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# Morphological changes in the myocardium of rats with chronic alcohol intoxication after treatment with new GABA- and glutamic acid derivatives

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## ABSTRACT

**Aim.** To study pathohistological changes in the myocardium of rats with chronic alcohol intoxication (CAI) after treatment with a new glutamic acid derivative glufimet (compound RSPU-238) and a new gamma-aminobutyric acid (GABA) derivative (compound RSPU-260).

**Materials and methods.** Experiments were performed on female Wistar rats aged 10 months. The rats were divided into the following groups: group 1 – intact females; group 2 – a control group which included animals after CAI simulated by replacing drinking water with 10% ethanol solution for 24 weeks; groups 3 and 4 – experimental groups, in which females were intraperitoneally administered with glufimet at a dose of 28.7 mg / kg and RSPU-260 at a dose of 25 mg / kg once a day for 14 days after cessation of alcohol solution consumption; group 5 – a group of animals receiving a reference listed drug mildronate at a dose of 50 mg / kg according to a regimen similar to that of the studied compounds. Changes in microstructural and morphometric parameters of the left ventricular myocardium were assessed using light microscopy.

**Results.** In animals after CAI, the cardiomyocyte volume fraction decreased, while the interstitial and vascular volume fractions increased. Degeneration of cardiomyocytes, such as their wave-like deformation, loss of transverse striation, foci of plasmolysis, and fragmentation of muscle fibers were revealed. In rats treated with glufimet, the structural changes in cardiomyocytes were minimal. Lower vascular plethora was observed; blood vessels were characterized by single stasis and sludge. The cardiomyocyte volume fraction was 9.7% greater than in control animals, while the interstitial and vascular volume fractions were 66.0 and 70.0% smaller, respectively. The animals treated with the RSPU-260 compound had no significant degenerative changes in cardiomyocytes and small vessels similar to the experimental animals injected with glufimet. Mildronate had a less pronounced cardioprotective effect.

**Conclusion.** Administration of new GABA and glutamic acid derivatives to animals with simulated chronic alcohol intoxication leads to improvement of the microstructure in cardiomyocytes compared with control rats. This indicates pronounced cardioprotective effects of the studied neuroactive amino acid derivatives.

**Keywords:** chronic alcohol intoxication, cardioprotective effect, GABA and glutamic acid derivatives

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# Морфологические изменения миокарда крыс после хронической алкогольной интоксикации на фоне лечения новыми производными ГАМК и глутаминовой кислоты

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**РЕЗЮМЕ**

**Цель** – изучение патоморфологических изменений миокарда крыс после хронической алкогольной интоксикации (ХАИ) на фоне лечения новыми производными глутаминовой кислоты – глуфиметом (соединение РГПУ-238), гамма-аминомасляной кислоты (ГАМК) – соединением РГПУ-260.

**Материалы и методы.** Эксперименты проведены на самках крыс линии Wistar, в возрасте 10 мес, разделенных на группы: 1 – интактные самки; 2 – контрольная группа, животные после ХАИ, которая моделировалась заменой питьевой воды на 10%-й раствор этанола в течение 24 нед; 3 и 4 – экспериментальные группы, в которых самкам вводили, соответственно, глуфимет в дозе 28,7 мг/кг и РГПУ-260 в дозе 25 мг/кг внутрибрюшинно, однократно в течение 14 сут после прекращения алкоголизации. Животные группы 5 получали препарат сравнения милдронат в дозе 50 мг/кг в аналогичном с исследуемыми соединениями режиме. Оценивали изменение микроструктурных и морфометрических параметров миокарда левого желудочка с использованием световой микроскопии.

**Результаты.** У животных после ХАИ выявлены уменьшение объемной доли кардиомиоцитов с увеличением таковой интерстиция и сосудов, а также деструктивные изменения кардиомиоцитов в виде их волнообразной деформации, потери поперечной исчерченности, очагов плазмолиза и фрагментации мышечных волокон. У крыс с терапией глуфиметом после ХАИ структурные изменения мышечных клеток были минимальны, сопровождались незначительным отеком, сосуды менее полнокровны с единичными стазами и сладжами, объемная доля кардиомиоцитов была на 9,7% выше, а интерстиция и сосудов – на 66,0 и 70,0% соответственно ниже. У животных, получавших соединение РГПУ-260 после алкоголизации, отсутствовали выраженные дегенеративные изменения кардиомиоцитов и нарушения микроциркуляции в миокарде аналогично самкам, которым вводили глуфимет. Милдронат оказывал менее выраженное кардиопротекторное действие.

**Заключение.** Введение животным с моделированной хронической алкогольной интоксикацией новых производных ГАМК и глутаминовой кислоты вызвало улучшение микроструктурных и морфометрических характеристик миокарда по сравнению с контрольными крысами, что свидетельствует о выраженных кардиопротекторных эффектах изучаемых производных нейроактивных аминокислот.

**Ключевые слова:** хроническая алкогольная интоксикация, кардиопротекторное действие, производные ГАМК и глутаминовой кислоты

**Конфликт интересов.** Авторы декларируют отсутствие явных и потенциальных конфликтов интересов, связанных с публикацией настоящей статьи.

**Источник финансирования.** Авторы заявляют об отсутствии финансирования при проведении исследования.

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## INTRODUCTION

Diseases associated with alcohol consumption remain some of the most studied pathological conditions due to their high social significance. According to the World Health Organization (WHO), alcohol consumption is a leading risk factor for premature death and disability among middle-aged and young people [1]. Recently, an increase in the consumption of ethyl alcohol has been recorded, especially during the COVID-19 pandemic [2].

The consequences of alcohol consumption are associated with cardiovascular and neuropsychiatric diseases, cancer, as well as with liver, kidney, and endocrine diseases [3]. According to numerous studies, ethanol exerts one of the most damaging effects on the heart. Alcoholic cardiomyopathy (ACM) is the most prevalent form of ethanol-induced heart damage in chronic alcohol intoxication (CAI) [4].

Ethanol has negative effects on cardiomyocytes (CMs) damaging their membrane, receptors, mitochondria, ribosomes, cytoskeleton, and DNA. This is due to the small size of the ethanol molecule, its high reactivity and large volume of distribution in the body. Ethanol causes impairment of the plasma membrane, activation of lipid peroxidation and apoptosis, and disruption of signaling mechanisms [5]. Ethanol affects the structure of the myocyte cytoskeleton, connexons, and desmosomes, which causes structural instability of cells [6].

As a result, swelling and destruction of mitochondria lead to energy deficiency in CMs. These processes are accompanied by disorders of lipid metabolism and fatty degeneration of the heart. Ion exchange disorders cause fragmentation of myofibrils. Hypoxia, energy deficiency, electrolyte imbalance, and oxidative stress lead to severe atrophy and death of CMs and compensatory replacement of myofibrils with connective tissue [7]. A decrease in the volume fraction of CMs and changes in their microstructure as well as disorders of the excitation – contraction coupling and contractile protein synthesis cause a decline in myocardial contractility and development of heart failure (HF).

Currently, the principles of treatment for ACM mainly include metabolic therapy (meldonium, mexidol, etc.) and are directed to compensation for already developed HF. However, there is still no pathogen-specific correction of morphological and functional disorders. In this regard, search for pharmacological agents which exert cardioprotective effect in CAI remains relevant.

New derivatives of glutamic acid and gamma-aminobutyric acid (GABA) can be considered as such agents. Previous studies have shown cardioprotective effects of the glutamic acid derivative glufimet (dimethyl ester of 3-phenyl glutamic acid hydrochloride, RSPU-238 compound, Fig. 1, *a*) and the GABA derivative compound RSPU-260 (a two-component composition of methyl-4-amino-3- phenylbutanoate (mefebut) and L-arginine hydrochloride in the ratio of 1:1, Fig. 1, *b*) in CAI. Recently, glufimet and RSPU-260 have demonstrated their membrane-protective and antihypoxic effects. Application of these compounds contributes to an increase in inotropic myocardial reserve, improves endothelium-dependent vasodilation, and reduces lipid peroxidation [8, 9].

In this regard, the aim of the study was to assess the alcohol-induced pathohistological changes in the rat myocardium after treatment with glufimet and RSPU-260.

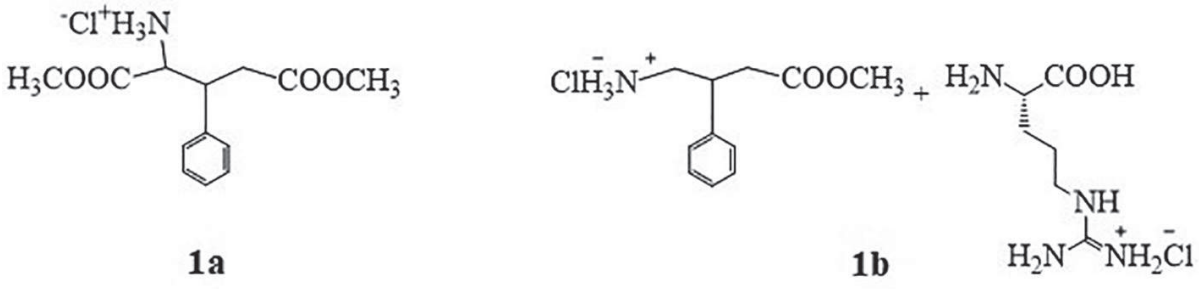


Fig. 1. Structural formulae of*: a*– glufimet, *b* – the compound RSPU-260

## MATERIALS AND METHODS

Experiments were carried out on female Wistar white rats, aged 10 months and weighing 280–320 g. The rats were delivered from the Stolbovaya animal resource center (Russia, Moscow region). The animals were housed and maintained under the standard vivarium conditions with free access to food and water in 12 : 12 light / dark cycle. The study was

carried out in compliance with the Good Laboratory Practice (GLP) requirements for preclinical trials in the Russian Federation (Ministry of Health of the Russian Federation, order no. 199n of April 1, 2016 "On the Adoption of Rules of Good Laboratory Practice"), the International Recommendations of the European Convention for the Protection of Vertebrate Animals Used in Experiments or for Other Scientific Purposes (1986), and the European Union Directive 2010/63/EU 22.09.2010 on the protection of animals used for scientific purposes.

To simulate CAI, the rats were provided with a 10% (v/v) ethanol solution (RFK, Russia) sweetened with sucrose (50 g / l) as the only source of drinking for 24 weeks [10].

The rats were divided into the following groups: group 1 – intact females ($n$ = 7); group 2 – control group, females after CAI ($n$ = 7) receiving 0.1 ml saline solution per 100 g of weight after discontinuation of alcohol consumption; groups 3 and 4 – two experimental groups in which the female rats after CAI were administered with glufimet ($n$ = 7) at a dose of 28.7 mg / kg and RSPU-260 ($n$ = 7) at a dose of 25 mg / kg; group 5 – a group of animals receiving the reference listed drug mildronate (Grindex, Latvia) at a dose of 50 mg / kg ($n$ = 7). Glufimet and RSPU-260 were synthesized at the Department of Organic Chemistry, Herzen Russian State Pedagogical University, St. Petersburg, Russia. Saline solution, the studied compounds (dissolved in saline), and the reference listed drug were injected intraperitoneally once a day for 14 days, starting from the day following CAI cessation.

The hearts were obtained from the lethally narcotized animals (chloral hydrate, 400 mg / kg). The muscle tissue blocks (0.5 x 0.8 cm) obtained from the left ventricle were fixed in 10% neutral buffered formalin for 24 hours. After being washed in running tap water for 6 hours, the tissue blocks were dehydrated and subsequently treated with xylene and placed in the HISTOMIX medium (Biovitrum, Russian Federation). Sectioning of the myocardial blocks was performed with the rotatory microtome HM340E (Thermo Fisher, USA), followed by mounting of the 5–6-μm slices on Polysine microscope adhesion slides (Thermo Scientific, USA). Subsequent to deparaffinization and rehydration, the myocardial sections attached to the microscope slides were stained with hematoxylin (NPF Abris+, Russia) and 0.5% alcoholic solution of eosin (Labiko LLC, Russian Federation). The stained sections on coverslips were mounted in the VitroGel medium (ErgoProduction LLC, Russian Federation) [11].

The morphometric examination involved the assessment of the digitized microphotographs of the rat myocardium processed with MCview software (LOMO-microsystems, Russian Federation). For the quantitative assessment of changes in the sections, we determined the volume fraction of CMs, vessels, and interstitium, thickness of CM wall, CM nuclear area in the longitudinal section, and CM cross-sectional area. To meet the data representativeness criterion, the morphometric measurements were taken in 10 randomly chosen fields of view for each section. The qualitative analysis of microstructural changes included the assessment of the following pathohistological features: the presence of focal and perivascular sclerotic lesions, atrophy (hypertrophy) of individual muscle fibers or their groups, wave-like deformation of CMs, foci of CM disintegration, foci of interstitial accumulation of lymphoid and lymphohistiocytic infiltrates, microhemorrhages, stasis and sludge in arterioles and venules, uneven coloration of CMs and their nuclei. We used the semi-quantitative method with a 1–4-point scale to assess the pathohistological changes [12].

The statistical analysis was performed using the Statistica 12.5 software. The Shapiro – Wilk test was used to evaluate the normality of data distribution. The Student – Newman – Keuls test was applied for pairwise comparison. The quantitative variables were presented as $M \pm SD$, where $M$ is the mean, and $SD$ is the standard deviation. The differences were considered statistically significant at $p < 0.05$.

## RESULTS

The microscopic examination of the intact rats revealed no pathological changes in the myocardium. The cross-striated sarcoplasm of muscle fibers was well defined, the nuclei with weak polymorphism were located in the central compartment of CMs which were surrounded by a thin layer of loose connective tissue with single erythrostasis (Fig. 2, *a*).

CAI resulted in degenerative changes in the rat myocardium, such as perivascular and intermuscular edema with moderate hypertrophy and wave-like deformation of CMs, loss of cross-striation, foci of plasmolysis, and fragmentation of muscle fibers. Round and oval nuclei were hyperchromic (Fig. 2, *b*). Disturbances of microcirculation were manifested by plethora of the vessels, aggregation of erythrocytes, and petechial hemorrhages. Pronounced leukocyte

infiltration was observed in the muscle tissue. The morphometric analysis revealed an increase in the CM cross-sectional area by 9.2% ($p < 0.05$), a significant decrease in the CM volume fraction, and an increase in the volume fractions of the interstitium (by 98.0%, $p < 0.05$) and vessels (by 11.1%) in the female rats with CAI compared with the intact animals (Table).

The animals receiving glufimet showed less pronounced changes (Fig. 2, *c*). Cross-striation of muscle fibers was preserved, no edema and wave-like deformation were noted in CMs separated by thin layers of loose connective tissue. We also found less plethoric vessels and insignificant phenomena of stasis and sludge of erythrocytes in the microvasculature. The mean cross-sectional area of left ventricular CMs in this group of animals was close to the value in the intact rats and by 9.5% smaller than in the control rats ($p < 0.05$) (Table). It is worth noting that treatment with glufimet after CAI resulted in the greater volume fraction of CMs (by 9.7%, $p < 0.05$) and reduced volume fraction of the interstitium and vessels (by 66.0 and 70.0%, respectively, $p < 0.05$). In addition, the mean nuclear area in CMs significantly increased by 26.2% ($p < 0.05$) compared with the female rats after CAI.

The compound RSPU-260 had pronounced cardioprotective effects. After treatment with RSPU-260, minimal pathological changes in CMs and microcirculation, such as minor petechial hemorrhages and stasis of erythrocytes, were noted (Fig. 2, *d*). The interstitial volume fraction was by 42.3% ($p < 0.05$) smaller, and the CM volume fraction was significantly increased compared with the control group.

In the animals treated with the reference listed drug mildronate, the pathomorphological changes were more pronounced than in the groups which were injected with the experimental compounds (Fig. 2, *e*). The microscopic examination revealed hypotrophy of muscle cells, which was confirmed by the morphometric analysis data – the mean cross-sectional area of CMs was by 17.8% smaller than in the rats after CAI and by 10.2% smaller than in the intact animals. In the microvasculature, stasis and sludge of erythrocytes and minor petechial hemorrhages were noted. However, the CM volume fraction was significantly greater and the interstitial volume fraction was significantly smaller than in the controls ($p < 0.05$) (Table).

Table

**Changes in the morphometric parameters of the left ventricular myocardium in the experimental rats after CAI in the context of treatment with the new GABA and glutamic acid derivatives, *M* ± *SD***

| Animal groups | CM cross-sectional area, μm2 | CM nuclear area, μm2 | CM thicknesses, μm | CM volume fraction, % | Vascular volume fraction, % | Interstitial volume fraction, % |
|---|---|---|---|---|---|---|
| Intact group | 241.4 ± 11.2 | 32.1 ± 2.7 | 12.8 ± 1.6 | 92.4 ± 1.4 | 2.7 ± 1.1 | 4.9 ± 0.8 |
| CAI + saline | 263.6 ± 14.3 * | 33.6 ± 1.6 | 12.5 ± 1.3 | 87.3 ± 1.2 * | 3.0 ± 0.9 | 9.7 ± 1.1 * |
| CAI + glufimet | 238.5 ± 9.2 # | 42.4 ± 4.6 # | 12.7 ± 0.9 | 95.8 ± 0.9 # | 0.9 ± 0.5 # | 3.3 ± 0.7 # |
| CAI + RSPU-260 | 245.3 ± 13.3 # | 30.7 ± 5.0 | 11.6 ± 0.7 | 92.2 ± 1.4 # | 2.2 ± 0.9 | 5.6 ± 1.1 # |
| CAI + mildronate | 216.7 ± 13.6 # | 28.7 ± 2.3 # | 11.1 ± 1.2 # | 92.5 ± 2.7 # | 2.4 ± 0.6 | 5.1 ± 1.1 # |

* $p < 0.05$ relative to the group of intact animals (Student's *t* test);
# $p < 0.05$ relative to the control group of animals with CAI receiving saline (Newman – Keuls test).

## DISCUSSION

Replacing drinking water with 10% ethanol solution for 24 weeks caused pathohistological changes in the left ventricular myocardium of the experimental rats. The morphometric analysis demonstrated a decrease in the CM volume fraction, which indicates a decrease in the number of functioning muscle cells and a rise in the interstitial volume fraction due to proliferation of fibroblasts and perivascular and intermuscular fluid accumulation. In the control group of animals after CAI, destructive changes in the CMs were revealed: they lost their cross-striation and had foci of plasmolysis and fragmentation of muscle fibers. Ethanol has damaging effects by directly affecting cellular structures and via production of reactive oxygen species and disruption of lipid metabolism and intracellular calcium homeostasis [13].

Ethyl alcohol impairs cell membrane integrity, increasing mobility and permeability of the phospholipid bilayer. These changes lead to disruption of membrane-associated proteins, resulting in disruption of their transport and signaling. Ethanol easily diffuses through the cell membrane and affects organelles in CMs, especially mitochondria. Ethyl alcohol reduces the membrane potential and activity of membrane respiratory chain complexes, which also damages mtDNA.

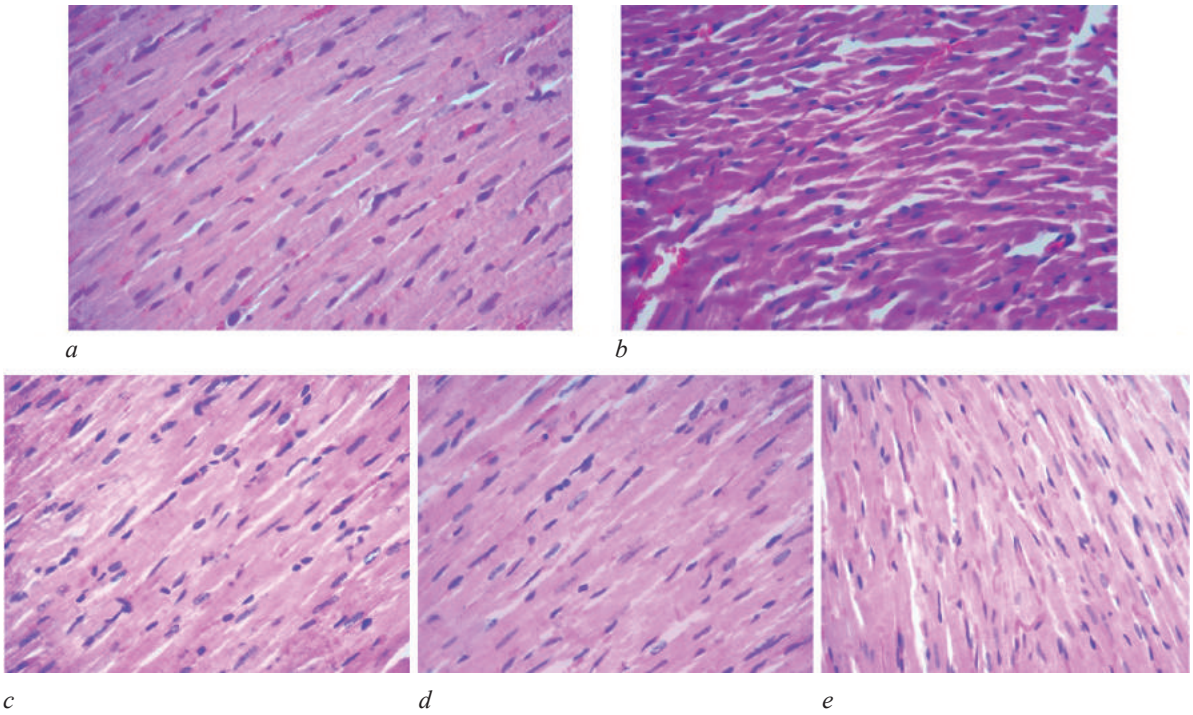


Fig. 2. Microscopic images of the left ventricular myocardium: *a* – in the intact rats, *b* – in the control group, *c* – after treatment with glufimet, *d* – after treatment with RSPU-260, *e* – after treatment with mildronate. Staining with hematoxylin and eosin, x400.

The described changes contribute to the development of hypoxia and ATP deficiency in alcohol-damaged cells. In support of this, the presented study showed a trend toward an increase in the vascular volume fraction which can be explained by stimulated angiogenesis under hypoxic conditions. Also, an increase in the cross-sectional area of CMs was revealed, indicating compensatory myocardial hypertrophy. In addition, mitochondrial dysfunction leads to active production of reactive oxygen species (ROS) involved in apoptosis of CMs [14], causing fragmentation of contractile proteins and dysfunction of the sarcoplasmic reticulum [15, 16].

In CAI, synthesis of CM structural proteins changes and the excitation – contraction coupling reduces [17]. Chronic alcohol consumption enhances expression of type I and III collagen in the myocardium, leading to fibrosis of the cardiac muscle tissue [18]. In addition, ethanol induces $Ca^{2+}$ leakage from the sarcoplasmic reticulum and decreases sensitivity of myofilaments to calcium [19, 20]. The described processes exacerbate hypoxia and induce the development of fatty infiltration in the myocardium, fragmentation of myofibrils, compensatory CM hypertrophy, as well as necrosis and apoptosis with the compensatory growth of connective tissue. As a result, the contractile function of the myocardium decreases and chronic HF develops leading to severe disability and death.

The new glutamic acid derivative glufimet limited the negative effects of ethanol on the myocardium. In the rats treated with glufimet, degenerative changes in CMs were minimal; they were accompanied by minor edema. The microvasculature was less plethoric with insignificant phenomena of stasis and sludge. The mean cross-sectional area of CMs treated with glufimet was significantly smaller than that in the control rats with CAI and similar to that in the intact animals. Cardioprotective effects of glufimet are probably associated with incorporation of glutamate and glycine fragments in its chemical formula.

Glutamic acid has a wide range of metabolic effects. This amino acid can improve myocardial tolerance to hypoxia due to intensification of anaerobic glycolysis in the cytosol and regeneration of $NAD^+$, participation in the malate – aspartate shuttle, and activation of the electron transport chain in mitochondria. In addition, glutamate probably contributes to restoration of oxidative metabolism due to replenishment of Krebs cycle intermediates, for example, α-ketoglutarate [21]. It is known that glycine is involved in detoxification reactions and reduces the intensity of lipid peroxidation, being a part of glutathione, a tripeptide with pronounced

antioxidant activity. It also has cytoprotective and anti-inflammatory effects [22].

The new GABA derivative RSPU-260 was as effective as glufimet. In the animals treated with RSPU-260 after 24-week alcohol consumption, no pronounced degenerative changes in CMs and disorders of myocardial microcirculation were revealed. GABA, like glutamic acid, has metabolic effects, being a precursor of succinate, a Krebs cycle intermediate. Therefore, an increase in succinate, the substrate of complex II of the electron transport chain, stimulates ATP synthase and formation of ATP in the cell [23]. In addition, GABA limits excessive sympathetic influences on the heart, supporting functional reserves of the myocardium. The RSPU-260 compound contains L-arginine, a substrate for synthesis of nitric oxide, a bioactive molecule with a number of cardio- and endothelium-protective effects.

Mildronate had a less pronounced cardioprotective effect. In the rats treated with the reference listed drug, muscle fiber atrophy and minor microcirculation disorders were observed. Mildronate regulates energy metabolism by reducing synthesis and biological activity of L-carnitine, stimulating glucose metabolism [24].

## CONCLUSION

The new GABA and glutamic acid derivatives stabilized the microstructural and morphometric parameters of the myocardium after CAI in the experimental animals. The results can be used for further research and development of new drugs to optimize pharmacotherapy of heart diseases associated with CAI.

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Nesterova A.A., Prokofiev I.I. – collection, processing, analysis, and interpretation of the data, drafting of the article. Perfilova V.N. – conception and design, analysis and interpretation of the data. Evsyukov O.Yu. – analysis and interpretation of the data. Kustova M.V. – collection, processing, and analysis of the data. Tyurenkov I.N. – conception and design, critical revision of the manuscript for important intellectual content, final approval of the manuscript for publication.

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