.F. conceptualized the study, contributed to the methodology, funding acquisition, and edited the manuscript. Bak P.G. curated the data and wrote the original draft. Abramov A.V. helped with visualization and investigation. Kucherenko L.I. supervised the study. Khromylova O.V. wrote, reviewed the text. All authors contributed to the article and approved the submitted version.

Funding

This work was supported by a project by the Ministry of Health of Ukraine. Project (No: 0117U000658) and SPA “Pharmatron”.

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It is known that doxorubicin generates ROS by affecting eNOS and penetrating into mitochondria, as well as enhancing Fe²⁺ - dependent reactions. Mitochondria damage results in the release of cytochrome C and in decrease of ATP production. ATP depletion decreases the affinity of Hsp90 for ErbB2, a cardioprotective protein. Overproduction of ROS and nitrogen leads to the initiation of apoptosis. Through B1-adrenergic receptors Hypertril exhibits a cardioprotective effect (normalization of heart rate and amplitude of the ventricular R wave and the amplitude of the T wave of repolarization, which, obviously, could be associated with an improvement in energy supply to ensure the contractile function of the heart, as well as a decrease in the ST segment). Also, in addition, Hypertril, in contrast to classical beta-blockers, has the property of preventing the dissociation of eNOS, thereby reducing ROS and preserving NO, which leads to inhibition of the ROS-dependent mechanism of apoptosis initiation. Metoprolol, B1 blocker without additional antioxidant properties, does not provide complete cardioprotection in experimental doxorubicin-induced CHF (Figure 1). ROS - reactive oxygen species; Cyt C - cytochrome C; eNOS - endothelial nitric oxide; NO is nitrogen monoxide; ATP - adenosine triphosphate; bcl-2 - anti-apoptotic protein; HSP90 - heat shock protein 90 kDa; ErbB2 is a protein tyrosine kinase that is part of several receptor complexes of the cell surface, Fe²⁺ / Fe³⁺ - ferrous or ferric iron.

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