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THE STATE OF THE MYOCARDIAL NITROOXIDERGIC SYSTEM DURING MODELLING OF DOXORUBICIN-INDUCED CHRONIC HEART FAILURE AND THE ADMINISTRATION OF BETA-BLOCKERS OF VARIOUS GENERATIONS

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Abstract

In this study, a 1-(β-phenylethyl)-4-amino-1,2,4-triazolium bromide (Hypertril) with β-blocker and NO-mimetic properties, which is of interest for the treatment of chronic heart failure (CHF) was examined. The purpose was to study the effect of various β-blockers on the indicators of the NO system in a CHF model. CHF was induced with doxorubicin at a cumulative dose of 14 mg/kg in 85 Wistar rats, which were intragastrically administered metoprolol succinate (15 mg/kg), carvedilol (50 mg/kg), bisoprolol (10 mg/kg), nebivolol (10 mg/kg) and Hypertril (3.5 mg/kg). In the heart, the expression of eNOS and iNOS and the concentration of stable metabolites NO, nitrotyrosine and SH groups were determined. Modelling of CHF leads to a decrease in the expression of eNOS and an increase in iNOS and nitrotyrosine, as well as a deficiency of NO and the SH group. Nebivolol, especially Hypertril, had the most pronounced normalising effect on the NO system. The advantage of Hypertril over basic β-blockers was revealed.

Rezumat

În acest studiu, a fost examinată o bromură de 1-(β-feniletil)-4-amino-1,2,4-triazoliu (Hypertril) cu proprietăți β-blocante și NO-mimetice, care prezintă interes pentru tratamentul insuficienței cardiace cronice (CHF). S-a studiat efectul diferitelor β-blocante asupra indicatorilor sistemului NO într-un model de CHF. ICC a fost indusă cu doxorubicină la o doză cumulativă de 14 mg/kg la 85 de șobolani Wistar, cărora li s-au administrat intragastric succinat de metoprolol (15 mg/kg), carvedilol (50 mg/kg), bisoprolol (10 mg/kg), nebivolol (10 mg/kg) și Hypertril (3,5 mg/kg). În inimă, a fost determinată expresia eNOS și iNOS și concentrația de metaboliți stabili NO, nitrotirozină și grupuri SH. Modelul murin de CHF presupune o scădere a expresiei eNOS și la o creștere a iNOS și a nitrotirozinei, precum și la o deficiență a NO și a grupului SH. Nebivololul și, în special Hypertril, au avut cel mai pronunțat efect modulator asupra sistemului NO. A fost evidențiat avantajul Hypertril față de β-blocantele tradiționale.

Keywords: chronic heart failure, Hypertril, 1-(β-phenylethyl)-4-amino-1,2,4-triazolium bromide, nitric oxide system markers

Introduction

Chronic heart failure (CHF) is an important medical and social problem [24]. About 1.5 - 2.5% of the world population suffers from clinically expressed CHF [50]. Taking into account asymptomatic left ventricular dysfunction, the prevalence of CHF can reach 4% [33]. Among people over 90 years of age, CHF is detected in 70% of cases [32]. The social significance of CHF is due to the significant financial costs required by inpatient treatment of patients [55]. Mortality from CHF remains very high: more than 50% of patients die within five years after diagnosis [45]. Despite the relative decline in the prevalence of most cardiovascular diseases in the world over the past 20 years, as well as the development and implementation of new treatment methods (including pharmacotherapeutic ones), the morbidity and mortality rate of patients with CHF continues to remain high and is not decreasing [12]. According to modern clinical guidelines, β-blockers are indicated for treating patients with CHF and high cardiovascular risk [17, 40, 46]. At the same time, the phenomenon of "class effect" in β-blockers is questioned due to the chemical and pharmacological heterogeneity [3]. Some caution is also caused by data on the connection of therapy with traditional β-blockers with unfavourable clinical outcomes, compared with other classes of antianginal drugs. The administration of traditional β-blockers (which influence only β-receptors) may be associated with a deterioration in quality of life and lipid and carbohydrate metabolism [8]. Thus, using traditional β-blockers can lead to the development of insulin resistance (risk of developing diabetes mellitus 2), increased triglyceride levels, decreased high-density lipoproteins against the background of impaired peripheral circulation and decreased cardiac output [16]. Traditional β-blockers can lead to

deterioration in cognitive function, sleep disturbances and erectile dysfunction [35, 44]. Modern β-blockers represent a heterogeneous group of drugs, including non-selective drugs (anaprilin, nadolol - first generation β-blockers), cardioselective drugs without vasodilating properties (metoprolol, bisoprolol, atenolol - second generation β-blockers), as well as vasodilating drugs that combine non-selectivity, cardioselectivity with the influence of α-receptors (carvedilol, althiopril, labetalol - third generation β-blockers), or cardio-selectivity with a NO-mimetic effect (nebivolol - third generation β-blocker). A well-known representative of this group is nebivolol [21, 22]. There is also a potential drug, Hypertril (1-(β-phenylethyl)-4-amino-1,2,4-triazolium bromide), exhibiting β-adrenergic blocking, antihypertensive and NO-mimetic effects [37]. β-Blockers with a NO-mimetic effect are of particular interest for treating CHF. This is due to the fact that the development of CHF is accompanied by disturbances in the pumping function of the heart, basal vascular tone, decreased perfusion and metabolism in tissues, as well as changes in the neurohumoral regulation of blood circulation both central and peripheral mechanisms [51]. Endothelial dysfunction is important in developing inadequate blood supply to tissues with the needs of their metabolism in CHF, in which an imbalance of vasoactive molecules occurs, especially NO and endothelin 1, accompanied by changes in the expression of NOS isoforms [58]. NO is involved in the multicomponent response of cells and tissues of the body in CHF. To date, evidence has been obtained of the importance of NO in the initiation of apoptosis and the role of apoptosis in the development and progression of CHF[20].

At the same time, data on changes in NO production in CHF remain controversial. There is almost no data on the influence of different classes of β-blockers on various functional areas of the NO system in CHF. The information listed above encourages a comparative assessment of the effect of β-blockers of different generations on the parameters of the NO system in CHF.

The study's purpose was to investigate the effect of β-blockers of various generations (bisoprolol, metoprolol, carvedilol, nebivolol and the new drug Hypertril) on the markers of the NO system in the model of doxorubicin-induced CHF.

Materials and Methods

The experimental study design

Animals. One hundred thirty white Wistar rats, weighing between 190 - 220 g, were used in the studies. They were taken from the vivarium of the Institute of Pharmacology and Toxicology of the Academy of Medical Sciences of Ukraine and the Institute of Physiology A.A. Bogomolets of the Academy of Medical Sciences of Ukraine. All animals were placed under a 14-day quarantine (acclimatisation period). Every animal under quarantine had daily examinations for behaviour and general health, and they were observed in their cages twice a day for morbidity and mortality. Animals that satisfied experiment's inclusion requirements were split into groups using the randomisation technique prior to the study's commencement. The animals were monitored during the experiment, their appearance was described, and their deaths were noted [54]. Upon receipt, each animal was examined by a qualified veterinarian to determine its state of health. The animals were placed in polycarbonate cages measuring 550 x 320 x 180 mm with galvanised steel covers measuring 660 x 370 x 140 mm and glass drinkers. Five rats were kept in each cage. Each cage was labelled with a study number, species, sex, animal numbers and dose. Cages were placed on racks according to the dose levels and cage numbers indicated on the labels. The following conditions were maintained in the room for keeping animals: temperature – 20 - 24°C, humidity – 30 - 70% and lighting cycle- 12 hours of light and 12 hours of darkness.

All rats were fed an *ad libitum* standard ration for laboratory animals supplied by Phoenix, Ukraine. Water from the municipal water supply network (after reverse osmosis and sterilisation by UV radiation) was given without restrictions. Previously treated by autoclaving, Alder sawdust (*Alnus glutinosa*) was used as litter.

All manipulations complied with the "European Applicable Protection of Vertebrate Animals used for Experimental and Scientific Purposes" and the regulations on gathering animals for biomedical investigations (Strasbourg, 1986, as revised in 1998). The Zaporizhzhia State Medical University Commission on Bioethics decided to adopt the experimental study protocols and outcomes (Protocol No. 3, dated March 22, 2021).

Experimental model. A doxorubicin model was used to induce the symptoms of chronic heart failure [5]. The most successful doxorubicin pharmacological model of chronic heart failure (CHF) can be thought to cause severe and progressive CHF in the majority of animals. Rats administered doxorubicin intraperitoneally (six injections over 14 days at a cumulative dose of 15 mg/kg) develop progressive CHF and exhibit a decrease in left ventricular contractility and eccentric remodelling.

Drugs and pharmacological agents. Doxorubicin 50 mg/25 mL (EBEWE Pharma Ges.mbH Nfg. KG, Austria) was administered in the study. Following a 14-day administration of doxorubicin – Hypertril (Scientific and technological complex "Institute of Single Crystals" of the National Academy of Sciences of Ukraine, certificate No. 2, series 020213) at an experimentally substantiated dose of 3.5 mg/kg [37], metoprolol succinate (Astra Zeneca UK Ltd., Sweden) - 15 mg/kg [5], nebivolol (Teva Pharmaceutical Industries, Ltd., Israel) 10 mg/kg [14], carvedilol (Salutas Pharma GmbH,

Germany) 50 mg/kg [13] and bisoprolol (Teva Pharmaceutical Industries, Ltd., Israel) 10 mg/kg [56], all preparations were given intragastrically once a day in the form of a suspension of 1% starch mucus for 30 days. The intact group consisted of 10 animals, while twenty animals comprised the control group, and there were 20 animals in each experimental group. The following substances were used in the research: bispoprolol pills, carvedolol pills, nebivolol pills, metoprolol pills and Hypertril substances. Hypertril is classified as a class IV toxic substance; when given intragastrically to rats, its LD50 is 683.4 mg/kg. The State Expert Centre of the Ukrainian Ministry of Health decided to approve and successfully complete the Hypertril clinical trial's Phase 1. Phase 2 clinical trials for Hypertril, an antihypertensive and antianginal medication, are presently underway.

Anaesthesia. Rats from all experimental groups were removed from the study and given thiopental anaesthesia (40 mg/kg). After that, blood samples from the celiac artery were taken for further examination.

Biological Material Preparation

Pre-treatment of biological material for biochemical and immunoenzyme studies was carried out according to unified, generally accepted methods. In a 1:10 ratio, cold 0.15 M KCl (4°C) was used to clean the heart. Once the extra fat and connective tissue were removed, along with the internal cavities' blood arteries and clots, the heart was again cleaned in a 1:10 ratio using 0.15 M KCl (4°C). It was then ground up in liquid nitrogen until it attained powder consistency. One hundred milligrams of cardiac tissue previously pulverised into a fine powder using liquid nitrogen was precisely weighed on a WT500 torsion balance (Moscow, Russia). Next, 10.0 mL of a medium kept at 2°C was thoroughly mixed with the powdered tissue. The concentration of these components in millimoles *per* litre (mmol/L) was 250 mmol/L of sucrose, 20 mmol/L of Tris-HCl buffer and 1 mmol/L of EDTA, all of which were adjusted to a pH of 7.4. After that, the homogenate was put through a pre-centrifugation process. Big cell fragments were extracted using a Sigma 3 - 30 k refrigerated centrifuge (Osterode am Harz, Germany) set at 4°C for 7 minutes at 1000 g. Using the same Sigma 3 - 30 k refrigerated centrifuge (Germany), the resultant supernatant was carefully collected and then put through a second centrifugation step at 4°C for 20 min at 17.000x g. After completing this process, the supernatant was gathered and frozen at -80°C. For additional research, the dense mitochondrial precipitate formed during resuspension was used. On the resuspended fraction and mitochondria, measurements of nitrotyrosine, iNOS, eNOS and the concentration of NO metabolites (NOx) were made. Cytosolic and mitochondrial fractions were used for biochemical and enzyme immunoassay studies.

Immunohistochemistry. For immunohistochemical analysis, for 24 hours, the heart's apex was immersed in Bouin's fixative. After the tissue was dehydrated and infiltrated with chloroform and paraffin, the myocardium was embedded in the paraplast (McCormick, Hunt Valley, MD, USA). On a Microm-325 rotary microtome (Microm Corp., Munich, Germany), serial histological slices with a thickness of 15 μm were made. These sections underwent ethanol and 0-xylene treatment before being used for real-time PCR analysis.

Histological sections of the heart were isolated from the paraplast and rehydrated. They were then three times washed for five minutes with phosphate buffer (pH = 7.4) and incubated for thirty minutes at 2N hydrochloric acid (37°C) to measure the intensity of expression of inducible (iNOS) and endothelial (eNOS) NO synthase. They were then incubated for 30 min with a 0.1% solution of trypsin in phosphate buffer (37°C), after which they were rinsed twice for five minutes with the phosphate buffer (pH = 7.4), twice for five minutes with borate Holmes buffer (pH = 8.4), and four times for five minutes with phosphate buffer (pH = 7.4). Following treatment, the sections were incubated with primary polyclonal antibodies of rabbits' IgG (1:500) eNOS (R-20 #SC-648) from Santa Cruz Biotechnology, Inc. (USA) for 24 hours in a humid chamber (4 - 6°C). The sections were washed four times for five minutes with phosphate buffer (pH = 7.4). Following incubation, the sections were cleaned four times in phosphate buffer (pH = 7.4) for 5 minutes each time. Then, secondary goat antibodies to the mouse IgG fragment, paired with a fluorescent dye (FITC) from Sigma-Aldrich (cat. no. F2266), were incubated with them for an hour (37°C). Primary polyclonal iNOS antibodies (C-20#SC-654 FITC) conjugated with a fluorescent dye (FITC) from Santa Cruz Biotechnology, Inc. (USA) were incubated for 24 hours in a humid chamber (4 - 6°C) to determine the expression of iNOS. Sections were also washed after incubation four times for 5 minutes with phosphate buffer (pH = 7.4). The sections were immersed in a glycerol-phosphate buffer mixture (9:1), following a final four-fold wash with phosphate buffer (pH = 7.4). The intensity of NOS isoform expression was measured by counting the number of iNOS and eNOS-positive cells in sections taken with a COHU-4922 video camera (USA) and fed into the VIDAS-386 digital image analysis system (Kontron Elektronic, Germany) using an Axioskop fluorescent microscope (Zeiss, Germany). Immersion oil Immersol 518F and immersion lens F-Fluar 40x/1.30 Oil (Carl Zeiss). The AxioCam-ERc 5s (8-bit camera) was used to capture all of the photographs, and AxioVision 4.8 LE (Carl Zeiss) was used to save the images as a computer file in TIFF format with a resolution of 2560 x 1920. The identical brightness, exposure and correction settings were applied to each photograph. ImageJ (NIH, USA) was used to analyse the images. Prior to analysis, the microscope's scale was considered to convert pixels to μm^2. We defined the regions of

interest (ROI) with significant fluorescence during the interactive mode analysis. We computed the ROI's absolute area (μm^2), immunoreactive material (IRM) and corrected total fluorescence (CTF, Uif), all of which are directly correlated with the amount of IRM present. We determined the IRM-specific area (SA, %) as the absolute area of IRM divided by the absolute area of ROI and the IRM concentration (CONC, mUif/μm^2) as the CTF divided by the absolute area of ROI with the goal of integrated assessment. In each group, we assessed at least 100 visual fields.

Enzyme-Linked Immunosorbent Assay (ELISA). The amount of nitrotyrosine in the heart's cytosol and mitochondria homogenate was measured using the solid phase immunoassay sandwich method of ELISA. The protocol was followed when using the ELISA Kit (Catalogue No. HK 501-02) from Hycult Biotech, Uden, Netherlands. Using the Cloud-Clone Corporation kit (Katy, TX, USA, #PAA868Ra01), the activity of endothelial nitric oxide synthase (eNOS) in the cytosol was assessed by enzyme immunoassay in accordance with the instructions. Using the MyBioSource kit (San Diego, CA, USA, #MBS023874), the activity of inducible nitric oxide synthase (iNOS) in the cytosol was measured by enzyme immunoassay in accordance with the instructions. An entire plate enzyme immunoassay analyser (SIRIO-S, Seac, Italy) was used for these analyses (Hanchev 2018, Ferreira 2012).

Biochemical Methods. The Griess method was used to measure the amounts of NO metabolites (NOx) in the hearts. After obtaining the 1.0 mL of the supernatant as previously described, 100 µL of 0.092 M zinc sulphate and 100 µL of 1 M NaOH were added, mixed and allowed for 30 - 40 min to deproteinise the mixture. Next, it was centrifuged with an Eppendorf™ 5430 G centrifuge (Hamburg, Germany) at 4000x g for 10 min (at 5°C). After that, 100 L of the resultant supernatant was moved to a microplate well, and each well received 0.5 mM of vanadium (III) chloride to help convert nitrate to nitrite. Next, 0.2 µm of N-1-(naphthyl) ethylenediamine and 50 µm of sulfonamide were added. The incubation mixture has a total volume of 300 µL. Subsequently, the samples were incubated at 370°C for 30 minutes, and the optical density was recorded at 540 nm. The content of NOx was ascertained using a linear standard curve covering the range of 0 - 50 µmol/L sodium nitrate. The tissues' NOx levels were expressed in µmol/L. The activity of total NOS was ascertained using a spectrophotometric approach based on the stoichiometric oxidation of NADPH during the reaction of NO production from L-arginine [25]. The quantity of NO, measured at 340 nm, equals the drop in NADPH. Using additional samples containing the NOS inhibitor N-nitro-L-arginine (1 mM), it was shown that the rate of NADPH oxidation can function as an indication of NOS activity. The reagents used were incubation mixture (pH 7.4), diluted with distilled water to 1 L, containing 25 mM Tris-HCl, 5 mM $MgCl_2$, 1 mM $CaCl_2$, 1 mM NADPH and 1 mM L-arginine. A quartz cuvette measuring 1 cm was filled with 2.9 mL of the incubation mixture (37°C). The reaction was initiated by mixing 0.1 mL of the cytosol and mitochondrial lysate. The spectrophotometer was at 340 nm immediately and four minutes later (Eppendorf BioSpectometr, USA). The expression for total NOS activity is nmol/mg/protein/min.

Ellman's method [30] was utilised to determine the concentration of SH groups in proteins. DTNB, or 5,5-dithiobis-2-nitrobenzoic acid, is based on the thiol-disulfide exchange reaction, which releases the 2-nitro-5-thiobenzoate anion, which has an absorption at 412 nm (molar extinction coefficient ε412 = 14000 M^{-1} cm^{-1}). The reagents were 4 mg of 5,5~-dithiobis-2-nitrobenzoic acid (DTNB) in 1 mL phosphate buffer; 0.1 M sodium phosphate, pH 8.0, with 1 mM EDTA. 0.25 mL of cytosol/mitochondrial lysate is added to a quartz cuvette (1 cm) along with 0.05 mL of DTNB and 2.5 mL of phosphate buffer to initiate the reaction. Spectrophotometer at 412 nm (Eppendorf BioSpectometr, USA) after 10 minutes at 37°C. Protein concentration is given in mmol/g. The Lowry technique was used to calculate the sample's protein concentration.

Statistical analysis

Statistical analyses were conducted using "Statistica® for Windows" (StatSOFT, Hamburg, Germany). ANOVA for repeated measures or one-way ANOVA, with post hoc Bonferroni correction or the Kruskal-Wallis criterion and Dunn correction, were used to evaluate group comparisons. A cutoff of $p < 0.05$ was used to define statistical significance.

Results and Discussion

Our studies have shown that when modelling CHF in the cytosolic and mitochondrial fractions of the heart of animals, there is a significant inhibition of the activity of total NOS (by 34.6% and 21%, respectively), a decrease in the level of stable NO metabolites - nitrites (by 30% and 33%) against the background deficiency of thiol compounds (by 74.1%). Such shifts in the NO/reduced thiol system led to a decrease in the bioavailability of NO and its conversion to peroxynitrite, as evidenced by an increase in nitrotyrosine in the cytosol and mitochondria of the myocardium (4.3 times and 3.1 times, respectively) (Table I and Table II). The results of biochemical studies were confirmed by immunohistochemistry (Figure 1 and Figure 2) and enzyme immunoassay, which demonstrated a decrease in the density of eNOS-positive cells by 59% ($p < 0.05$) and an increase in the density of iNOS-positive cells by 142% ($p < 0.05$) compared with similar indicators of the intact group (Table III), as well as a decrease in eNOS expression in the myocardial cytosol by 65% ($p < 0.05$) and an increase in iNOS by 3.9 times ($p < 0.05$) (Table IV).

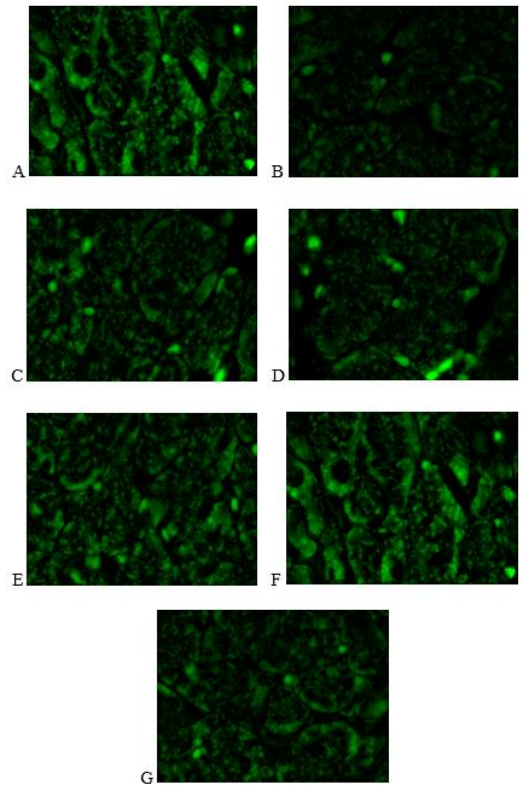

Figure 1.
Distribution of IRM to eNOS in the apical section of the Wistar rats myocardium of (A) intact; B. Control (CHF); C. CHF + bisoprolol; D. CHF + metoprolol; E. CHF + nebivolol; F. CHF+ Hypertril; G. CHF + carvedilol (indirect immunofluorescence 630x)

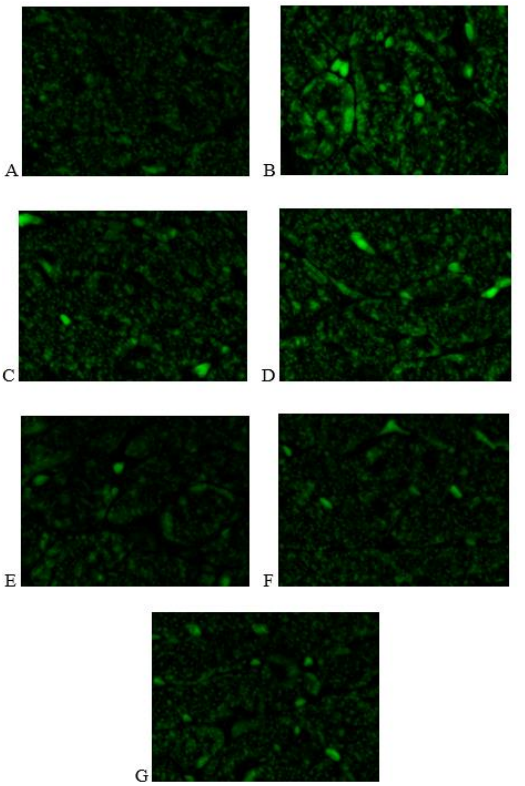

Figure 2.
Distribution of IRM to iNOS in the apical section of the Wistar rats myocardium of (A) intact; B. Control (CHF); C. CHF + bisoprolol; D. CHF + metoprolol; E. CHF + nebivolol; F. CHF + Hypertril; G. CHF + carvedilol (indirect immunofluorescence 630x)

Table I

Influence of the studied β-blockers on the activity of the nitric oxide system in the cytosolic fraction of the heart tissue of animals with CHF

Group of animals	NOS, nmol/min/mg protein	NO Metabolites (NOx), µmol/L	Reduced thiols, mmol/g protein	Nitrotyrosine, pg/mL
Intact (n = 10)	12.7 ± 1.10	5.4 ± 0.38	457.2 ± 27.1	18.2 ± 1.3
CHF (control) (n = 6)	8.3 ± 0.52[1]	3.8 ± 0.22[1]	118.2 ± 10.4[1]	128.2 ± 14.5[1]
CHF+ Hypertril, 3.5 mg/kg (n = 19)	11.5 ± 0.92 *	5.2 ± 0.31 *	195.5 ± 18.1 *	61.5 ± 5.5 *
CHF + carvedilol, 50 mg/kg (n = 10)	8.7 ± 0.77	4.4 ± 0.11*	168.5 ± 14.2 *	87.2 ± 8.2 *
CHF + nebivolol, 10 mg/kg (n = 16)	10.7 ± 0.68 *	4.8 ± 0.22 *	157.3 ± 12.7 *	100.5 ± 8.9 *
CHF + bisoprolol, 10 mg/kg (n = 16)	7.7 ± 0.82	3.7 ± 0.15	115.3 ± 10.7	110.2 ± 15.7
CHF + metoprolol, 15 mg/kg (n = 12)	7.9 ± 0.68	3.9 ± 0.28	119.2 ± 12.5	112.3 ± 10.7

Legend: "n" is the number of animals that survived at the end of the experiment; * - changes are significant in relation to animals in the control group ($p < 0.05$); 1 - changes are significant in relation to animals of the intact group ($p < 0.05$)

Table II

Influence of the studied β-blockers on the activity of the nitric oxide system in the mitochondrial fraction of the heart tissue of animals with CHF

Group of animals	NOS, nmol/min/mg protein	NO Metabolites (NOx), µmol/L	Nitrotyrosine, pg/mL
Intact (n = 10)	3.3 ± 0.2	2.1 ± 0.11	7.98 ± 0.67
CHF (control) (n = 6)	2.6 ± 0.2[1]	1.4 ± 0.10[1]	33.7 ± 4.2[1]
CHF + Hypertril, 3.5 mg/kg (n = 19)	3.2 ± 0.1 *	1.8 ± 0.09 *	15.3 ± 1.2 *
CHF + carvedilol, 50 mg/kg (n = 10)	2.4 ± 0.3	1.5 ± 0.12	21.8 ± 4.3 *
CHF + nebivolol, 10 mg/kg (n = 16)	2.8 ± 0.1	1.5 ± 0.17	24.7 ± 2.2 *
CHF + bisoprolol, 10 mg/kg (n = 16)	2.0 ± 0.4	1.2 ± 0.17	31.3 ± 3.8
CHF + metoprolol, 15 mg/kg (n = 12)	1.6 ± 0.1	1.3 ± 0.14	32.7 ± 4.5

Legend: * - changes are significant in relation to animals in the control group ($p < 0.05$); 1 - changes are significant in relation to animals of the intact group ($p < 0.05$)

Table III

Influence of the studied β-blockers on the density of NOS-positive cells in the myocardium of experimental animals with CHF

Group of animals	Density of eNOS-positive cells, mEif/mm^2	Density of iNOS-positive cells, mEif/mm^2
Intact (n = 10)	512.7 ± 38.4	114.6 ± 12.0
CHF (control) (n = 6)	210.5 ± 20.2[1]	276.3 ± 21.4[1]
CHF + Hypertril, 3.5 mg/kg (n = 19)	412.5 ± 34.2 *	122.5 ± 12.2 *[1]
CHF + carvedilol, 50 mg/kg (n = 10)	248.3 ± 17.2 *	203.3 ± 18.2 *
CHF + nebivolol, 10 mg/kg (n = 16)	283.7 ± 21.7 *	203.8 ± 11.7 *
CHF + bisoprolol, 10 mg/kg (n = 16)	229.8 ± 43.2	269.2 ± 18.2
CHF + metoprolol, 15 mg/kg (n = 12)	211.4 ± 22.4	280.2 ± 14.4

Legend: * - changes are significant in relation to animals in the control group ($p < 0.05$); 1 - changes are significant in relation to animals of the intact group ($p < 0.05$)

Table IV

The influence of the studied β-blockers on the expression of NOS isoforms in the cytosol of the myocardium of experimental animals with CHF

Group of animals	iNOS, pg/mL	eNOS, pg/mL
Intact (n = 10)	22.4 ± 1.8	55.6 ± 3.7
CHF (control) (n = 6)	87.4 ± 7.4[1]	19.5 ± 2.3[1]
CHF + Hypertril, 3.5 mg/kg (n = 19)	24.2 ± 2.3*	58.5 ± 4.2*
CHF + Carvedilol 50 mg/kg (n = 10)	68.5 ± 5.4*	30.7 ± 4.7*
CHF + nebivolol, 10 mg/kg (n = 16)	42.8 ± 3.4*	47.5 ± 3.2*
CHF + bisoprolol, 10 mg/kg (n = 16)	75.8 ± 10.3	27.7 ± 3.7*
CHF + metoprolol, 15 mg/kg (n = 12)	82.0 ± 9.4	17.7 ± 3.3

Legend: * - changes are significant in relation to animals in the control group ($p < 0.05$); 1 - changes are significant in relation to animals of the intact group ($p < 0.05$)

Our data do not contradict the results of other researchers who showed that with CHF in rats, there was a decrease in NO synthesis in the aortic wall, a decrease in eNOS activity and an increase in iNOS activity in the heart. The affinity of eNOS for doxorubicin is significantly higher than that of other myocardial reductases; therefore, doxorubicin causes its inhibition both in the cytosol and in mitochondria, with a subsequent decrease in the formation of NO and increased formation of ROS [28, 41]. Since endotheliocytes represent the first barrier to the penetration of doxorubicin into the myocardium, they are exposed to higher concentrations compared to cardiomyocytes, which explains the early sensitivity of coronary vessels to doxorubicin compared to myocardial contractile function.

However, an increase in the expression of iNOS in CHF does not compensate for the overall NO synthase activity. The resulting NO is quickly transformed into cytotoxic peroxynitrite [5], which is involved in the chemical destruction of protein structures of ion channels, receptors and, especially, mitochondria, as evidenced by a greater increase in nitrotyrosine in mitochondria hearts of rats with CHF. Using histo-immunochemical studies, we found a decrease in the density of eNOS-positive cells in the myocardium of animals with CHF and an increase in the density of iNOS-positive cells. These observations indicate that the formation of CHF after long-term administration of doxorubicin occurs against the background of suppression of the expression of eNOS, which has a pronounced cardioprotective effect and enhances the expression of iNOS, which is involved in the implementation of the apoptosis program indirectly through the activation of nitrosating stress. Similar changes were confirmed by enzyme immunoassay data (Table IV). Administration of β-blockers of various generations to experimental animals after the formation of CHF demonstrated the following results. Hypertril and nebivolol turned out to be the most attractive from the standpoint of their influence on the nitroxidergic system of the myocardium, which had a significant effect on most of the determined parameters. Carvedilol and bisoprolol are of particular interest. Metoprolol did not affect the myocardial nitroxidergic system in the doxorubicin model of CHF.

Thus, administration of Hypertril to animals with experimental CHF leads to normalisation of NOS activity in the cytosol and mitochondria of the myocardium due to an increase in eNOS (increase in the density of eNOS-positive cells by 95.6% and an increase in eNOS expression by three times compared to the control group, ($p < 0.05$) (Tables I - IV), thus increasing the formation of NO (increase in nitrites by 37% and 28% in the cytosol and mitochondria of the myocardium ($p < 0.05$) (Table I and Table II). The initiation of Hypertril treatment led to the normalisation of iNOS expression in the myocardium (a decrease in the density of iNOS-positive cells by 56% and a decrease in iNOS expression by 72% ($p < 0.05$) (Table III and Table IV). Normalisation of the ratio between eNOS and iNOS under the influence of Hypertril leads to inhibition

reactions of nitrosating stress, which is manifested in a significant decrease, compared with the group of untreated animals, of nitrotyrosine by 36% in the cytosol and by 55% in the myocardial mitochondria of animals with CHF. Another important mechanism for the positive effect of Hypertril on the nitrooxidegic system of the myocardium is the increase in the bioavailability of NO. This property is closely related to its antioxidant effect - an increase in the level of reduced thiols - NO transport molecules - in CHF. Thus, in the group of animals with CHF treated with Hypertril, inhibition of the consumption of reduced thiols in the myocardial cytosol in oxidative stress reactions was observed. In the Hypertril group, this indicator was higher than the control indicator by 65.4% ($p < 0.05$). Notably, in the cytosol and mitochondria of animals with CHF treated with Hypertril, the concentration of NOx was 37% and 28% ($p < 0.05$), respectively, higher than in the control group.

The administration of nebivolol led to effects similar in direction, but with less pronounced effects on the nitroxidergic system of the myocardium. Thus, nebivolol increased the activity of NOS in the cytosol of the myocardium of rats with CHF by 29%, the concentration of eNOS by 143%, the density of eNOS and the cells in the myocardium by 34.7%, accompanied by an increase in NO metabolites by 26% ($p < 0.05$) compared with the control group. Nebivolol did not affect the parameters of the nitroxidergic system of myocardial mitochondria. At the same time, a significant antioxidant effect was found in nebivolol. The drug reduced the nitrotyrosine level in the myocardium's cytosol and mitochondria by 21.6% and 26.4% ($p < 0.05$). It increased the level of reduced thiols by 33% ($p < 0.05$) in the cytosol of the myocardium of rats with CHF. Nebivolol also normalised the expression of iNOS in the myocardium - the concentration of iNOS decreased by 51% and the density of iNOS and the cells by 26.4%.

We found that administering carvedilol to rats with doxorubicin-induced CHF leads to a significant antioxidant effect, higher than nebivolol but lower than Hypertril. Thus, in the cytosol and mitochondria of the myocardium of rats with CHF treated with carvedilol, a decrease in nitrotyrosine by 32% was found and 35%, respectively ($p < 0.05$) and a 42% increase in total thiol groups ($p < 0.05$) compared to the control group. Carvedilol, possibly due to its rather powerful antioxidant effect, influenced some indicators of the nitrooxidergic system. In terms of the strength of its effect on the studied parameters of the NO system, carvedilol was inferior to Hypertril and nebivolol. Thus, the administration of carvedilol led to a significant ($p < 0.05$) increase in the concentration of eNOS by 57% and the density of eNOS and the cells, as well as a decrease in the concentration of iNOS by 21% and the density of iNOS and the cells by 18% in the myocardium of rats with CHF. Carvedilol also increased the level of NO metabolites by 26% ($p < 0.05$) in the cytosol compared to the control group.

Bisoprolol had a significant effect only on the concentration of eNOS in the myocardium of rats with CHF. In terms of its effect on this indicator, it was inferior to Hypertril, nebivolol and carvedilol. The administration of metoprolol to rats with CHF did not significantly affect the parameters studied in the myocardial nitrooxidegic system.

Discussion

Several investigations have demonstrated that substantial disruptions in the cardiac nitrooxydergic system accompany CHF. The dynamics of the final metabolites of NO in CHF demonstrate that depression of NO generalisation is typical for endothelial dysfunction associated with cardiovascular pathology. This depression is linked to inhibition of the endothelial NO synthase gene, a lack of cofactors for NO synthesis, a decrease in L-arginine and the main cofactor NOS-tetrahydropterin, oxidation of very low-density lipoproteins, increased local concentration of peroxynitrite in the vascular wall, decreased antioxidant protection and an increase in endogenous NO inhibitors [15]. As NO is a crucial mediator of vasodilation, a shortage in it can lead to several conditions, including arterial hypertension, altered arterial tone, diminished coronary reserve, left ventricular hypertrophy and the development of diastolic dysfunction in the left ventricle. Modifications in the endogenous nitric oxide system have been found in CHF patients. The two forms of nitric oxide synthases (NOS) expressed by cardiomyocytes are eNOS and iNOS. Whereas iNOS is activated by cytokines, eNOS activity is controlled by the myocardium's contractile state. NO deficit is seen in CHF due to ROS-induced reductions in eNOS expression and the concentration of essential cofactors for NO synthase [41]. Numerous research findings demonstrate that iNOS, IL-1β, TNF-α and INF promote NO production in cardiomyocytes. Nevertheless, NO degradation in the myocardium and vascular wall increases in conjunction with the suppression of eNOS expression and the activation of oxidative stress because it is converted into peroxynitrite by ROS, as demonstrated by an increase in nitrotyrosine [5]. Studies have also shown that a major adverse effect of myocardial ischemia is the loss of NO-mediated effects, such as the inhibition of monocyte activation by so-called adhesion molecules, the reduction of platelet aggregation and the suppression of cell proliferation. The phenomena of immunoinflammatory activation are largely implemented *via* the latter mechanism. In developing the latter phenomenon, a significant factor is multidirectional changes in the expression of two NOS isoforms – endothelial (decreased, as mentioned above) and inducible (increased). iNOS, unlike eNOS, does not require Ca^{2+} and calmodulin for its synthesis and produces NO in concentrations significantly higher than those formed under the

influence of eNOS. In addition, unlike eNOS, iNOS is expressed only under pathological conditions - in response to activation by proinflammatory cytokines and ROS [47, 48].

The active synthesis of iNOS by the latter is a key factor in the hyperproduction of the same (according to the principle of a vicious circle) TNF-α and other pro-inflammatory cytokines, increased formation of free radicals with subsequent damage and apoptosis of target tissue cells. The myocardium is directly harmed by cytotoxic forms of NO, which also trigger the interstitial growth and fibrosis processes. This leads to an increase in the negative inotropic effect of NO on the myocardium and geometric remodelling of the heart. Further upregulation of iNOS expression results in increased cytokine-dependent NO generation and reduces contractility. Transforming growth factor-β1 (TGF-β1) is responsible for the transition of fibroblasts into myofibroblasts. ROS and NO, specifically peroxynitrite, regulate TGF-β1 expression, which is linked to the start of smooth muscle α-actinin (α-SMA) and desmin expression [53]. Information was gathered regarding the relationship between TGF-β1 expression NADPH oxidase and iNOS activity [9]. Myofibroblasts perform important physiological functions in tissue injury and healing. An increased number of myofibroblasts is observed in the heart's various fibrotic and sclerotic processes. In the clinic, an increase in the activity of general NOS and an increase in the level of nitrites is observed in decompensated CHF [34]. Therefore, it makes perfect sense for individuals with CHF to take medications that stimulate the manufacture of NO. When combined with basic therapy consisting of antioxidants, glutathione system modulators, antagonists, angiotensin receptor modulators and statins, there is evidence of a favourable effect on the nitrooxidergic system and endothelial function in diabetes, hypertension and nitrate tolerance [15]. In patients with CHF, β-blockers have been shown in several recent studies to reduce the incidence of serious complications (including overall and cardiovascular mortality); however, research is still being done to determine how individual β-blockers affect NO synthesis in patients with cardiovascular diseases [18, 36]. Considering the latter, it is worth reporting the following. The results we obtained on the effect of β-adrenergic blockers on the myocardium's nitro-oxydergic system in doxorubicin-induced CHF align with the existing various experimental studies. Thus, Carvedilol can increase eNOS expression during cadmium cardiotoxicity [49] and normalise the eNOS/iNOS ratio during liver ischemia/reperfusion. Moreover, this effect is due to the antioxidant effect of the drug [27]. Another study found that carvedilol increased NO formation due to its effect on P2Y purine receptors of glomerular endothelial cells. Moreover, the potency of carvedilol was comparable to nebivolol [31]. Many authors associate the metabolithotropic, endotheliotropic effects of carvedil in arterial hypertension, type 2 diabetes mellitus, hyperthyroidism and CHF with its antioxidant mechanism (to a greater extent, an inhibitor of lipid peroxidation and, to a lesser extent, a ROS scavenger). Moreover, the strength of the antioxidant effect of carvedilol is significantly superior to metoprolol and bisoprolol [4, 7, 47, 52]. Data on the effect of bisoprol on the NO system are limited and contradictory. There is evidence that bisoprolol exhibits endothelial protective activity and increases production in diabetes mellitus. However, regarding the strength of such an action, it is significantly inferior to nebivolol [5]. There is evidence that bisoprolol does not have this effect [1].

Nebivolol is currently the only sufficiently studied β-blocker with a NO-mimetic effect. It modulates eNOS expression, leading to excess NO without causing oxidative stress. What is very important when treating patients with CHF [10] is that nebivolol normalises the eNOS/iNOS ratio and limits oxidative stress during ischemia/repression [29]. Nebivolol exhibits NO-dependent endothelial protective activity in smokers in patients with arterial hypertension and coronary heart disease. Nebivolol stimulates endothelial production of adenosine triphosphate, increases endothelial calcium levels through P2Y receptors and determines calcium-dependent activation of eNOS. Nebivolol inhibits the oxidative inactivation of eNOS and reduces the level of circulating dimethylarginine, an eNOS inhibitor. Nebivolol exhibits antioxidant activity and reduces the release of superoxide and peroxynitrite by the endothelium. Nebivolol can inhibit the formation of ROS by the energy-producing systems of mitochondria [39].

The effect of Hypertril on the nitroxidergic system of the myocardium in doxorubicin-induced CHF revealed in this study, is also confirmed by studies in rats with spontaneous hypertension. The additional positive effect of Hypertril on the NO system against the background of its β-adrenergic blocking effect leads not only to normalising blood pressure but also to an improvement in myocardial metabolism and inhibition of oxidative stress in spontaneously hypertensive rats [11, 38]. Data were obtained for the first time on the effect of Hypertril on the activity of eNOS in the blood of rats due to doxorubicin-induced CHF [6]. The effect of Hypertril on the NO system in rats with doxorubicin-induced CHF leads to an improvement in the morpho-functional characteristics of cardiomyocytes [2] and to the most pronounced improvement in the bioelectric activity of the heart compared to carvedilol, nebivolol, metoprolol and bisoprolol [23]. Also, the effect of Hypertril on the expression of eNOS may be associated with this effect on the thiol-disulfide system and the increase in the level of reduced thiols that we detected. As is known, a deficiency of glutathione intermediates in this system can contribute to eNOS deprivation [42]. Compounds that increase the level

of SH groups (such as acetylcysteine) lead to the normalisation of DNA methylation at the eNOS promoter [57]. We believe that Hypertril's positive modulation of eNOS expression, confirmed by immuno-enzyme methods and immunohistochemistry, is based on its antioxidant effect. We have established that 1,2,4-triazole derivatives are ROS and peroxynitrite scavengers [5]. According to the concept of "kindling radicals" or "bonfire" hypothesis, ROS and peroxynitrite are active mechanisms of eNOS uncoupling [15]. Anti-oxidant modulation of eNOS expression in cardio-vascular pathology is quite attractive. Targets for anti-oxidant modulation of eNOS may be oxidative depletion of tetrahydrobiopterin, oxidative modification of the zinc-sulphur complex of the eNOS dimer, glutathione deficiency, ROS-dependent oxidation of cysteine residues and phosphorylation of Thr495/Tyr657, ROS-dependent increase in asymmetric dimethylarginine [5, 15]. Thus, we have demonstrated the effect of β-adrenergic blockers on the activity of the myocardial nitrooxydergic system in the doxorubicin model of CHF. We found that nebivolol and, especially, Hypertril exerted the most pronounced effect. Carvedilol has a less pronounced effect on the myocardial NO system. The effect of bisoprolol on the NO system is quite weak. Metoprolol does not affect the NO system in CHF.

The drugs can be ranked as follows: Hypertril > nebivolol > carvedilol > bisoprolol. In our opinion, the effect on the NO system of the studied β-adrenergic blockers is due to the antioxidant effect, namely the ability to regulate the formation of ROS and peroxy-nitrite and not to inhibit the processes of lipid per-oxidation. Since ROS can lead to oxidative inactivation of eNOS, it reduces the bioavailability of NO and SH/ROS-mediated expression of eNOS [5, 15, 57].

Conclusions

Thus, the results obtained demonstrated the undoubted advantage of the new original molecule (Hypertril) over basic β-adrenergic blockers (metoprolol, nebivolol carvedilol, bisoprolol) and experimentally justify further in-depth study to create a drug based on it for the treatment of CHF.

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Conflict of interest

The authors declare no conflict of interest.

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