



Pharmacological correction of the sequelae of acute alcohol-induced myocardial damage with new derivatives of neuroactive amino acids coupled with the blockade of the neuronal NO synthase isoform

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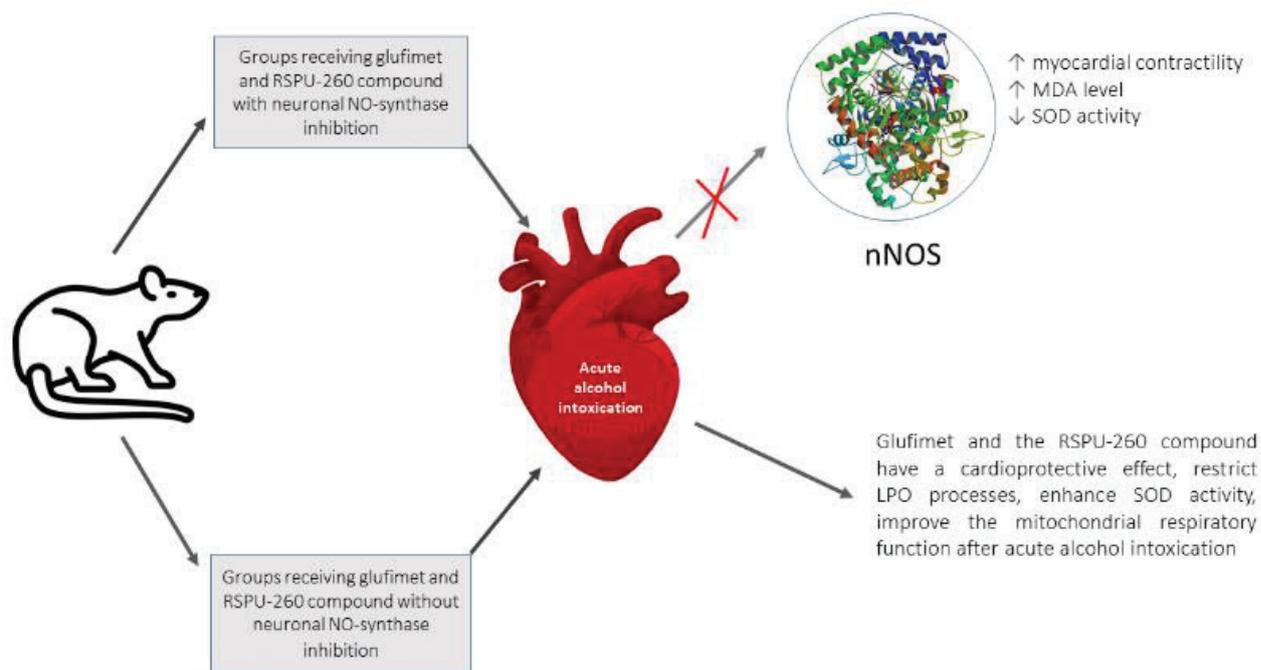
Abstract

Introduction: Acute alcohol intoxication (AAI) induces a number of myocardial disorders, which result in mitochondrial dysfunction in cardiomyocytes, oxidative stress, and decreased cardiac contractility. Nitric oxide produced by the nNOS is one of the major modulators of cardiac activity. New derivatives of GABA (RSPU-260 compound) and glutamate (glufimet) can be potentially regarded as such agents as the interaction between the NO system and the GABA and glutamatergic systems has been proved.

Materials and methods: All the studies were performed on female white Wistar rats, aged 10 months, whose weight was 280–320g. AAI intoxication was modeled of 32% ethanol (gavage, 4g/kg).

Results and discussion: Glufimet and the RSPU-260 compound caused a significant improvement in myocardial contractility, increased oxygen consumption in the V3 state according to Chance, raised the respiratory control ratio and decreased the intensity of LPO intensity. Their effectiveness exceeded that of mildronate, their comparator. nNOS inhibition resulted in a pronounced aggravation of oxidative stress implicated in MDA accumulation in cardiac mitochondria and decreased activity of SOD; myocardial contractility and mitochondrial function indicators did not show a significant difference from the control group. The compounds under study coupled with nNOS inhibition had a cardioprotective effect.

Conclusion: Glufimet and the RSPU-260 compound, derivatives of neuroactive amino acids, have a pronounced cardioprotective effect, restrict LPO processes, enhance SOD activity, improve the mitochondrial respiratory function after acute alcohol intoxication when coupled with neuronal NO-synthase inhibition, the expression of which persists after AAI.

Graphical abstract:**Keywords**

acute alcohol intoxication, cardioprotection, derivatives of GABA and glutamic acid, neuronal NOS

Introduction

Alcohol abuse has become a serious problem of national healthcare as it leads to a number negative sequelae, such as low quality of life, injuries, cognitive deficit, early disability and death. Alcohol is directly or indirectly related to more than 30% of deaths in men under 30 years of age (Lopez-Caneda et al. 2019).

Excessive alcohol consumption increases the risk of fatal arrhythmias, acute myocardial infarction, and congestive cardiac failure (Whitman et al. 2017). It brings about an increase in sympathetic activity and a decrease in parasympathetic activity, which results in vegetative imbalance, especially, in the period following acute intoxication (Brunner et. al. 2021; Greenlund et.al. 2021).

At the molecular level, alcohol-induced contractile dysfunction is implicated in protein and calcium homeostasis disturbances, increased oxidative stress, and apoptosis. Ethanol oxidation by alcohol dehydrogenase, catalase and CYP2E1 gives rise to reactive oxygen species (ROS), which cause the disruption of cell biomacromolecules. Ethanol has a negative effect on mitochondria, impairs the structure and functions of their membranes, which results in a higher ROS production and cell energy deficit, increased apoptosis and necrosis, and a decreased share of contractile cardiomyocytes (Alleynes and Dopico 2021).

Nitric oxide (NO) acts as one of the regulators of myocardial contractility modulating sympathetic and parasympathetic effects on the heart, ion channel performance, mitochondrial respiratory function, and the performance of other organelles of cardiomyocytes. NO is the simplest compound, which is formed in the body as a result of oxidation of L-arginine catalyzed by NO-synthase (NOS). The neuronal isoform of NOS (nNOS) regulating Ca^{2+} -ATPase (SERCA) and reuptake of intracellular Ca^{2+} is constitutively expressed in the sarcoplasmic reticulum of cardiomyocytes. Moreover, nNOS is also the only NOS isoform which is expressed in intrinsic cardiac neurons from vegetative nerves and ganglia and controls the parasympathetic and sympathetic regulation of the cardiac rhythm and contractility. NO produced with the help of nNOS weakens basal cardiac inotropy and modulates the activity of L-type Ca^{2+} -channels in the plasma membranes by means of cGMP and nitrosylation mechanisms. nNOS regulates the activity of constitutive cardiac oxidases (xanthine oxidoreductase, NADPH-oxidase) and decreases the level of intracellular superoxide and other ROS (Zhang et al. 2014; Carnicer et. al. 2017). Furthermore, nNOS can impact myocardial contractility, regulating mitochondrial functions. However, there is no consensus about the mechanisms underlying the regulation of mitochondrial protein activity by nitric oxide. As is

known, **nitric oxide** has an inhibitory effect on mitochondrial respiration in the heart and brain (Brown and Borutaite 2007). However, a number of authors have reported recently that **NO** positively regulates the respiratory chain activity (Dedkova and Blatter 2009; Dholakia et al. 2018; Sakamuri et al. 2020).

Therefore, to date strong evidence of the positive role of the neuronal isoform of NO-synthase in the functioning of cardiomyocytes has been gathered. In this regard, its decreased activity after AAI necessitates a search for agents affecting **NO** production to restrict cardioneegative sequelae of alcohol consumption (Tapia-Rojas et al. 2017). Glutamic acid and GABA derivatives may be regarded as such compounds. The earlier findings have demonstrated the cardioprotective effect of glufimet, a new glutamate derivative, and the RSPU-260 compound (a new GABA derivative), which increase the cardiac functional reserve in a time of chronic alcohol intoxication (Kustova et al. 2021; Perfilova et al. 2021). Moreover, the effect of neuroactive acid derivatives on the **nitric oxide** system in case of acute stress stimulation has been demonstrated (Borisov et al. 2017; Tyurenkov et al. 2017). From this perspective, a study of the effect of glufimet and the RSPU-260 compound, new derivatives of neuroactive amino acids, coupled with the NOS neuronal isoform blockade, on the performance of rats' myocardium after acute alcohol intoxication is of great interest.

Materials and methods

Experimental animals

The experiments were conducted on female Wistar rats aged 10 months. Their average weight was 280–320g. The animals were housed under standard vivarium conditions in accordance with the guidelines of *The European Convention for the Protection of Vertebrate Animals used for Experimental and other Scientific Purposes* (The European Convention, 1986) and *The European Union Directive* (EU Directive 2010/63/EU). They had free access to water and food and 12 hours of daylight.

The protocol of the experimental study was reviewed by the Regional Independent Ethics Committee (Regional Research Ethics Committee of Volgograd Region, minutes N. 2095-2019 of 25.01.2019).

Drugs and treatment

The drugs under study – glufimet (dimethyl ether of hydrochloride 3-phenylglutamic acid, the RSPU-238 compound) and the RSPU-260 compound (two-component crystal structure of hydrochloride methyl-4-amino-3-phenylbutanoate and L-arginine hydrochloride in a 1:1 ratio) (Fig. 1) were synthesized at the Department of Organic Chemistry of A.I. Herzen University (St. Petersburg, Russia); **mildronate**, their comparator agent, (active substance

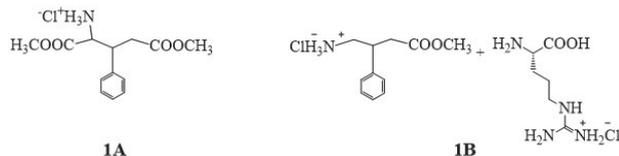


Figure 1. Structural formulas of glufimet (1A) and the RSPU-260 compound (1B).

is meldonium) was used as a ready-for-use solution for injections (Grindex, Latvia). **Saline** solution was applied as a solvent for the studied agents and **7-nitroindazole (7NI)** (Sigma-Aldrich, USA). The investigated compounds and **7NI** were introduced intraperitoneally 10 minutes before alcoholization (glufimet at a dose of 28.7 mg/kg, the RSPU-260 compound – 25 mg/kg, **mildronate** – 50 mg/kg, **7NI** – 50 mg/kg).

Experimental design

Acute alcohol intoxication (AAI) was modeled by intragastric administration of 32% **ethanol** at a rate of 4g of 95% ethyl alcohol solution (ZAO “RFK”, Russia) per 1kg of the animal weight (Jeon et al. 2020). 9 groups with 8 animals in each were formed: 1 – intact group; 2 – control group, animals with AAI receiving **saline** solution; 3, 4 and 5 – experimental groups: animals with AAI, to which glufimet, RSPU-260, and **mildronate**, a comparator, were administered, respectively; 6 – group of alcoholized females receiving **7NI** and **saline** solution; 7, 8, 9 groups – animals with AAI which received glufimet, the RSPU-260 compound and **mildronate**, respectively, which were coupled with nNOS inhibition.

Assessment of myocardial contractility

10 hours after the alcoholization, the animals were narcotized (**chloral hydrate**, 350 mg/kg) and preoperative preparation was performed (Perfilova et al. 2021). A catheter connected to a pressure sensor (Biopac Systems, USA) was introduced through the heart apex to the left ventricle to register the following indicators: myocardial contraction rate ($+dP/dt_{max}$, mmHg/sec), myocardial relaxation rate ($-dP/dt_{max}$, mmHg/sec), left ventricular pressure (LVP), and heart rate (HR) (beats per minute). After the period of stabilization (10 min) and registration of baseline indicators, load tests were conducted: volume load test (intravenous bolus administration of 0.9% **NaCl** solution at a rate of 0.3mg per 100g of the animal weight), adrenoreactivity test (IV introduction of **adrenaline** at a dilution of 10^{-7} g/L at a dose of 0.1 ml per 100g of the animal weight) and isometric load test (occlusion of the ascending aorta for 30 seconds). Maximum intensity of structural performance (MISP) was calculated as shown below:

$$x = \frac{\text{avg} \cdot \text{LVP} \times \text{avg} \cdot \text{HR}}{\text{left ventricular mass} + 1/3 \text{ of the interventricular septum}}$$

It was expressed in mm Hg/mg*min.

Isolation of mitochondria

After the load tests had been performed, the heart was washed in ice-cold saline solution and homogenized at a temperature of 4 °C in a Potter-Elvehjem homogenizer in an isolation medium containing 220mM mannite, 100mM sucrose, 1mM EDTA, 4mM KH_2PO_4 , 20mM HEPES, pH=7.3. Mitochondria were obtained using standard differential centrifugation (Lanza and Nair 2009). The homogenates were centrifuged at 600 G for 10 minutes to sediment debris and intact cells. The supernatant was centrifuged for 20 minutes (8000 G). The sediment was resuspended and used as a mitochondrial fraction, in which respiration intensity, the concentration of lipid peroxidation products and antioxidant enzyme activities were determined.

Assessment of mitochondrial function

Mitochondrial oxygen consumption rate was determined by means of polarography using an Oxytherm System polarograph with the Clark electrode (Hansatech Instruments, UK). The functional state of mitochondria was investigated based on the protocol described by Lanza and Nair (2009). Previously, all the solutions (except ADP) were thermostated for 20 minutes at 33 °C. The reagents required to make solutions were obtained from Sigma-Aldrich (USA). To explore the metabolic states according to Chance, 100 μl of the following substances were consecutively introduced into a 1ml thermostated polarographic cell with the polarographic medium (0.5 mM EDTA, mM $\text{MgCl}_2 \cdot 6\text{H}_2\text{O}$, 20 mM taurine, 10 mM KH_2PO_4 , 20 mM HEPES, 110 mM sucrose, 1 g/L BSA, pH=7,4): V1 (baseline respiratory rate) – mitochondrial suspension; V2 (substrate-dependent respiratory rate) – malate/glutamate (5mM/5mM), a substrate of complex I of the respiratory chain; V3(I) (oxygen consumption rate in the presents oxidation substrate of complex I and ADP, oxidation substrates of complex I), namely, in the state of oxidative phosphorylation involving the activation of Complex I – ADP (200 μM); V3(I + II) – succinate (5mM), a substrate of Complex II of the respiratory chain, then ADP; V3(II) – rotenone, an inhibitor of complex I (0.5 μM), and ADP; V4 – oligomycin, an inhibitor of ATP-synthase (2.5 mM); V(uncoupling) – carbonyl cyanide-4-(trifluoromethoxy)phenylhydrazone (FCCP), a proton ionophore (0.05 mM). The rate of oxygen consumption was expressed in $\text{nmol O}_2/\text{min}/\text{mg}$ protein. To assess the coupling of respiration and phosphorylation, we calculated the respiratory control ratio (RCR) (V3/V4 ratio) (Brand and Nicholls 2011).

Determination of the level of lipid product oxidation and antioxidant enzyme activity

The aliquots of mitochondrial suspension were exposed to a single freeze-thaw cycle for mitochondria to be disrupted. The concentration of malondialdehyde (MDA)

was determined, after which the activity of antioxidant enzymes was assessed: catalase (Oliveira et al. 2007), glutathione peroxidase (Oliveira et al. 2007), and superoxide dismutase (SOD) (Kostyuk 1990).

Our assessment of catalase activity was based on a decrease in hydrogen peroxide (H_2O_2) in the reaction mixture compared to the blank sample. The enzyme degraded H_2O_2 into water and oxygen within 20 minutes in a phosphate buffer at pH of 6.8. Then the reaction was stopped by adding 4% ammonium molybdate, after which the mixture was centrifuged at 8000 rpm for 10 minutes using a CM-50 centrifuge (Elmi, Latvia). A Helios γ spectrophotometer (Thermo Electron Corporation, UK) was employed to measure the supernatant at a wavelength of 410 nm. The enzyme activity was expressed in $\text{H}_2\text{O}_2/\text{min}/\text{mg}$ protein.

The reaction of glutathione oxidation induced by tert-butyl hydroperoxide underlay the elucidation of glutathione peroxidase activity. After 5-minute incubation of the mixture containing 40mM of glutathione, 10mM of tert-butyl hydroperoxide and mitochondrial suspension in a Tris-HCl buffer, glutathione oxidation was stopped by adding 20% trichloroacetic acid solution. After that it was centrifuged at 3000 rpm for 10 min using a CM-6M centrifuge (Elmi, Latvia). We also determined the level of reduced glutathione in the supernatant with the help of 0.4% 5,5'-Dithiobis (2-nitrobenzoic acid) at 412 nm using a Helios γ spectrophotometer (Thermo Electron Corporation, UK). The difference between the concentrations of reduced glutathione and comparable blank samples (containing 20% TCA solution) was used to calculate the enzyme activity per time unit. The specific activity was expressed in $\mu\text{mol}/\text{min}/\text{mg}$ protein.

The method of determining MDA content is based on measuring the amount of TBC-reactive products, which are formed when 0.7% thiobarbituric acid solution is boiled with isolated mitochondria in acidic medium (with 1.3% H_3PO_4 added). We estimated the optical density of the obtained solution at 532 nm using a Helios γ spectrophotometer (Thermo Electron Corporation, UK). The MDA level was expressed in μmol MDA/mg protein.

Protein concentrations in the samples were assessed using a Pierce BCA Protein Assay Kit (Thermo Scientific, USA).

Statistical analysis

We applied GraphPad Prism 9 software and standard statistical criteria for statistical procession of our findings. The data were presented as Mean \pm SD. Normality of distribution was tested using Kolmogorov-Smirnov and Shapiro-Wilk tests. Equal SDs were assessed using Brown-Forsythe and Welch tests. For equal SDs, we used a one-way ANOVA test (with post-hoc Sidak test). If SDs showed significant differences, Kruskal-Wallis and Dunn's tests were employed with a post-hoc test. The differences were assumed statistically significant at $p < 0.05$.

Results

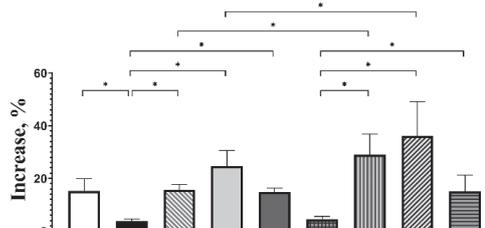
Effect of the studied compounds on rats' cardiac contractility during AAI and nNOS blockade

In the intact animals, the maximum increases in the rates of contraction, relaxation and LVP due to rised preload amounted to 15.3, 22.5, and 18.9%, respectively, compared to the baseline values. In the rats with AAI, the increases in these indicators were 4, 2.2 and 1.8 times as low as in the intact group ($p < 0.05$). The females receiving glufimet showed an increase in +dP/dt, -dP/dt, and LVP, which was 4.1, 4.7, and 1.7 times higher ($p < 0.05$), respectively, in relation to the group of AAI + saline solution. The maximum increases in the experimental group of animals, to which the RSPU-260 compound was administered, were also higher compared to the control group (6.5, 2.7, and 1.6 times ($p < 0.05$), respectively). The females with AAI receiving mildronate, a comparator, demonstrated a greater rise in the rates of contraction and relaxation in response to increased preload (3.9 and 2.3 times ($p < 0.05$), respectively). However, increase in LVP did not show any significant differences vs. the values of the alcoholized animals (Fig. 2).

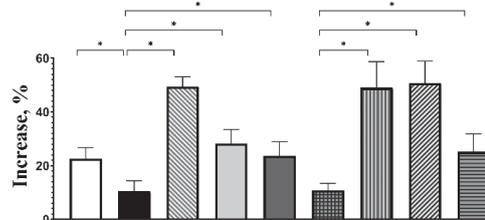
The response to the increased preload in the group of AAI + 7NI + Saline was comparable to that in the alcoholized animals of the control group without nNOS blockade. The females from the experimental groups receiving 7-nitroindazole and the studied compounds displayed higher increases in the rates of contraction, relaxation and LVP vs. those with AAI + 7NI + saline solution: for the animals of the AAI + 7NI + glufimet group, they were 6.3, 4.5, and 2.7 times ($p < 0.05$), respectively; for the AAI + 7NI + RSPU-260 group – 7.8, 4.7, and 3.3 times ($p < 0.05$), respectively; for AAI + 7NI + mildronate – 3.3, 2.3, and 2.6 times ($p < 0.05$), respectively. We have found that in the experimental groups receiving 7-nitroindazole and compounds under study, the response to increased preload was more noticeable in relation to similar groups without nNOS blockade: for the females receiving 7-nitroindazole and glufimet, a rise in contraction rate was 1.8 times as high ($p < 0.05$), LVP – 1.6 times ($p < 0.05$); the AAI + 7NI + RSPU-260 rats also showed a bigger increase in +dP/dt, -dP/dt and LVP (1.5, 1.8, and 1.4 times ($p < 0.05$), respectively). No significant differences in these increases were registered in the animals with AAI receiving mildronate compared to the group with nNOS blockade (Fig. 2).

When an adrenergic test was conducted in rats of the intact group, the maximum increases in the rates of contraction, relaxation and LVP amounted to 52.7%, 48.9%, and 42.7%, respectively, in relation to the baseline values. The control group females demonstrated a rise in these indices, which was 3.6, 3.1, and 2.2 times lower ($p < 0.05$), respectively; the animals of the experimental groups receiving glufimet showed increases, which were 2.3, 3.5, and 1.9 times higher ($p < 0.05$), respectively; the RSPU-260 compound – 2.0, 3.0, and 1.4 times higher ($p < 0.05$), respectively; mildronate – 2.5, 3.0, and 1.2 times higher ($p < 0.05$), respectively, compared to the negative controls (Fig. 3).

2A



2B



2C

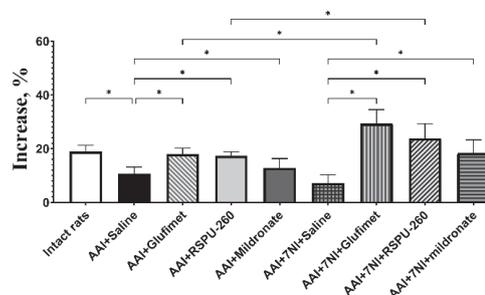


Figure 2. Effect of the studied compounds on cardiac contractility in rats after AAI (acute alcohol intoxication) and with nNOS blockade associated with increased preload. The figure shows: **A.** +dP/dt increase (increase in myocardial contraction rate); **B.** -dP/dt increase (increase in myocardial relaxation rate); **C.** LVP (left ventricular pressure) increase. **Note:** 7NI – 7-nitroindazole; * – the differences are statistically significant at $p < 0.05$.

Increases in the rates of contraction, relaxation and LVP in the rats of the AAI + 7NI + Saline group during the adrenergic test did not show any significant differences from those in the control group animals. When nNOS was blocked in the females with AAI, to which glufimet was administered, a rise in +dP/dt, -dP/dt, and LVP was 4.0, 2.8, and 2.0 times higher, respectively, ($p < 0.05$); when the RSPU-260 compound was administered – 3.2, 4.2, and 1.6 times higher, respectively, ($p < 0.05$) compared to the AAI + 7NI + Saline group. In the females of the experimental group, only an increase in the contraction rate was 2.5 times greater ($p < 0.05$) in relation to the control group with nNOS blockade; increases in the relaxation rates and LVP showed no noticeable differences. However, rises in +dP/dt and LVP in the rats of the AAI + 7NI + glufimet group were 2.0 and 1.3 times higher ($p < 0.05$), whereas in the AAI + 7NI + RSPU-260 group they were 1.8 and 1.3 times as high ($p < 0.05$), an increase in the rate of relaxation was 1.4 times higher ($p < 0.05$) compared to

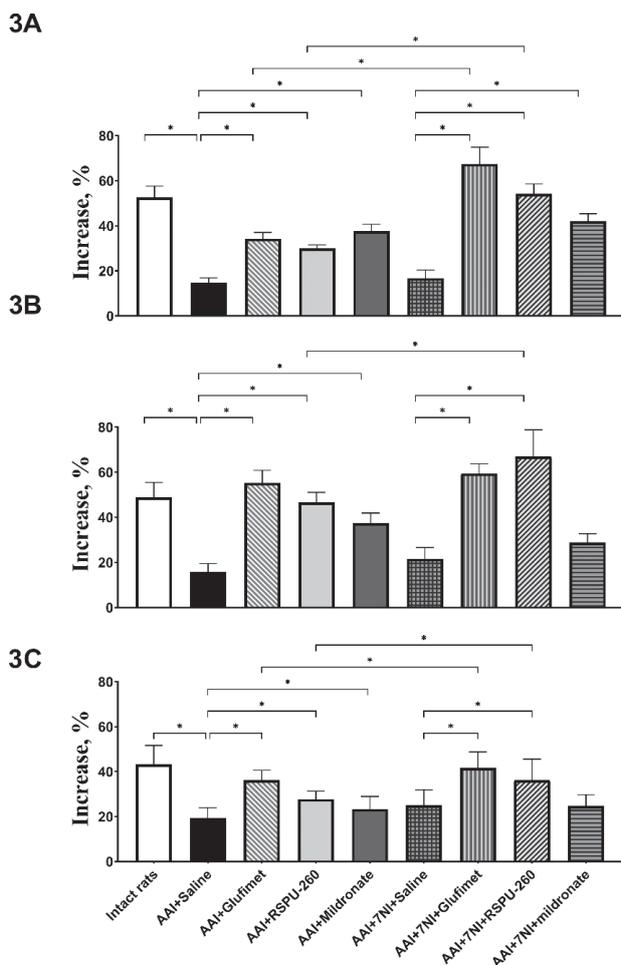


Figure 3. Effect of the studied compounds on cardiac contractility in rats after AAI (acute alcohol intoxication) and with nNOS blockade during the adrenoreactivity test. The figure shows: **A.** +dP/dt increase (increase in myocardial contraction rate); **B.** -dP/dt increase (increase in myocardial relaxation rate); **C.** LVP (left ventricular pressure) increase. **Note:** 7NI – 7-nitroindazole; * – the differences are statistically significant at $p < 0.05$.

the similar experimental group without nNOS blockade. The experimental animals with AAI receiving **mildronate** demonstrated comparable increases during the adrenoreactivity tests (Fig. 3).

When a maximum isometric load test was performed in rats of the intact group, increases in rates of contraction, relaxation and LVP vs. baseline values were 137.8%, 123.8%, and 139.7% on the 5th second of occlusion and 104.4%, 117.3%, and 114.6% on the 30th second, respectively. The females with AAI showed lower increases in similar indices on the 5th second (1.7, 1.5, and 1.3 times, $p < 0.05$) and on the 30th second (1.7, 2.2, and 1.3 times, $p < 0.05$), respectively. Rises in +dP/dt, -dP/dt and LVP in the rats receiving glufimet were significantly higher compared to the control group both on the 5th second (1.7, 1.5, and 1.6 times, $p < 0.05$) and on the 30th second of exposure to the maximum isometric load (1.6, 2.1, and 1.6 times, $p < 0.05$), respectively. The animals with AAI, to which the RSPU-260 compound was administered, also exhibited more noticeable increases in the indices under

study in relation to the negative controls – on the 5th second – 1.6, 1.5, and 1.2 times ($p < 0.05$), on the 30th second – 1.7, 2.0, and 1.3 times ($p < 0.05$), respectively. In the rats receiving **mildronate**, these values were 1.6, 1.4, and 1.4 times higher ($p < 0.05$) on the 5th second and 1.4, 1.5, and 1.3 times higher ($p < 0.05$) on the 30th second of occlusion compared to the AAI + **Saline** group (Fig. 4).

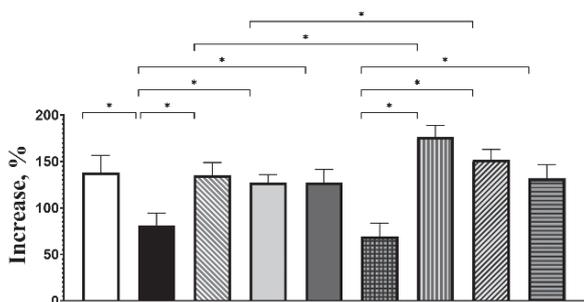
Furthermore, increases in the rates of contraction, relaxation and LVP in response to the ascending aorta occlusion in the females with AAI, to which **7-nitroindazole** and the investigated compounds were administered, were more significant than in the rats of the corresponding control group with nNOS blockade. The animals receiving AAI + **7NI** + glufimet showed 2.5-, 2.7-, and 1.7-fold rises ($p < 0.05$) on the 5th second, respectively, and 3.0-, 3.5-, and 1.7-fold rises ($p < 0.05$) on the 30th second in relation to the negative controls with nNOS blockade. In the experimental group of the females receiving **7-nitroindazole** and the RSPU-260 compound, an increase in the indices under study was 2.2, 2.4 and 1.4 ($p < 0.05$) on the 5th second and 2.2, 2.5, and 1.3 times ($p < 0.05$) on the 30th second compared to the AAI + **7NI** + **Saline** group. In the experimental animals receiving **7-nitroindazole** and **mildronate**, the increases in the investigated indicators were 1.9, 2.6, and 1.4 times as high ($p < 0.05$) on the 5th second and 2.0, 2.0, and 1.4 times as high ($p < 0.05$) on the 30th second, respectively, vs. the corresponding control group (Fig. 4).

We have found that rises in the studied indices in the animals with nNOS blockade receiving the investigated compounds were more noticeable than in corresponding groups without **NO** deficiency. We registered an increase ($p < 0.05$) in the rates of contraction and relaxation on the 5th second in the AAI + **7NI** + glufimet group, which was, on average, 1.3 times more. However, there was no significant increase in LVP. On the 30th second, the indices were 1.7, 1.3, and 1.2 times ($p < 0.05$) higher compared to those in the AAI + glufimet group. The experimental group of females with nNOS blockade, to which the RSPU compound was administered, demonstrated rises in +dP/dt, -dP/dt and LVP both on the 5th and 30th seconds, which were, on average, 1.2 times higher ($p < 0.05$) compared to the similar group without nNOS inhibition. No significant differences in the studied indices were registered in the rats with AAI receiving **mildronate** (Fig. 4).

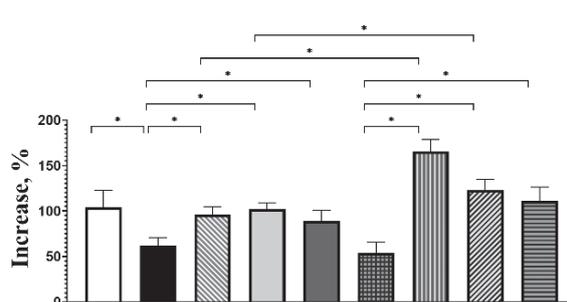
An increase in MISP both on the 5th and 30th seconds of occlusion of the ascending aorta in rats with AAI was 1.5 times lower ($p < 0.05$) compared to the intact group. The experimental group of females receiving glufimet, the RSPU-260 compound, and **mildronate**, demonstrated rises in MISP on the 5th second of the maximum isometric load, which were 1.5, 1.4, and 1.6 times higher ($p < 0.05$) and on the 30th second – 1.5, 1.3 and 1.6 times higher ($p < 0.05$), respectively (Table 1).

The AAI + **7NI** + **Saline** group displayed an increase, which did not significantly differ from the negative control rats. In females from the experimental groups receiving

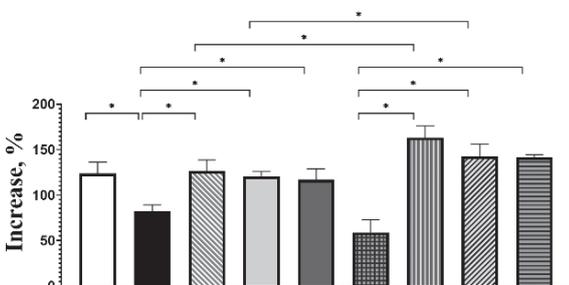
4A



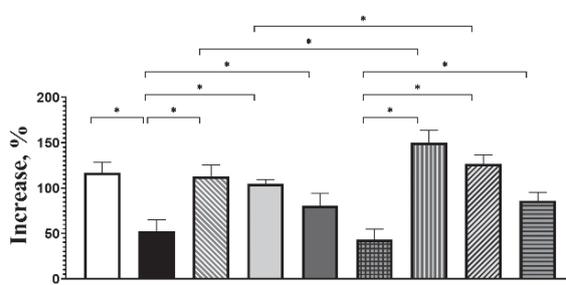
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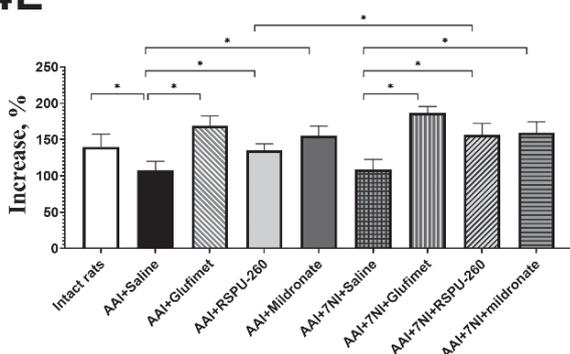
4C



4D



4E



4F

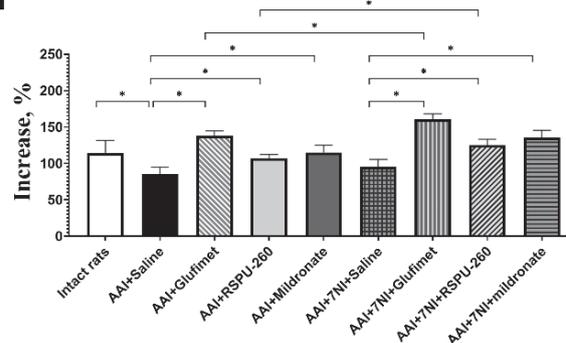


Figure 4. Effect of the studied compounds on cardiac contractility of rats after AAI (acute alcohol intoxication) with nNOS blockade during the maximum isometric load test. The figure shows: **A.** +dP/dt increase (increase in myocardial contraction rate) on the 5th and **B.** 30th seconds of the ascending aorta occlusion; **C, D.** -dP/dt on the 5th and 30th seconds (increase in myocardial relaxation rate); **E, F.** LVP (left ventricular pressure) increase on the 5th and 30th seconds of load, respectively. **Note:** 7NI – 7-nitroindazole; * – the differences are statistically significant at p<0.05.

the studied compounds and 7-nitroindazole, the MISP values were significantly higher compared to the control group on the 5th second of occlusion: in the AAI + 7NI + glufimet group – 1.7 times (p<0.05), in the AAI + 7NI + RSPU-260 group – 1.4 times (p<0.05) and mildronate

– 1.6 times (p<0.05). On the 30th second, no noticeable differences in MISP were observed either in the animals from the experimental groups receiving the investigated agents and 7-nitroindazole or in those without nNOS blockade (Table 1).

Table 1. Effect of the studied compounds on MISP in rats with AAI and nNOS blockade

Animal groups	MISP, mm Hg/mg*min		
	Initial value	5 th s	30 th s
Intact rats	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 + RSPU-260	68.9±12.3	212.4±41.12 § (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 + 7NI + Saline	65.3±12.3	157.5±34.2 (140.8±25.2)	155.0±31.4 (137.2±15.4)
AAI + 7NI + Glufimet	72.8±8.3	249.7±42.1 (242.6±38.6) ‡	219.1±43.3 (200.3±43.6)
AAI + 7NI + RSPU-260	66.8±7.3	207.4±28.9 (211.2±32.3) ‡	172.2±22.2 (159.0±31.1)
AAI + 7NI + Mildronate	59.8±11.0	201.3±39.3 (236.7±22.6) ‡	179.6±39.2 (200.8±33.9)

Note: AAI – acute alcohol intoxication; MISP – maximum intensity of structural performance; 7NI – 7-nitroindazole; the differences are statistically significant compared to (p<0.05): † – Intact group; ‡ – AAI + 7NI + Saline group; § – AAI + Saline group. In brackets, an increase in relation to baseline values is shown, %.

Effect of the studied compounds on the functional activity of rats' cardiac mitochondria after AAI and with nNOS blockade.

In the rats with AAI, the rates of baseline and substrate-dependent respiration were 1.2 (p<0.05) and 1.4 (p<0.05) times lower compared to the intact group. Oxygen consumption in the V3 state according to Chance in a time of isolated activity of complex I of the respiratory chain in the females with AAI was 1.2 times lower (p<0.05); when both complexes I and II were stimulated – 1.7 times lower (p<0.05); and when only complex II was at work – 1.5 times lower (p<0.05). However, V4 rate was 1.4 times higher (p<0.05) vs. the intact group. RCRs for complex I, jointly stimulated complexes I and II and isolated activity of complex II were 2.2, 2.3, and 2.0 times lower (p<0.05) in the control group of rats than in the intact animals, which implies a pronounced uncoupling of respiration and phosphorylation processes (Table 2).

The compounds under study promoted an improvement in the functional state of mitochondria: the rate of stimulated respiration when an oxidation substrate of complex I was added was 1.4 times higher (p<0.05) in the AAI + glufimet rats, and in the animals of the AAI + RSPU-260 group, it was 1.5 times higher. Furthermore, when succinate was added, the females of these groups showed, on average, a rise in oxygen consumption, which was 1.8 times as high (p<0.05); when rotenone was administered – a 1.5 times as high (p<0.05), whereas in the V4 condition, it was 1.5 times as low (p<0.05) as in the control group. The rats with AAI receiving glufimet demonstrated a 2.2-time higher increase in RCR for complex I in relation to the alcoholized animals. In the rats of the AAI + RSPU-260 group, there was a 2.2-time greater rise (p<0.05). RCR for joint activity of complexes I and II in the rats of these groups was 2.5 times higher (p<0.05) and for complex II –

2.2 times higher (p<0.05). The females with AAI receiving mildronate also demonstrated a higher oxygen consumption rate compared to the control group in the V3 state: 1.5 times (p<0.05) for complex I, 1.7 times (p<0.05) for I + II, and 1.4 times (p<0.05) for complex II. The V4 rate was 1.6 times lower (p<0.05). For isolated activity of complexes I and II, on average, RCR was 2.2 times higher (p<0.05), for joint activity of complexes I and II – 2.5 times higher (p<0.05) in rats of the experimental group receiving mildronate vs. those in the AAI + Saline group (Table 2).

The females with AAI receiving 7-nitroindazole displayed no significant differences in the rates of oxygen consumption in the V3 and V4 states and RCR from those in the control group without nNOS blockade. In the experimental groups of animals which had received the studied compounds alongside with 7-nitroindazole, the functional state of mitochondria was improving. The AAI + 7NI + glufimet group showed 1.4, 1.7, and 1.6 times greater rises in oxygen consumption rate in the V3 state for complexes I, I + II, and II (p<0.05), respectively; in the V4 state it was 1.4 time lower (p<0.05) compared to the group of animals with nNOS blockade. In the rats of the experimental groups with AAI receiving 7-nitroindazole and the RSPU-260 compound or mildronate, the rate of stimulated respiration in the presence of ADP and malate, on average, was 1.3 times higher (p<0.05); when succinate was added, it was 1.5 times higher (p<0.05); when rotenone was added, it was 1.5 times greater (p<0.05) compared to the AAI + 7NI + Saline group. Moreover, in the experimental group with AAI receiving 7-nitroindazole and the RSPU-260 compound, V4 was 1.4 times higher (p<0.05) than in the AAI + 7NI + saline rats, whereas the rats of the similar group receiving mildronate did not show any statistically significant differences. The AAI + 7NI + glufimet, AAI + 7NI + RSPU-260 and AAI + 7NI + mildronate groups exhibited 2.0, 1.8 and 1.5 times higher increases (p<0.05) in RCR I; 2.3, 2.1 and 1.7 times greater increases in RCR I + II (p<0.05); and 2.2, 2.1 and 1.8 times higher rises (p<0.05) in RCR II compared to the rats with nNOS blockade. When oxygen consumption rates and RCR were contrasted in the corresponding states according to Chance, no significant differences between the experimental groups of rats with AAI receiving the studied compounds and 7-nitroindazole without nNOS blockade were revealed (Table 2).

Effect of the studied compounds on MDA and the activity of antioxidant enzymes of cardiac mitochondria in rats after AAI and with nNOS blockade

MDA concentration in cardiac mitochondria of the control group rats was 1.3 times as high (p<0.05) as the values of the intact group. In the experimental group of animals with AAI receiving glufimet, it was 1.4 times lower (p<0.05); and in the AAI+RSPU-250 group, it was 1.3 times as low (p<0.05) as in the control group. The AAI females receiving 7-nitroindazole showed no differences in the MDA content in relation to the controls without nNOS blockade (Table 3).

Table 2. Effect of the studied compounds on mitochondrial oxygen consumption rate in rats with AAI and nNOS blockade

Animal groups	V1	V2	V3 (I)	V3(I+II)	V3 (II)	V4	RCR (I)	RCR (I+II)	RCR (II)
Intact rats	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 + RSPU-260	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 + 7NI + Saline	17.8±2.1	20.6±3.5	23.5±4.0	26.0±4.1	19.2±2.8	18.9±4.9	1.4±0.3	1.7±0.3	1.1±0.2
AAI + 7NI + Glufimet	21.7±2.5‡	25.5±3.5	33.3±4.2‡	42.9±5.2‡	30.0±4.6‡	13.0±1.8‡	2.6±0.3‡	3.3±0.3‡	2.3±0.3‡
AAI + 7NI + RSPU-260	22.1±1.7‡	25.3±2.2	30.4±1.8‡	38.6±6.1‡	28.4±1.7‡	13.5±3.7‡	2.4±0.6‡	3.0±0.6‡	2.2±0.5‡
AAI + 7NI + Mildronate	21.1±1.3‡	24.6±1.6	30.1±3.3	38.4±4.1‡	29.1±5.4‡	15.9±3.1	1.9±0.4	2.5±0.3	1.9±0.3

Note: AAI – acute alcohol intoxication; MISP – maximum intensity of structural performance; 7NI – 7-nitroindazole; the differences are statistically significant compared to (p<0.05): † – Intact rats; ‡ – AAI + 7NI + Saline group; § – AAI + Saline group.

SOD activity in rats of the AAI + Saline was 1.7 times lower (p<0.05) than in the intact animals. The experimental groups receiving glufimet or the RSPU compound before AAI displayed a higher SOD activity (1.7 times and 1.8 times (p<0.05), respectively) vs. the control group. In the AAI + 7NI + Saline rats, SOD was less active than in the control group (1.7 times lower, p<0.05). The animals of the experimental groups, in which nNOS was inhibited and the studied compounds were introduced, demonstrated a higher SOD activity: in the AAI + 7NI + glufimet group – 2.5 times (p<0.05); in the AAI + 7NI + RSPU-260 animals – 2.6 times and in the AAI + 7NI + mildronate group – 2.4 times (p<0.05) compared to the corresponding experimental groups, which did not receive 7-nitroindazole (Table 3).

Discussion

As can be seen from the above, acute alcohol intoxication results in decreased cardiac functional reserve, which was implicated in a smaller rise in myocardial contractility parameters (+dP/dt, -dP/dt, LVP) when load tests were conducted. Alcohol abuse is a common cause of death associated with impaired myocardial contractility and cardiac arrhythmias. The main mechanisms of alcohol-induced damage include direct cardiotoxicity of ethanol and acetaldehyde, its major metabolite, increased production of ROS, disturbed intracellular calcium homeostasis, accumulation of ethyl esters of fatty acids, as well as mitochondrial dysfunction (Li et al. 2012).

A negative inotropic effect after acute alcohol intoxication is associated with impaired excitation-contraction coupling in cardiomyocytes. Ethanol induces Ca²⁺ release from sarcoplasmic reticulum (SR) through ryanodine type 2 receptors (RyR2), initiates SERCA-mediated Ca²⁺ uptake in SR and decreases the myofilament calcium sensitivity (Mustroph et al. 2018; Alleyne and Dopico 2021). These processes result in a decreased amount of Ca²⁺ released from SR in response to stimulation and diminished myocardial contractility.

ROS also play an important role in cardiodepressive effects of acute alcohol intoxication. The major source of ROS in cardiomyocytes is the production of superoxide anion by NADPH-oxidase 2 (NOX2). It has been demonstrated that NOX2 knockout in mice prevents the activation of Ca²⁺/calmodulin-dependent protein kinase

Table 3. Effect of the studied compounds on MDA concentration and the activity of antioxidant enzymes of rats' cardiac mitochondria with AAI and nNOS blockade

Animal groups	MDA, μmol/mg protein	Catalase, nmol H ₂ O ₂ /min/mg protein	Glutathione peroxidase, μmol/min/mg protein	SOD, % inhibition/mg protein
Intact rats	26.6±3.6	154.6±22.7	2.1±0.6	48.1±6.7
AAI + Saline	40.9±4.0†	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 + RSPU-260	32.5±3.4§	143.5±23.7	2.3±0.4	51.7±5.6§
AAI + Mildronate	34.2±4.4	143.6±35.3	2.6±0.3	38.9±4.9
AAI + 7NI + Saline	47.9±4.1	169.6±34.8	2.8±0.5	16.6±4.3
AAI + 7NI + Glufimet	42.9±6.5	164.2±29.7	2.9±0.3	41.2±6.4‡
AAI + 7NI + RSPU-260	43.2±4.5	137.9±32.6	1.8±0.5	43.1±6.1‡
AAI + 7NI + Mildronate	35.4±6.2‡	134.8±22.8	2.6±0.5	39.7±11.8‡

Note: AAI – acute alcohol intoxication; MISP – maximum intensity of structural performance; 7NI – 7-nitroindazole; the differences are statistically significant compared to (p<0.05): † – intact group; ‡ – AAI + 7NI + Saline group; § – AAI + Saline group.

(CaMKII), phospholamban phosphorylation and Ca²⁺ release from SR caused by ethanol and mediated by excessive NOX2-derived production of ROS. Furthermore, the inhibition of CaMKII, the activity of which rises due to ethanol exposure, causes a considerable decline in Ca²⁺ release in cells (Brandt et al. 2016). The findings of our study are consistent with the literature data: we have demonstrated an increase in the concentration of MDA, a lipid peroxidation (LPO) product, as well as a statistically relevant decrease in the activity of superoxide dismutase, a first line antioxidant defense enzyme. A number of publications have provided the evidence of diminished activity of SOD associated with oxidative stress in mitochondria, disturbed synthesis of mitochondrial proteins and higher intensity of lipid peroxidation during acute and chronic alcohol intoxication (Vucevic et al. 2013; Matyas et al. 2016; Steiner and Lang 2017).

The myocardium requires substantial energy supply to be able to contract, especially when exposed to the toxic effects of ethyl alcohol. This fact initiated a study of the functional state of mitochondria in cardiomyocytes. Our work has demonstrated that acute ethanol intoxication causes a decline in the oxygen consumption rate in the oxidative phosphorylation state (V3 according to Chance), both in isolated and joint activity of respiratory complexes I and II. These findings are consistent with previously published data indicating disrupted mechanisms of oxidative control in mitochondria, decreased activity of complexes I, II, and IV of the respira-

tory chain alongside with structural changes in mitochondria (Steiner and Lang 2017; Fernández-Solà 2020).

The administration of **7-nitroindazole**, an inhibitor of the neuronal isoform of NO-synthase, in rats has shown ambiguous effects. In the rats with nNOS blockade, myocardial contractility tended to increase during load tests compared to the animals with AAI receiving **saline** solution. This effect is likely to be due to a negative inotropic effect of **nitric oxide** as the lack of its production by the neuronal NO-synthase caused a slight increase in cardiac contractility after alcohol intoxication. **NO** produced by nNOS has a direct regulatory effect on myofilament proteins, myosin in particular, and the activity of ATPase (Kerrick et al. 2018). However, it is worth noting that the animals receiving **7-nitroindazole** showed a statistically significant rise in the level of MDA in cardiac mitochondria and a decline in SOD activity, which indicates a developing oxidative stress. The latter may subsequently lead to mitochondrial dysfunction, energy supply deficit in cells, and impaired contractility.

New derivatives of neuroactive amino acids, such as glufimet (the RSPU-238 compound) and the RSPU-260 compound, had a noticeable cardioprotective effect, which was implicated in enhanced contractility parameters during volume load, adrenergic activity and isometric load tests. Glufimet, a glutamic acid derivative, is crucial for myocardial metabolism. Although the role of amino acids as energy substrates is not significant, glutamate is of key importance for restoring oxidative metabolism in cardiomyocytes. The involvement of glutamate in malate-aspartate shuttle promotes the restoration of the mitochondrial pool of hydrogen protons, regeneration of the cytosolic NAD^+ content required for anaerobic glycolysis (Jiang et al. 2020). Glutamic acid is also a source of α -ketoglutarate, an intermediate of the Krebs cycle. Moreover, glutamate is of great significance for the central and peripheral control of the cardiovascular system performance, as it causes sympathetic activation of the myocardium (Neckel et al. 2012). A glufimet molecule comprises glycine residue, which is involved in the synthesis of proteins, such as glutathione tripeptide having a potent antioxidant action, and in detoxification reactions. Glycine has a wide range of anti-inflammatory and cytoprotective properties (Pérez-Torres et al. 2017). The above-listed mechanisms are likely to underlie improvements of mitochondrial function of the heart and a decrease in the LPO intensity. It should be noted that the effects demonstrated by glufimet are comparable to those of **mildronate**, a comparator agent, whereas some of its indicators were superior.

GABA, on the basis of which the RSPU-260 compound was derived, also has a wide range of metabolic effects on a cell: it acts as an important link in the GABA shunt, in which its transamination occurs eventually giving rise to succinate, an intermediate of the Krebs cycle and substrate of complex II mitochondrial respiratory chain. This causes an increase in the proton gradient and, consequently, stimulates ATP-synthase bringing about a rise in ATP synthesis, which will be used by hexokinase bound to the outer mitochondrial membrane, thus ensuring glucose phosphorylation. The described events promote the production of glucose-6-phosphate in cytosol and ADP, which is used as a substrate for mitochondrial

ATP-synthase. ATP recirculation results in a decrease in mitochondrial membrane potential and, consequently, ROS production related to it (Cavalcanti-de-Albuquerque et al. 2022). In our study, we have demonstrated that the RSPU-260 compound considerably enhanced the coupling of oxidative phosphorylation processes in rats' cardiac cells after AAI increasing the rate of ADP-induced oxygen consumption by mitochondria. Diminished mitochondrial dysfunction caused by the RSPU compound was implicated in a noticeable decline in the level of LPO products, as mitochondria are the major source of ROS in cells. It can also be assumed that this compound being a GABA derivative restricts excessive sympathetic effects on the heart, preserves the myocardial functional reserve, which is indicated by higher increases in the rates of myocardial contraction and relaxation, as well as left ventricular pressure.

When nNOS was inhibited, the studied derivatives of neuroactive amino acids also restricted a cardioneuroactive effect of alcohol, which was implicated in increased rises in contractility parameters during load tests, higher respiratory control ratios for mitochondrial complexes I and II, a decreased MDA level, and an enhanced activity of SOD in relation to the similar indices of the animals receiving **7-nitroindazole** only. The effects of the compounds under study are likely not to be directly mediated by the neuronal NO-synthase. However, it is probable that the RSPU-260 compound may act as a substrate for NO synthesis as its molecule comprises L-arginine. The current literature data on the interaction of glutamate- and GABA-ergic systems with the **nitric oxide** system and the earlier research findings demonstrating *in vitro* and *ex vivo* decrease in the concentration of inducible NO-synthase and cGMP in lipopolysaccharide-activated peritoneal macrophages of mice receiving glufimet and phenibut (a GABA derivative) (Borisov et al. 2017) make it possible to assume that the studied compounds have a direct or indirect effect on the expression of iNOS, which is excessively activated during alcohol intoxication (Bielenichev et al. 2017). **Nitric oxide** produced by this NOS in large quantities can be easily converted into peroxynitrite, a pro-oxidant compound, which can be involved in oxidative, radical-mediated reactions with biomacromolecules. This can result in changes in cell functions from disturbed cell signaling to necrosis and apoptosis. The findings of a number of studies have demonstrated that the activation of different metabotropic glutamate receptors (mGluR2, mGluR3, mGluR5) reduces iNOS expression by decreasing the activity of NF- κ B, NADPH-oxidase, and the permeability of Ca^{2+} -channels of plasma membrane (Foreman et al. 2005; Yao et al. 2005; Loane et al. 2013). Moreover, it has been reported that a glufimet molecule includes glycine residue, which can also inhibit NF- κ B activation and iNOS expression (Mauriz et al. 2001). Some evidence has been found that GABA can inhibit iNOS by decreasing interleukin-6 and tumour necrosis factor- α (TNF α) (Lee et al. 2011).

Conflict of interests

The authors declare no conflict of interests.

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