



## STUDY OF THE PHARMACOLOGICAL ACTIVITY OF NOVEL EPOR/CD131 HETERORECEPTOR AGONISTS IN MICE WITH ENDOTHELIAL-SPECIFIC EXPRESSION OF MUTANT POLG GENE

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**The aim** of the research was to study antiatherosclerotic and endothelial kinds of a protective activity of peptides mimicking an erythropoietin a-helix B tertiary structure with laboratory codes EP-11-1 (UEHLERLNSS), EP-11-2. (UEQLERLNCS), EP-11-3 (UEQLERLNST).

**Materials and methods.** The study was conducted on 96 C57Bl/6J male double transgenic Polg<sup>mut/mut</sup>/Cdh5-CRE mice. Atherosclerosis was induced by a balloon injury accompanied by Western diet. Then, for 27 days, the drugs under study were administered once per 3 days at the dose of 20 µg/kg. On the 28<sup>th</sup> day, the animals were euthanized and the area of atherosclerotic plaques was collected for an assessment. The expression of genes associated with the processes of inflammation, apoptosis, and angiogenesis was determined in the tissues of the aorta. In addition, the endothelial protective effect of peptides in isolated segments of the thoracic aorta of wild and transgenic ransgenic Polg<sup>mut/mut</sup> mice was studied.

**Results.** The assessment of the plaque size in the animals with the Polg<sup>mut/mut</sup>/Cdh5-CRE genotype against the background of the peptides under study did not reveal statistically significant differences in comparison to control. However, a quantitative PCR showed a statistically significant decreased expression of pro-apoptotic factors p-53 and Bax, and also increase the expression of anti-apoptotic factor Bcl-2 against the background of the peptides EP-11-1 and EP-11-2 administration. The administration of EP-11-1 and the original peptide pHBSP resulted in a statistically significant decrease in the Bax/Bcl-2 ratio. Compounds EP-11-1, EP-11-2, and EP-11-3 were more effective than the original peptide pHBSP, in reducing the increased expression of genes for inflammatory markers iNos, intercellular adhesion molecules Icam-1, Vcam-1 and E-selectin. The use of EP-11-1 led to a more efficient, in comparison with pHBSP, restoration of endothelial-dependent vasodilation of the aortic segments in mice with endothelial-specific overexpression of the mutant Polg gene.

**Conclusion.** The study carried out on a murine model of the endothelial-specific expression of mutant gamma polymerase has shown that derivatives of the pHBSP peptide with laboratory codes EP-11-1, EP-11-2, EP-11-3, obtained by BLAST-searching for groups of pHBSP related peptides, have atheroprotective and endothelial protective kinds of a protective activity, which is more pronounced in comparison with the original peptide pHBSP.

**Keywords:** atherosclerosis; erythropoietin derivatives; pHBSP derivatives; atheroprotective effect; endothelial protective effect

**Abbreviations:** PCR – polymerase chain reaction; pHBSP – pyroglutamate helix B surface peptide; HBSP – helix B surface peptide; iNOS – inducible NO synthase; Polg – polymerase gamma; ICAM-1 – Inter-Cellular Adhesion Molecule 1; VCAM-1 – Vascular cell adhesion molecule-1; EPO – erythropoietin; EpoR – erythropoietin receptor; mRNA – matrix ribonucleic acid; HUVEC – Human Umbilical Vein Endothelial Cells; AKT1 – RAC-alpha serine/threonine-protein kinase ; eNOS / NOS3 – endothelial nitric oxide synthase; NO – nitrogen oxide; Ach – acetylcholine; PI3K – phosphoinositide 3-kinase; NP – sodium nitroprusside

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## ИЗУЧЕНИЕ ФАРМАКОЛОГИЧЕСКОЙ АКТИВНОСТИ НОВЫХ АГОНИСТОВ ГЕТЕРОРЕЦЕПТОРА EPOR/CD131 У ЖИВОТНЫХ С ЭНДОТЕЛИОСПЕЦИФИЧНОЙ ЭКСПРЕССИЕЙ МУТАНТНОГО ГЕНА POLG

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**Цель.** Изучение антиатеросклеротической и эндотелиопротективной активности пептидных производных, имитирующих α-спираль В эритропоэтина под лабораторными шифрами EP-11-1 (UEHLERALNSS), EP-11-2, (UEQLERALNCS), EP-11-3 (UEQLERALNTS).

**Материалы и методы.** Исследование проведено на 96 самцах мышей с генотипом Polg<sup>mut/mut</sup>/Cdh5-CRE на фоне C57Bl/6J. Атеросклероз моделировали путем баллонного повреждения сосудистой стенки у животных, находящихся на западной диете. Затем в течение 27 дней вводили изучаемые соединения 1 раз в 3 дня в дозе 20 мкг/кг. На 28-й день животных эвтаназировали и оценивали площадь атеросклеротических бляшек. Также в тканях аорты определяли экспрессию генов, связанных с процессами воспаления, апоптоза и ангиогенеза. Кроме того, было изучено эндотелиопротективное действие пептидов на изолированных кольцах грудной аорты диких и трансгенных мышей Polg<sup>mut/mut</sup>.

**Результаты.** При оценке размера бляшки у животных с генотипом Polg<sup>mut/mut</sup>/Cdh5-CRE на фоне применения изучаемых пептидов мы не обнаружили статистически значимого изменения размера бляшки. При проведении количественной полимеразной цепной реакции показано, что пептиды EP-11-1 и EP-11-2 статистически значимо в сравнении с контрольной группой животных, снижают экспрессию проапоптотических факторов p53 и Bax, а также увеличивают экспрессию антиапоптотического фактора Bcl-2. Введение соединения EP-11-1 и исходного пептида rHBSP привело к статистически значимому снижению соотношения Bax/Bcl-2). Соединения EP-11-1, EP-11-2 и EP-11-3 более эффективно, чем исходный пептид, rHBSP, снизили повышенную на фоне баллонной травмы экспрессию генов воспалительных маркеров iNOS, молекул межклеточной адгезии ICAM-1, VCAM-1 и E-селектина. Применение EP-11-1 привело к более эффективному, в сравнении с rHBSP, восстановлению эндотелийзависимой вазодилатации кольца аорты мышей с эндотелий специфической гиперэкспрессией мутантного гена Polg.

**Заключение.** В исследовании, проведенном на мышинной модели эндотелиоспецифичной экспрессии мутантной полимеразы гамма, нами показано, что производные пептида HBSP с лабораторными шифрами EP-11-1, EP-11-2, EP-11-3, полученные путем поиска групп родственных пептидов к молекуле rHBSP с помощью программы BLAST, обладают атеропротективной и эндотелиопротективной активностью, более выраженной в сравнении с исходным пептидом rHBSP.

**Ключевые слова:** атеросклероз; производные эритропоэтина; производные rHBSP, атеропротективное действие; эндотелиопротективное действие

**Список сокращений:** ПЦР – полимеразная цепная реакция; rHBSP – pyroglutamate helix B surface peptide / пироглутаматный поверхностный пептид спирали В; HBSP – helix B surface peptide / поверхностный пептид спирали В; iNOS – индубельная NO-синтаза; Polg – polymerase gamma / полимеразы гамма; ICAM-1 – Inter-Cellular Adhesion Molecule 1 / межклеточная молекула адгезии – 1; VCAM-1 – Vascular cell adhesion molecule 1 / молекула адгезии клеточного эндотелия 1; ЭПО – эритропоэтин; Epor – рецептор эритропоэтина; мРНК – матричная рибонуклеиновая кислота; HUVEC – Isolation of Human Umbilical Vein Endothelial Cells / культура эндотелиальных клеток из пупочной вены человека; AKT1 – RAC-alpha serine/threonine-protein kinase / RAC-альфа-серин/треонин-протеинкиназа; eNOS/NOS3 – эндотелиальная синтаза оксида азота; NO – оксид азота; AX – ацетилхолин; PI3K – phosphoinositide 3-kinase / Фосфоинозитид-3-киназа; НП – нитропруссид натрия

## INTRODUCTION

Erythropoietin (EPO) is one of the hormones produced by the kidneys. It was originally identified as a critical regulator of the hematopoiesis. Recombinant erythropoietin is widely used in the treatment of anemia associated with a chronic kidney disease, heart failure, and cancer [1]. In the human body, EPO stimulates the production of about 200 billion red blood cells daily. After being produced in the kidneys, EPO is secreted into the bloodstream and targets erythroid progenitor cells in the bone marrow [2, 3]. EPO acts by binding to its specific receptor on the surface of erythrocyte progenitor cells. The knockout of Epo (Epo<sup>-/-</sup>) or the EPO receptor (EpoR<sup>-/-</sup>) in mice leads to the embryo death caused by the development of severe anemia [4, 5].

Over the past decade, many non-hematopoietic effects of erythropoietin including its antiatherosclerotic action, have been identified [6, 7]. When non-hematopoietic effects are realized, locally produced hypoglycosylated erythropoietin acts in a paracrine-autocrine pathway and transmits signals mediated by the interaction with the tissue protective heterodimeric erythropoietin receptor EPOR/CD131 [8, 9]. The availability of recombinant EPO influenced the appearance of works devoted to the study of the non-hematopoietic activity of EPO, including its protective effect on endotheliocytes and neurons. Reports on the presence of EPO receptors (EpoR), expression of EpoR mRNA and/or EpoR protein besides the erythropoietic organs suggest the possibility of a non-hematopoietic receptor effect of EPO [3]. As reported, human umbilical vein endothelial cells became the first non-hematopoietic cells to express EpoR, bind erythropoietin and show a proliferative response to the EPO administration *in vitro* [8, 10]. It was found out that EPO protects rat brain microvascular endothelial cell cultures from the anoxia-induced damage by activating AKT1, maintaining mitochondrial membrane potential, and preventing oxidative stress-induced apoptosis [12]. An important function of endothelial cells is the expression of endothelial nitric oxide synthase (eNOS/NOS3), catalyzing the synthesis of nitric oxide (NO), the main regulator of vascular homeostasis. Using a cell culture of endotheliocytes, it was found out that a combination of a reduced oxygen content and EPO pretreatment in the cell culture increases the expression of mRNA and EpoR protein, increases the expression of eNOS, and thereby stimulates the production of NO [13]. In experimental models in transgenic mice with high hematocrit, it was shown that arterial hypertension does not develop due to a significant increased level of eNOS and NO in the vascular tissue and in the bloodstream [14].

Currently, it is clear that the thrombosis-related side effects of recombinant EPO prevent its clinical use in non-anemic patients [15]. To prevent thrombotic complications associated with the EPO therapy, EPO derivatives lacking a hematopoietic activity but having a tissue-protective effect, have been obtained. The 11-amino acid peptide imitating the tertiary structure of

the erythropoietin B chain (HBSP) is one of EPO derivatives that exhibits a non-hematopoietic activity comparable to recombinant erythropoietin [16–19].

To search for new compounds with atheroprotective and endothelial protective effects, the HBSP amino acid sequence was changed by searching for groups of related peptides of the original compound, using the BLAST program. As a result, 3 compounds that mimic the a-helix B of erythropoietin, were obtained: EP-11-1 (UEHLER-ALNSS), EP-11-2, (UEQLERALNCS), EP-11-3 (UEQLERAL-NTS).

**THE AIM** of this research was to study the antiatherosclerotic and endothelial kinds of a protective activity of peptides EP-11-1, EP-11-2, EP-11-3.

## MATERIALS AND METHODS

### Animals and diet

The study comprised 96 C57Bl/6J double transgenic Polg<sup>mut/mut</sup>/Cdh5-CRE male mice obtained from the Institute of Gene Biology, Russian Academy of Sciences. The requirements of the Law of the Russian Federation “On the Protection of Animals from Cruelty” dated June 24, 1998, the rules of laboratory practice during preclinical studies in the Russian Federation (GOST 3 51000.3-96 and GOST R 53434-2009), European Community directives (86/609 EU), the rules of the International Recommendations of the European Convention for the Protection of Vertebrate Animals used in experimental research (1997) and the Rules of laboratory practice adopted in the Russian Federation (order of the Ministry of Health of the Russian Federation No.708 dated 29.08.2010) were followed. The experiments were approved by the local ethics committee of Belgorod State National Research University, Belgorod, protocol No.19/23. The Polg<sup>mut/mut</sup>/Cdh5-CRE genotype is associated with the endothelial-specific expression of the mutant Polg gene encoding the polymerase gamma enzyme with the D257A mutation, leading to the absence of 3'exonuclease activity and the accumulation of mutations during the mitochondrial genome replication. The expression of the mutant protein leads to the development of a mitochondrial dysfunction with the formation of disturbances in the processes occurring in the vascular endothelium. 2 weeks before the start of the experiment, the animals were placed on a Western 2% cholesterol diet [20].

### Modeling of balloon damage to vascular wall

The surgical procedure was performed on a heated platform under a preparative microscope. Under anesthesia (zolazepam 2.5 mg/100 g (Virbac, France) + xylazine 2 mg/100 g (Biogel, Russia) intraperitoneally), a common femoral artery was isolated through an incision in the medial femoral region, a balloon injury of endothelium was applied as described before. To alleviate the postoperative pain syndrome, within 3 days after the operation, the animals received metamizole sodium with drinking water *ad libitum* at the concentration of 50 mg

metamizole sodium (Pharmstandard-Ufavita, Russia) per 100 ml of water [20–22].

### Study design and drugs administration

The list of the studied peptides, their laboratory codes and amino acid sequences are presented in Table 1.

Animals with genotype Polg<sup>mut/mut</sup>/Cdh5-CRE were divided into 6 equal groups:

- 1) Intact;
- 2) Control – the animals with modeling of balloon damage on a western diet;
- 3) pHBSP – the animals with modeling of pathology, administrated with pHBSP peptide (subcutaneously, s/c) from the 1<sup>st</sup> day at the dose of 20 µg/kg once per 3 days for 28 days (the total dose – 180 µg/kg);
- 4) EP-11-1 – the animals with modeling of pathology, administrated with EP-11-1 peptide (subcutaneously, s/c) from the 1<sup>st</sup> day at the dose of 20 µg/kg once per 3 days for 28 days (the total dose – 180 µg/kg);
- 5) EP-11-2 – the animals with modeling of pathology, administrated with EP-11-2 peptide (subcutaneously, s/c) from the 1<sup>st</sup> day at the dose of 20 µg/kg once per 3 days for 28 days (the total dose – 180 µg/kg);
- 6) EP-11-3 – the animals with modeling of pathology, administrated with EP-11-3 peptide (subcutaneously, s/c) from the 1<sup>st</sup> day at the dose of 20 µg/kg once per 3 days for 28 days (the total dose – 180 µg/kg).

The dose and route of administration of the studied peptides were selected according to previous experimental data obtained in the study of the pharmacological activity of several compounds based on pHBSP, with added tripeptide motifs RGD, KGD, and PGP to the original peptide [20].

### Measurement of atherosclerotic plaque area

A macroscopic examination of atherosclerotic aortic plaques was performed using the material from 4 animals from each group. For this purpose, on the 28<sup>th</sup> day after modeling the balloon injury, the animals were euthanized by an overdose of anesthesia (zoletil 10 mg/100 g intraperitoneally) and the abdominal aorta was thoroughly removed from the bifurcation to the area at the level of the diaphragm.

Then the preparations were cut longitudinally, straightened on a foam pad, washed with a 50% ethanol solution, and immersed in the Oil Red O solution for 15 minutes. After that, the preparations were washed with distilled water and digital photographs were taken. Using the imageJ program, the ratio of the atherosclerotic plaque area (stained in red) to intact tissue was calculated [20].

### Quantitative PCR

After euthanasia, the aortic tissue in the area of balloon injury was sampled from the rest of the animals, homogenized, and incubated for 10 minutes at 37°C in the Extract RNA solution. After lysis of the sample in the reagent, it was subjected to chloroform purification; the supernatant sample was collected and washed with iso-

propyl alcohol and 70% ethanol. The concentration of the obtained RNA, was measured on an IMPLENNano-Photometer<sup>®</sup> spectrophotometer and adjusted to the concentration of 300 ng/µl. A reverse transcription was performed using the MMLVRTSK021 kit in accordance with the manufacturer's protocol (Evrogen, Russia). The study was carried out in accordance with the previously described methodology [20]. The list of the primers used in quantitative PCR, is presented in Table 2.

### Study of effect of aortic ring preparations on vascular endothelium

For the experiment, the following experimental groups were formed (n=8 animals per group):

- 1) Wildtype mice;
- 2) Polg<sup>mut/mut</sup>/Cdh5-CRE mice;
- 3) Polg<sup>mut/mut</sup>/Cdh5-CRE mice treated with pHBSP 20 µg/kg;
- 4) Polg<sup>mut/mut</sup>/Cdh5-CRE mice treated with EP-11-1 20 µg/kg;
- 5) Polg<sup>mut/mut</sup>/Cdh5-CRE mice treated with EP-11-2 20 µg/kg;
- 6) Polg<sup>mut/mut</sup>/Cdh5-CRE mice treated with EP-11-3 20 µg/kg.

The compounds under study – innovative peptides with laboratory codes EP-11-1, EP-11-2, EP-11-3 – were administered intraperitoneally at the indicated doses for 7 days. On the 8<sup>th</sup> day from the beginning of the experiment, the experimental animals were anesthetized using the intraperitoneal injection of chloral hydrate at the dose of 300 mg/kg. Further on, in anesthetized mice, the thorax was opened to remove the thoracic aorta. The thoracic aorta was placed in a modified ice-cold Krebs Henseleit solution (118 mM NaCl, 4.7 mM KCl, 1.2 mM Na<sub>2</sub>PO<sub>4</sub>, 0.5 mM MgCl<sub>2</sub>, 1.12 mM CaCl<sub>2</sub>, 25 mM NaHCO<sub>3</sub>, 0.03 mM EDTA, 11 mM glucose) with pH 7.4. The aorta was carefully removed from the surrounding adipose and connective tissue and cut into short 2 mm transverse segments. The aortic rings were suspended in an organ bath (Biopac Bas System Station, Biopac systems, USA) containing 10 ml of a K-H solution maintained at 37° C, and 95% O<sub>2</sub> and 5% CO<sub>2</sub> were bubbled between two parallel stainless steel hooks. The isometric tension during the experiments was measured and recorded using the Biopac Systems USA software and hardware complex. The data acquisition and processing were performed using the Biopac Icq 4.2 software. Each aortic segment had been gradually stretched to an initial tension of 0.8 g and allowed to equilibrate in a standard 10 ml organ bath for 60 minutes prior to the experiment. After the scales equilibration, the segments were first contracted with 60 mM KCl to induce their contractile response and achieve a reproducible maximum contractile response, then they were washed with Krebs-Henseleit solution three times to restore the tension to the basal level. The response of the aortic segments contraction to the submaximal concentration of phenylephrine (1 µmol/L) was induced 30 min after the restoration of the basal

level. On the plateau of the epinephrine-induced contraction, the tests for endothelium-dependent and endothelium-independent vasodilation were performed. Acetylcholine ( $10^{-9}$ - $10^{-5}$  M) was cumulatively added to the aortic bath as an agent causing endothelium-dependent vasodilation, and sodium nitroprusside ( $10^{-9}$ - $10^{-5}$  M) was added as an agent causing endothelium-independent vasodilation. Sensitivity was defined as a relaxation percentage of the baseline value obtained at the epinephrine administration plateau.

### Statistical processing

Statistical processing was performed using the Statistics 10.0 software. The obtained data were checked for the normality of distribution using the Shapiro-Wilk test and the Spiegelhalter test (the normtest library), the assessment of the equality of variances – using the Leuven test (the lawstat library). Depending on the type of the feature distribution and the equality of variances, the significance of the results obtained was assessed using a parametric (ANOVA) or nonparametric (the Kruskal-Wallis test) one-way analysis of variance. The unpaired Student's t-test was used as a post-hoc analysis to identify differences in intergroup comparisons, or the Mann-Whitney test, respectively, with the Benjamini-Hochberg correction for a multiple hypothesis testing. The results were considered significant at  $p \leq 0.05$ .

## RESULTS

### Macroscopic evaluation of plaque size

In accordance with the experiment design, a macroscopic assessment of the balloon injury-induced plaque in wildtype (intact group) and Polg<sup>mut/mut</sup>/Cdh5-CRE animals (the control group) was carried out. It was found out that in the control group of Polg<sup>mut/mut</sup>/Cdh5-CRE animals, lipid deposits serving a marker of atherosclerosis,

were visualized in all preparations stained with Oil Red O. That resulted in an increase in the size of the plaque in the control group by more than 11 times. Against the background of the test peptides, when processing the data obtained in the assessment of the plaque size in the animals with the Polg<sup>mut/mut</sup>/Cdh5-CRE genotype, no statistically significant change in the plaque size was found out (Fig. 1).

### Quantitative PCR

In addition to the macroscopic plaque assessment, a molecular biological analysis of atherosclerotic plaque tissue after the balloon-induced injury was performed in all experimental groups. Fig. 2 shows that against the background of balloon injury modeling, in the animals with the Polg<sup>mut/mut</sup>/Cdh5-CRE genotype, the expression of the markers of programmed cell death p53 and Bax is to a significant degree increased and the expression of the antiapoptotic marker Bcl-2 is decreased. As the heat map presented in Figure 2A shows, peptides EP-11-1 and EP-11-2 statistically significantly compared with the control group of animals ( $p < 0.05$ ), reduce the expression of pro-apoptotic factors p-53 and Bax, as well as increase the expression of anti-apoptotic factor Bcl-2 ( $p < 0.05$ ). The most effective in terms of changing the expression of apoptosis factors was EP-11-1 – the expression values of the p53, Bax and Bcl-2 genes did not differ from those in the control group (Fig. 2A).

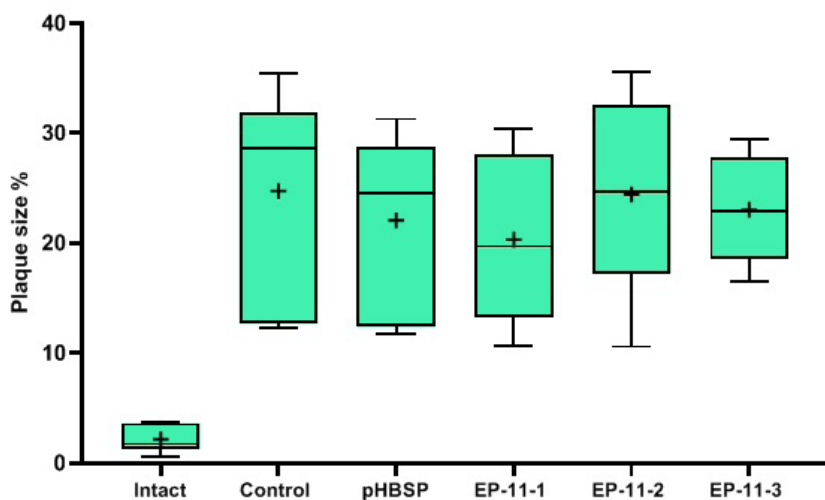
Fig. 2B shows the Bax/Bcl-2 ratio characterizing the integral pro-apoptotic orientation of the cell; the higher it is the more pronounced the activation of programmed cell death cascades. The figure shows that in the animals with the genotype Polg<sup>mut/mut</sup>/Cdh5-CRE, the Bax/Bcl-2 ratio is significantly increased, and the introduction of a compound with the laboratory code EP-11-1 and the initial peptide pHBSP statistically significantly reduce the Bax/Bcl-2 ratio (Fig. 2B).

**Table 1 – Amino acid sequence of test compounds**

Laboratory code	Amino acid sequence
pHBSP	QEQLERALNSS
EP-11-1	UEHLERALNSS
EP-11-2	UEQLERALNCS
EP-11-3	UEQLERALNTS

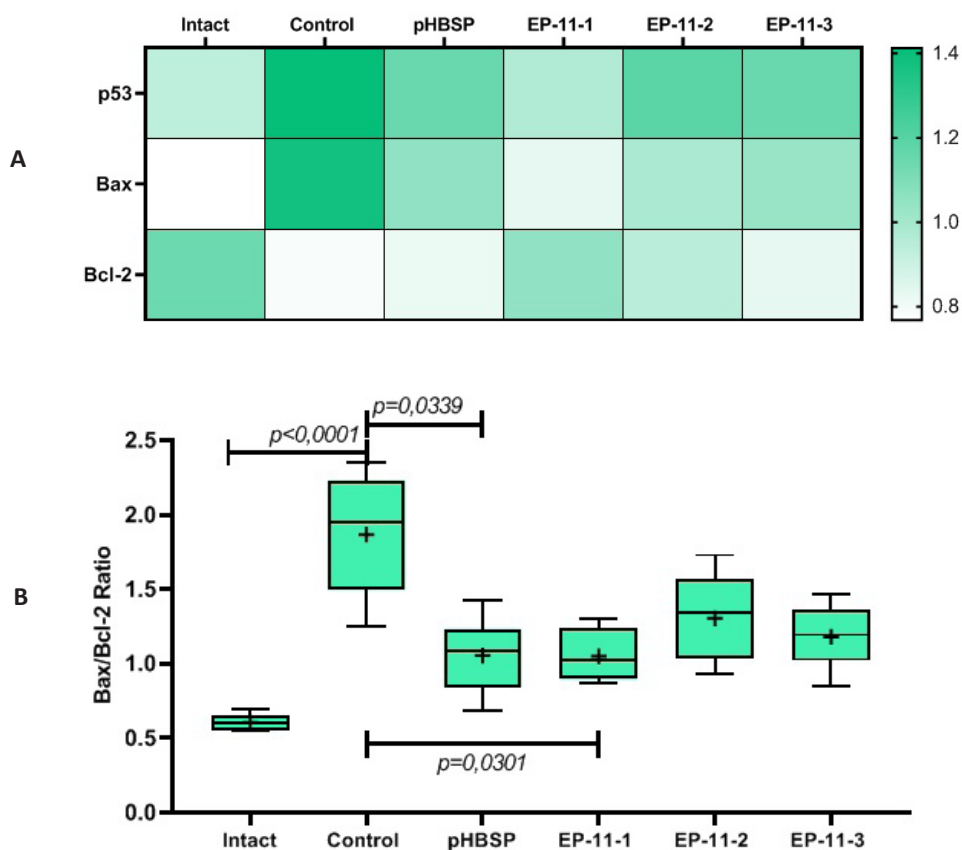
**Table 2 – Primers used for quantitative PCR**

Gene	F-primer	R-primer	Product length	GenBank
Trp53 (p53)	CGACTACAGTTAGGGGGCAC	CCATGGCAGTCATCCAGTCT	95	NM_001127233.1
Bcl2	TCACCCCTGGTGGACAACAT	TTCCACAAAGGCATCCCAGC	102	NM_009741.5
Bax	CCCGAGCTGATCAGAACCAT	GAGGCCTTCCCAGCCAC	96	NM_007527.3
Vegfa (VEGF-A)	GGGCCTCCGAAACCATGAA	TGCAGCCTGGGACCACTTG	95	NM_001025250.3
Flt-1 (VEGFR-1)	CCCATCGGCAGACCAATACA	CGGTGCACTTGAGGACAAGA	96	NM_001363135.1
Hif-1a	AGAACAACCTTGAGCTGGCGT	TGGAGGTGAAGTCTGTCTGT	103	NM_001092957.1
Nos2 (iNOS)	GCTCTAGTGAAGCAAAGCCCA	GGGATTCTGGAACATTCTGTGC	103	NM_001313921.1
Icam-1	CTCCGGACTTTCGATCTTCCA	CCTTCCAGGGAGCAAAACAAC	98	NM_010493.3
Vcam-1	TACTGTTTGCACTCTCAAGC	CGTAGTGCTGCAAGTGAGGG	101	NM_011693.3
Sele (E-selectin)	GGGAAGAAGACTGTCTAGCC	AGGGGAGCTGGCTTCTAAG	96	XM_006496715.3
Gapdh	GGGTCCAGCTTAGGTTTCATC	CCCAATACGGCCAATCCGT	100	NM_001289726.1



**Figure 1 – Atherosclerotic plaque size in groups of wildtype and  $Polg^{mut/mut}/Cdh5-CRE$  animals against the background of studied peptides use**

Note: the “+” sign on the graph shows the arithmetic mean for each experimental group



**Figure 2 – Influence of test drugs on relative expression of apoptosis markers (2A) and Bax/Bcl-2 ratio (2B)**

Note: the + sign on the graph shows the arithmetic mean for each experimental group

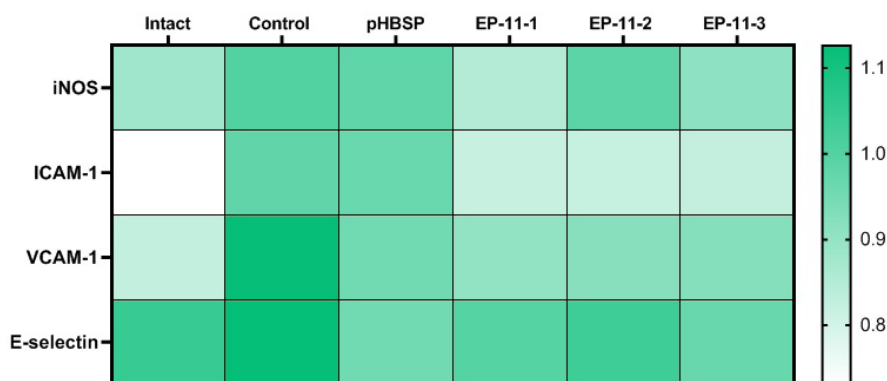


Figure 3 – Influence of test-compounds on inflammatory markers relative expression

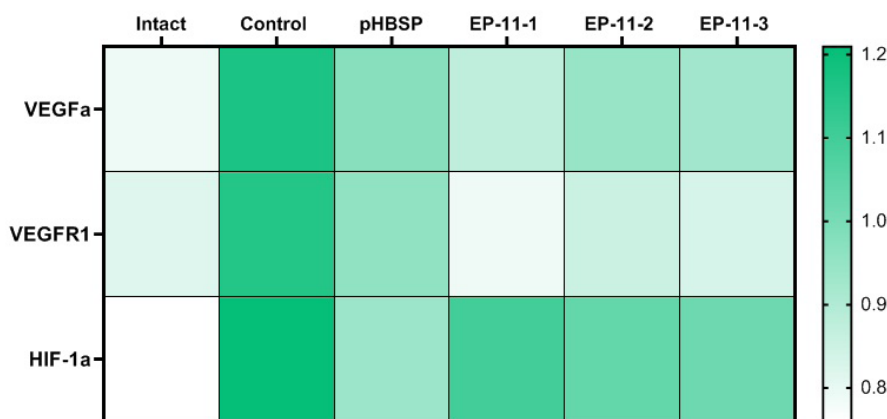


Figure 4 – Influence of test compounds on relative expression of related to angiogenesis factors

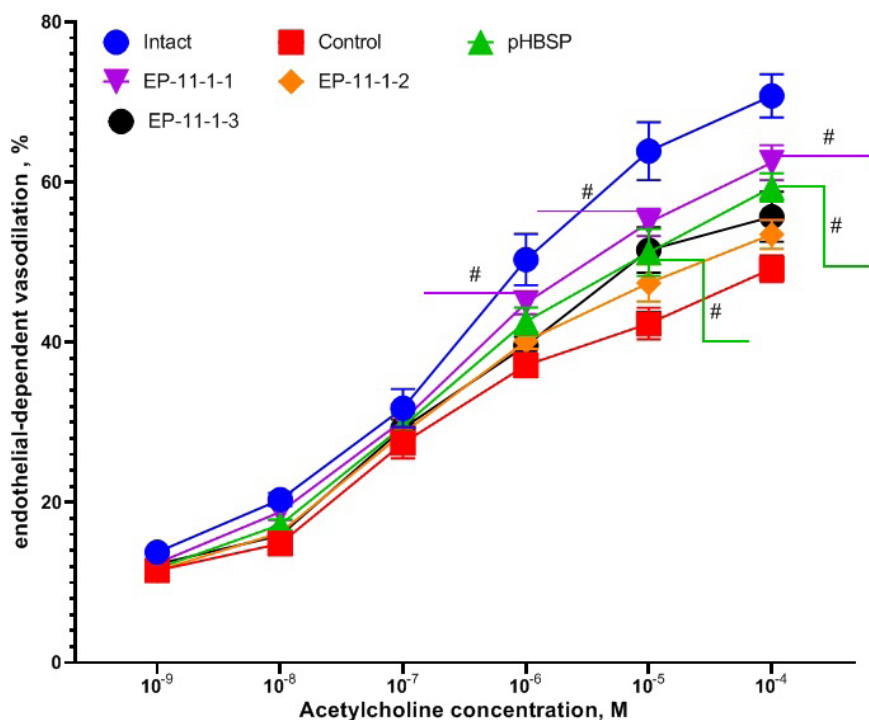
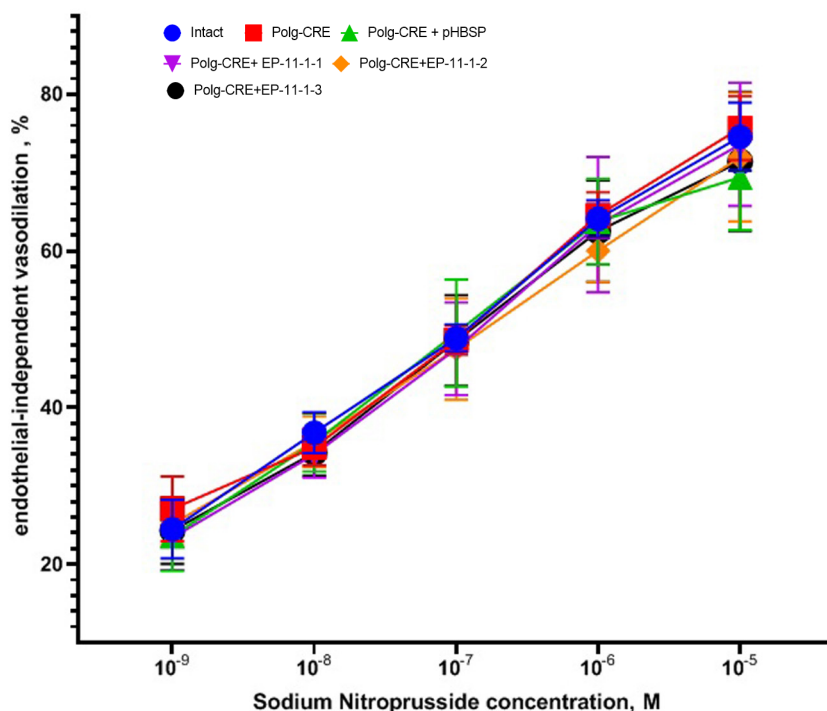


Figure 5 – Results of a test with endothelial-dependent vasodilation in response to ACh on the isolated segments of thoracic aortas of the Polg<sup>mut/mut</sup>/Cdh5-CRE mice  
 Note: # – at p < 0.05 in comparison with the untreated group



**Figure 6 – Results of endothelial-independent vasodilation test in response to SN on isolated segments of thoracic aortas of Polg<sup>mut/mut</sup>/Cdh5-CRE mice**

The study of the expression of the inflammatory markers group showed a significantly increased expression of iNOS, Icam-1, Vcam-1, and E-selectin in the control group of the animals with the Polg<sup>mut/mut</sup>/Cdh5-CRE genotype (Fig. 3). The test compounds with laboratory codes EP-11-1, EP-11-2, and EP-11-3, reduced the increased expression of the inflammatory genes iNOS, Icam-1, Vcam-1 and E-selectin more effectively than the initial peptide pHBSP. The most pronounced effect was obtained in the group with the use of EP-11-1 (Fig. 3).

In addition, the test compounds with laboratory codes EP-11-1, EP-11-2, EP-11-3 were more effective in reducing the expression of the factors associated with angiogenesis than the original peptide pHBSP (Fig. 4). As in the previous series, EP-11-1 was the most effective in this test (Fig. 4).

#### Assessment of vascular endothelium functioning on preparations of isolated aortic rings

The effect of the compounds on the vascular endothelium functioning, was carried out on isolated segments of the thoracic aorta of Polg<sup>mut/mut</sup>/Cdh5-CRE mice, kept on western diet. The endothelial function was assessed by performing endothelial-dependent vasodilation in response to ACh. As Fig. 5 shows, acetylcholine-induced endothelial-dependent vasodilation was significantly reduced in Polg<sup>mut/mut</sup>/Cdh5-CRE mice. Notably, the impairment of endothelial-dependent vasodilation caused by ACh in Polg<sup>mut/mut</sup>/Cdh5-CRE mice,

was restored after the peptide EP-11-1 administration. The degree of the aortic segments relaxation upon administration of ACh at the concentrations of 10<sup>-6</sup>M, 10<sup>-5</sup>M and 10<sup>-4</sup>M in this group, was significantly higher in comparison to the control group (Fig. 5). The administration of the original peptide pHBSP also led to an increase in vascular relaxation in response to the administration of ACh at the concentrations of 10<sup>-5</sup>M and 10<sup>-4</sup>M. The administration of peptides EP-11-2 and EP-11-3 did not significantly affect the response degree of the vascular segment to ACh (Fig. 5).

At the same time, the assessment of endothelium-independent vasodilation in response to SN did not reveal statistically significant differences between the experimental groups (Fig. 6).

#### DISCUSSION

The biological effects of recombinant EPO are wide and multifaceted, and much attention of researchers was attracted by its non-hematopoietic effects. In particular, the authors were interested in atheroprotective and endothelial protective effects. In vivo studies have shown that EPO reduces the manifestations of an ischemia/reperfusion damage to cardiomyocytes, which is partially explained by an increase in NO production and an acute response to an increase in hematocrit. The same study reported that the EPO-mediated eNOS activation is associated with PI3K signaling, while the EPO-associated reduction in cardiomyocyte ischemia



is not observed in the mice with the eNos  $-/-$  genotype [23]. It is assumed that the erythropoietin-induced NO production by endotheliocytes is mediated primarily by the induction and activation of eNOS, especially at a reduced oxygen content [24, 25]. In addition, the mice with ectopic transgenic EPO expression showed an increased eNOS activity and increased plasma NO levels, which prevent cardiovascular diseases such as hypertension and thrombosis, while the inhibition of NO synthase leads to cardiovascular diseases and deaths [3].

In terms of the known limitation of the recombinant EPO use in erythropoietic doses, the problem of finding new derivatives of tissue protective EPO with lack of hematopoietic properties, is relevant in modern medicine and pharmacology. The peptides that are agonists of the EPOR/CD131 heteroreceptor, trigger EPO-associated cytoprotective cascades, but do not have an erythropoietic effect. The previous studies have demonstrated that a peptide imitating the spatial structure of the erythropoietin B chain pHBSP, has a pronounced endothelial protective effect in modeling L-NAME-induced endothelial dysfunction in rats [26, 27]. However, in this study, a prothrombotic effect of pHBSP has also been shown. In view of the above, the need for further modifications of this molecule is obvious. In our opinion, the pHBSP modification to improve its pharmacokinetic and pharmacodynamic parameters may become a further promising development of pharmacotherapy for cardiovascular diseases based on short-chain peptides [28].

The search for such compounds can be solved in several ways, including the attachment of amino acid motifs with anticoagulant properties to the amino acid sequence or by searching for groups of related peptides of the original compound using the BLAST program. At the first stage of the study, an attempt to enrich the original pHBSP molecule by adding tripeptide motifs RGD, KGD, and PGP, having antiaggregant effect, was made. As a result, fundamentally new compounds that combine cytoprotective [29] and antiplatelet effects, were obtained [30]. It has also been shown that the EPO-based peptides are able to improve the functional state of the vascular wall against the background of atherosclerotic lesions and can ameliorate the pathobiological processes associated with a mitochondrial dysfunction. In addition, the studied peptides have a pronounced endothelial protective effect against the background of *in vitro* modeling of oxidative stress [20].

In this research, the pharmacological activity of 3 peptides that mimic the spatial structure of the EPO  $\alpha$ -helix (EP-11-1 (UEHLERALNSS), EP-11-2. (UEQLERALNCS), EP-11-3 (UEQLERALNTS)), obtained by searching for groups of related peptides to the pHBSP molecule using the BLAST program, has been studied.

For the study, a line of animals with an endothelial-specific expression of the mutant Polg gene, was selected. Polymerase gamma is an enzyme that plays a key role in mitochondrial DNA replication. The pathology of

this enzyme leads to the inclusion of “wrong” nucleotides without a subsequent correction, which causes a mitochondrial dysfunction with a subsequent increase in the production of active radicals and a cell damage. Homozygous animals with the systemic Polg mutation do not survive; therefore, in this work, the endothelial specific expression of an inducible transgene was used [20].

In the presented model, atherosclerosis is associated with a traumatic effect on the vessel against the background of damage to endothelial cells due to the mitochondrial dysfunction. The study of an atheroprotective activity showed that the studied peptides, as well as the original peptide pHBSP, did not significantly ameliorate the histological structure and size of the atherosclerotic plaque in the pathology model. The maximum reduction in the size of the atherosclerotic plaque, was established in the group of the animals treated with EP-11-1, which, however, was not statistically significant. Perhaps, in the further studies the effect of drugs on the histological structure and size of atherosclerotic plaques should be assessed in a model of atherogenesis that is not associated with a physical damage to the endothelium.

Using a molecular biological analysis of plaque samples, it was found out that the studied peptides EP-11-1 and EP-11-2 significantly reduced the expression of the pro-apoptotic factors p-53 and Bax, and also increased the expression of the anti-apoptotic factor Bcl-2. When calculating the ratio of Bax to Bcl-2 expression, it was found out that in the animals with the Polg<sup>mut/mut</sup>/Cdh5-CRE genotype, the Bax/Bcl-2 ratio statistically significantly increased by more than 3 times. In addition, the introduction of a compound with a laboratory code EP-11-1 and the original peptide pHBSP statistically significantly reduced the Bax/Bcl-2 ratio by 57.2 and 56.4%, respectively. These findings are consistent with other studies showing that the administration of EPO for 10 weeks considerably decreases the Bax/Bcl-2 protein ratio in the aortic tissue of apolipoprotein E deficient mice fed a high-fat diet [31]. Along with the anti-apoptotic effect, the studied compounds with laboratory codes EP-11-1, EP-11-2, and EP-11-3 were more effective than the original peptide, pHBSP, in terms of the decrease of the iNos, Icam-1, Vcam-1 and E-selectin expression. At the same time, the maximum efficiency was found in the group of the animals that received the peptide with the laboratory code EP-11-1. The anti-inflammatory effect of EPO and its derivatives is widely known and has been studied [32], and this study confirmed the retention of this type of activity in derivatives that mimic the erythropoietin B chain.

The study of the peptides pharmacological effect on isolated segments of the pulmonary aorta in Polg<sup>mut/mut</sup>/Cdh5-CRE mice showed that the endothelium-dependent vasodilation induced by Ach, was significantly reduced (70.78% at the acetylcholine concentration  $10^{-4}$  M) compared to intact wild-type mice (49.2% at the

acetylcholine concentration  $10^{-4}$  M). The use of peptide EP-11-1 led to the restoration of endothelium-dependent vasodilation induced by ACh at concentrations  $10^{-6}$ M,  $10^{-5}$ M и  $10^{-4}$ M in mice with endothelial specific overexpression of the mutant gene Polg. The administration of the original pHBSP peptide also led to an increase in the vascular relaxation in response to the administration of ACh at concentrations  $10^{-5}$ M и  $10^{-4}$ M. The introduction of peptides with laboratory codes of another leader compound EP-11-2 and EP-11-3, did not statistically significantly affect the degree of response of the vascular segment to ACh. Noteworthy, no changes in the response of endothelium to independent vasodilation in any of the experimental groups were found. This fact confirms that in this study, the tissue protective effect of the peptides is in the normalization of the function of the vascular endothelium which determines the pronounced endothelial protective activity of these compounds.

### CONCLUSION

At the first stage of the search for new EPO derivatives with tissue protective properties without manifest-

ing a hematopoietic activity, the original pHBSP peptide was enriched by adding tripeptide motifs RGD, KGD, and PGP. The resulting compounds combined cytoprotective and antiplatelet effects, had an endothelial protective activity, and were able to attenuate atherosclerotic lesions. In the present study, the second pool of compounds – derivatives of the pHBSP peptide with laboratory codes EP-11-1 (UEHLERALNSS), EP-11-2. (UEQLERALNCS), EP-11-3 (UEQLERALNNTS), obtained by BLAST-searching for groups of related to pHBSP peptides, was tested. In the study carried out on a mouse model of the endothelium-specific expression of the mutant Polg gene, it has been shown that the most active compound with laboratory code EP-11-1 has a more pronounced atheroprotective and endothelial protective activity than the original peptide pHBSP.

The results of this study, in combination with the previously obtained data characterizing the pharmacological activity of pHBSP derivatives containing RGD, KGD, and PGP, prove the effectiveness of this approach and reveal the prospects for further search for new EPO-derived nonhematopoietic peptides with tissue protective properties

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### CONFLICT OF INTEREST

The authors declare no conflict of interest.

### AUTHORS' CONTRIBUTION

Mikhail V. Korokin – main idea, study planning, study conducting, statistical proceeding, article writing; Marina V. Kubekina – preparation of experimental animals, extraction of RNA, reverse transcription, analysis of the expression of targeted genes; Alexey V. Deykin – RNA extraction, reverse transcription, analysis of the expression of targeted genes; Oleg V. Antsiferov – observation, care and handling of animals, drugs administration, research of pharmacological activity; Vladimir M. Pokrovskii – observation, care and handling of animals, drugs administration, research of pharmacological activity; Liliya V. Korokina – statistical processing, development of research design; Pobeda A.S. – statistical processing, article writing, references formalization; Valeria A. Soldatova – observation, care and handling of animals, drugs administration, research of pharmacological activity; Natalia L. Kartashkina – reverse transcription, analysis of the expression of targeted genes; Elena V. Kuzubova – observation, care and handling of animals, drugs administration, research of pharmacological activity; Alexandra I. Radchenko – observation, care and handling of animals, drugs administration, research of pharmacological activity; Mikhail V. Pokrovskii – research planning, management of experimental work, quality assurance.

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