



## 11-AMINO ACID PEPTIDE IMITATING THE STRUCTURE OF ERYTHROPOIETIN $\alpha$ -HELIX B IMPROVES ENDOTHELIAL FUNCTION, BUT STIMULATES THROMBOSIS IN RATS

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An 11-amino acid peptide imitating the natural structure of B erythropoietin  $\alpha$ -helix (P- $\alpha$ B), has a specific affinity to the heterodimeric complex EPOR/CD131.

**The aim** of the study was to test whether P- $\alpha$ B can be positioned as a preventing and treating agent for cardiovascular diseases.

**Materials and methods.** The study was performed on sexually mature male Wistar rats. Endothelial dysfunction was modulated by a 7-days intraperitoneal administration of L-NAME at the dose of 2.5 mg/100 g. P- $\alpha$ B, or erythropoietin (EPO), was used for therapy at the dose of 2.5  $\mu$ g/100 g  $\times$  3 times for 7 days, the total dose was 7.5  $\mu$ g/100 g. The function of endothelium was estimated by an endothelium-dependent and endothelium-independent vasodilatation. In addition, a histological assessment of the abdominal aortic wall state and the analysis of *eNos*, *Tnf* and *Il-1 $\beta$*  genes expression were performed. To estimate prothrombotic properties, P- $\alpha$ B and EPO were administered, at the doses of 2.5 and 5  $\mu$ g/100 g (3 times a day for 7 days, the total doses were 7.5  $\mu$ g/100 g and 15  $\mu$ g/100 g, respectively) and on the 8<sup>th</sup> day, the time of ferric (III) chloride-induced carotid artery thrombosis was estimated.

**Results.** The results of the functional tests for endothelium-dependent and endothelium-independent vasodilatation, as well as the histological picture of the aorta have evidenced that P- $\alpha$ B and EPO do not affect L-NAME-induced hypertension but improve the endothelium function. At the same time, P- $\alpha$ B shows a significantly higher endothelial-protective activity, reducing the coefficient of endothelial dysfunction from  $5.1 \pm 0.15$  to  $2.72 \pm 0.12$ . In addition, P- $\alpha$ B has significantly increased the expression of *eNos* and reduced the expression level of *Tnf* and *Il-1 $\beta$*  mRNA genes. Carrying out Ferric (III) chloride-induced carotid artery thrombosis has revealed that P- $\alpha$ B (5  $\mu$ g/100 g  $\times$  3 times a day for 7 days, total dose was 15  $\mu$ g/100 g) has a lower but statistically significant prothrombotic activity than EPO.

**Conclusion.** P- $\alpha$ B can be positioned as an atheroprotector because of its ability to prevent the death of endothelial cells, as well as to reduce remodeling and proinflammatory activation of the vascular wall. However, the prothrombotic properties of P- $\alpha$ B limit its use as a preventing and treating agent for atherosclerosis-associated diseases.

**Keywords:** atherosclerosis, erythropoietin, rats, P- $\alpha$ B, cibenitide, endothelium

**Abbreviations:** P- $\alpha$ B –  $\alpha$ -helix of B erythropoietin; EPO – erythropoietin; L-NAME – N( $\omega$ )-nitro-L-arginine methyl ester; eNOS – endothelial nitric oxide synthase; ED – endothelial dysfunction; EDC – endothelial dysfunction coefficient; SBP – systolic blood pressure; DBP – diastolic blood pressure.

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## 11-АМИНОКИСЛОТНЫЙ ПЕПТИД, ИМИТИРУЮЩИЙ СТРУКТУРУ А-СПИРАЛИ В ЭРИТРОПОЭТИНА, УЛУЧШАЕТ ФУНКЦИЮ ЭНДОТЕЛИЯ, НО СТИМУЛИРУЕТ ТРОМБООБРАЗОВАНИЕ У КРЫС

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**Цель.** 11-аминокислотный пептид, имитирующий природную структуру  $\alpha$ -спирали В эритропоэтина (Р- $\alpha$ В) обладает специфическим сродством к гетеродимерному комплексу EPOR/CD131. В нашем исследовании мы решили проверить может ли Р- $\alpha$ В позиционироваться в качестве средства для профилактики и лечения сердечно-сосудистых заболеваний.

**Материалы и методы.** Исследование выполнено на половозрелых самцах крыс линии Wistar. Дисфункцию эндотелия моделировали путем 7-дневного внутрибрюшинного введения L-NAME в дозе 2,5 мг/100 г. В качестве терапии использовали Р- $\alpha$ В или эритропоэтин (ЕРО) в дозе 2,5 мкг/100 г  $\times$  3 раза в течение 7 дней, суммарная доза 7,5 мкг/100 г. Функцию эндотелия оценивали путем проведения эндотелийзависимой и эндотелийнезависимой вазодилатации. В дополнение к этому проводили гистологическую оценку состояния стенки абдоминальной аорты и анализ экспрессии генов eNos, Tnf и Il-1 $\beta$ . Для оценки протромботических свойств Р- $\alpha$ В и ЕРО вводили в дозах 2,5 и 5 мкг/100 г (3 раза в течение 7 дней, суммарная доза 7,5 мкг/100 г и 15 мкг/100 г, соответственно) и на 8-й день оценивали время железа (III) хлорид-индуцированного тромбоза сонной артерии.

**Результаты.** Р- $\alpha$ В и ЕРО не влияют на L-NAME-индуцированную гипертензию, однако улучшают функцию эндотелия, о чем свидетельствуют результаты функциональных проб на эндотелийзависимую и эндотелийнезависимую вазодилатацию, а также гистологическая картина аорты. При этом Р- $\alpha$ В демонстрирует значительно большую эндотелиопротективную активность, снижая коэффициент эндотелиальной дисфункции с  $5,1 \pm 0,15$  до  $2,72 \pm 0,12$ . Кроме того, Р- $\alpha$ В значительно увеличил экспрессию eNos, и снизил уровень экспрессии мРНК генов Tnf и Il-1 $\beta$ . При проведении железа (III) хлорид-индуцированного тромбоза сонной артерии обнаружено, что Р- $\alpha$ В (в дозе 5 мкг/100 г  $\times$  3 раза в течение 7 дней, суммарная доза 15 мкг/100 г) обладает меньшей, чем ЕРО, но статистически значимой протромботической активностью.

**Заключение.** Р- $\alpha$ В может позиционироваться в качестве атеропротектора ввиду способности предотвращать гибель эндотелиоцитов, а также снижать ремоделирование и провоспалительную активацию сосудистой стенки. Тем не менее протромботические свойства Р- $\alpha$ В ограничивают его применение в качестве средства для профилактики и лечения атеросклероз-ассоциированных заболеваний.

**Ключевые слова:** атеросклероз, эритропоэтин, крысы, Р- $\alpha$ В, цибенитид, эндотелий

**Список сокращений:** Р- $\alpha$ В –  $\alpha$ -спираль В эритропоэтина; ЕРО – эритропоэтин; L-NAME – N-нитро-L-аргинин метиловый эфир; eNOS – эндотелиальная синтаза оксида азота; ЭД – эндотелиальная дисфункция; КЭД – коэффициент эндотелиальной дисфункции; САД – систолическое артериальное давление; ДАД – диастолическое артериальное давление.

### INTRODUCTION

The permanent growth of atherosclerosis-associated diseases in the overall structure of the death causes and the disability rate in the developed countries, necessitates their in-depth study and the improvement of the correction methods [1, 2]. Hereby the experience accumulated since the first works by N.N. Anichkov [3] shows that the atherosclerotic damage of the vascular

wall is a long-term multifactorial process [4]. According to the modern understanding of cardiovascular disease pathogenesis, endothelial dysfunction (ED) plays an integral role in the development of atherosclerosis and related complications [5–7]. The changes in the spectrum of molecules secreted and expressed by endothelium, and disruption of its barrier function, eventually lead to the vascular wall infiltration by atheromatous masses

and formation of atherosclerotic plaques [8]. The emerging pathogenetic cascade becomes an actual target for pharmacological influence [1, 2].

An effective way to prevent the endothelium injury is the use of molecules with a universal cytoprotective activity [9]. One of such molecules is an endogenous glycoprotein – erythropoietin (EPO) [10]. A number of our studies have demonstrated that EPO is capable of significantly improving the morphofunctional state of the vascular wall when simulating ED in rats [11–14]. Nevertheless, a long-term experience shows that promising results of EPO preclinical studies are poorly translated into clinical reality and its main niche is still treatment of anemia [15–17].

In 2004, Michael Brines et al. [17] proved that non-hematopoietic effects of EPO are realized via the heterodimeric complex of EPOR/CD131. The discovery of the fact that the erythropoietic and tissue protective properties of EPO are realized by means of two different receptor systems, has led to the creation of prerequisites for a fundamentally new direction in the search for innovative molecules with a cytoprotective activity.

In 2008, the same authors [18] presented the generalized results of the study of the cytoprotective activity of an 11-amino acid peptide based on erythropoietin  $\alpha$ -helix B. It imitates the spatial part of the molecule that interacts with the heterodimeric EPOR/CD131 receptor, but does not interact with the homodimeric EPOR/EPOR receptor.

This compound (P- $\alpha$ B, cibemotide, PubChem CID: 91810664) demonstrated the ability to significantly improve the morphofunctional state of tissues in diabetic macular edema, a renal ischemia/reperfusion injury, and significantly improve cognitive functions in the model of galantamine-induced amnesia in the absence of any effect on erythropoiesis.

**THE AIM** of the research is to evaluate the endothelial- and atheroprotective activity of P- $\alpha$ B and to evaluate the prothrombotic properties of this molecule in order to identify the obstacles in the clinical use of P- $\alpha$ B as a preventing and treating agent for cardiovascular accidents.

## MATERIALS AND METHODS

### Animals

The animals were obtained from the Charles River Laboratories Kennel (Massachusetts, USA). They were kept at the preclinical research center of the Research Institute of Pharmacology of Living Systems. After 14 days of quarantine, the rats were stratified by weight and placed by 9 individuals in separate conventional cages according to their experimental group. Before and during the study, the animals were kept in rooms with artificial lighting (12h/12h mode) at 21–23°C, the humidity of 38–50%, and had a free access to food and water. The number of the conclusion of the Independent Ethical Committee is 06-09/02-1 dated 16 May 2019.

The experiment was performed on 76 male rats (200–220 g) of the Wistar line. The requirements of the Law of the Russian Federation “On Protection of Animals from Cruelty” dated 24 June 1998, the rules of laboratory practice during preclinical studies in the Russian Federation (GOST 3 51000.3-96 and GOST R 53434-2009), Directives of the European Community 86/609EU and the Rules of laboratory practice adopted in the Russian Federation (Order of the Ministry of Health of the Russian Federation No 708 dated 29 August 2010), were observed.

### Endothelial dysfunction modeling

Endothelial dysfunction was modeled by a 7-days' intraperitoneal administration of the blocker of endothelial nitric oxide synthase L-NAME (Sigma Aldrich, USA) at the dose of 2.5 mg/100 g. To estimate the endothelial-protective effect of P- $\alpha$ B in comparison with EPO, 4 groups of 9 animals each, were formed of 36 male Wistar rats (200–220 g):

- 1) ED + EPO (LLC “Pharmapark”) (2.5  $\mu$ g/100 g, 3 times a day for 7 days, the total dose was 7.5  $\mu$ g/100 g);
- 2) ED + P- $\alpha$ B (Pharmapark LLC) (2.5  $\mu$ g/100 g, 3 times a day for 7 days, the total dose was 7.5  $\mu$ g/100 g);
- 3) ED + Solvent (0.9% sodium chloride solution 0.1 ml/100 g, 3 times a day for 7 days, the total dose was 0.3 ml/100 g);
- 4) Intact + Solvent (0.9% sodium chloride solution 0.1 ml/100 g, 3 times a day for 7 days, the total dose was 0.3 ml/100 g).

Exactly 24 hours after the last administration of L-NAME to each animal under anesthesia (Zolazepam (Virbac (France)) 6 mg/100 g + Chloral hydrate (Panreac (Spain)) 15 mg/100 g), the left carotid artery was catheterized for intravascular blood pressure monitoring using Biopac MP150. Endothelium-dependent (Acetylcholine, 4  $\mu$ g/100 g) and endothelium-independent (Sodium nitroprusside, 3  $\mu$ g/100 g) kinds of vasodilation were stimulated against the background of continuous blood pressure monitoring. Vasoactive agents were injected at the intervals of 15 minutes through a catheter installed in the femoral vein. During all manipulations, the animal was assigned a unique code and the surgeon did not know the animal's belonging to the group. The ratio of the area above the pressure drop curve during the administration of sodium nitroprusside to the area above the pressure drop curve during the administration of acetylcholine, was taken as the endothelial dysfunction coefficient (EDC). After the functional vascular tests had been performed, the animals were euthanized by exsanguination and the abdominal part of the aorta was taken for histological studies, as well as for the evaluation of *eNos*, *Il-1b* and *Tnf* mRNA genes expression.

### Histology

The samples of the abdominal aorta were fixed in

a 10% formaldehyde solution and then embedded into paraffin wax in a carousel-type machine "STP 120 (Microm International GmbH, Germany). Embedding of blocks with a standard orientation of pieces was carried out at the station for embedding biological material into paraffin wax EC 350 (Microm International GMBH, Germany). To ensure standardization, wax embedding was carried out in the form of multiblocks of 5–6 pieces each.

The sections 5 microns thick for histological examination, were made on a semi-automatic rotary microtome with the system of transportation and distribution of sections "HM 340 E" (Microm International GmbH, Germany).

Hematoxylin and eosin staining was carried out in a machine for staining histological sections and smears (Microm International GmbH, Germany).

Hematoxylin and eosin staining was carried out in a machine for staining histological sections and smears (Microm International GmbH, Germany). A descriptive study of histological preparations was performed under the microscope Axio Scope A1 (Carl Zeiss Microimaging GmbH, Germany).

#### Determination of eNos, Tnf and Il-1 $\beta$ expression by quantitative polymerase chain reaction (qPCR)

The aortic tissue was taken out, homogenized and incubated in an "Extract RNA" solution for 10 minutes at 37°C. After the sample lysing in the reagent, it was chloroform cleaned, and the resulting RNA precipitate was washed with isopropyl alcohol and 70% ethyl alcohol. The concentration of the RNA was measured on an IMPLENNanoPhotometer®. The RNA yield was approximately 1000 ng/ $\mu$ l. A reverse transcription was performed using MMLVRTSK021 kit in accordance with the protocol of the manufacturer (Evrogen). The mixture was carefully mixed and heated for 2 minutes at 70°C to melt down the secondary RNA structures and the subsequent annealing of the OligoDT primer. Then the samples were transferred to ice. The entire reaction mixture was incubated for 60 min. at 40 °C in the T100™ ThermalCycler (Bio-Rad). To stop the reaction, the mixture was heated at 70°C for 10 minutes. The obtained cDNA was diluted to the concentration of 1 ng/ $\mu$ l. The gene expression level was estimated relative to the Gapdh reference gene values. The calculation of the expression at a specific point was carried out by the formula: Gene expression =  $2^{-(Ct(Gapdh)-Ct(Gene\ of\ interest))}$  (Table 1).

#### Prothrombotic Activity Study

40 rats, divided into five equal groups:

- 1) EPO (2.5  $\mu$ g/100 g, 3 times a day for 7 days, the total dose was 7.5  $\mu$ g/100 g);
- 2) EPO (5  $\mu$ g/100 g, 3 times a day for 7 days, the total dose was 15  $\mu$ g/100 g);
- 3) P- $\alpha$ B (2.5  $\mu$ g/100 g, 3 times a day for 7 days, the total dose was 7.5  $\mu$ g/100 g);

4) P- $\alpha$ B (5  $\mu$ g/100 g, 3 times a day for 7 days, the total dose was 15  $\mu$ g/100 g);

5) 0.9% sodium chloride solution (0.1 ml/100 g, 3 times a day for 7 days, the total dose was 0.3 ml/100 g).

24 hours after the last administration of the drug (or a solvent for the control group), the animals were anesthetized and fixed to the operating table. Then, a 10 mm incision was made to the left of the neck middle line, the common carotid artery was isolated and carefully separated from the surrounding tissues without damaging the vagus nerve. Using the ultrasound Minimax-Doppler (St. Petersburg, Russia), the best point for the signal was determined on the selected artery.

After that, a cotton wool moistened with a 50% ferric (III) chloride solution was applied, and the time was had been fixed until the initial signal was reduced to  $\approx$ 10%. In all manipulations, the animal was assigned a unique code and the surgeon did not know the animal's belonging to the group.

#### Statistical analysis

Statistical processing was performed using the R programming language. In the statistical sample, the data distribution type was determined using the Shapiro-Wilk test and the Spiegelhalter test ('normtest' package), the evaluation of the variance equality was determined using the Levene's test ('lawstat' package). Depending on the data distribution type and the variance equality, the significance of the obtained results was assessed using a parametric (ANOVA) or nonparametric (Kruskal–Wallis H test) one-way analysis of variance. The unpaired Student's t-test or Wilcoxon–Mann–Whitney test, respectively, were used as post-hoc analyses to identify the differences in the intergroup comparisons, with Benjamini–Hochberg procedure to decrease the false discovery rate. The results were considered statistically significant at  $p \leq 0.05$ .

## RESULTS

#### Estimation of endothelial dysfunction

An 8-day administration of L-NAME (2.5 mg/100 g) resulted in a significant increase in blood pressure. At the same time, EPO and P- $\alpha$ B therapy had no statistically significant effect on systolic and diastolic blood pressure values (Table 2).

At the same time, the functional tests with acetylcholine and sodium nitroprusside revealed a significant effect of the therapy on the endothelium NO-producing function (Fig. 1A). The group treated with L-NAME and 0.9% sodium chloride solution, demonstrated almost 5-time increasing in EDC ( $5.1 \pm 0.15$  c.u. in contrast to  $1.21 \pm 0.09$  in intact animals). In the group treated with EPO and P- $\alpha$ B, this indicator was  $3.81 \pm 0.14$  and  $2.72 \pm 0.12$ , respectively.



Table 1 – Primers for determination of mRNA expression of target and reference genes

Primer name	Nucleotide sequence 5'->3'	Melting point (°C)	PCR product size (nucleotide pairs)
Il-1b F	TCGTGCTGTCTGACCCATGT	61.47	126
Il-1b R	AGGCCACAGGGATTTTGTCG	60.61	
eNos F	GCCAACTCAAGGCAGGAGAC	60.96	129
eNos R	ATCCCCGGAAGGGTGCAATA	60.99	
Tnf-alpha F	TGAACTTCGGGGTGATCGGT	61.19	152
Tnf-alpha R	CGCTTGGTGGTTTGCTACGA	61.2	
Gapdh F	AGTGCCAGCCTCGTCTCATA	60.68	141
Gapdh R	TGAGGTCAATGAAGGGGTCGT	61.11	

Table 2 – Influence of EPO and 11-amino acid peptide P-αB on rat blood pressure in modeling L-NAME-induced endothelial dysfunction

Group	SBP (mm Hg)	DBP (mm Hg)
Intact	121.5±3.4	97.5±3.1
ED + NaCl (0.9%)	189.9±4.8	133.2±4.1
ED + EPO	186.0±5.1	133.2±4.5
ED + P-αB	190.3±4.3	134.3±3.9

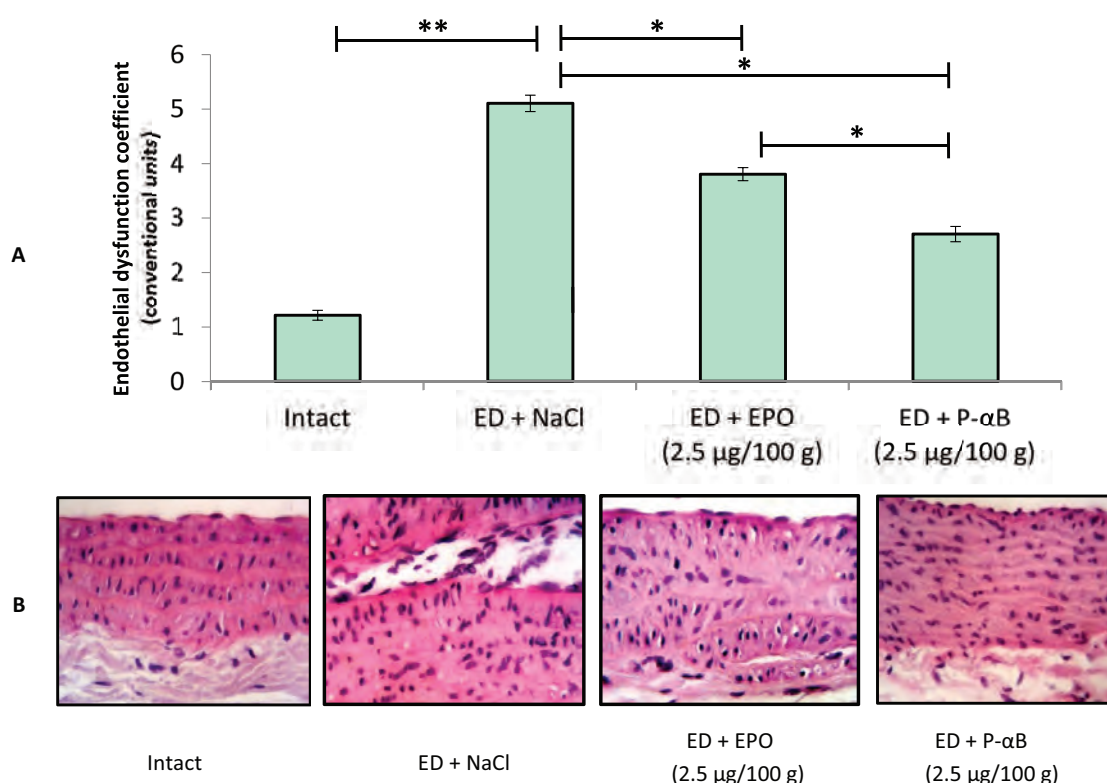


Figure 1 – Morphofunctional state of the vascular wall

Note: A) Influence of EPO and P-αB on the endothelial dysfunction coefficient calculated as the ratio of the area above the pressure drop curve during endothelium-independent vasodilation to the area above the pressure drop curve in endothelium-dependent vasodilation; B) Histological picture of the abdominal aorta wall. Intact – Endothelial lining is continuous, endothelial cells are flat. There are no signs of swelling or infiltration. Architectonics is not broken, the ratio of layers is preserved. ED + 0.9% sodium chloride solution – There is swelling of the outer shell, round cell infiltration of the middle shell and vacuolar dystrophy of smooth myocytes. The cell density is high. The ratio of layers is changed in comparison with that of intact animals, endotheliocytes are swollen, most of them are exfoliated from the surface of the basal membrane. ED + EPO – Against the background of disturbances of the architectonics of the middle shell and the initial signs of fibrosis, polymorphocytic infiltration of the outer shell of the vessel is observed; ED + P-αB – In the preparation, a complete safety of the architectonics of the vessel wall layers is visualized. The endothelial lining is kept safe, and the endothelial cells are located on the basal membrane in one layer. There are no signs of pericellular and perivascular edema (stained with hematoxylin and eosin. × 400). \* –  $p \leq 0,05$ ; \*\* –  $p \leq 0,01$ .

### Histological picture of the aortic wall

In the histological study, a similar trend characterizing the endothelial-protective activity of EPO and P- $\alpha$ B, was determined (Fig. 1B).

– L-NAME + 0.9% sodium chloride solution. In the group of the animals with L-NAME-induced ED significant morphological changes in comparison with intact animals were revealed. They consisted in the presence of perivascular and pericellular edema signs, inflammation of all the membranes of the vessel wall (aorta). Single diapedemic haemorrhages were observed in the perivascular tissue, and there were mural microthrombi in the vascular lumen. In the area of endothelial lining, there was swelling of endothelial cells and their exfoliating from the surface of the basal membrane. The nuclei were located along the wall of the blood vessel in small areas with the preserved endothelium. In single endothelial cells, karyolysis, vacuolization of cytoplasm and wrinkling of the cells were observed up to their death. There was a round cell infiltration of the middle and outer shells. Vacuolar dystrophy was observed in the middle shell, in smooth myocytes. In the outer shell, there was deflaking and signs of swelling. The density of the cells in both middle and outer shells was high.

– L-NAME+EPO (2.5  $\mu$ g/100 g). In this group, polymorphocytic infiltration of the outer shell of the vessel was observed against the background of minor disturbances in the architectonics of elastic membranes, the presence of functionally active fibroblasts (cells with dark-basophilic cytoplasm, large sized, visualized in the center of the middle shell), which may indicate the initial signs of fibrosis. A small number of reactively altered smooth myocytes located between the elastic fenestrated membranes, were visualized. At the same time, the majority of endotheliocytes had a flattened form and were located continuously, their nuclei were oriented parallel to the basal membrane.

– L-NAME+ P- $\alpha$ B (2.5  $\mu$ g/100 g). In the study of histological slices in the group of the animals after the pharmacological correction by P- $\alpha$ B, almost a complete safety of the architectonics of the vessel wall layers was revealed. The ratio of the layer thicknesses did not differ visually from that in the intact group of the animals. The endothelial lining was kept safe, the endothelial cells were placed on the basal membrane in one layer, the cells were shaped flat, the cytoplasm was slightly oxyphilic. The stick-shaped nuclei were oriented along the blood vessel. No signs of pericellular or perivascular edema were visualized.

A slightly increased cell density per unit of the section area was revealed, but it was without any signs of destruction, observed in the animals without pharmacological therapy.

### Expression of eNos, Tnf and Il-1 $\beta$ according to quantitative PCR

When modeling L-NAME-induced ED in comparison with the intact animals, an increase in the relative expression of the eNos mRNA gene was revealed in all groups. At the

same time, the eNos expression grows in the following series: ED + 0.9% sodium chloride solution < ED + EPO < ED + P- $\alpha$ B. The level of proinflammatory cytokines mRNA genes *Tnf* and *Il-1 $\beta$*  is characterized by the highest increase in the group of ED without therapy, and the use of EPO and P- $\alpha$ B reduces the degree of their expression (Fig. 2).

### Estimation of prothrombotic activity

When estimating the time of the onset of ferric (III) chloride-induced thrombosis in the animals treated with EPO and P- $\alpha$ B for 8 days, a dose-dependent reduction of the thrombosis time was found out. A more significant prothrombotic effect was demonstrated by EPO, which reduced the thrombosis time to 16.7 $\pm$ 1.2 min. (2.5  $\mu$ g/100 g) and 14.2 $\pm$ 1.3 min. (5  $\mu$ g/100 g) compared to 19.5 $\pm$ 0.9 min. in the group not treated with any preparations. P- $\alpha$ B at the dose of 1.25  $\mu$ g/100 g did not significantly affect the thrombosis time, and at the dose of 2.5  $\mu$ g/100 g, it accelerated the carotid artery thrombosis time up to 16.2 $\pm$ 1.1 min. (Fig. 3).

### DISCUSSION

Our work has shown that the 11-aminoacid peptide P- $\alpha$ B, which has a selective affinity to the heterodimeric receptor EPOR/CD131, is able to reduce the endothelial damage in the L-NAME-induced nitrogen oxide deficiency. The eNOS blockade led to persistent hypertension, which provoked morphological changes in the vessel wall. It was manifested in its hypertrophy, necrosis and architectonics disorders.

With the use of a molecular-biological analysis, an increase in the mRNA expression of the eNos gene, which is probably a compensatory response to hypertension, has been found. At the same time, the level of the proinflammatory cytokine markers – TNF- $\alpha$  and IL-1 $\beta$  – has increased. The triple application of P- $\alpha$ B at the dose of 2.5  $\mu$ g/100 g did not affect arterial hypertension associated with the introduction of L-NAME, but led to a more pronounced increase in the expression of the eNos mRNA gene. This may be explained by the fact that P- $\alpha$ B has increased the number of functioning cells capable of synthesizing eNOS due to its antiapoptotic properties. It is noteworthy that in the EPO group, despite the decrease in EDC, the eNOS mRNA level did not significantly increase with respect to the control. This is consistent with the data obtained by Sultan F. et al. showing that EPO is capable of suppressing the expression of eNOS [19]. Apparently, this property qualitatively distinguishes the vasotropic activity of P- $\alpha$ B from other erythropoietin preparations.

The results obtained also show that P- $\alpha$ B is able to prevent an inflammatory activation in the L-NAME-induced ED model. This phenomenon is most likely associated with both the antiapoptotic activity of the compound and the intrinsic anti-inflammatory activity of erythropoietin molecules. This property is very important for the potential atheroprotector, as a decrease in the cytokine activation is necessary to stabilize the atherosclerotic plaque and prevent its rupture.

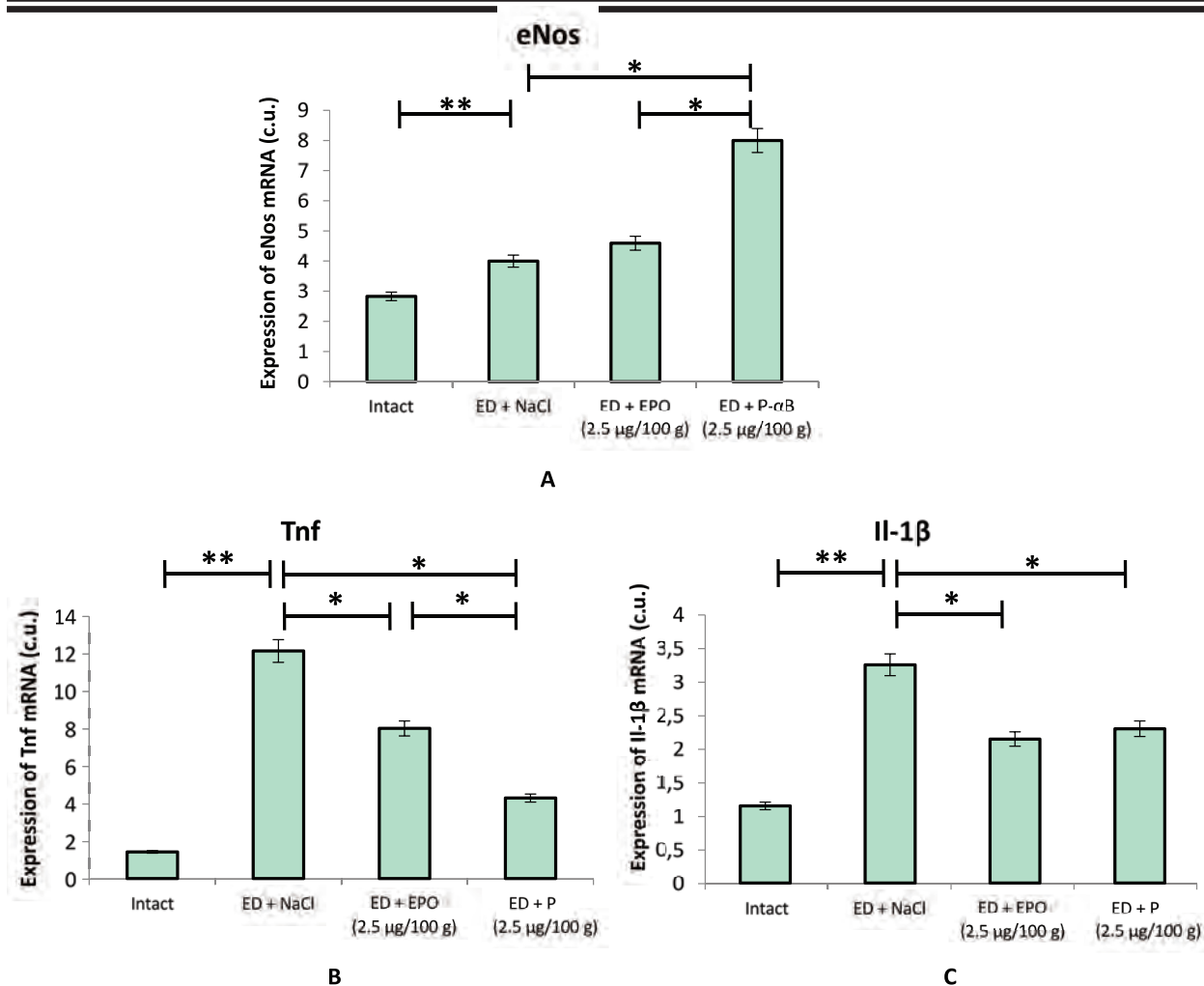


Figure 2 – Influence of EPO and P-αB on the expression of eNos, Tnf, and Il-1β mRNA genes in the abdominal aorta against the background of L-NAME-induced ED modeling (based on quantitative PCR data)

Note: \* –  $p \leq 0,05$ ; \*\* –  $p \leq 0,01$ .

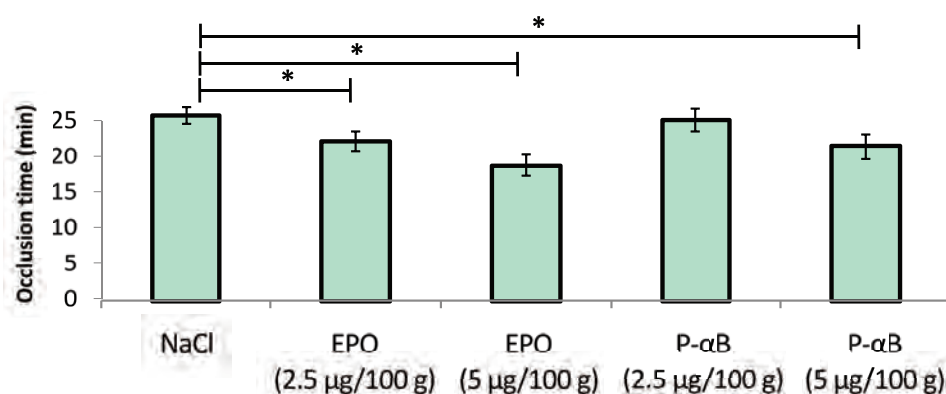


Figure 3 – Influence of EPO and P-αB on the time of  $\text{FeCl}_3$ -induced thrombotic occlusion of the carotid artery (reduction of Doppler signal to the level of  $\approx 10\%$  from the initial one)

Note: \* –  $p \leq 0,05$ .

Finally, the last stage of the study with the use of FeCl<sub>3</sub>-induced carotid artery thrombosis in the rats has revealed that P-αB has prothrombotic properties. The prothrombotic activity of P-αB is less pronounced than that of EPO and has not appeared in the doses at which it demonstrates an endothelial-protective action (2.5 μg/100g × 3 for 7 days). However, this property is a significant limitation in the positioning of P-αB as a preventing and treating agent for cardiovascular diseases associated with atherosclerosis.

We see a perspective in the modification of P-αB by adding peptide motifs with an antiaggregant activity. To eliminate a prothrombotic activity, the amino acid sequences Arg-Gly-Asp and Lys-Gly-Asp, can be added to P-αB. Arg-Gly-Asp and Lys-Gly-Asp are known to have pronounced antiaggregant properties [20–22]. In a number of domestic studies, antithrombotic and antiplatelet properties of another auxiliary amino acid tripeptide, Pro-Gly-Pro, have also been revealed [23–25]. In addition, Pro-Gly-Pro stabilizes the molecule in the biological environment by inhibiting the activity of proteolytic enzymes [26] and has the ability to block

angiotensin-converting enzyme [27], one of the most important proatherogenic factors that catalyzes the reaction of angiotensin II formation and promotes vascular wall remodeling [28].

The character (position, linkers, etc.) for the amino acid sequences in the base molecule still remains the key problem. The bioinformatics analysis will make it possible to determine the most optimal localizations for the tripeptide addition, allowing not to influence the interesting pharmacophores, preserving both endothelial-protective and antiplatelet kinds of activity.

### CONCLUSION

The 11-amino acid peptide imitating erythropoietin α-helix B, has a pronounced endothelial-protective and potentially atheroprotective effect due to its ability to prevent the death of endothelial cells, as well as to reduce remodeling and pro-inflammatory activation of the vascular wall. However, the prothrombotic activity of P-αB limits its use as a preventing and treating agent for atherosclerosis-associated diseases and necessitates further modifications of this molecule.

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### AUTHORS' CONTRIBUTION

**M.V. Korokin** – writing the article, the development of the research design, a sample preparation for the histological study, a morphological description of aortic wall sections; **V.O. Soldatov** – writing the article, developing a research design; **Alesia A. Tietze** – peptide synthesis, literature analysis; **I.V. Golubev** – the administration of drugs to animals, modeling a L-NAME-induced endothelial dysfunction; **A.E. Belykh** – mRNA isolation, reverse transcription reaction, analysis of mRNA expression of eNos, Tnf and Il-1β genes, translation of the article. **M.V. Kubekina** – mRNA isolation, reverse transcription reaction, analysis of mRNA expression of eNos, Tnf, and Il-1β genes, formalization of the literature list, work with graphic material; **O.A. Puchenkova** – work with the animals in all stages, separation of RNA, sampling for histological study. **T.A. Denisyuk** – participation in the evaluation of the endothelial dysfunction coefficient; **V.V. Gureyev** – estimation of the endothelial dysfunction coefficient; **T.G. Pokrovskaya** – consultation on planning, methodology and implementation of the experiment; **O.S. Gudyrev** – prothrombotic activity assessment, literature analysis; **M.A. Zhuchenko** – peptide synthesis, literature analysis; **M.A. Zatolokina** – preparation of samples for histological examination, morphological description of aortic wall sections; **M.V. Pokrovskiy** – the idea, research planning, consultation on the implementation of the individual phases of the pilot works.

### CONFLICT OF INTERESTS

The authors declare no conflict of interest.

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