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Research Article

The study of the endothelial protective properties of the L-norvaline combination with mexidol in the simulation of L-NAME-induced NO deficiency

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Abstract

Introduction: The endothelium is considered as a target for the prevention and treatment of pathological processes leading to cardiovascular diseases.

Materials and methods: Endothelial dysfunction was simulated in male rats using an e-NOS inhibitor L-NAME (25 mg/kg/day intraperitoneally, 7 days). This pathology was then corrected by administering mexidol at a dose of 60 mg/kg and L-norvaline at a dose of 10 mg/kg, as well as the combination of both at the same doses, intragastrically, through an atraumatic probe, once a day.

Results and discussion: A combination of L-norvaline at a dose of 10 mg/kg and mexidol at a dose of 60 mg/kg against the background of L-NAME-induced endothelial dysfunction has a pronounced endothelial protective effect. This is reflected in the prevalence of endothelial-dependent vascular relaxation, a significant decrease in the coefficient of endothelial dysfunction, as well as the prevention of a decline in the concentrations of stable nitric oxide metabolites and of a decrease in the level of the inflammatory changes marker in the vascular wall. The combination of L-norvaline and mexidol, with different pathogenetic action mechanisms, has a pronounced endothelial protective effect because of their antioxidant properties (mexidol) and donation of nitric oxide (L-arginine).

Conclusion: The investigated drugs showed pronounced endothelial protective activity and can be recommended for further pre-clinical studies.

Keywords

Endothelium, endothelial dysfunction, mexidol, L-norvaline, nitric oxide.

Introduction

The discovery of an active role of the endothelium has led to significant progress in the field of vascular research.

It is known that endotheliocytes have a pronounced metabolic activity and synthesize substances that provide: atrombogenicity and thrombogenicity of the vascular wall, regulation of the vascular wall adhesion, regulation of vascular tone and regulation of vascular growth (Félétou and Vanhoutte 2006).

The impaired synthesis of nitric oxide (NO) results in changes of the vasoregulating function of the endothelium, followed by vasoconstriction.

The barrier role of the vascular endothelium as an active organ determines its main role in the human body: maintaining homeostasis by regulating the equilibrium state of opposite processes – vascular tone (vasodilation/vasoconstriction), anatomical structure of the vessels (synthesis/inhibition of proliferation factors), hemostasis (synthesis and inhibition of fibrinolysis factors and of platelet aggregation), and local inflammation (production of pro- and anti-inflammatory factors).

The manifestation of failure of any of these functions leads to a violation of the integral regulation of peripheral blood flow, which in clinical practice corresponds to the established term "endothelial dysfunction" (ED).

Endothelial dysfunction is considered as a predictor of a number of diseases, including hypertension, coronary heart disease, chronic heart failure, as well as a pathogenetic component of organ damage in diabetes, hypoestrogenic and several other pathological conditions (Félétou and Vanhoutte 2006).

In cardiovascular diseases, the ability of endothelial cells to release relaxing factors decreases, and the formation of vasoconstrictors is maintained or increased, i.e. an imbalance is formed between the mediators. These normally ensure the optimal course of all endothelium-dependent processes, such as relaxation and constriction, anti- and pro-thrombogenesis, antiproliferation and proliferation. The main mechanisms in the development of ED are the insufficient production of nitric oxide (Félétou and Vanhoutte 2006), as well as the impaired synthesis of NO, which results in changes of the vasoregulating function of the endothelium, and is finally followed by vasoconstriction. Nitric oxide is involved in the regulation of almost all endothelium functions, and, moreover, is the factor most sensitive to damage. The most important factor in the disruption of the formation and/or of bioavailability of nitric oxide is the excessive formation of reactive oxygen species (ROS), which is observed in many diseases (Förstermann at al. 2017, Mason 2016). Nitric oxide inhibits adhesion, platelet aggregation and the growth of a forming thrombus. It can stimulate angiogenesis, which is potentially important in conditions of myocardial ischemia (Chernomortseva et al. 2010, Pokrovsky et al. 2008).

Endothelial dysfunction associated with the impaired nitric oxide production is one of the initial metabolic disorders in the development of cardiovascular diseases. NO is formed from the amino acid L-arginine. Metabolism of L-arginine in cells proceeds in two ways. L-arginine is hydrolyzed by arginase to ornithine and urea. The other way of converting L-arginine to nitric oxide and citrulline is catalyzed by NO-synthase. The enzymes of arginase and NO-synthase compete with each other for the common substrate L-arginine (Johnson et al. 2005, Zhu et al. 2017). An increase in the activity of the arginase enzyme leads to a decrease in the formation of the main vasodilator – NO, that is, to the development of ED (Belenkov et al. 2009, Pokrovsky et al. 2008, Tsepeleva et al. 2011, Xiong et al. 2014).

The use of arginase inhibitors is currently considered one of the promising ways to correct ED. Their mechanism of action is to block the enzyme arginase, and, consequently, to disturb the conversion of L-arginine into ornithine and urea. As a result, more L-arginine is cleaved by the action of NO synthase to form nitric oxide. Thus, arginase inhibitors prevent the development of nitric oxide deficiency, as one of the most important etiological factors of endothelial dysfunction (Poljanskaja and Pokrovsky 2014).

Arginase inhibitors can be selective and non-selective. Some of the arginase inhibitors have already been studied in vitro. The least studied in the group of non-selective arginase inhibitors is L-norvaline (Tsepeleva et al. 2011).

In this regard, the correction of free radical processes in ED is extremely important, since it makes it possible to prevent the accelerated degradation of NO, to restore the activity of NO synthase, to express NO in the bloodstream and to prevent the development of endothelial dysfunction (Bulakhova 2006).

The basis of disorders leading to endothelial dysfunction is the change in the production of biologically active compounds synthesized by vascular endothelial cells (EC), among which reactive oxygen species are of greatest importance, from the point of view of the problem under consideration (Chernomortseva et al. 2010, Sibal et al. 2010, Ragulina 2017). This group of highly reactive molecules includes molecular oxygen and a number of its derivatives, which are formed in all aerobic cells.

Excessive reactive oxygen species cause significant changes in the function of the vascular endothelium, for example; inhibition of endothelium-dependent vasodilation and an increase in the synthesis of adhesive molecules leads to adhesion and penetration of monocytes into the vascular wall, turning them into macrophages; an increase in the production of various growth factors, an increase in platelet aggregation and thrombus formation, apoptosis activity, etc. In general, severe dysfunction of the vascular endothelium occurs (Félétou and Vanhoutte 2006, Mason 2016).

A promising antioxidant drug is a 3-hydroxypyridine derivative mexidol, which regulates metabolic processes in the myocardium and the vascular wall. It reduces the manifestations of oxidative stress, inhibits free radical lipid peroxidation and increases the activity of the antioxidant system of enzymes (Bulakhova 2006, Ragulina 2017).

An integrated practical treatment of impaired endothelial functions may be of great practical interest when therapy combines drugs that affect several pathogenetic links of endothelial dysfunction development.

Aim of the study

To study the endothelil protective effects of the combination of an arginase inhibitor L-norvaline and antioxidant mexidol.

Materials and methods

The experiments were performed on 70 Wistar male rats (250 ± 50 g), in compliance with the requirements of the federal law of the Russian Federation *On Protection of Animals Against Cruel Treatment* dated June 24, 1998, the rules of laboratory practice in preclinical studies in the Russian Federation (GOST 3 51000.3-96 and GOST R 53434-2009), European Community directives (86/609 EU), the rules and the international recommendations of *The European Convention for the Protection of Vertebrate Animals Used in Experimental Studies* (1997) and *The Laboratory Practice Rules* adopted in the Russian Federation (order of the Ministry of Healthcare of the Russian Federation No. 708 of August 29, 2010) (GOST 33044-2014 2015).

Simulation of endothelial dysfunction was performed by injections of the NO-synthase inhibitor N-nitro-L-arginine methyl ester (L-NAME, Sigma) to males intraperitoneally once a day at a dose of 25 mg/kg for seven days. The animals of the intact group were injected with saline (0.9% NaCl) in the same volume (Pokrovsky et al. 2006).

The following experimental groups of male rats were formed: 1) intact – (n = 10); 2) group of L-NAME-induced deficiency of nitrogen oxide (n = 10); 3) L-NAME+L-norvaline10 mg/kg (n = 10); 4) L-NAME+mexidol 60 mg/kg (n = 10); 5) L-NAME+L-norvaline10 mg/kg+mexidol 60 mg/kg (n = 10).

L-norvaline and mexidol, as well as their combination, were injected to the experimental animals intragastrically daily (through an atraumatic probe) at a dose of 10 mg/kg/day and 60 mg/kg/day against the injection of L-NAME to them.

Functional vascular and cardiac tests were performed by insertion of a catheter into the left carotid artery of the rats to record hemodynamic parameters on the 8th day of the experiment. The animals were at that moment under anesthesia. Bolus dosing of vascular and cardiac samples was in the right femoral vein. The hemodynamic parameters, namely, systolic blood pressure (SBP), diastolic blood pressure (DBP), and heart rate (HR) were measured continuously by means of a TSD104A sensor and a Biopac MP150 system. Acetylcholine (40 μg/kg) was injected intravenously to determine endothelium-dependent vasodilatation (EDV), and sodium nitroprusside (30 μg/kg) was injected intravenously to determine endothelium-independent vasodilation (ENDV) (Pokrovsky et al. 2006).

The coefficient of endothelial dysfunction (CED) was calculated as the ratio of the area of the triangle above the blood pressure (BP) recovery curve in the endothelium-independent vasodilation to the area of the triangle above the BP recovery curve in the endothelium-dependent vasodilation in terms of mean arterial pressure.

On the model of L-NAME-induced ED after vascular samples, myocardial contractility was studied in anesthetized males in controlled breathing. The left ventricular cavity was accessed with a needle through the apex of the heart, the left ventricular pressure was recorded by means of a TSD104A sensor of L-154 ADC and a Biopac MP150 system; stress tests were performed to measure adrenaline reactivity (by injecting adrenaline intravenously 1·10–5 mol/L in volume of 0.1 ml/100 g per body weight) and load resistance (by compressing the ascending part of the aortic arch for 30 seconds) (Pokrovsky et al. 2008).

The total nitrates and nitrites were measured in one step by using a modified method (Metelskaya et al. 2004). The level of C-reactive protein was determined by immunoturbodimetric measurement at a wavelength of 340 nm, by means of nonlinear calibration using HUMAN reagents (Germany) on a Vitalab Flexor biochemical analyzer.

The data obtained as a result of the research was processed statistically, using parametric and non-parametric methods. The significance of differences in the mean values in the case of a normal distribution in two samples was evaluated by Student's t-test, in the case of an abnormal distribution – by Wilcoxon test. In order to identify hidden factors affecting statistical correlations, a factor analysis was carried out, using the method of basic components. Differences at $p < 0.05$ were recognized as statistically significant. Statistical processing was performed using MS Excel and Statistica 10 software (Glants 1999).

Results and discussion

The effect of L-norvaline and mexidol, as well as their combined use at doses of 10 mg/kg and 60 mg/kg, respectively, on the baseline blood pressure indicators in anesthetized rats with L-NAME-induced pathology simulation are presented in Table 1.

A seven-day administration of L-NAME, according to the above scheme, was found to cause arterial hypertension 190.3 ± 6.7/145.0 ± 3.9 mmHg. Processing the experimental data obtained when performing functional tests for endothelium-dependent and endothelium-independent vascular relaxation in the experimental animals made it possible to establish a 5-time increase in CED in the group with NO-synthase blockade (Fig. 1).

The values of indicators of SBP and DBP in the group of intact animals were considered as the target values of blood pressure. The studied drugs prevented the development of severe hypertension. The values of SBP and DBP were significantly lower than the corresponding values of the animals with L-NAME-simulated pathology. The experiment revealed that the combined use of L-norvaline and mexidol, in contrast to monotherapies with these drugs, had a hypotensive effect, which was significantly different from that in the animals treated with monotherapies with these substances against the background of L-NAME.

The results of functional tests for endothelium-dependent (acetylcholine 40 μg/kg) and endothelium-independent (Sodium nitroprusside 30 μg/kg) vascular relaxation in animals with L-NAME-induced pathology with the combined therapy with mexidol (60 mg/kg) and L-norvaline (10 mg/kg) made it possible to establish their significant endothelial protective effect, which was expressed

Table 1. Model of L-NAME-induced NO Deficiency. Changes in Hemodynamics and Functional Tests When Using a Combination of L-norvaline (10 mg/kg) and Mexidol M (60 mg/kg) Against the Background of L-NAME-induced Simulation of Endothelial dysfunction (M ± m, n = 10).

Group of animals	Functional test	SBP, mmHg	DBP, mmHg	HR, b/min
Intact	Source data	137.7 ± 3.7	101.9 ± 4.3	453.8 ± 11.6
	Acetylcholine	84.3 ± 4.5	38.7 ± 2.8	448.3 ± 11.0
	Sodium nitroprusside	83.0 ± 3.7	42.1 ± 4.4	464.1 ± 11.5
L-NAME (25 mg/kg)	Source data	190.3 ± 6.7*	145.0 ± 3.9*	417.0 ± 11.7
	Acetylcholine	110.6 ± 5.2*	82.8 ± 6.6*	399.8 ± 15.6*
	Sodium nitroprusside	88.7 ± 4.7	50.8 ± 4.2	401.7 ± 11.8
L-NAME (25 mg/kg)+ L-norvaline (10 mg/kg)	Source data	180.0 ± 4.7*	144.6 ± 5.1*	371.4 ± 11.0**
	Acetylcholine	106.7 ± 4.9*	56.1 ± 1.8**	344.2 ± 15.0**
	Sodium nitroprusside	129.3 ± 5.1**	64.6 ± 2.5*	358.3 ± 12.0**
L-NAME (25 mg/kg)+ mexidol (60 mg/kg)	Source data	168.1 ± 1.3*	135.6 ± 1.2*	361.3 ± 8.1**
	Acetylcholine	80.4 ± 2.6**	51.3 ± 4.3**	353.0 ± 6.9**
	Sodium nitroprusside	80.7 ± 5.1*	45,7 ± 2.0*	379.2 ± 7,3**
L-NAME (25 mg/kg)+ L-norvaline (10 mg/kg)+ mexidol (60 мг/кг)	Source data	160.2 ± 1.5**	131.0 ± 3.5*	399.0 ± 19.0**
	Acetylcholine	85.5 ± 3.5**	62.6 ± 3.6**	346.1 ± 10.0**
	Sodium nitroprusside	87.4 ± 4.7*	51.8 ± 4.6*	357.4 ± 12.0**

Note: SBP – systolic blood pressure, DBP – diastolic blood pressure, HR – heart rate; * – $p < 0.05$ – in comparison with intact animals, ** – $p < 0.05$ – in comparison with L-NAME.

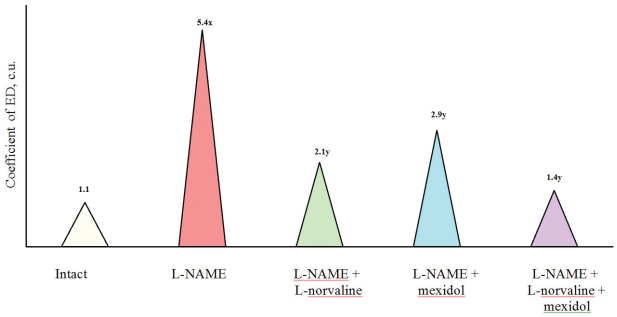

Figure 1. Effect of mexidol and L-norvaline on CED of male rats in the simulation of L-NAME-induced NO deficiency (M±m). **Note:** * – $p < 0.05$ compared to the intact rats; y – $p < 0.05$ compared to the L-NAME group of animals; CED – coefficient of endothelial dysfunction.

in the predominance of endothelium-dependent vascular relaxation (Fig. 1).

At the same time CED decreased to 1.4 ± 0.2 c.u., which was significantly different from a monotherapy with mexidol (2.9 ± 0.2 c.u.). It is noteworthy that this is somewhat better than with a L-norvaline monotherapy (2.1 ± 0.2 c.u.).

In combination with the information described above about the beneficial effect of the combined use of an arginase inhibitor L-norvaline and a synthetic antioxidant mexidol on the original blood pressure indicators, the data obtained indicate the effectiveness of the combined use of L-norvaline with mexidol for the correction of L-NAME-induced pathology, which allowed a moderate decrease in blood pressure values, along with effective CED reduction.

When conducting functional stress-tests on the model of L-NAME-induced NO deficiency, there was an increase in the absolute values of left ventricular pressure in the adrenoreactivity test to 247.3 ± 4.8 mm Hg, which was significantly higher than the values of the intact animals. The use of the combination of L-norvaline (10 mg/kg) and mexidol (60 mg/kg) contributed to the prevention of an increase in adrenoreactivity caused by L-NAME-induced pathology (Tab. 2).

A decrease in myocardial reserve was recorded in the test for load resistance. In the intact males, the left ventricular pressure decreased to 83.6 ± 2.1% by the 25th second of the aortic compression, and in the animals with L-NAME-induced ED – to 66.0 ± 3.2% (Table 2). Using the combination of mexidol and L-norvaline leads to an increase in myocardial reserve.

The introduction of an endothelial NO-synthase blocker L-NAME caused a disturbance in the synthesis of nitric oxide (nitrite ions (NOx) concentration is 2.3 ± 0.7 μmol/l), and the combination of the drugs restored the level of NO necessary for normal functioning of endothelium.

The concentration of nitrite ions under the influence of the combined use of L-norvaline and mexidol increased to 5.8 ± 0.4 μmol/l and was not statistically and significantly different from those of intact animals – 7.2 ± 0.6 μmol/l.

Monotherapies with L-norvaline and mexidol also led to an increase in NOx concentration in serum to 4.3 ± 0.6 and 4.7 ± 0.3 μmol/l, respectively, but not as much as when compared with the combination of the drugs. The results obtained on the NO-producing function of the endothelium make it possible to suggest that both drugs have an impact on preventing a decrease in the concentration of nitrite ions NOx in plasma of the laboratory animals under L-NAME-induced NO deficiency.

In addition to the positive effects of drugs aimed at increasing the level of nitric oxide, the study conducted also proved an anti-inflammatory component of their endothelial protective action. The level of the most important regulator of inflammatory and immune processes – C-reactive protein (CRP) in plasma in the intact group was 0.4 ± 0.1 mg/dl. Simulation of nitric oxide deficiency naturally increased the level of CRP to 2.4 ± 0.2 mg/dl. The introduction of L-norvaline resulted in a decrease in the level of this cytokine, which was 0.9 ± 0.1 mg/dl. It is notewor-

Table 2. Effect of Using Mexidol and L-norvaline on Functional Parameters in Male Rats When Simulating L-NAME-induced NO Deficiency (M±m).

Indicator	Intact	L-NAME	L-NAME+ L-norvaline 10 mg/kg	L-NAME+ mexidol 60 mg/kg	L-NAME+ L-norvaline 10 mg/kg+ mexidol 60 mg/kg
ADR, mmHg	199.2 ± 8.3	247.3 ± 4.8*	239.6 ± 2.2**	220.2 ± 5.2**	191.4 ± 8.5**
LR, %	83.6 ± 2.1	66.0 ± 3.2*	87.6 ± 1.1**	84.2 ± 1.3**	91.0 ± 4.5**

Note: ADR – adrenoreactivity, LR – load resistance. * – $p < 0.05$ compared with the intact group of animals; ** – $p < 0.05$ compared with L-NAME group of animals.

thy that mexidol, both in the form of monotherapy and in combination with L-norvaline, significantly reduced the concentration of CRP in the blood of the experimental animals to 0.6 ± 0.1 mg/dl with the same efficiency. The latter suggests that it is mexidol that has the main influence on reducing the concentration of CRP.

Recent years have been marked by the intensive development of fundamental and clinical research on the role of the vascular endothelium in the genesis of cardiovascular diseases (CVD). A theoretical basis was created for a new direction of fundamental and clinical research – to study the role of endothelial dysfunction in the pathogenesis of CVD and to search for ways to effectively correct it (Belenkov et al. 2009, Chernomortseva 2010, Ragulina 2017).

The main objective of the current study was to investigate the possibility of correcting L-NAME-induced endothelial dysfunction in the experiment by combining the arginase inhibitor L-norvaline with the synthetic antioxidant mexidol.

Monotherapy with the tested drugs did not increase the blood pressure indicators to a given level and, therefore, mexidol and L-norvaline cannot be considered as monotherapy on a model with L-NAME-induced arterial hypertension.

The initial levels of blood pressure when simulating L-NAME-induced arterial hypertension were significantly lower than in the groups with L-norvaline and mexidol monotherapies, which suggests that the basis of the proposed endothelioprotective effect on the two different pathogenetic links.

On the one hand, mexidol inhibits free radical oxidation, activates superoxide dismutase, increases the content of polar fractions of lipids in the membrane, reduces the viscosity of the lipid layer, increases the membrane fluidity, activates the energy exchange in the cell and thus protects the NO-synthase, increasing nitric oxide production (Belenkov et al. 2009, Bulakhova 2006, Ragulina 2017, Siasos at al. 2018).

On the other hand, L-norvaline inhibits the enzyme that destroys the substrate for the formation of nitric oxide, and compensates for violations of NO production in the endothelium (Tsepeleva et al. 2011). L-novaline also exhibits its protective effects due to its ability to inhibit the ribosomal protein kinase S6K1 involved in the synthesis of adhesion molecules, in particular E-selectin, VCAM-1 and ICAM-1, inhibiting the formation of thromboxane A2 and platelets, fibrin complex, accelerating the plasmin formation and fibrin destruction, reducing blood viscosity; reducing the formation of free radicals and ensuring their removal from endothelial cells (Mason 2016, Poljanskaja and Pokrovsky 2014).

The results of the experiments conducted in the present study indicate that the combination of mexidol and L-norvaline has a pronounced therapeutic effect in the restoration of the endothelial regulatory function and helps to restore the activity of NOS synthase, as judged by an increase in the concentration of total nitrate and nitrite ions, which are final metabolites of nitric oxide (Bulakhova 2006, Tsepeleva et al. 2011). This can be due to an increase in the bioavailability of NO, since antioxidants, as well as a reduced oxidative stress, prevent the degradation of nitric oxide, while blocking arginase leads to an increase in the endogenous synthesis of L-arginine and the restoration of NO production (Bulakhova 2006, Poljanskaja and Pokrovsky 2014). In addition to the positive effects of L-norvaline and mexidol aimed at increasing the level of nitric oxide, the present study also proved the anti-inflammatory component of their endothelioprotective action.

The above aspects of this problem can serve as a basis for the development of new preventive and therapeutic measures to be used in addition to the existing methods of treating cardiovascular diseases.

Monotherapy with L-norvaline and mexidol showed significantly less pronounced endothelioprotective properties than the combined use of these drugs. Thus, the combined use of a non-selective arginase inhibitor L-norvaline and a synthetic antioxidant mexidol to achieve endothelial protective effects should be considered as more efficient. This, in turn, make it possible to recommend a wide clinical study to be conducted due to the pathogenetically justified effects on the antioxidant and antiarginase systems.

Conclusion

The combination of L-norvaline at a dose of 10 mg/kg and mexidol at a dose of 60 mg/kg against the background of L-NAME-induced endothelial dysfunction has a pronounced endothelial protective effect. It is reflected in the prevalence of endothelial-dependent vascular relaxation and a significant decreasing of the Coefficient of endothelial dysfunction, as well as in preventing the decline of concentrations of nitric oxide stable metabolites and a decrease in the level of the inflammatory changes marker in the vascular wall.

Conflict of interests

The authors have no conflict of interest to declare.

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Authors contribution

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