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Gudyrev O.S.²**PHARMACOLOGICAL EFFICACY OF AN INHIBITOR OF ARGINASE-2
KUD975 WITH L-NAME-INDUCED ENDOTHELIAL DYSFUNCTION**¹Moscow State University M.V. Lomonosov, 1 Leninskie Gory St., p.3, Moscow, 119234, Russia²Belgorod State University, 85 Pobedy St., Belgorod, 308015, Russia, e-mail: mkorokin@mail.ru

Annotation. The study of endothelial- and cardioprotective activity of finished dosage forms – tablets, film-coated, containing 10 mg of phenolic nature KUD-975, was shown a statistically significant decrease in blood pressure and the coefficient of endothelial dysfunction on the background of simulation L-NAME-induced pathology. In the group of animals treated with KUD975 found a statistically significant increase in the concentrations of stable metabolites of nitric oxide, which in addition to the normalization of vasodilation says about endothelioprotective action of the compounds studied. The initial values of the functioning of the left ventricle of the heart during treatment with KUD975 found hypodynamic action, which resulted in a decrease in the absolute values of the heart left ventricle pressure, $+ dp / dt$, $-dp / dt$. Results of the study of the functional state of the myocardium during exercise testing, and histological evaluation of the myocardium revealed distinct cardioprotective effects in the study of KUD975 at a dose of 3 mg / kg, expressed in preventing the increase adrenoreactivity, exhaustion myocardial reserve during the sample to the load resistance and hypodynamic restructuring physiological response to the report on hypoxia / reoxygenation compared with animals that have modeled the endothelial dysfunction.

Keywords: endothelial dysfunction, arginase inhibitors, the compounds of phenolic nature.

Introduction.

The endothelial cells line the luminal surface of all blood vessels and are involved in numerous regulatory functions, such as the control of contraction and proliferation of vascular smooth muscle, adhesion of leucocytes and platelets, permeability and inflammatory responses. The endothelium also possesses thrombolytic and fibrinolytic properties. In addition, its metabolic activity regulates the oxidation of plasma lipids, the formation of angiotensin II and the degradation of circulating catecholamines and kinins [1].

Although nitric oxide appears to be the major vasodilator released by endothelial cells in a vast majority of blood vessels, other substances, some of them still unknown, may play a role as well. Furthermore, soon after Dr Furchgott's discovery, it became clear that endothelial cells not only release relaxing factors but also produce contracting substances including endothelin. The release of endothelium-derived vasoactive substances is controlled by a host of neuromediators and by shear forces exerted by the blood flowing in the blood vessel. Under physiological conditions, a precise and balanced release of relaxing and contracting factors contributes to appropriate organ perfusion. However,

this balance is altered in diseases such as atherosclerosis, diabetes, chronic heart failure, coronary artery disease or hypertension [1].

A decrease in nitric oxide (NO) bioavailability has been proposed to contribute to endothelial dysfunction and increased peripheral resistances during essential arterial hypertension. Given that arginine is a substrate for both arginase and NO synthase, arginase activity may be a critical factor in NO bioavailability [2].

Because of high activity of arginase – the enzyme destroying L-arginine in a mucous membrane of thin intestine, 40% of arginine arriving with food destroyed in the course of absorption, and its remaining quantity arrives into a portal vein. Accepting the comprehensibility of L-arginine connected with protein for 90%, it is possible to consider, that only 50% of alimentary arginine goes to system circulation. The arginase is an enzyme of urea cycle, that hydrolyze L-arginine to ornithine and urea. There are two isoforms of this enzyme. Arginase I is constitutive, and "extrahepatic" arginase (arginase II) is induced in vessel endothelium cells by lipopolysaccharids and interferon. [3].

Using the concept of preferred structures and methods of computer modeling techniques developed

synthesis of phenolic compounds containing a heteroatom directly related and heterocyclic structural fragments. The design of the molecular structures of these low molecular weight compounds performed using modern medical and chemical approaches, and involves a high endothelial- and cardioprotective activity of compounds obtained. During *in vitro* enzymatic studies assay was designed to find inhibitors of arginase I and arginase II. Extensive 100K screening to find inhibitors of arginase II was conducted. For one of the most active hits performed studies allowed the development of a formulation, study its pharmacological activity and toxicological safety.

The purpose of research – the study of cardioprotective activity endothelial- and finished dosage forms – tablets, film-coated, containing 10 mg of phenolic nature KUD-975.

MATERIAL AND METHODS OF RESEARCH

The experiments were performed on white rats of males of the Wistar line weighting 180-200 g (N = 10 animals in the group). To model endothelial dysfunction, N-nitro-L-arginine methyl ester (L-NAME) was administered intraperitoneally at a dose of 25 mg / kg / day. The finished dosage form-film-coated tablets containing 10 mg of KUD975 were administered intragastrically, 30 minutes prior to administration of L-NAME, in a dose 3 mg / kg once a day for 7 days. Intact animals were injected intragastrically with a 1% starch solution at a dose of 10 ml / kg for 7 days.

On the 8th day from the beginning of the experiment, under anesthesia (chloral hydrate 300 mg / kg), a catheter was inserted into the left carotid artery to record blood pressure values (BP), bolus administration of pharmacological agents was performed in the femoral vein. The parameters of hemodynamics: systolic blood pressure (SBP), diastolic blood pressure (DBP) and heart rate are measured continuously through the "Biopac" hardware-software complex and the computer program "Acqknowledge 3.8.1". In addition to blood pressure measurement, a number of functional tests were performed, followed by an assessment of changes in hemodynamic parameters (SBP, DBP, heart rate) in response to intravenous administration of a solution of acetylcholine (AH) at a dose of 40 µg / kg at a rate of 0.1 ml per 100 g of body weight of the animal, as well as changes in hemodynamic parameters in response to intravenous administration of sodium nitroprusside (NP) solution at a dose of 30 mcg / kg at the rate of 0.1 ml per 100 g of animal body weight [4, 5, 6, 7].

The degree of endothelial dysfunction in experimental animals, as well as the degree of its

correction by the studied drugs, was assessed by the calculated coefficient of endothelial dysfunction (CED). This coefficient was calculated using the formula: $CED = S_{BP\ NP} / S_{BP\ AH}$, where $S_{BP\ NP}$ is the area of the triangle above the curve of recovery of the blood pressure, and the points of the smaller leg are the point of maximum fall of the blood pressure and the point of exit of the blood pressure level to the plateau when the functional test is performed with sodium nitroprusside, $S_{BP\ AH}$ – the area of the triangle above the curve of recovery of blood pressure when carrying out a sample with acetylcholine, and for the smaller of the legs take the difference between the end point of the bradycardic cardiac component and the recovery point of AD.

Biochemical markers of endothelial dysfunction were the indices of concentration of stable metabolites of nitric oxide (Total NOx) and expression of endothelial NO synthase (eNOS). A modification of the method for the determination of stable metabolites of NO was used, which allows one-step quantitative determination of total nitrates and nitrites after deproteinization of blood serum. The principle of the method is the simultaneous reduction of nitrates to nitrites in the presence of chloride bath tubing and the reaction of diazotization followed by the development of color. The level of expression of endothelial nitric oxide synthase (e-NOS) was determined in a cell lysate by the method of Hendrickson (Metelskaya VA, Gumanova NG, 2002). Detection of the eNOS band was performed by the enhanced chemiluminescence (ECL) method [4, 6].

A study of myocardial contractility after pathology modeling was carried out in anesthetized rats, which were on controlled respiration. The cavity of the left ventricle was probed with a needle through the apex of the heart and the parameters of cardihaemodynamics (left ventricular pressure, maximum speed of reduction (+ dp / dt max), maximum speed were recorded by means of the hardware complex "Biopac" (USA) and computer program "Acqknowledge 3.8.1" Relaxing (-dp / dt max), heart rate (heart rate).

To assess the functional capabilities of the myocardium in animals, loading tests were performed in the sequence shown:

1. Test for adrenoactivity – intravenous one-stage administration of a solution of adrenaline hydrochloride $1 \cdot 10^{-5}$ mol / l, calculated on the basis of 0.1 ml per 100 g of body weight. When this sample was performed, an assessment was made of the maximum elevation of left ventricular pressure (LVP) in response to the administration of epinephrine.

2. Resistance load – clamping of the ascending aorta by 30 sec. After this test, the expiration of the

myocardial reserve (expressed as a percentage) was calculated, which is equal to the ratio of the LVP increment at the 5th second of the aortic clamping to the increase in the LVP at the 25th second of the aortic clamping

3. 3-minute hypoxia followed by reoxygenation. After the test, the maximum LDL was evaluated in response to the resumption of pulmonary ventilation.

Histological examination of the heart is carried out for morphological confirmation of the development of simulated pathological processes and in the complex evaluation of the effectiveness of substances. The material is fixed in 10% formalin, followed by pouring into paraffin. The stains of hematoxylin and regimen are used to detect early damage to cardiomyocytes, according to Van Gison. With the morphometry of the heart, a method for determining the diameter of cardiomyocytes will be used.

Reliability of changes in absolute parameters was determined by the difference method of variational statistics with finding the mean values of the shifts (M), the mean of the arithmetic mean ($\pm m$), and the probability of possible error (p) from the

Student's tables. Differences were assessed as significant at $p < 0.05$. Statistical calculations were carried out using Microsoft Excel 7.0.

RESULTS

According to the study design, endothelial dysfunction was simulated daily for 7 days by intraperitoneal injection of L-NAME (25 mg / kg). Animals in the groups were randomized by sex and weight.

The investigated KUD975 tablets were administered intragastrically with a probe, once a day 30 minutes before L-NAME for 7 days.

According to the protocol on the eighth day, an anesthetized animal was taken into an experiment.

The effect of test compounds on baseline blood pressure in anesthetized rats with modeling of L-NAME-induced pathology is presented in Figure 6.1.

Daily, for 7 days, intraperitoneal injection of L-NAME resulted in a statistically significant increase in blood pressure (Fig. 1)

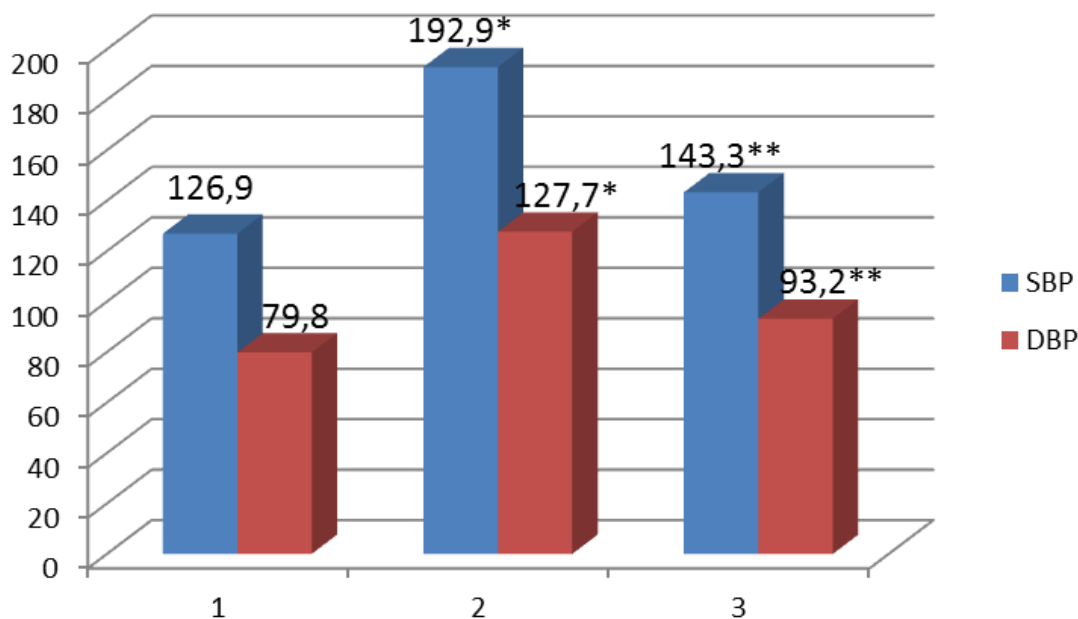


Figure 1. Effect of KUD975 on blood pressure in modeling endothelial dysfunction (mmHg, N = 10 animals in the group)
1- Intact animals; 2 -L-NAME; 3 - L-NAME + KUD975

Notes: here and below:

1* – $p < 0.05$ – in comparison with the group of intact animals;

2** – $p < 0.05$ – in comparison with the group of animals that received L-NAME

It was found that the KUD-975 GLF statistically significantly reduces the initial arterial pressure in the background of modeling of L-NAME induced endothelial dysfunction.

In the figure 2. The results of functional tests on the endothelium-dependent (acetylcholine 40 μg / kg IV)

and endothelium-independent (nitroprusside 30 mg / kg IV) vascular relaxation in animals with L-NAME induced pathology in the background of treatment with the drugs studied.

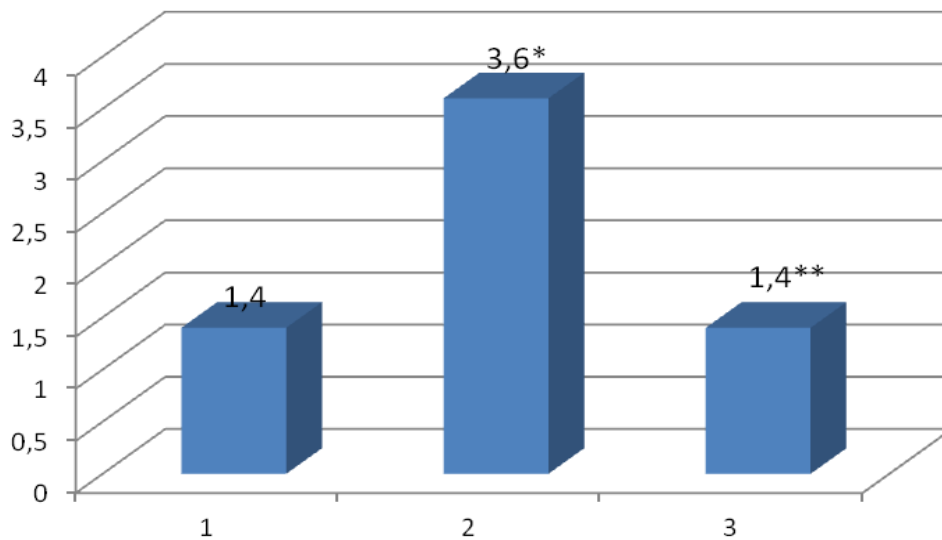


Figure 2. The values of the coefficient of endothelial dysfunction in the simulation of L-NAME induced deficiency of nitric oxide and its correction with the help of the developed KUD975 (standard units, N = 10 animals in the group)
1 – Intact animals; 2 –L-NAME; 3 – L-NAME + KUD975

The processing of the obtained experimental data made it possible to establish that the developed finished dosage form at a dose of 3 mg / kg had an endothelioprotective effect, which resulted in a statistically significant decrease in the endothelial dysfunction coefficient and an absolute achievement by the coefficient of endothelial dysfunction in the group of animals receiving KUD975 of the CED level of intact animals.

When analyzing the absolute values of areas over the blood pressure restoration curves, it was

found that the optimal ratio of endothelium dependent and endothelium independent vasodilatation (close to the animals in the control series) was characteristic of the group of animals receiving KUD-975 tablets. This fact indicates the pronounced effect of KUD-975 on endotheliocytes and the nitric oxide synthesis system.

The results of studying the effect of test compounds on the expression of endothelial NO synthase are presented in Figure 3.

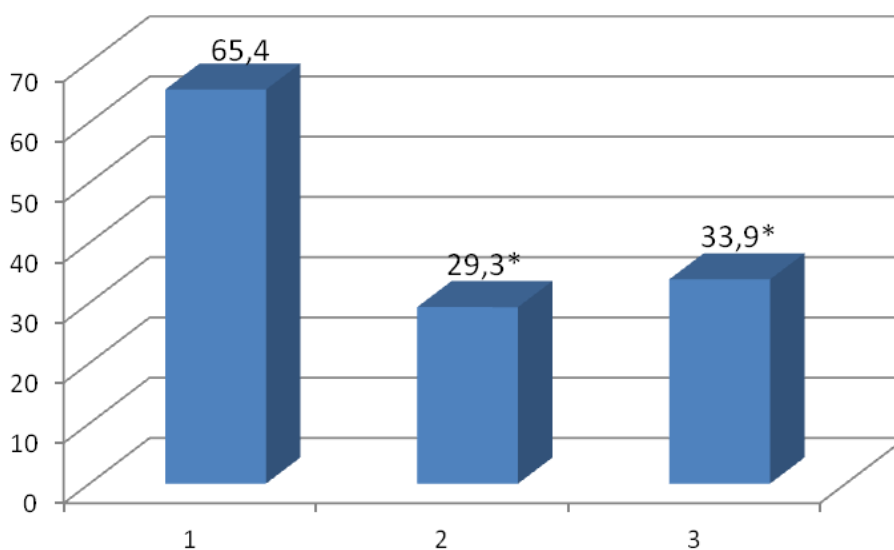


Figure 3. Influence of the developed KUD975 on eNOS expression (% Of control, N = 10 animals per group).
1- Intact animals; 2 – L-NAME; 3 – L-NAME + KUD975.

It was found that the use of the developed tablets KUD975 did not lead to a significant increase in eNOS expression.

The NO-producing function of the endothelium was studied on the basis of the concentration of stable NOx metabolites (Figure 4).

Modeling of L-NAME induced endothelial dysfunction led to a decrease in the concentration of stable metabolites of the oxide by more than 2 times. A statistically significant increase in the

concentration of stable metabolites of nitric oxide was observed in the group of animals receiving KUD975 (Figure 4). A marked increase in the concentration of the final stable metabolites of nitric oxide was found in the group of animals that received KUD975, which, along with the normalization of vasodilatation, indicates a pronounced endothelioprotective effect of the finished drug formulation studied.

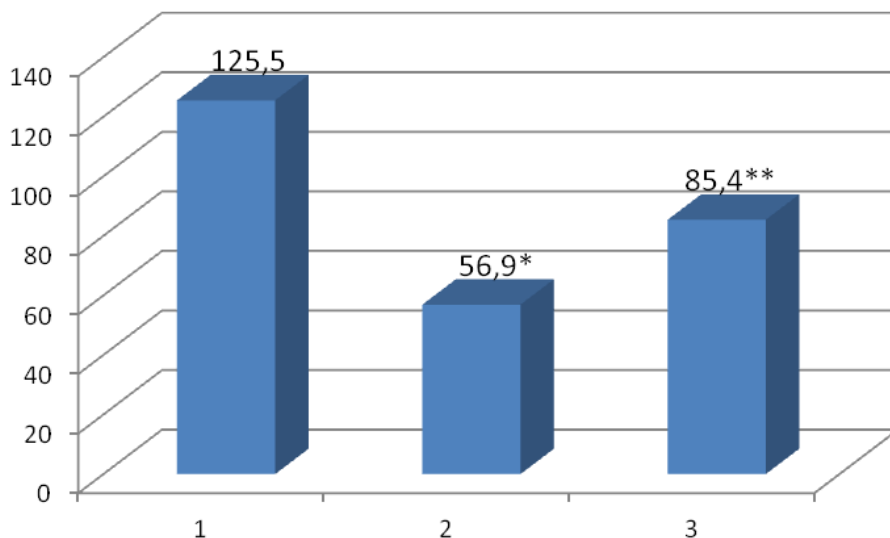


Figure 4. Effect of test compounds on the concentration of stable metabolites of nitric oxide (NOx) (μmol / L, N = 10 animals in the group).
1 – Intact animals; 2 – L-NAME; 3 – L-NAME + KUD975.

When transferring animals to controlled respiration and catheterization of the left ventricular cavity of the heart, it was found that L-NAME-induced deficiency of nitric oxide was characterized by hyperdynamic shifts in the initial cardiohemodynamics (Table 1). The test for adrenoactivity, resistance loading and posthypoxic reactivity of the myocardium were accompanied by significantly higher values of LVP and velocity indices (Tables 1, 2, 3).

The effect of the finished dosage form on initial parameters of cardihaemodynamics is presented in Table 1. The hypodynamic action of the test compounds was revealed in the use of tablets KUD975, which resulted in a decrease in absolute LVP values, + dp / dt, -dp / dt (Table 1).

Load evaluation was performed to evaluate the functional capabilities of the myocardium in animals with L-NAME induced endothelial dysfunction.

The adrenoactivity test was characterized by a pronounced increase in the absolute values of the LVP, + dp / dt, -dp / dt.

Table 1
Changes in the ratios of left ventricular contractility of the rat heart in the modeling of L-NAME induced endothelial dysfunction and its correction with the help of KUD975 (M ± m)

Group of animals	LVP mm Hg.	+dp/dt _{max} mm Hg /s	-dp/dt _{max} mm Hg /s	Heart rate
Intact animals	112,2±8,6	6707,4±154,1	4594±151,6	350,8±11,7
L-NAME	189,8±8,0*	8274,8±250,6*	5266±87,2*	358,4±8,7
L-NAME + KUD-975	126,9±3,0**	7238,4±105,2**	5171,5±50,3*	351,4±9,2

Notes

1 * – p <0,05 – in comparison with the group of intact animals

2 ** – p <0.05 – in comparison with the group of animals that received L-NAME

The analysis of the obtained experimental data made it possible to establish a statistically significant decrease in the maximum LVP during the

adrenoreactivity test in a series of experiments with the KUD975. (Figure 5).

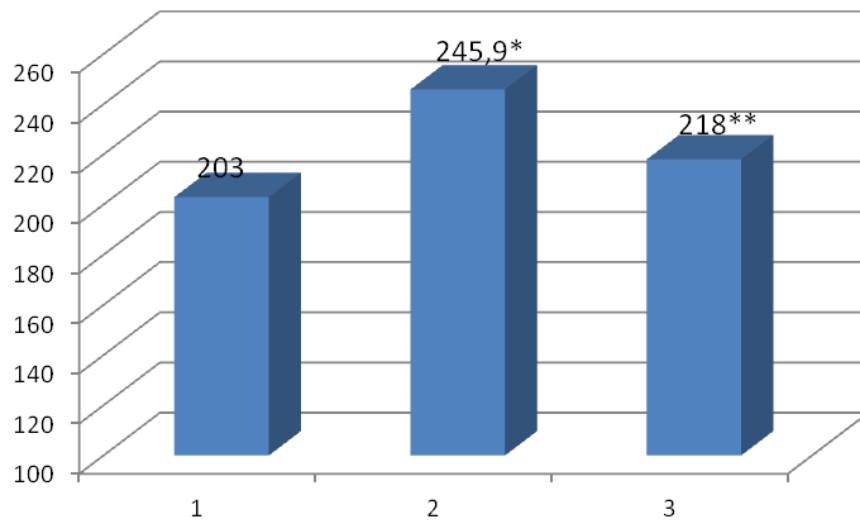


Figure 5. Effect of KUD975 on the maximum arterial pressure in the cavity of the left ventricle of the heart when carrying out a sample for adrenoreactivity. (Mm Hg, N = 10 animals per group).
1 – Intact animals; 2 – L-NAME; 3 – L-NAME + KUD975

When carrying out the sample for the resistance load, the results are comparable with those of the

adrenoreactivity test (Figure 5).

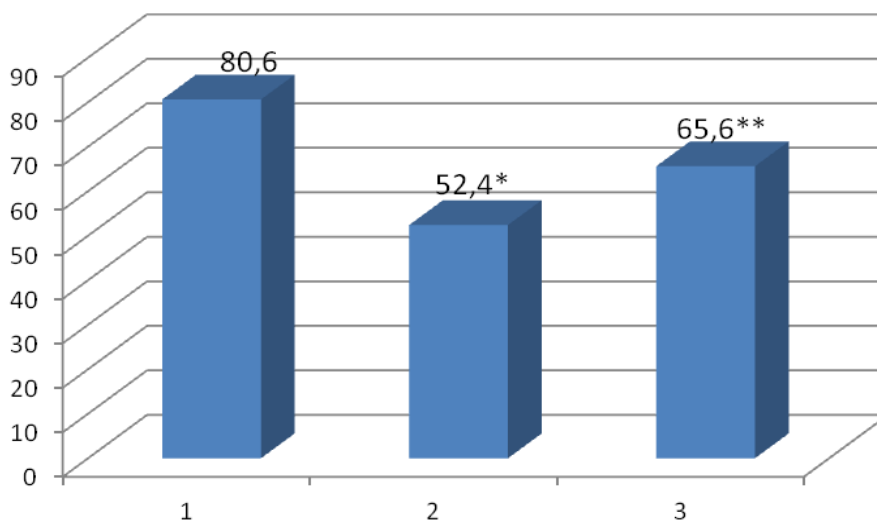


Figure 6. Effect of the KUD975 GLF on the depletion of the myocardial reserve when carrying out the sample on the resistance load (% , N = 10 animals in the group)
1 – Intact animals; 2 – L-NAME; 3 – L-NAME + KUD975

The results of the hypoxic test are presented in Table 2. Modeling of L-NAME induced endothelial dysfunction led to an increase in the numbers of maximum LVP with simultaneous, statistically significant reduction in heart rate and rate of myocardial contractility at the peak of reoxygenation.

As can be seen from the presented data, in the group of animals that received the tablets KUD975

values of the maximum rate of contraction and relaxation, the IPS at the peak of reoxygenation exceeded those in the L-NAME-induced pathology group, and the LVP value in these groups was significantly lower than in animals with Modeled pathology. A lower LVP value with an increase in the rate and heart rate indicates an effective adjustment of the vascular tone regulation under the action of the phenolic compounds studied (Table 2).

Table 2

Changes in the ratios of left ventricular contractility in rat hearts in a sample with acute hypoxia and reoxygenation in the modeling of L-NAME-induced endothelial dysfunction and its correction with the help of KUD975 tablets (M ± m)

Group of animals	LVP mm Hg.	+dp/dt _{max} mm Hg /s	-dp/dt _{max} mm Hg /s	Heart rate
Intact animals	191,7±5,5	8076,6±562	5044,8±499,2	242,2±8,5
L-NAME	229,6±4,2*	7001,8±729,4	4184,3±234,7	198,9±5,4*
L-NAME + KUD-975	194,1±4,4**	8205,5±571,6	4622,5±439,8	256,9±9,7**

Notes:

1 * – p < 0,05 – in comparison with the group of intact animals

2 ** – p < 0,05 – in comparison with the group of animals that received L-NAME

Morphological examination of the heart showed no signs of myocardial damage in the group of animals treated with KUD975. Myocardium had a homogeneous structure with a uniform density of cardiomyocyte location and stroma. Cardiomyocytes with a fine-fibrillar cytoplasmic structure, distinguishable by transverse striation, centrally

located monomorphic nuclei with moderately condensed chromatin. Stroma streaks are fine-fibrous, with uniform saturation with fibroblastic elements. There were no differences in different zones of the myocardium (subendocardial, subepicardial, middle regions). (Figure 7).

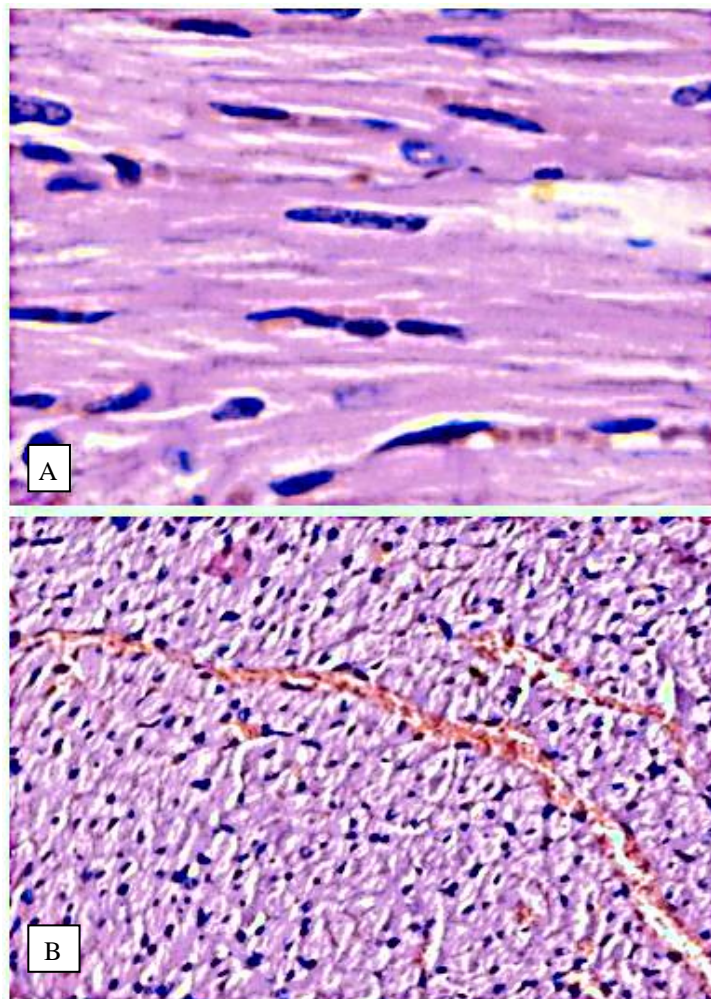


Figure 7. Histological structure of the myocardium in animals receiving KUD975 at a dose of 3 mg / kg on day 8 of the experiment: a homogeneous structure of the myocardium with a uniform blood filling of capillaries between cardiomyocytes, moderately pronounced dilatation and plethora of venules in the subepicardial zone (B). X200

The process of NO synthesis involves firstly the oxidation of arginine to NG-hydroxy-L-arginine (NHA) using nicotinamide adenine dinucleotide phosphate (NADPH) and O₂ catalyzed by NOS [8]. The second step involves the production of NO when NHA is converted to L-citrulline via NOS. The actions of NOS are accelerated by the cofactors flavin adenine dinucleotide (FAD), flavin mononucleotide (FMN), and tetrahydrobiopterin (BH₄). In the absence of its substrate L-arginine or its cofactor BH₄, eNOS uncouples and produces ROS, making it one of the four major enzymes involved in the production of vascular ROS. (The others are xanthine oxidase, NADH/NADPH, the mitochondrial electron transport chain, and eNOS). NOS uncoupling is an important contributor to endothelial dysfunction and plays a crucial role in the cardiovascular phenotype. Arginase, a critical urea cycle enzyme, also utilizes L-arginine. It thereby directly competes with eNOS for their common substrate L-arginine and constrains its availability to eNOS, compromising NO production and increasing the production of ROS by NOS uncoupling [9, 10, 11]. Arginase, which is present in two isoforms (arginase I in the liver and arginase II extrahepatic) catalyzes the final step of the urea cycle yielding L-ornithine and urea from L-arginine. Arginase II appears to be the predominant isoform in human endothelial cells [12] and is highly compartmentalized. There appear to be at least three distinct pools of L-arginine that are spatially confined and regulated by different transporters and enzymes [13].

In this regard, an actual problem is to find novel compounds, selective inhibitors of arginase II study and correction capabilities farmakological correction endothelial dysfunction with these compounds.

This study shows that the developed tablets, film-coated, containing 10 mg of phenolic nature KUD-975, have a pronounced endothelioprotective effect. Thus, we can talk about endothelio- and cardioprotective effects of this group of substances and their term used to treat cardiovascular disease.

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