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THE ROLE OF iNOS INHIBITION IN THE MECHANISM OF THE CARDIOPROTECTIVE EFFECT OF NEW GABA AND GLUTAMIC ACID DERIVATIVES IN THE MODEL OF ACUTE ALCOHOLIC MYOCARDIAL INJURY IN RATS

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The cardioprotective effects of new derivatives of glutamic acid (glufimet) and GABA (mefargin) were studied in rats exposed to acute alcohol intoxication (AAI) under conditions of selective blockade of inducible NO-synthase (iNOS). AAI induced a pronounced decrease in the contractile function of the myocardium during exercise tests (load by volume, test for adrenoreactivity, isometric exercise), caused mitochondrial dysfunction and increased processes of lipid peroxidation (LPO) in heart cells. A decrease in NO production during iNOS inhibition and AAI improved the respiratory function of mitochondria, a decreased the level of LPO products, and increased mitochondrial superoxide dismutase activity of heart cells. This led to an increase in myocardial contractility. The studied compounds, glufimet and mefargin, caused a statistically significant increase in the rates of myocardial contraction and relaxation, left ventricular pressure, and also reduced NO production. This was accompanied by a decrease in the intensity of LPO processes and an increase in the respiratory control ratio (RCR), reflecting the coupling between respiration and phosphorylation processes during activation of the respiratory chain complexes I and II. The decrease in NO concentration during selective blockade of iNOS and administration of the studied substances was less pronounced than without blockade of the enzyme. This suggests the putative effect of new derivatives of neuroactive amino acids on the NO system.

Key words: alcoholic myocardial injury; GABA and derivatives glutamic acid; iNOS blockade

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INTRODUCTION

Acute alcohol intoxication (AAI) is a leading cause among household poisonings in Russia; it can lead to the development of severe arrhythmias, coronary heart disease, myocardial infarction, and heart failure [1].

AAI causes a decrease in myocardial contractility due to sarcomere damage as a result of the development of oxidative stress, apoptosis of cardiomyocytes, and impaired action potential formation [2, 3]. It is known that the ethanol metabolite acetaldehyde selectively inhibits the vagus nerve effect on the heart and activates the sympathetic nervous system in the first 24 h after alcohol intoxication [4]. It is involved in the uncoupling of mitochondrial respiration and phosphorylation and it induces oxidative stress and death of cardiomyocytes [5]. Ethanol consumption has a significant impact on expression and activity of NO-synthases (NOS) producing nitric oxide, one of the most important regulators of the cardiovascular system. Chronic ethanol intake is accompanied by altered expression and activity of various types of NOS; this results in the development of endothelial dysfunction [6]. Zhang et al. reported, that endothelial cells treated with high doses of ethanol secreted less nitric oxide [7]. In addition, ethanol stimulated NO synthesis by neutrophils [8]. At the same time, oxidative stress caused by AAI leads to a decrease in the activity of endothelial nitric oxide synthase (eNOS), mitochondrial dysfunction, accompanied by increased generation of reactive oxygen species (ROS) [9]. Activation of the inducible isoform of NO-synthase (iNOS), producing large amounts of NO, causes further impairments in lipid peroxidation (LPO) processes due to formation of the prooxidant compound peroxynitrite.

Thus, the search for substances capable of influencing the nitric oxide system, particularly iNOS, and correcting the multiple manifestations of the cardio-negative effect of ethanol on the heart still remains an important issue.

Previous studies revealed cardioprotective effects of new derivatives of neuroactive amino acids. For example, under conditions of acute stress a glutamic acid derivative, glufimet, improved the functional state of rat heart mitochondria, reduced the intensity of LPO processes in cardiomyocytes, and contributed to the maintenance of myocardial functional reserves [10]. Similar results were obtained on the model of chronic alcohol intoxication (CAI) [11]. In addition, it is known that glufimet inhibits iNOS expression [12]. The GABA derivative mefargin, a composition of mefebut and L-arginine, has pronounced cardioprotective effects in CAI;

these include an increase in the inotropic reserves of the heart, antioxidant, antiaggregant, and anticoagulant effects [13]. The assumption about the effect of mefargin on the NO system is based on the presence of the amino acid L-arginine, which is a substrate for NO synthesis.

The aim of this study was to investigate the role of iNOS inhibition in the implementation of the cardioprotective effect of new GABA and glutamic acid derivatives in acute alcohol intoxication in rats.

MATERIALS AND METHODS

The experiments were carried out on 72 ten-month-old female Wistar rats weighing 280-320 g, obtained from the nursery of laboratory animals Rappolovo (Leningrad region). CAI was modeled by intragastric administration of 32% ethanol solution at a dose of 4 g of 95% ethanol solution (RFK, Russia) per 1 kg [14]. The studied compounds glufimet (3-phenylglutamic acid hydrochloride dimethyl ester, RGPU-238) and mefargin (a two-component crystalline composition of methyl 4-amino-3-phenylbutanoate hydrochloride and L-arginine hydrochloride in a 1:1 ratio, RGPU-260) were synthesized at the Department of Organic Chemistry of the Herzen Russian State Pedagogical University. The reference drug, mildronate (with meldonium as the active substance), was used as a ready-made solution for injections (Grindex, Latvia). The studied compounds and the iNOS inhibitor aminoguanidine (AG) (Sigma-Aldrich, USA) were administered intraperitoneally 10 min before the onset of alcoholization. Saline solution was used as a solvent for the test substances and aminoguanidine. The following groups (8 animals each) were formed: 1 — intact; 2 — control — AAI + saline; 3, 4 and 5 — groups of animals with AAI, which were administered, respectively, glufimet (28.7 mg/kg), mefargin (25 mg/kg) and the reference drug mildronate (50 mg/kg), 6 — a group of alcoholic rats treated with AG (50 mg/kg) + saline; groups 7, 8, 9 — animals with AAI, which were injected with glufimet, mefargin and mildronate, respectively, under conditions of iNOS inhibition by AG. This work represents a fragment of the study using the inhibition of various NO-synthases. In this regard, intact, control, and experimental groups with AAI, treated with glufimet, mefargin, and mildronate were common to all experiment [15].

Ten hours after alcoholization, the animals were anesthetized (chloral hydrate, 350 mg/kg), and surgical preparation was performed [11]. Under *in vivo* conditions, a catheter connected to a pressure sensor (Biopac Systems, USA) was inserted through the apex of the heart into the left ventricle for registration of the myocardial contraction rate (+dp/dtmax, mm Hg/s), myocardial relaxation rate (-dP/dtmax, mm Hg/s), left ventricular pressure (LVP) (mm Hg) and heart rate (HR) (bpm). After a period of stabilization (10 min) and recording of initial parameters, load tests were performed. These included volume load (intravenous bolus injection of 0.9% NaCl solution, at a dose of 0.3 ml per 100 g), a test for adrenoreactivity (intravenous administration of adrenaline in dilution 10-7 g/l at a dose of 0.1 ml/100 g) and isometric loading (occlusion of the ascending aortic arch for 30 s). The maximum intensity of structural performance (MISP) was determined by calculation as (MISP) = ((LVPav × HRav) / (left ventricular mass + 1/3 interventricular septum mass)), which was expressed in mm Hg/mg min.

After that, the heart was removed, washed in ice-cold saline and homogenized in a Potter-Elvehjem homogenizer at 4°C in the isolation medium containing 220 mM mannitol, 100 mM sucrose, 1 mM EDTA, 4 mM KH_2PO_4, 20 mM HEPES, pH 7,3. The resultant homogenate was centrifuged for 10 min at 600 *g* (using a refrigerated centrifuge 2-16PK, Sigma, Germany) to sediment debris and unbroken cells. The supernatant was again centrifuged for 20 min (8000 *g*). The resultant supernatant was kept for subsequent determination of the concentration of final NO metabolites, and the sediment was resuspended and used as a mitochondrial fraction [16]. The rate of mitochondrial oxygen uptake was determined by the polarographic method using a Clark type electrode and an Oxytherm System polarograph (Hansatech Instruments, UK). The functional state of mitochondria was studied according to the protocol described by Lanza et al. and based on changes in oxygen uptake by isolated mitochondria in various metabolic states (according to Chance) [16]. Before polarographic measurements all solutions (except ADP) were thermostated for 20 min at 33°C. The reagents required for the preparation of solutions were obtained from Sigma-Aldrich (USA). The metabolic states according to Chance were studied using a temperature-controlled cell of the polarograph. The medium for the polarographic measurements (1 ml) contained 0.5 mM EDTA, 3 mM $MgCl_2 \times 6H_2O$, 20 mM taurine, 10 mM KH_2PO_4, 20 mM HEPES, 110 mM sucrose, 1 g/l fatty acid-free BSA, pH 7.4). The following additions have been successively added in (100 μl each) to record V_1 (basic respiration rate) — mitochondrial suspension; V_2 (rate of substrate-dependent respiration) — complex I substrates of malate/glutamate (5 mM/5 mM); V_3 (I) — rate of oxygen consumption in the presence of oxidation substrates and ADP, i.e. (in the state of oxidative phosphorylation upon complex I activation) — ADP (200 μM); V_3 (I+II) — substrate of the complex II of the respiratory chain — succinate (5 mM) and then ADP; V_3 (II) — rotenone, inhibitor of complex I (0.5 μM), then ADP; V_4 — oligomycin, ATP synthase inhibitor (2.5 mM); V(uncoupled) — carbonyl cyanide-4-(trifluoromethoxy) phenylhydrazone (FCCP), proton ionophore (0.05 mM). The oxygen uptake rate was expressed in nmol O_2/min/mg of protein.

Coupling of mitochondrial respiration and phosphorylation was assessed using the respiratory control ratio (RCR) (V_3/V_4) [17]. After recording mitochondrial respiration parameters, aliquots of the mitochondrial suspensions were exposed to a single freeze-thaw cycle to disrupt mitochondria. These preparations were used for determination of malondialdehyde (MDA) and activity of mitochondrial antioxidant enzymes: catalase [18], glutathione peroxidase [19], and superoxide dismutase (SOD) [19].

Catalase activity was determined by H_2O_2 decomposition assessed versus blank. The enzyme reduced H_2O_2 to water and oxygen for 20 min in phosphate buffer, pH 6.8. The reaction stopped by 4% ammonium molybdate; the mixture was centrifuged for 10 min at 8000 rpm in a CM-6M centrifuge (Elmi, Latvia). The supernatant was photometried at 410 nm using a Helios γ spectrophotometer (Thermo Electron Corporation, UK), The enzyme activity was expressed as mg of H_2O_2/min per 1 mg of protein.

Determination of glutathione peroxidase activity was based on the oxidation reaction of glutathione with *tert*-butyl hydroperoxide. After a 5-min incubation in a mixture of 40 mM glutathione (GSH), 10 mM *tert*-butyl hydroperoxide, and mitochondrial suspension in Tris-HCl buffer, the glutathione oxidation reaction was stopped by adding a 20% trichloroacetic (TCA) acid solution. The samples were centrifuged for 10 min at 3000 rpm in a CM-6M centrifuge. The level of reduced glutathione in the supernatant was determined with 0.4% 5,5′-dithiobis(2-nitrobenzoic acid) on a Helios γ spectrophotometer at 412 nm. The difference in concentrations of reduced glutathione with similar blank samples containing 20% TCA solution was used to calculate the activity of the enzyme per unit time. The specific activity of the enzyme was expressed in μmol GSH/min/mg protein.

The SOD activity was evaluated by the decrease in the optical density of 1.4 μM quercetin solution in 0.015 M phosphate buffer (pH 7.8) in the presence of 0.8 mM tetramethylethylenediamine (TMED) immediately and 20 min after addition of the mitochondrial suspension. SOD inhibits quercetin oxidation by TMED and this results in a slower rate of the optical density decrease. SOD activity was expressed as % inhibition per 1 mg of protein.

The method of the MDA content of determination is based on measuring the amount of TBA-reactive products formed by boiling a 0.7% solution of thiobarbituric acid with isolated mitochondria in an acidic medium (addition of 1.3% H_3PO_4). The optical density of the resultant solution was determined at 532 nm using a Helios γ spectrophotometer. The MDA level was expressed in μmol MDA/mg protein.

The protein concentration in the samples was determined using the Pierce™ BCA Protein Assay Kit (Thermo Scientific, USA).

The concentration of final nitric oxide metabolites, nitrite, and nitrate ions, in serum and heart homogenates was determined by the screening method modified by Metelskaya and Gumanova [20], based on the simultaneous reduction of nitrates to nitrites by means of vanadium(III) chloride (Sigma, USA) and the reaction of diazotization with sulfanilamide nitrite resulted in the development of the color evaluated by subsequent spectrophotometry.

Statistical processing was carried out in the GraphPad Prism 9 program using standard statistical criteria. Data are presented as mean ± standard deviation (SD). The distribution was tested for normality using the Kolmogorov-Smirnov and Shapiro-Wilk tests. Equality of variances (equal SDs) was assessed using the Brown-Forsythe test and the Welch test. If the variances were equal, one-way analysis of variance (Tukey's test) was used; if the SD differed significantly, the Kruskal-Wallis test with post-hoc Dunn's test was used. Differences were considered as statistically significant at $p<0.05$.

RESULTS AND DISCUSSION

The Effect of the Studied Compounds on the Cardiac Contractile Function of Rats with AAI and iNOS Blockade

The study of the inotropic reserves of the heart under the loading tests conditions has shown that AAI caused a pronounced decrease in the contractile function of the myocardium. An increased preload of animals of the AAI+saline group, resulted in lower increments in +dP/dt, -dP/dt and LVP, which were 4.0, 2.2, and 1.8 times lower as compared to intact rats, in which the increments of these parameters were 15.3%, 22.6%, and 18.9% versus the initial data, respectively (Fig. 1).

In animals of the experimental groups treated with the test compounds and mildronate before AAI, there was a more pronounced increase in +dP/dt, -dP/dt and LV in response to an increase in preload as compared to the negative control. In animals of the AAI+glufimet group, the increase in +dP/dt was 4.1 times, -dP/dt — 4.7 times, LVP — 1.7 times higher ($p<0.05$) than in animals of the AAI+saline group. In the experimental group of animals treated with mefargin, the maximum increases in the studied parameters were 6.5, 2.7, and 1.6 times ($p<0.05$), respectively, higher than in the control group. Animals with AAI treated with mildronate showed a markedly higher increase in +dP/dt and -dP/dt in response to an increase in preload — 3.9 and 2.3 times ($p<0.05$), respectively; at the same time, the increase in LVP did not differ significantly as compared to the values of alcoholized animals in the control group (Fig. 1).

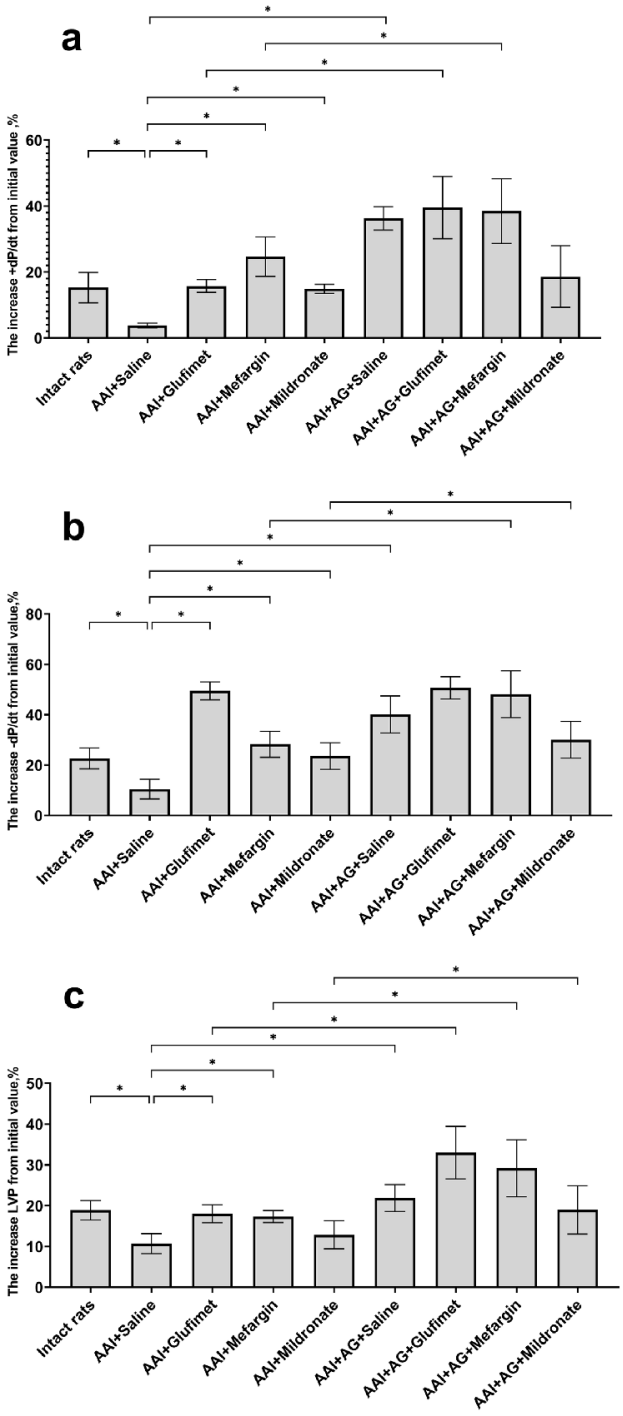

Figure 1. The influence of the tested compounds on the contractile function of the heart of AAI animals with of iNOS inhibition during an increase in preload. * – differences are statistically significant at $p<0.05$ (the Kruskal-Wallis test with the post-hoc Dunn test).

In animals treated with AG before AAI, the increases in the studied parameters in response to the introduction of the volume were significantly higher than in the control group: by 9.6, 3.9, and 2.1 times ($p<0.05$), respectively. In the experimental groups of animals treated with the test compounds and AG before AAI, higher increases in the studied parameters were also observed as compared to similar experimental groups without iNOS inhibition: in the AAI+AG+glufimet group, the +dP/dt increase was 2.5 times higher, LVP — 1.8 times higher ($p<0.05$), in the AAI+AG+mefargin group — +dP/dt increase 1.5 times higher ($p<0.05$), -dP/dt — 1.7 times higher ($p<0.05$), LVP — 1.7 times higher ($p<0.05$). The parameters in the group of animals treated with AAI+AG+saline, the increments of +dP/dt, -dP/dt, and LVP were slightly higher, but did not reach the level of statistical significance (Fig. 1).

In animals of the intact group, the test for adrenoreactivity revealed the increase in +dP/dt of 52.7%, -dP/dt of 48.9%, and the increase in LVP of 42.7%. In rats of the negative control group, lower increases in the studied parameters in response to the adrenaline administration were found (3.5, 3.1, and 2.2 times lower; $p<0.05$). In the experimental group AAI+glufimet, the increase in +dP/dt was 2.3 times ($p<0.05$), -dP/dt — 3.5 times ($p<0.05$), and LVP — 1.9 times higher than in the control group. In animals treated with mefargin before alcoholization, the increments of +dP/dt and -dP/dt were 2.3 and 1.4 times ($p<0.05$) higher as compared to the negative control. Animals with AAI treated with mildronate showed a higher increase: +dP/dt — 2.5 times ($p<0.05$), -dP/dt — 2.4 times as compared with the animals the AAI+saline group (Fig. 2).

Animals with AAI treated with AG+saline demonstrated significantly more pronounced increases in the adrenoreactivity test as compared to the negative control group: +dP/dt — 3.3 times ($p<0.05$), -dP/dt — 3.5 times ($p<0.05$), and LVP — 1.6 times ($p<0.05$) (Fig. 2). The increase in the studied parameters in the experimental groups treated with the studied compounds and AG insignificantly differ from the parameters of animals treated with ethanol+AG+saline. In animals of the experimental groups treated with the new derivatives of neuroactive amino acids and AG there was a tendency to the increase in the studied parameters as compared with animals with functioning iNOS (Fig. 2).

At 5 s and 30 s of performing the maximum isometric load, negative control animals demonstrated lower increases in the rates of contraction and relaxation of the myocardium and LV as compared to intact animals: 1.7, 1.5, and 1.3 times lower at 5 s ($p<0.05$), 1.7, 2.2, and 1.3 times lower at 30 s ($p<0.05$). In the experimental groups treated with the test compounds before AAI, higher increases in the corresponding indicators were noted during the maximum isometric load as compared to the control group. The increase in +dP/dt, -dP/dt, and LVP in alcoholic animals treated with glufimet, at 5 s of the isometric load was 1.7, 1.5, and 1.6 times higher, respectively ($p<0.05$), at 30 s it was 1.6, 2.1, and 1.6 times higher ($p<0.05$); in animals treated with mefargin, at 5 s of the isometric load, the increase of these parameters was 1.6, 1.5, and 1.2

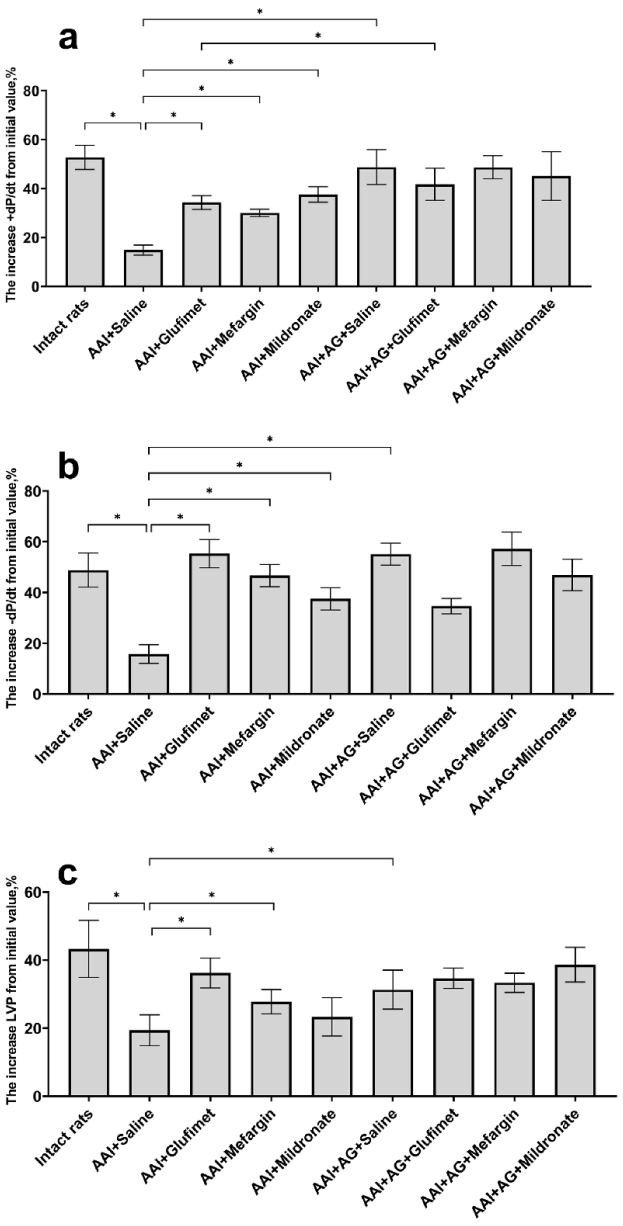

Figure 2. The influence of the tested compounds on the contractile function of the heart of AAI animals with iNOS inhibition during the test for adrenoreactivity. * – differences are statistically significant at $p<0.05$ (the Kruskal-Wallis test with the post-hoc Dunn test).

times higher ($p<0.05$), at 30 s — it was 1.7, 2.0, and 1.3 times higher ($p<0.05$). In animals treated with mildronate, the values at 5 s of the isometric load were 1.6, 1.4, and 1.4 times ($p<0.05$), and at 30 s they were 1.4, 1.5, and 1.3 times ($p<0.05$) as higher compared to the animals of the AAI+saline group (Fig. 3).

Under conditions of iNOS inhibition AAI did not lead to a significant decrease in contractility during the maximum isometric load: in animals the AAI+AG+saline group, the +dP/dt increase at 5 s was 1.8 times higher ($p<0.05$), -dP/dt was 1.9 times higher ($p<0.05$), LVP was 1.6 times higher ($p<0.05$) compared with the negative control group. At 30 s of the isometric load, the increases in the studied parameters were 2.0, 2.8, and 1.7 times ($p<0.05$) higher as compared to the animals of the AAI+saline group, respectively (Fig. 3).

The contractility parameters in the groups of animals with iNOS inhibition, which were treated with studied compounds before AAI, were slightly higher as compared to those in the AAI+AG+saline group; however, they did not reach the level of statistically significance.

In animals of the AAI+AG+mefargin group, the increases in the studied parameters were higher as compared to animals of the same group without iNOS inhibition: the +dP/dt and -dP/dt increases at 5 s were higher by 1.2 times ($p<0.05$), and LVP by 1.3 times; at 30 s they were 1.3, 1.4, and 1.4 times ($p<0.05$), respectively, higher as compared with the AAI+mefargin group. In animals treated with glufimet and AG before AAI, a higher increase in +dP/dt (1.4 times; $p<0.05$) was observed at 30 s; at 5 s and 30 s of the isometric load, -dP/dt was higher by 1.3 and 1.4 times ($p<0.05$) (Fig. 3).

Acute alcohol intoxication led to a decrease in the MISP parameter with an increase in afterload: the increase in MISP both at 5 s and at 30 s of occlusion of the ascending aortic arch in AAI rats was 1.5 times lower ($p<0.05$) as compared with the intact group. In rats of the experimental groups treated with glufimet, mefargin and the reference drug mildronate, the increase in MISP at 5 s of the maximum isometric load was 1.5, 1.4, and 1.6 times higher ($p<0.05$), at 30 s MISP was 1.5, 1.3, and 1.6 times higher ($p<0.05$), respectively (Table 1).

In animals treated with AG before alcoholization, AAI led to a less pronounced decrease in MISP as compared with animals in the negative control group: the increase in this parameter at 5 s was 1.6 times ($p<0.05$), at 30 s it was 1.9 times ($p<0.05$) higher versus control rats. In animals of the experimental groups with iNOS inhibition, the increases in MISP did not differ from those in the group of AAI+AG+saline and showed a tendency to increase when compared with similar experimental groups of animals that were not treated with AG (Table 1).

The results considered above suggest that AAI leads to a significant decrease in myocardial contractility, which is consistent with the literature data [3, 21]. The recognized decrease in the rate of relaxation, even without changes in the LVP, may indicate incomplete relaxation in diastole, deterioration of ventricular filling [22]. Presumably, the reason for the decrease in contractility is the Ca^{2+} leakage from the sarcoplasmic reticulum, caused by the activation of NADPH oxidase-2 (NOX2) and Ca^{2+}/calmodulin-dependent protein kinase (CaMKII) by ethanol [23]. It is known that AAI causes the development of mitochondrial dysfunction and oxidative stress, apoptosis, followed by the onset of necrosis [3]. The mechanism of the damaging effect of ethanol in AAI involves a decrease in the activity

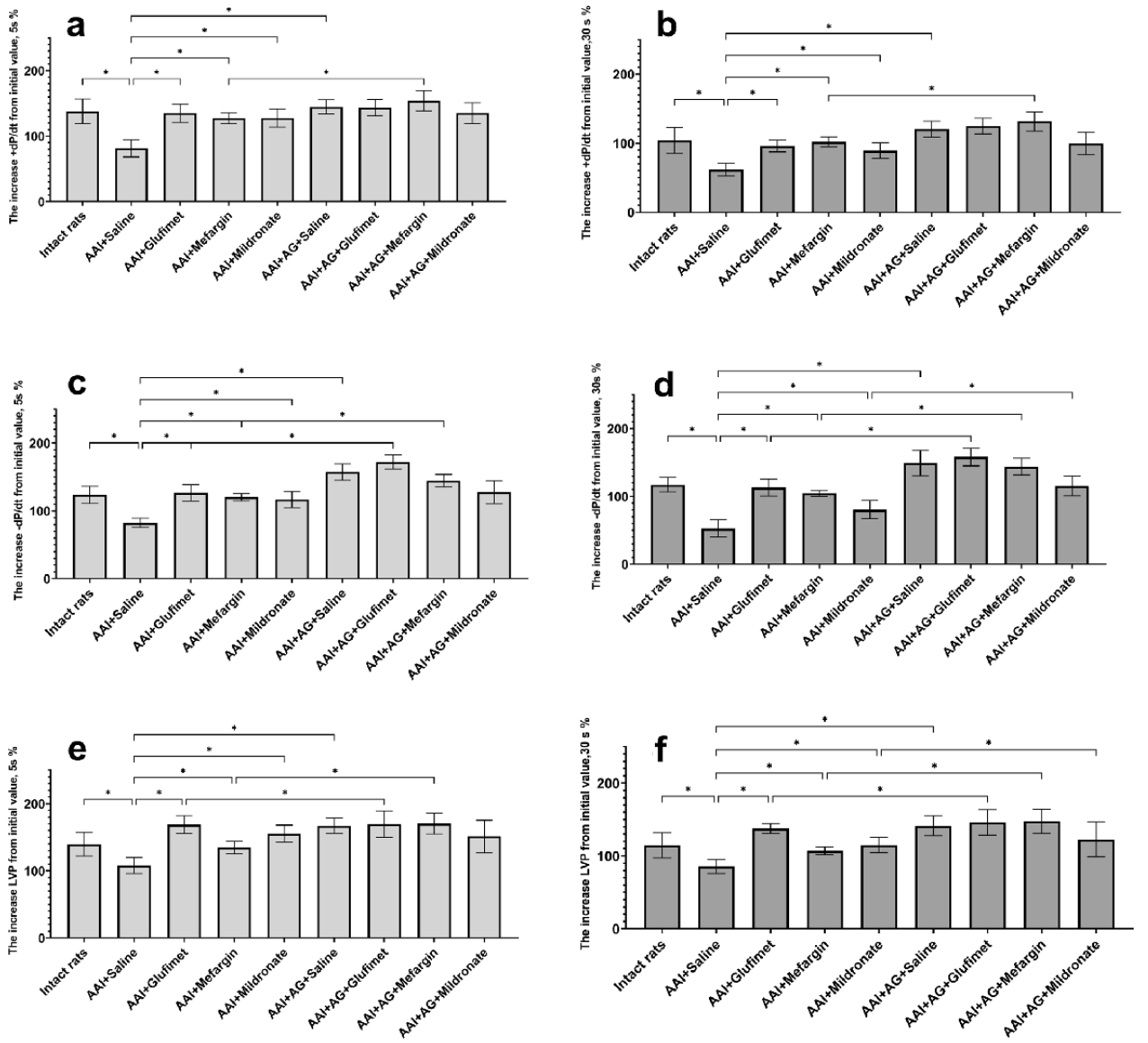

Figure 3. The influence of the tested compounds on the contractile function of the heart of AAI animals with blockade of iNOS during the implementation of the maximum isometric load. * – differences are statistically significant at $p<0.05$ (the Tukey's test with equal variances, the Kruskal-Wallis test with the post-hoc Dunn's test if variances are not equal).

Table 1. The effect of the tested compounds on the maximum intensity of structural performance (MISP) in AAI rats with iNOS inhibition

Group of animals	MMISP, mm Hg/mg min (percent of the parameter increase versus initial values is shown in brackets)		
	Initial value	5 s occlusion	30 s occlusion
Intact	73.9±18.0	237.6±54.2 (222.9±24.6)	197.4±46.1 (169.2±31.7)
AAI+saline	74.1±19.5	185.0±49.5 (149.9±20.6)*	159.7±51.2 (113.1±25.2)*
AAI+glufimet	69.3±15.1	226.4±51.1 (226.3±28.4)#	190.3±46.8 (173.4±19.0)#
AAI+mefargin	68.9±12.3	212.4±41.1 (209.1±25.7)#	166.4±37.8 (150.7±23.7)#
AAI+mildronate	70.7±19.8	236.7±48.3 (242.8±44.3)#	198.3±64.5 (178.2±40.1)#
AAI+AG+saline	66.0±11.2	226.3±20.1 (241.0±28.4)#	211.3±44.4 (219.2±35.3)#
AAI+AG+glufimet	65.7±12.2	230.2±45.6 (251.6±43.4)	203.9±40.1 (212.3±45.7)
AAI+AG+ mefargin	70.9±13.2	233.6±47.4 (231.7±54.0)	217.0±58.1 (205.6±59.6)
AAI+AG+mildronate	59.7±7.2	198.5±28.7 (233.3±31.9)	171.0±35.2 (188.2±56.3)

Statistically significant differences ($p<0.05$) as compared with: * – intact group; # – AAI+saline group (the Tukey test).

of the Akt system, which also increases the nuclear translocation of NF-κB, which, in turn, activates iNOS and type 2 cyclooxygenase (COX-2) [24]. Nitric oxide is also an important link in the pathogenesis of alcoholic heart disease in AAI. It is known that the use of ethanol leads to an increase in the synthesis of asymmetric dimethylarginine (ADMA), which inhibits eNOS, while iNOS activity increases [6]. In large quantities, nitric oxide exhibits a negative inotropic effect: as can be seen from the results obtained, in groups of animals with AAI +dP/dt, -dP/dt and LVP, it is lower than in intact animals. In addition, when iNOS was inhibited, the studied parameters were significantly higher than in the control group of animals. The latter confirms the fact of a decrease in myocardial contractility during excessive NO production.

New derivatives of neuroactive amino acids, glufimet and mefargin, had a pronounced cardioprotective effect. This was expressed in an increase in the rates of contraction, relaxation, LVP, and MISP during volume loading, adrenoreactivity tests, and isometric loading. Glufimet and mefargin are derivatives of glutamic acid and GABA, respectively; each of which plays a key role in the cardiac cell metabolism. Glutamate is involved in the restoration of the mitochondrial proton pool and cytosolic NAD^+ [25] and is a source of the Krebs cycle intermediates. As an excitatory amino acid, glutamate activates sympathetic effects on the myocardium [26]. GABA also has a wide range of metabolic effects in the cell and probably limits excessive sympathetic influences on the heart, thus maintaining its functional reserves. Glufimet has an NO-ergic component in its mechanism of action, which is manifested in a decrease in the expression of inducible iNOS in mouse peritoneal macrophages activated with lipopolysaccharide *in vitro* and *ex vivo*, and also in the realization of cardioprotective effects in the case of AG administration under acute stress [12]. GABA (the derivative of which is mefargin) can inhibit iNOS by suppressing synthesis of interleukin-6 and tumor necrosis factor-α (TNF-α) [27].

The reference drug, mildronate, inhibits synthesis of carnitine, increases the cell content of gamma-butyrobetaine, which stimulates NO production [28]. However, this drug had a less pronounced cardioprotective effect as compared to animals treated with glufimet and mefargin. This is probably due to the fact that, in addition to the effect on the NO system, the studied compounds are involved in energy metabolism in the cell and provide antioxidant protection, which was also demonstrated by the results of this work.

The Study of the Effects of Glufimet and Mefargin on the Respiratory Function of Mitochondria in AAI and iNOS inhibition

As described above, one of the reasons for the decrease in the inotropic function of the heart in AAI may be mitochondrial dysfunction, and therefore we have investigated the effect of glufimet and mefargin on the rate of oxygen uptake by mitochondria in various metabolic states.

In mitochondria from AAI animals, the rates of basal and substrate-dependent respiration were 1.2 ($p<0.05$) and 1.4 ($p<0.05$) times lower as compared with mitochondria from animals of the intact group. In mitochondria of animals with AAI state V_3 respiration during the isolated work of the respiratory chain complex I was 1.2 times lower ($p<0.05$) than in the intact group. In the case of joint stimulation of complexes I and II state V_3 respiration was 1.7 times lower ($p<0.05$) and in the case of complex II stimulation it was 1.5 times lower ($p<0.05$). The V_4 rate was 1.4 times ($p<0.05$) higher than in mitochondria from animals of the intact group. The RCR values for complex I, joint stimulation of complexes I and II, and isolated work of complex II were 2.2, 2.3, and 2.0 times ($p<0.05$) lower in animals mitochondria of the control as group compared to intact animals. This suggests a pronounced uncoupling of the processes of respiration and phosphorylation (Table 2).

Table 2. The effect of the tested compounds on the rate of oxygen uptake by rat heart mitochondria

Group of animals	V_1	V_2	V_3 (I)	V_3 (I+II)	V_3 (II)	V_4	RCR (I)	RCR (I+II)	RCR (II)
Intact	21.0±2.0	28.7±4.0	37.8±3.3	40.9±3.7	25.6±4.2	14.4±3.0	2.7±0.5	3.0±0.8	1.9±0.6
AAI+saline	17.5±1.3*	20.6±2.6*	23.2±3.4*	26.1±5.8	17.4±2.3*	19.7±3.2*	1.2±0.3*	1.4±0.4*	1.0±0.3
AAI+glufimet	21.5±0.9#	24.4±2.3	33.5±6.0#	43.8±9.6#	26.5±5.7#	13.3±2.5#	2.5±0.5#	3.3±0.7#	2.0±0.4#
AAI+mefargin	21.1±1.8#	24.8±3.2	35.8±5.9#	43.9±9.7#	26.8±5.2#	13.3±0.8#	2.7±0.7#	3.3±0.9#	2.0±0.7#
AAI+mildronate	21.1±2.3#	26.3±2.6#	35.3±6.0#	42.2±6.4#	24.5±5.3	12.7±2.9#	2.9±0.6#	3.4±0.7#	1.9±0.5#
AAI+AG+saline	19.0±1.5	26.5±4.7	35.0±5.3#	41.1±7.2#	22.1±6.5	15.0±3.5	2.4±0.6#	2.8±5.6#	1.5±0.2
AAI+AG+glufimet	21.9±2.0&	26.5±3.4	33.5±4.4	43.1±6.7	30.6±5.4	12.4±1.8	2.9±0.7	3.7±0.7	2.5±0.3&
AAI+AG+mefargin	21.5±2.1	30.2±6.5	37.3±7.2	45.3±9.0	30.6±4.9	15.6±5.7	2.5±0.4	3.0±0.7	2.0±0.5
AAI+AG+mildronate	20.8±1.8	27.0±5.1	35.7±6.0	46.3±8.1	30.4±5.0	14.3±1.5	2.5±0.4	3.2±0.5	2.1±0.4

Statistically significant differences ($p<0.05$) as compared with: * – intact group; # – negative control group; & – AAI+AG+saline group (the Tukey test).

Administration of the tested compounds was accompanied by improvement of the functional state of mitochondria. The respiration rate after addition of the complex I substrate was 1.4 times higher in mitochondria from animals of the AAI+glufimet group ($p<0.05$), and in 1.5 times higher in mitochondria from animals of the AAI+mefargin group. In the mitochondria from animals of this group oxygen consumption after succinate addition was 1.8 times higher ($p<0.05$) and after rotenone addition it was 1.5 times higher ($p<0.05$) than mitochondria of animals from the control group. On the contrary, the rate of state V_4 respiration was 1.5 times ($p<0.05$) lower as compared to mitochondria from animals of the control group. The RCR values were 2.1 times and 2.2 times higher (in both cases $p<0.05$) in mitochondria from AAI animals treated with glufimet or mefargin, respectively. In the case of concerted functioning of complex I and complex II, the RCR value was 2.5 times higher ($p<0.05$), while in the case of complex II substrate it was 2.2 times higher ($p<0.05$). Animals with AAI treated with mildronate also showed a higher rate of state V_3 respiration as compared to mitochondria from animals of the control group: 1.5 times higher ($p<0.05$) for complex I, 1.7 times higher ($p<0.05$) for complex I+II, and 1.4 times higher for complex II. The rate of state V_4 respiration was 1.6 times lower ($p<0.05$). The RCR value for the isolated work of complex I and complex II was about 2.2 times higher ($p<0.05$), and for the joint work of complex I and complex II 2.5 times higher ($p<0.05$) in rats of the experimental group treated with mildronate as compared with AAI + saline (Table 2).

Mitochondria from animals of the AAI+AG+saline group were characterized by a 1.4 times higher rate ($p<0.05$) of state V_3 respiration on complex I substrate, and a 1.7 times higher rate ($p<0.05$) in the case of the joint work of complex I and complex II as compared with the negative control group. No changes were found in V_3 (II) and V_4 respiration as comparted with the corresponding parameters of the control group mitochondria (Table 2).

Mitochondria from rats with AAI were characterized by a 2-fold lower RCR values both in the case of separate and joint substrate stimulation of complex I and complex II ($p<0.05$) as compared with mitochondrial parameters of intact animals. This suggests uncoupling of respiration and oxidative phosphorylation in cardiac mitochondria. Glufimet and mefargin improved the functional state of mitochondria, manifested as a more than 2-fold increase in the RCR values ($p<0.05$) compared with the negative control group. Inhibition of iNOS in AAI animals also led to an improvement in mitochondrial respiration: using the substrates of complexes I and I + II, the RCR values were 2 times higher and in the case of the complex II substrate they were 1.5 times higher ($p<0.05$) as compared with these parameters of AAI animals in the control group. In animals with AAI and iNOS inhibition treatment with mefargin was not accompanied by changes in the RCR as compared with AAI animals treated with AG only (i.e. without test compounds) (Table 2). However, a pronounced increase in RCR in mitochondria from AAI animals treated with glufimet under conditions of iNOS inhibition (by AG) is probably associated with a significant increase in the coupling processes of substrate oxidation and ATP synthesis, accompanied by a decrease in electron leakage from the respiratory chain. During hypoxia, the leakage of electrons from the respiratory complexes increases. Under conditions of decreased ROS production due to iNOS inhibition, this effect may be attributed to the antihypoxic and metabolic properties of glufimet.

Thus, AAI led to pronounced mitochondrial dysfunction, manifested in a decrease in the state V_3 respiration rate during joint and separate substrate activation of respiratory complexes I and II, and an increase in V_4 under similar conditions. The described changes were characterized by a corresponding decrease in the RCR values. This suggests uncoupling of oxidative phosphorylation and ATP synthesis, leading to energy deficiency in the cell. This is consistent with the literature data indicating impairments of the mechanisms of oxidative control in mitochondria, a decrease in the activity of complexes I, II, IV and structural changes in mitochondria [29, 30].

Animals pretreated with glufimet and mefargin before AAI showed a pronounced improvement in the functioning of heart mitochondria: the RCR values during substrate activation of respiratory complexes I, II, and I + II were statistically significantly higher than those in animals of the control group. It is possible that the effects of the studied substances are related to their metabolic function. Glufimet is a derivative of glutamic acid, which is crucial for the restoration of oxidative metabolism in cardiomyocytes, in particular, the mitochondrial pool of protons, and the regeneration of the cytosolic NAD^+ concentrations [25]. Glutamic acid is also a source of α-ketoglutaric acid, a Krebs cycle intermediate. GABA serves as an important link in the functioning of the Roberts shunt, in which succinate, a substrate of complex II of the mitochondrial respiratory chain, is formed; it increases the proton gradient and, consequently, stimulates ATP synthase, leading to an increase in ATP synthesis.

Inhibition of iNOS led to an increase in state V_3 respiration and RCR during joint and separate substrate activation of complexes I and II of the respiratory chain. NO, synthesized by iNOS in large quantities, is easily converted into the prooxidant molecule, peroxynitrite. It is possible, that the absence of excess NO synthesis limited the development of oxidative stress and reduced oxidative damage of mitochondrial biomolecules in heart cells.

In this context it was relevant to investigate the effect of new amino acid derivatives on lipid peroxidation (LPO) under conditions of iNOS inhibition.

The Study of the Effect of Glufimet and Mefargin on the Concentration of LPO Products and Activities of Antioxidant Enzymes in Hearts of Animals Exposed to AAI under Conditions of iNOS inhibition

AAI was accompanied by intensification of LPO in heart mitochondria, as evidenced by an increase in the concentration of MDA in the control group by 1.3 times ($p<0.05$) as compared to its level in mitochondria of intact animals. In the AAI+glufimet group and in the AAI+mefargin group the MDA concentration was 1.4 times ($p<0.05$) and 1.3 times ($p<0.05$) lower as compared with the control group, respectively. Under conditions of iNOS inhibition, 1.3 times ($p<0.05$) lower MDA values were observed in animals with AAI as compared to mitochondria of rats of the control group (Table 3).

The study of antioxidant enzyme activities, no statistically significant changes in the activity of catalase and glutathione peroxidase were found. The activity of SOD in animals of the AAI+saline group was 1.7 times ($p<0.05$) lower than in the animals of the intact group. In experimental groups of AAI animals treated with glufimet or mefargin, SOD activity was 1.7 ($p<0.05$) and 1.8 times ($p<0.05$), respectively, higher than in rats of the control group (Table 3).

Obviously, AAI leads to an increase in LPO processes, as evidenced by the accumulation of secondary LPO products and a decrease in the activity of SOD, the antioxidant enzyme of the first line of defense. The main source of ROS in cardiomyocytes is the production of superoxide anion by NADPH oxidase 2 (NOX2), which is capable of both independently damaging and reacting with NO to form peroxynitrite [31]. The resultant reactive oxygen and nitrogen species directly contribute to the fragmentation of contractile proteins, dysfunction of the sarcoplasmic reticulum, and a decrease in the activity of myofibrillar ATPase [32]. All these processes, along with changes in cell bioenergetics, reduce the functional reserves of the heart and cause the development of heart failure.

The tested compounds significantly limited the intensity of LPO processes. Glufimet and mefargin decreased the concentration of MDA, probably by improving the functional state of mitochondria, the main source of free radicals. In addition, the glufimet structure contains a glycine residue, which is involved in the synthesis of peptides and proteins, for example, the tripeptide glutathione, exhibiting a potent antioxidant effect.

In AAI animals treated with AG, the concentration of LPO products was statistically significantly lower. The selective inhibitor of iNOS prevented excessive NO production, peroxynitrite formation, and oxidative damage of lipids (with MDA formation). It should be noted that under conditions of iNOS inhibition the tested compounds also had an antioxidant effect. This effect is less pronounced in glufimet; this is consistent with the glufimet effect on iNOS activity and thus provides its cardioprotective effect.

Taking into consideration probable involvement of the nitric oxide system in the cardioprotective action of the tested compounds, based on earlier obtained results [12, 33], it seemed necessary to study the effect of glufimet and mefargin on the concentration of final nitric oxide metabolites in the serum and heart homogenate of animals with AAI under conditions of iNOS inhibition.

The influence of New Derivatives of Neuroactive Amino Acids on the Level of Final NO Metabolites in Alcoholized Animals under Conditions of iNOS Inhibition

AAI led to an increase in the total concentration of nitrite and nitrate ions in serum and heart homogenates by 68% ($p<0.05$) and 22%, respectively. Glufimet and mefargin reduced this parameter in blood by 19% and 28% ($p<0.05$) and in heart homogenates

Table 3. The effect of the studied compounds on the concentration of malondialdehyde (MDA) and the activity of antioxidant enzymes in heart mitochondria of rats with AAI

Group of animals	MDA, μmol/mg of protein	Catalase, mg H_2O_2/min/mg of protein	Glutathione peroxidase, μmol GSH/min/mg of protein	SOD, % inhibition/mg of protein
Intact	26.6±3.6	154.6±22.7	2.1±0.6	48.1±6.8
AAI+saline	40.9±2.7*	168.2±30.2	2.7±0.6	28.5±4.5*
AAI+glufimet	28.7±4.3#	138.6±27.4	2.7±0.3	47.4±5.3#
AAI+mefargin	32.5±3.4#	143.5±23.7	2.3±0.4	51.7±5.6#
AAI+mildronate	34.2±3.9	143.6±35.3	2.6±0.3	38.9±4.9
AAI+AG+saline	30.3±2.9#	161.6±18.1	2.8±0.4	39.1±6.1
AAI+AG+glufimet	25.6±2.7	170.9±32.3	2.8±0.6	36.6±4.8
AAI+AG+mefargin	31.3±4.2	178.2±30.4	2.5±0.6	45.1±5.0
AAI+AG+mildronate	32.2±3.6	157.4±29.1	1.5±0.6	35.4±5.2

Statistically significant differences ($p<0.05$) as compared with: * – intact group; # – AAI+saline group (the Tukey test).

by 14% (p<0.05) and 20% (p<0.05), respectively. Mildronate statistically significantly reduced the concentration of final NO metabolites only in the heart cells (15%, p<0.05) as compared with animals of the control group (Fig. 4).

Inhibition of iNOS by AG caused a significant decrease in NO levels in serum and heart homogenates by 46% (p<0.05) and 40% (p<0.05), respectively. In groups of animals treated with new glutamate and GABA derivatives under conditions of iNOS inhibition, concentrations of final metabolites of nitric oxide in serum and heart homogenates were higher by 14% and 44% (in the group of AAI animals treated with glufimet) and by 48% and 59% (in animals of the AAI+AG+mefargin group), respectively, as compared with the group of AAI animals treated with the iNOS inhibition (Fig. 4).

Nitric oxide has a wide range of biological properties. It is involved in the regulation of myocardial contractility; NO has a modulating effect on the functioning of cardiomyocyte mitochondria and oxidative status. From the obtained results, it follows that AAI leads to an increase in the NO synthesis, probably due to the activation of iNOS, which is confirmed by the literature data [34]. It is known, that metabolism of one ethanol molecule is accompanied by reduction of 2 NAD^+ molecules. This significantly increases the NADH/NAD^+ ratio in the cell and causes a decrease in the expression of NAD-dependent deacetylase SIRT-1. This enzyme is primarily localized in the cell nucleus and modulates activity of various molecular targets; particularly, it suppresses iNOS expression [35].

Glufimet and mefargin reduced concentrations of final NO metabolites in serum and heart homogenates of AAI animals. In this regard, it can be assumed that improving the coupling of processes in the respiratory chain, glutamic acid and GABA derivatives, replenish the pool of NAD^+, which serves as a substrate for SIRT-1. The latter, in turn, inhibits the iNOS expression, stabilizes HIF-1α, thus exhibiting anti-inflammatory, anti-apoptotic, and antioxidant effects, thereby increasing the inotropic reserves of the heart. In addition, another mechanism for the implementation of the cardioprotective effect of glufimet may include activation of various metabotropic glutamate receptors (mGluR2, mGluR3, mGluR5); this activation decreases iNOS expression by reducing the activity of NF-κB, NADPH oxidase, as well as the permeability of plasma membrane Ca^{2+} channels [36-38]. Glycine included in the structure of glufimet is also able to inhibit NF-κB activation and iNOS expression [39]. There is evidence that GABA can inhibit iNOS by reducing synthesis of interleukin-6 and TNF-α [27]. The NO-ergic mechanism of action of the studied compounds is also supported by the absence of a decrease in the NO level in animals with iNOS inhibition, which is especially pronounced in the case of mefargin. This composition includes L-arginine, which is a substrate for NO synthesis by constitutive NO synthases (endothelial and neuronal). It can be assumed that this compound increases their activity and the NO synthesis in basic amounts, which have modulatory effects in cells. A close functional relationship between GABA and the NO ergic system is known [40]. Being a derivative of glutamic acid glufimet probably exerts its effects by stimulating ionotropic glutamate receptors, in particular the NMDA subtype, by increasing cytosolic Ca^{2+}, which, after binding to calmodulin, activates the neuronal isoform of NOS [41]. This is supported by a decrease in nNOS activity upon blockade of the NMDA receptor [42].

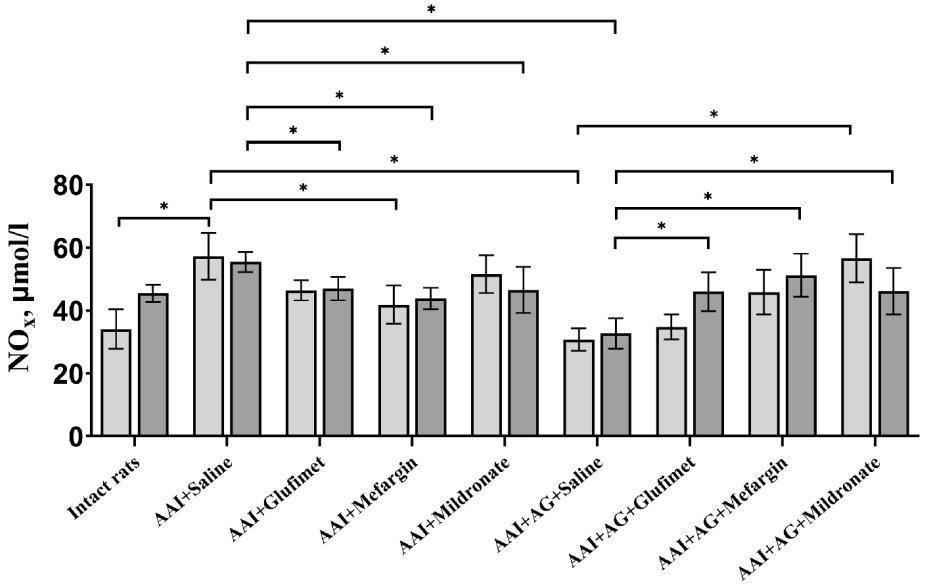

Figure 4. Changes in concentrations of final nitric oxide metabolites in serum and heart homogenates of AAI animals induced by glufimet and mefargin under conditions of iNOS inhibition. The shaded columns are the studied parameter in serum, the black columns are the studied parameter in the heart homogenate. Differences are statistically significant at p<0.05 (the Kruskal-Wallis test with the post-hoc Dunn test).

CONCLUSIONS

Acute alcohol intoxication leads to a pronounced decrease in myocardial contractility, the development of mitochondrial dysfunction, and oxidative stress. Ethanol impairs protein and calcium homeostasis, potentiates oxidative damage of heart biomacromolecules; it has a negative impact on the structure and function of membranes, particularly, mitochondria. This contributes to the development of apoptosis, a decrease in the volume of contractile cardiomyocytes and heart failure. The studied derivatives of glutamic acid and GABA, glufimet and mefargin, promote the preservation of the functional reserves of the heart, improve the respiratory function of mitochondria, and reduce the ROS production. In the presented work, we focused on the putative involvement of nitric oxide in the cardioprotective effect of the studied compounds, as evidenced earlier by the data of previous studies and the results obtained. Glufimet and mefargin led to a significant decrease in the final metabolites of NO in blood serum and heart tissue. Their level was higher in AAI animals, probably due to inhibition of iNOS. However, the decrease in NO concentrations during selective blockade of iNOS and administration of the tested compounds was less pronounced than without iNOS blockade. It can be assumed that the glutamate derivative glufimet and the GABA derivative mefargin reduce the expression/activity of iNOS or induce the expression of constitutive NO synthases involved in the modulation of various metabolic pathways in the cell and the contractile function of the heart. The results obtained can serve as a prerequisite for the development of cardioprotective drugs based on derivatives of neuroactive amino acids that modulate the nitric oxide system in cardiomyocytes for effective pharmacotherapy of the consequences of the cardio-negative effects of alcohol.

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COMPLIANCE WITH ETHICAL STANDARDS

The animals were kept in a vivarium with free access to water and food, with a 12-hour daylight hours in accordance with the recommendations of the National Standard of the Russian Federation GOST R-33044-2014 “Principles of Good Laboratory Practice”, International recommendations of the “European Convention for the Protection of Vertebrate Animals used for experiments or for other scientific purposes” (1986) and Directive 2010/63/EU of the European Parliament and of the Council of the European Union of September 22, 2010 on the protection of animals used for scientific purposes. The study protocol was approved by the Regional Research Ethics Committee of the Volgograd Region (protocol 2095-2019 of January 25, 2019).

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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РОЛЬ ИНГИБИРОВАНИЯ iNOS В МЕХАНИЗМЕ КАРДИОПРОТЕКТОРНОГО ЭФФЕКТА НОВЫХ ПРОИЗВОДНЫХ ГАМК И ГЛУТАМИНОВОЙ КИСЛОТЫ НА МОДЕЛИ ОСТРОГО АЛКОГОЛЬНОГО ПОВРЕЖДЕНИЯ МИОКАРДА У КРЫС

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Изучены кардиопротекторные эффекты новых производных глутаминовой кислоты (глуфимета) и ГАМК (мефаргина) при острой алкогольной интоксикации (ОАИ) на фоне селективной блокады индуцибельной NO-синтазы (iNOS). Выявлено, что ОАИ приводит к выраженному снижению сократительной функции миокарда при проведении нагрузочных проб (нагрузка объёмом, проба на адренореактивность, изометрическая нагрузка), митохондриальной дисфункции и усилению процессов перекисного окисления липидов (ПОЛ) в клетках сердца. Снижение продукции NO при ингибировании iNOS на фоне ОАИ вызывает улучшение дыхательной функции митохондрий, уменьшение уровня продуктов ПОЛ и повышение активности супероксиддисмутазы в митохондриях клеток сердца, что приводит к увеличению сократимости миокарда. Исследуемые соединения — глуфимет и мефаргин — вызывают статистически значимый прирост скоростей сокращения и расслабления миокарда, левожелудочкового давления, а также уменьшают продукцию NO. Это сопровождается снижением интенсивности процессов ПОЛ и увеличением коэффициентов дыхательного контроля, отражающих сопряжение процессов дыхания и фосфорилирования, при активации комплексов I и II дыхательной цепи. Снижение концентрации NO при селективной блокаде iNOS и введении изучаемых веществ было менее выражено, чем без блокады фермента, что указывает на вероятное влияние новых производных нейроактивных аминокислот на систему оксида азота.

Полный текст статьи на русском языке доступен на сайте журнала (http://pbmc.ibmc.msk.ru).

Ключевые слова: алкогольное повреждение миокарда; производные глутаминовой кислоты и ГАМК; блокада iNOS

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