

MARKERS OF OXIDATIVE STRESS AND ENERGY METABOLISM IN THE RAT MYOCARDIUM DURING PHYSICAL EXERTION AND DURING THE INTRODUCTION OF AN ANTIOXIDANT – 8-BENZYLAMINOTHEOPHYLLINYL -7-ACETIC ACID HYDRAZIDE (C-3)

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One of the tasks of modern pharmacology is the development and creation of remedies that not only increase physical endurance, but also are able to protect target organs (heart, brain, kidneys, etc.) from hypoxia [1,2,3]. A significant increase in physical activity leads to an imbalance between the energy supply of the myocardium and its metabolic needs, i.e. to working hypoxia. During this period, there is an increase in the production of reactive oxygen species (ROS) by the bioenergetic reactions of mitochondria [3,4,5,6]. Oxidative stress develops during insufficient activity of antioxidant systems and an excess of ROS. In the process of oxidative stress, damage of the structures of cell membranes, protein structures of ion channels and receptors occurs, which leads to impaired ATP synthesis, increased fatigue and slows down the recovery process. ROS and free radicals lead to an increase in the permeability of the mitochondrial pore, a decrease in the charge of the mitochondrial membrane, and a decrease in its ATP-producing function [7, 8, 9]. In addition, damaged mitochondria are a source of pro-apoptotic factors. In recent years, it has been shown that prolonged intense physical exertion can cause apoptosis of human blood cells and myocardial cells in experimental animals, which will certainly negatively affect performance, in particular aerobic [10,11]. ROS lead to the oxidative modification of active centers of enzymes, including antioxidant (superoxide dismutase) and regulating energy metabolism (malate dehydrogenase), which occurs during physical exertion, at the level of the whole organism lengthens the recovery period after training sessions and complicates the formation of the necessary tension of adaptive mechanisms in athletes [12]. Even these not numerous facts reflect the metabolic basis of the need to use antioxidant drugs during physical exertion. In sports medicine, antioxidants such as Mexidol, Emoxypine, Thiotriazoline have found application [3, 12, 13, 14]. However, to date, in the arsenal of a modern doctor there are no remedies that provide cardioprotection during increased physical exertion and at the same time increase endurance. Approaches to the rational metabolic correction of working myocardial hypoxia, which occurs during a sharp change in the type of physical training, increase in physical activity, require development. In this regard, xanthine derivatives are of interest. In the 80s of the past century, the works established a pronounced actoprotective and antioxidant activity of xanthine derivatives [3, 15, 16]. Using our own software methods, as a result of virtual screening, we selected a substance — hydrazide of 8-benzylaminotheophyllinyl-7-acetic acid (code C-3) with potential properties of NO scavenger [17, 18]. Under the conditions of experimental in vitro and in vivo studies, these properties were confirmed [16, 19], which makes the further study of this substance promising.

The aim of this study was to study the antioxidant and energotropic properties of 8-benzylaminotheophyllinyl-7-acetic acid hydrazide (code C-3) on working hypoxia models

Materials and methods. The studies were performed on a sufficient number of experimental animals, and all manipulations were carried out in accordance with the provision on the use of animals in biomedical experiments (Strasbourg, 1986, as amended in 1998) and the "The general ethical principles of animal experimentation" (Kyiv, 2001) which are consistent with the provisions of the "European Convention for the Protection of Vertebrate Animals, which are used for experimental and scientific purposes." The protocols of experimental studies and their results were approved by the decision of the Bioethics Commission of ZSMU (protocol No. 2 of 04/14/2015)

Hydrazide 8-benzylaminotheophyllinyl-7-acetic acid (code C-3) was synthesized at the Department of Biological Chemistry under the guidance of prof. E.V. Alexandra. LD₅₀ C-3 for intragastric administration to rats - 2100 ± 142.8 mg / kg (toxicity class V). The experiments were performed on 80 Wistar rats weighing 160-170 g. Rats were obtained from the nursery of the Institute of Pharmacology and Toxicology of the Academy of Medical Sciences of Ukraine. The duration of the quarantine (acclimatization period) for all animals was 14 days. During quarantine, each animal was examined daily (behavior and general condition), animals were observed in cells twice a day (morbidity and mortality). Before the start of the study, animals meeting the criteria for inclusion in the experiment were divided into groups using the randomization method. Non-eligible animals were excluded from the study during quarantine. Experimental animals were kept on the same rations, under ordinary conditions in vivarium.

Evaluation of the antioxidant and anti-ischemic effects of substance C-3 was also evaluated under conditions of acute working hypoxia in rats under physical exertion on a treadmill in anaerobic mode. The animals were forced to run on a treadmill to complete exhaustion ("failure") at a belt speed of 50 m / min. at the angle of inclination is 30°. Animals were withdrawn from the experiment under thiopental anesthesia (40 mg / kg) at the time of "refusal" of physical activity [21,22]. Substance C-3 was administered intragastrically using a metal probe at a dose of 100 mg / kg 30 minutes before the experiment. Mildronate, Grindex (Latvia) was administered according to the same scheme at a dose of 250 mg / kg [3]. In this series of experiments, there were four groups of animals:

- 1) intact - without physical activity (10 rats);
- 2) control - with physical activity without drug administration (10 rats);
- 3) animals with physical activity receiving substance C-3 (10 rats);
- 4) animals with physical activity treated with Mildronate (10 rats).

Acute working hypoxia was also modeled by preliminary (30 min. prior) intraperitoneal administration of a coronary spastic agent, pituitrin, at a dose of 1 Unit / kg (10 animals in each group). We used pituitrin for injection manufactured by AB Endokrininiai (Lithuania).

Animals were removed from the experiment under thiopental anesthesia (40 mg / kg) at the time of "refusal" of physical activity. Cardioprotective activity was evaluated by reduction of biochemical markers of ischemic myocardial damage.

Substance C-3 was administered intragastrically using a metal probe at a dose of 100 mg / kg 30 minutes before the experiment. Mildronate, Grindex (Latvia) - according to the same scheme in a dose of 250 mg / kg. In this series of experiments, there were four groups of animals:

- 1) intact - with physical activity without the administration of pituitrin (10 rats);
- 2) control - with physical activity with the administration of pituitrin without the administration of drugs (10 rats);
- 3) animals with physical activity with the administration of pituitrin and substance C-3 (10 rats);
- 4) animals with physical activity with the administration of pituitrin and Mildronate (10 rats).

Animals were withdrawn from the experiment 6 hours after the formation of working hypoxia under thiopental sodium anesthesia (40 mg / kg), blood was taken from the

abdominal aorta and the heart was removed for biochemical studies. The heart was washed with cooled 0.15 M KCl (4° C) 1:10. The washed heart was cleaned of fat, connective tissue, blood vessels were cut out, blood clots were removed from the internal cavities and was washed again with 0.15 M KCl (4° C) 1:10. Then it was ground in liquid nitrogen to a powder state and homogenized in a 10-fold volume of medium at (2° C) containing (in millimoles): sucrose - 250, Tris-HCl buffer - 20, EDTA -1 (pH 7.4) [23]. At a temperature of + 4° C, the cytosolic and mitochondrial fractions were isolated by differential centrifugation on a Sigma 3-30k refrigerator centrifuge (Germany). To clean the mitochondrial fraction from large cell fragments, centrifugation was preliminarily performed for 7 minutes at 1000 g, and then the supernatant was re-centrifuged for 20 minutes at 17000g. The supernatant was discarded and stored at -80° C. A protein-free extract was obtained by adding an accurately weighted heart tissue homogenate to perchloric acid (0.6 M), followed by neutralization with 5.0 M potassium carbonate. As a biochemical marker of ischemic myocardial damage in blood serum, a cardio-specific isoenzyme creatine phosphokinase (CK-MB) was determined. The activity of CK-MB was determined on a Prestige 24i automated biochemical analyzer. To assess the intensity of oxidative stress in the myocardium, markers of oxidative modification of the protein — aldehyde phenylhydrazine (APH) and carboxyphenyl hydrazine (CPH) and nitrotyrosine — were determined. The state of the antioxidant system was evaluated by the activity of Superoxide dismutase (SOD). The state of energy metabolism was determined by the level of the most significant intermediates - ATP, lactate, pyruvate, malate. SOD activity was determined according to the method described by Chevvari et al. using phenazine methosulfate and nitro blue tetrazolium [24]. Indicators of oxidative protein modification in brain tissues were determined according to the B. Halliwell method by the interaction of oxidized amino acid residues with 2,4-dinitrophenylhydrazine (2,4-DNPH) and the formation of aldehyde phenylhydrazine (APH) and carboxylphenylhydrazine (CPH) having an absorption spectrum at 274 nm 363 nm, respectively [23,24]. Nitrotyrosine was determined by solid-phase immunosorbent sandwich ELISA method, ELISA Kit (Cat. No. HK 501-02) from Hycult Biotech and expressed in nm / g of tissue. The amount of malate was determined by the Hohorst method by the decrease of NADH at 340 nm [24]. The pyruvate content was determined by the Soch-Lamprecht method by the decrease of NADH at 340 nm [24]. The lactate content was determined by the Hohorst method by increase of NADH at 340 nm [24]. Adenyl nucleotides were determined by thin layer chromatography. Protein concentration was estimated by the Bradford method. The results of the study were calculated using the standard statistical package of the licensed program STATISTICA® for Windows 6.0 (StatSoftInc., No.AXXR712D833214FAN5), as well as SPSS 16.0 and Microsoft Office Excell 2003. Normality of distribution was assessed by the Shapiro-Wilk test. Data are presented as mean values. The significance of differences between the mean values was determined by the Student criterion with a normal distribution. In the case of a non-normal distribution or analysis of ordinal variables, the U Mann-Whitney test was used. For comparison of independent variables in more than two samples, analysis of variance (ANOVA) with normal distribution or the Kruskal-Wallis criterion for a distribution other than normal were used. For all types of analysis, differences $p < 0.05$ (95%) were considered statistically significant.

Results. Modeling of acute working hypoxia led to ischemic myocardial damage - ATP energy deficiency, increased lactate, decreased pyruvate and malate levels, CK-MB hyperenzymemia, increased markers of oxidative modification of the protein APH, CPH and nitrotyrosine during a decrease in SOD activity.

Table 1

The actoprotective and cardioprotective effect of substance C-3 and the reference drug under conditions of severe anaerobic exercise (acute working hypoxia)

Research indicators	Intact group (n = 10)	Control group (run- ning till ex- haustion) (n = 10)	Running till exhaustion + C-3, 100 mg / kg (n = 10)	Running till ex- haustion + Mil- dronate, 250 mg / kg (n = 10)
Duration of running, min	—	11,8±2,6	32,8±5,75*	17,5±3,43*
CK-MB, IU/l	15,2±1,15	31,5±2,5	18,2±1,2 * ¹	24,1±1,8*
APH, c.u./g of protein	8,5±0,77	14,3±1,01	8,52±0,68*	11,1±1,03*
CPH, c.u./g of protein	11,06±1,1	21,8±1,7	11,8±1,22*	14,8±1,22*
SOD, c.u./g of protein	205,7±20,08	125,4±11,0	195,7±19,9* ¹	131,6±12,7
Nitrotyrosine, nm/g of protein	21,36±1,98	37,8±3,9	24,47±2,45* ¹	35,9±2,44
ATP, µm /g of tissue	2,9±0,11	2,11±0,10	2,83±0,24*	2,57±0,14*
Lactate, µm /g	2,42±0,38	4,72±0,41	2,67±0,29* ¹	5,11±0,54
Pyruvate, µm /g	0,176±1,01	0,137±0,008	0,161±0,007*	0,172±0,008*
Malate, µm /g	0,71±0,03	0,489±0,03	0,75±0,04* ¹	0,505±0,05*

Note: * -P <0.05 compared to the control group

¹ - P <0.05 compared to the mildronate group

The prophylactic administration of substance C-3 had a significant antioxidant effect, aimed at suppressing oxidative stress reactions (Table 1). This conclusion is confirmed by the antioxidant effects of C-3, both in in vitro experiments and previously obtained results [20]. So, in the myocardium of experimental animals receiving C-3, a decrease in markers of oxidative modification of the protein — APH by 40.4%, CPH — by 45.8% and nitrotyrosine by 35.2% was observed. The administration of substance C-3 led to an increase in SOD activity in the myocardial cytosol by 56.0%. The administration of substance C-3 also had an anti-ischemic effect, contributing to a more “economical” expenditure of myocardial energy resources during working hypoxia caused by a sharp anaerobic exercise. Thus, the administration of substance C-3 to experimental animals led to an increase in the ATP content in the myocardium by 34.1%. Substance C-3 had a beneficial effect on the energy metabolism of the myocardium - it reduced the lactate content by 43.4% and increased the content of malate by 53.3% and pyruvate by 17.5% in the myocardium. The energotropic effect of C-3, most likely, consists in the activation of compensatory mitochondrial-cytosolic energy shunts and the reduction of low-productivity anaerobic glycolysis. We associate the positive effect of 8-benzylaminotheophyllinyl-7-acetic hydrazide on energy metabolism with its antioxidant effect and a decrease in oxidative damage to mitochondrial membranes. It is known that xanthine derivatives can affect the metabolism of adenylyl nucleotides and regulate ATP resynthesis [3, 14]. The inhibition of oxidative stress and improved myo-

cardial energy metabolism during working myocardial hypoxia under the influence of C-3 resulted in an increase in exercise tolerance by 178.1% and a decrease in CK-MB hyperenzymemia by 42.2% (which also indicates its cardioprotective effect). The prophylactic administration of mildronate had a less pronounced antioxidant effect. Mildronate did not have a significant effect on the activity of SOD and on the content of nitrotyrosine in the myocardium of during exhausting exercises and was inferior to C-3 in these indicators. Mildronate, unlike C-3, increased ATP production in the myocardium due to anaerobic glycolysis (increased lactate). C-3 surpasses Mildronate in the degree of lactate reduction and malate increase. Modeling of acute working hypoxia by administration of a coronary spastic agent - pituitrin - led to more pronounced than in the first case, consequences of oxidative stress and ischemic damage of the myocardium – increase of markers of oxidative modification of protein APH, CPH and nitrotyrosine at the inhibition of SOD activity, affect ATP energy level, increase in lactate level, decrease in pyruvate and malate levels, increase in CK-MB hyperenzymemia, and also decrease in exercise tolerance (The running duration till complete exhaustion decreased in few times) (Table 2).

Table 2

The actoprotective and cardioprotective effect of substance C-3 and the reference drug under conditions of severe anaerobic exercise (acute working hypoxia) together with pituitrin administration

Research indicators	Intact group (n = 10)	Control group (run- ning till ex- haustion) (n = 10)	Running till exhaustion + C-3, 100 mg / kg (n = 10)	Running till ex- haustion + Mil- dronate, 250 mg / kg (n = 10)
Duration of running, min	11,88±2,6	2,66±0,67	6,2±0,48* ¹	3,53±0,37
CK-MB, IU/l	15,2±1,15	45,5±3,1	23,1±1,7 * ¹	34,8±3,1*
APH, c.u./g of protein	8,5±0,77	18,35±1,8	12,6±1,15*	16,94±1,13
CPH, c.u./g of protein	11,06±1,1	30,6±2,12	18,45±1,32*	26,0±3,13
SOD, c.u./g of protein	205,7±20,8	103,7±8,9	184,5±13,6* ¹	117,5±14,8
Nitrotyrosine, nm/g of protein	21,3±1,98	49,43±3,8	30,1±3,5* ¹	40,9±3,7
ATP, µm /g of tissue	2,9±0,11	1,92±0,10	2,52±0,08*	2,27±0,05*
Lactate, µm /g	2,427±0,58	5,56±0,47	2,82±0,26*	7,07±0,61*
Pyruvate, µm /g	0,17±0,01	0,13±0,01	0,159±0,007*	0,165±0,013*
Malate, µm /g	0,71±0,03	0,469±0,04	0,61±0,02* ¹	0,483±0,03

Note: * -P <0.05 compared to the control group

¹ - P <0.05 compared to the mildronate group

As can be seen from the data presented in table 2, the preliminary administration of substance C-3 to animals subjected to physical activity (acute working hypoxia) during coronary spasm exerted an antioxidant effect, reducing the formation of markers of protein oxidative modification in the myocardium - APH by 31.3%, CPH by 39.7% and nitrotyrosine by 39.1%. Substance C-3 led to an increase in SOD activity in the myocardium under conditions of acute working hypoxia by 77.9%. The introduction of substance C-3 also had anti-ischemic effects, improving myocardial energy metabolism at the peak of physical fatigue. Thus, the ATP content in the myocardium of animals treated with C-3 increased by 31.2%, the lactate content decreased by 49.2%, while the content of malate increased by 30% and pyruvate by 22.3%. Under the conditions of reproduction of this model of working hypoxia, we also consider antioxidant mechanism to be dominant of action of C-3. As a result of the implementation of the antioxidant and anti-ischemic action of C-3 in a more stringent model of working hypoxia, an increase in exercise tolerance by 133% in animals of this group also appeared. Prophylactic administration of C-3 also leads to a decrease in CK-MB hyperenzymemia by 49.2% after physical exertion concomitantly with coronary spasm. C-3 is superior to Mildronate in this model of working hypoxia in such indicators as an increase in SOD activity, a decrease in nitrotyrosine, an increase in the content of malate and a decrease in lactate. C-3 is also superior to Mildronate in increasing treadmill running and lowering CK-MB hyperenzymemia. Thus, an increase in physical activity in the experiment leads to the formation of working hypoxia, which is especially pronounced together with of pituitrin administration, to an imbalance between the energy supply of the myocardium and its metabolic needs, i.e. to working hypoxia. At the time of the imbalance between the energy supply of the myocardium and its metabolic needs, there is a sharp overproduction of ROS and NO by the bioenergetic reactions of mitochondria [12, 14, 24]. It is mitochondria that are the primary source of ROS. ROS, especially superoxide, are formed under conditions of ischemia and hypoxia in the so-called parasitic reactions, in the initial section of the respiratory chain of mitochondria ($\text{CoQH}_2\text{-NAD}^+$) with the participation of NADH-CoQH_2 -reductase, the activity of which increases with blockade of the cytochrome C-dependent receptor on the outer surface of mitochondrial membranes in connection with increase of reduced flavins. In addition to superoxide, a key role in the development of mitochondrial disorders is played by NO and its more aggressive form - peroxynitrite, whose marker nitrotyrosine was discovered by us in the animal myocardium after physical exertion in an amount several times higher than the initial ones. Peroxynitrite nitrosylates guanine, which leads to the breaking of DNA chains and to mutations or triggering of apoptosis [13, 25, 26]. Excess NO inhibits the enzymes responsible for DNA repair; it shows the effect on alkyl transferase, formamidopyrimidine-DNA glycosylase and ligase. NO activates PARP and ADP-ribosylation, especially in the setting of ATP deficiency and the accumulation of reduced pyridine nucleotides. NO positively affects the synthesis of p53 protein, which induces the expression of Bax, Fas, p53AIP (apoptosis inducing protein) and other apoptogenic proteins, and moves to mitochondria during apoptosis, which may be one of the causes of ROS production and a decrease in the transmembrane potential on the inner membrane [8, 12, 13, 27]. Oxidative stress and initiation of apoptosis of cardiomyocytes and blood cells negatively affects the parameters of performance, in particular aerobic and training modes [1, 26, 28, 29]. It is considered advisable to use antioxidants, namely NO scavengers, to correct mitochondrial dysfunction [29]. As a result of numerous studies using the computer program that we have developed, as a result of virtual screening, we selected the C-3 substance. Experimental attempts to use antioxidant C-3 under conditions of excessive physical exertion and the formation of working hypoxia were crowned with therapeutic success. In our opinion, the mechanism of suppression of the reactions of nitrosating stress in C-3 is associated with the peculiarities of its chemical structure, which suggests that the substance under study plays the role of a "spin trap" when

interacting with the NO radical. As a proof of this hypothesis, the quantum mechanical energy descriptors of the boundary molecular orbitals were calculated at the Department of Medical Informatics, ZSMU: the energy of the highest occupied molecular orbital (E_{HOMO}) and the energy of the lowest unoccupied molecular orbital (E_{LUMO}) in the WinMopac software package (ver 7.2, descriptors - HOMOEnergy, LUMOEnergy, the semi-empirical method AM1, with the settings: Calculation = SinglePoint, WaveFunction = ClosedShell (RHF). As a result, it was found that they show that the E_{HOMO} parameter (HOMOEnergy descriptor) at the largest degree affects the value of AOA [33-34]. The mechanism of interaction of the C-3 substance and NO can be performed by transferring an electron from the highest occupied molecular orbital of the "spin trap" to the lowest unoccupied molecular orbital of the radical with the formation of a more stable radical complex.

Conclusion. As a result of the studies, it was found that the prophylactic single administration of substance C-3 at a dose of 100 mg / kg intraperitoneally increased exercise tolerance, improved myocardial energy metabolism, reduced ischemic changes in the heart, and inhibited the reactions of oxidative stress.

The administration of substance C-3 accompanied by coronary spasm caused by pituitrin increased exercise tolerance, improved myocardial energy metabolism, reduced ischemic changes in the heart, and inhibited the reactions of oxidative stress. As the dominant mechanism of action of C-3, we consider the antioxidant, aimed at reducing the oxidative modification of protein structures, possibly also mitochondrial membranes and key enzymes of energy metabolism. Prophylactic administration of Mildronate had a less pronounced antioxidant and anti-ischemic effect. The results obtained indicate the presence of pronounced antioxidant and anti-ischemic properties of substance C-3.

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Ethical considerations

All investigations conformed to the ethical of research and were approved by the Bioethics Committee of Zaporizhzhia State Medical University.

Conflict of interest

All authors declare that no conflict of interest exists.

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