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Research Article

Preclinical study of innovative peptides mimicking the tertiary structure of the α-helix B of erythropoietin

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Abstract

Introduction: The aim of this study was to examine the effectiveness of innovative peptides obtained by addition of polypeptide motifs with antiaggregation activity (Arg-Gly-Asp, Lys-Gly-Asp and Pro-Gly-Pro) to a peptide mimicking the tertiary structure of the α-helix B of erythropoietin pHBSP (Pyr-Glu-Gln-Leu-Glu-Arg-Ala-Leu-Asn-Ser-Ser).

Materials and methods: The cytoprotective activity of innovative peptides mimicking the tertiary structure of the α-helix B of erythropoietin at the doses of 5 μg/ml, 30 μg/ml and 50 μg/ml was studied on human endothelial cell culture in a simulated oxidative stress. An ADMA-like model of preeclampsia was simulated in the experiment. The study was conducted in 260 female Wistar rats, weighing 250–300 g.

Results and discussion: Innovative peptides mimicking the tertiary structure of the α-helix B of erythropoietin retain their cytoprotective activity in a simulated oxidative stress in HUVEC cell culture at the doses of 5 μg/ml, 30 μg/ml, and 50 μg/ml. The compounds with laboratory codes P-αB1 and P-αB3 had the most pronounced cytoprotective activity. Administration of N-nitro-L-arginine-methyl ether to pregnant females for 7 days causes the morphofunctional changes similar to clinical changes in preeclampsia. The innovative peptide under laboratory code P-αB4 at the dose of 50 μg/kg mimicking the tertiary structure of the α-helix B of erythropoietin shows the most pronounced protective properties.

Conclusion: Innovative peptides mimicking the tertiary structure of the α-helix B of erythropoietin have a pronounced positive influence on the morphofunctional disorders in animals with ADMA-like preeclampsia.

Keywords

erythropoietin derivative, preeclampsia, endothelial dysfunction, microcirculation, placenta.

Introduction

Preeclampsia is a complex multi-factorial disease and is traditionally characterized by a combined manifestation of edema, hypertension, and proteinuria. According to various authors, it affects from 2% to 10% of all pregnancies (Un Nisa et al. 2019; Messerli et al. 2019; Tomimatsu et al. 2019). In 0.03–0.055%, it develops into eclampsia

(Olaoye et al. 2019). After bleeding, it is the next leading cause in the structure of maternal and child morbidity and mortality in developed and developing countries (Ponmozhi et al. 2019, Lee et al. 2019), and especially in low-income countries (Olaoye et al. 2019). In the Russian Federation, arterial hypertension complicates 5–30% of the total number of pregnancies. The incidence of arterial hypertension, edema and proteinuria during pregnancy in the Russian Federation is approximately 17% of the total number of pregnancies; preeclampsia and eclampsia are diagnosed in about 2% of all pregnancies.

Despite a large number of studies on the prevention and treatment of preeclampsia, the results of a drug treatment are still unsatisfactory. Currently, delivery is the only radical treatment for preeclampsia. Other methods of treatment are aimed at the correction the mother's condition, which in turn contributes to improvement of the fetal condition. They include prevention of the convulsive syndrome, hypotensive therapy, correction of disorders of the electrolyte balance and hemostasis system. Other areas of the therapy are not yet yielding the expected result.

Many authors consider placental ischemia, endothelial dysfunction, and platelet aggregation disorders as the main pathogenetic links in the pathogenesis of preeclampsia (Granger et al. 2018; Jakobsen et al. 2019; Seamon et al. 2020).

Based on the main features of preeclampsia, one of the promising directions for creating new drugs for the treatment and prevention of preeclampsia is the use of drugs with anti-ischemic and cytoprotective activities. Such a pharmacological agent is 11-amino acid peptide pHBSP (PubChem CID: 91810664), which is an amino acid chain QEQLERALNSS (Pyr-Glu-Gln-Leu-Glu-Arg-Ala-Leu-Asn-Ser-Ser), having a selective affinity for the heterodimer complex EPOR/CD131 (Hache et al. 2016). The short peptide chain allows its modification by adding the polypeptide motifs RGD (Arg-Gly-Asp), KGD (Lys-Gly-Asp) and PGP (Pro-Gly-Pro), which are known for their anti-aggregation properties (Pastorova et al. 2001; Lyapina et al. 2007; Liapina et al. 2010; Obergan et al. 2019; Kuo et al. 2019). The key issue remains the retention of protective properties of innovative peptides mimicking the tertiary structure of the α-helix B of erythropoietin after embedding RGD, KGD and PGP motifs into the base molecule. The presented provisions determined the aim of the research.

Aim of the study: to study innovative peptides that mimic the tertiary structure of the α-helix B of erythropoietin in experimental preeclampsia.

Materials and methods

Study of cytoprotective activity

A modification of the method described by Eric A. Jaffe was used to obtain primary cultures of endotheliocytes from the human umbilical cord vein. The vein cavity was first aseptically washed with DPBS (Thermo FS) until blood was completely removed, then filled with 0.2% collagenase solution in DPBS with the addition of 0.9 mM $CaCl_2$, 0.493 mM $MgCl_2$, 5.56 mM glucose, 0.327 mM sodium pyruvate, and subjected to enzymatic dissociation of the inner layer of the vein – intima. To do this, the umbilical cord was transferred to a glass with DPBS and incubated at 37°C for 20 minutes. A collagenase solution containing cells was collected in a 50 ml tube by perfusion of the vein cavity with 20 ml of medium 199 (Sigma-Aldrich). The obtained endothelial cells were cultured in DMEM medium with the addition of 20 mM of HEPES buffer, 5 U/ml of heparin, 200 mcg/ml of E CG F (Sigma-Aldrich), 10% embryonic veal serum (FBS) at 37 °C in a humid atmosphere containing 5% CO_2.

The cells were cultured in 96-well plates covered with gelatin, with a density of 5 thousand cells per well. After 24 hours of incubation, the studied peptides (P - αB, P - αB 1, P - αB 2, P - αB 3, P - αB 4, P - αB 5, P - αB 6) were introduced in 3 concentrations – 5 mcg/ml, 30 mcg/ml, and 50 mcg/ml. Three hours after peptides introduction, hydrogen peroxide (H_2O_2) was added to the wells of the plate at a final concentration of 200 mcM. After 24 hours, the culture medium containing H_2O_2 was replaced with a normal one.

Cell viability was measured using an MTT test. The principle of the method is based on the ability of succinate dehydrogenase, an enzyme of the mitochondrial membrane, to reduce the yellow salt 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT) to purple formazan crystals that accumulate as a result of this reaction in the cytoplasm of living cells. Thus, the level of mitochondrial respiration of the cell was judged by the intensity of accumulation of formazan crystals in the cytoplasm, which is an indicator of its viability. The amount of formazan formed in the cell monolayer is proportionally correlated with the number of living cells in the test sample.

At the end of the experiment, the medium was sampled, and the solution of 3-(4,5-dimethylthiazole-2-yl)-2,5-diphenyl-tetrazolium bromide (MTT, Sigma) in PBS (Sigma), containing 0.9 mM $CaCl_2$ and 0.5 mM $MgCl_2$, was added to the wells of the plate at the rate of 10 mg MTT per well. After a 4-hour incubation at 37 °C in a humid atmosphere with 5% CO_2, the plate was centrifuged at 600g using a centrifuge R4810 (Eppendorf, Germany). The supernatant was carefully aspirated.

DMSO, 250 µl/well, was used to dissolve the formed formazan crystals. After complete dissolution of the crystals, 150 µl, without affecting the cell layer on the gelatin, was transferred to a new plate. The optical density of the formazan solution in DMSO was measured using a spectrophotometer Multiscan EX at a wavelength of 570 nm. By changing the optical density, the cytoprotective activity of peptides was judged. The MTT test results were evaluated by comparing the optical density in the experimental and control wells. Survival in each group was evaluated in relation to intact cells as the ratio of optical densities (Zal et al. 2018).

In vivo experiments

The experimental study was performed in The Research Institute of Pharmacology of Living Systems of Belgorod State National Research University (BelSU). The study was performed in accordance with the requirements of GOST ISO/IEC 17025-2009, GOST R ISO 5725-2002 and "Good Laboratory Practices", approved by order No. 199n of the Ministry of Health of the Russian Federation dated 1 April 2016, in compliance with The European Convention for the Protection of Vertebral Animals Used for Experimental and Other Scientific Purposes (Directive 2010/63/EU). All the experiments were approved by the Bioethical Commission of The Research Institute of Pharmacology of Living Systems of BelSU. Vivisection was performed in accordance with the ethical principles of handling laboratory animals – The European Convention for the Protection of Vertebral Animals Used for Experimental and Other Scientific Purposes. CETS No. 170.

The experimental animals were held in individually ventilated cages Tecniplast for small laboratory animals. The flooring was sawdust sterilized by UV irradiation. Feed was granulated for small laboratory animals – rodents. Water was purified and sterilized by UV irradiation. The microclimate was created and maintained by a system of individually ventilated cages. Acclimatization and selection of animals for research were carried out after a quarantine for at least 10 days. Distribution by groups was according to animals' body weight. Identification of the animals was performed by writing an individual label on the body.

At the time of the study, the animals were healthy, with no changes in behavior, appetite, or sleep-wake schedule. For 18 hours before the experiments, the animals were completely deprived of food, with free access to water.

The study was performed in 260 white female Wistar rats, weighing 250–300 g. The animals with specific gestation periods were obtained by introducing males to females in a ratio of 2:3. Twenty-four hours later, the animals were separated, and on the 10th day, under sedation, pregnancy was determined by palpation. Simulation of ADMA-like preeclampsia was performed by intraperitoneal administration of a non-selective NOS blocker N-nitro-L-arginine-methyl ether (L-NAME) (25 mg/kg/day) from the 14th to the 20th day of pregnancy. On the 21st day, under anesthesia (chloral hydrate 300 mg/kg), hemodynamic parameters were registered, and endothelial function was studied using a Biopac (USA) hardware complex (Gureev et al. 2014; Gureev et al. 2015; Korokin et al. 2015).

The concentration of stable metabolites of nitric oxide (total NOx) was a biochemical marker of endothelial dysfunction. The level of NO metabolites (i.e. the total concentration of nitrates and nitrites, NOx) was determined by a colorimetric method based on the development of color in the diazotization reaction with sulfanilamide nitrite, which is part of the Griss reagent (Metel'skaia et al. 2005).

To obtain the data on the state of microcirculation in the placenta on the 21st day of pregnancy, the level of microcirculation was measured under anesthesia, at 4 points at a distance of 1 mm from the edge of the placental disk. To obtain the data on the state of microcirculation in the placenta, we used the equipment manufactured by Biopac systems: a MP100 polygraph with a LDF100C laser Doppler flowmetry module and a TSD144 invasive needle sensor, which was placed directly on the projection of the placental disc. Registration and processing of LDF results was performed using the AcqKnowledge program version 3.8.1.; the values of microcirculation were expressed in perfusion units (PU) (Gureev et al. 2015; Severinova et al. 2019; Korokin et al. 2020).

Urine collection was performed for 12 hours, using special metabolic cages. Determination of the amount of protein in daily urine was performed by the pyrogall method, using a PE-5400B spectrophotometer (Yalamati et al. 2015).

To study the liquid content in the greater omentum, it was weighed and then dried at 37 °C for 24 hours and reweighed (Ivanova et al. 2012; Gureev et al. 2015).

To conduct a morphometrical study, the material was fixed in 10% formalin with subsequent embedding in paraffin. Slices of the kidneys were made perpendicular to the main axis of the organ through the pelvis. Histological slices of the placenta were made in a strictly vertical direction through the middle of the placental disk, all the layers of the placenta and the wall of the uterine horn. The study of microsections, photo protocoling and morphometry was performed using a Leica DM4000B microscope, with a video recording and image processing system. In all the morphological studies, hematoxylin and eosin staining was used.

To assess the growth and weight indicators, the fetuses were extracted from the uterine cavity, weighed, and their growth was measured (craniocaudal size), followed by the calculation of the growth and weight coefficient (Mironov et al. 2012).

According to the aim, all the animals were divided into the following groups:

1. Intact group (animals with oral administration of NaCl in equivalent doses from the 14th to the 20th day of pregnancy).
2. Simulation of experimental preeclampsia (control) (L-NAME (25 mg/kg once per day intraperitoneally) from the 14th to the 20th day of pregnancy).
3. Simulation of experimental preeclampsia + methyldopa (2 × 0.043 g/kg/day intragastrically).
4. Simulation of experimental preeclampsia + P-αB (50 μg/kg).
5. Simulation of experimental preeclampsia + P-αB1 (50 μg/kg).
6. Simulation of experimental preeclampsia + P-αB2 (50 μg/kg).
7. Simulation of experimental preeclampsia + P-αB3 (50 μg/kg).

8. Simulation of experimental preeclampsia + P-αB4 (50 μg/kg).
9. Simulation of experimental preeclampsia + P-αB5 (50 μg/kg).
10. Simulation of experimental preeclampsia + P-αB6 (50 μg/kg).
11. Simulation of experimental preeclampsia + P-αB4 (10 μg/kg).
12. Simulation of experimental preeclampsia + P-αB4 (10 μg/kg) + methyldopa (2 × 0.043 g/kg/day intragastrically).
13. Simulation of experimental preeclampsia + P-αB4 (50 μg/kg) + methyldopa (2 × 0.043 g/kg/day intragastrically).

Table 1. Amino acid sequence of the studied peptides.

Laboratory code	Amino acid sequence
P-αB	(U/Q)EQLERALNSS (Pyr/Gln)-Glu-Gln-Leu-Glu-Arg-Ala-Leu-Asn-Ser-Ser) **RGD** (Arg-Gly-Asp) **KGD** (Lys-Gly-Asp) **PGP** (Pro-Gly-Pro)
P-αB1	**RGD**QEQLERALNSS
P-αB2	(U/Q)EQLERALNSS**RGD**
P-αB3	**KGD**QEQLERALNSS
P-αB4	(U/Q)EQLERALNSS**KGD**
P-αB5	**PGP**QEQLERALNSS
P-αB6	(U/Q)EQLERALNSS**PGP**

Statistical processing of research results

Statistical processing was performed using the computing software environment R. The pattern of feature distribution in the statistical sample was determined using the Shapiro-Wilk test and the Spiegelhalter test (normtest library), and the equality of variances was estimated using the Levene's test (lawstat library). Depending on the type of feature distribution and equality of variances, the significance of the results was evaluated, using parametric (ANOVA) or non-parametric (Kruskal-Wallis test) one-way analysis of variance, and as a post-hoc analysis to identify differences in intergroup comparisons, the Student's unpaired t-test or the Mann-Whitney test, respectively, with the Benjamini-Hochberg correction for multiple hypothesis testing. The results were considered significant at $p \leq 0.05$.

Results and discussion

Study of cytoprotective activity of peptides mimicking the tertiary structure of the α-helix B of erythropoietin

Introduction of the studied innovative peptides to the HUVEC cell culture, against the background of oxidative stress, resulted in a statistically significant increase in optical density. This indicates an increase in mitochondrial activity and cell survival in the cell culture. The increase in the level of cytoprotective activity with an increase in the concentration of innovative peptides was not statistically significant, and the compounds with laboratory codes PaB1 and PaB3 had the most pronounced cytoprotective activity (Fig. 1).

Study of the effect of peptides mimicking the α-helix B of erythropoietin on the development of morphofunctional disorders in ADMA-like preeclampsia

After L-NAME administration to pregnant rats, there was a significant increase ($p < 0.05$) in systolic and diastolic blood pressure from 128.8 ± 2.61 mm Hg and 82.2 ± 3.27 mm Hg to 202.8 ± 6.86 mm Hg and 138.40 ± 2.95 mm Hg, respectively. There was a disruption of the regulatory mechanisms of vascular tone, as evidenced by an increase in the CED from 1.25 ± 0.04 to 3.11 ± 0.20 ($p < 0.05$). In the placenta, microcirculation decreased from 541 ± 23.4 PU to 218 ± 6.4 PU ($p < 0.05$). In the blood plasma, there was a decrease in the concentration of terminal NO metabolites from 2.22 ± 0.04 mmol/DL to 1.36 ± 0.05 mmol/DL, which indicates a decrease in the NO-synthesizing function of the endothelium. Simulated ADMA-like preeclampsia caused an increase in proteinuria and swelling of the greater omentum tissues.

Histological examination of the placental microsections on the 21st day of gestation in the animals with ADMA-like preeclampsia revealed changes of ischemic genesis. The morphological pattern was accompanied by a statistically significant ($p < 0.05$) decrease in the thickness of the fetal part from 0.950 ± 0.128 microns to 0.645 ± 0.0091 microns and an increase in the thickness of the maternal placenta ($p < 0.05$) from 0.325 ± 0.005 to 0.532 ± 0.009 microns. In addition, there was a decrease in the density of the cell pool of both the maternal and fetal parts of the placenta and a decrease in the diameter of the villi. A study of the fetal development in the animals with ADMA-like preeclampsia revealed fetal hypotrophy.

Intraperitoneal administration of the peptide (P-αB) mimicking the α-helix B of erythropoietin (50 μg/kg) during the period from the 10th to 20th days of pregnancy led to a statistically significant ($p < 0.05$) decrease in systolic and diastolic blood pressure to 142.8 ± 1.98 and 90.40 ± 5.21 mm Hg, respectively ($p < 0.05$) compared to the group of "untreated" animals (Table 2). The coefficient of endothelial dysfunction decreased to 2.0 ± 0.06, and the microcirculation index increased to 343 ± 6.0 PU ($p < 0.05$).

The most pronounced hypotensive effect after administration of the innovative peptides mimicking the α-helix B of erythropoietin was observed in P-αB4 (Table 2). Systolic and diastolic blood pressure after its administration reached a level not statistically distinguishable from that in the group of intact animals. In addition, a marked decrease in blood pressure was observed under the influence of peptides: P-αB1 and P-αB3, but it did not reach the level of the intact animals. The least pronounced effect was observed for peptides P-αB2 and P-αB6.

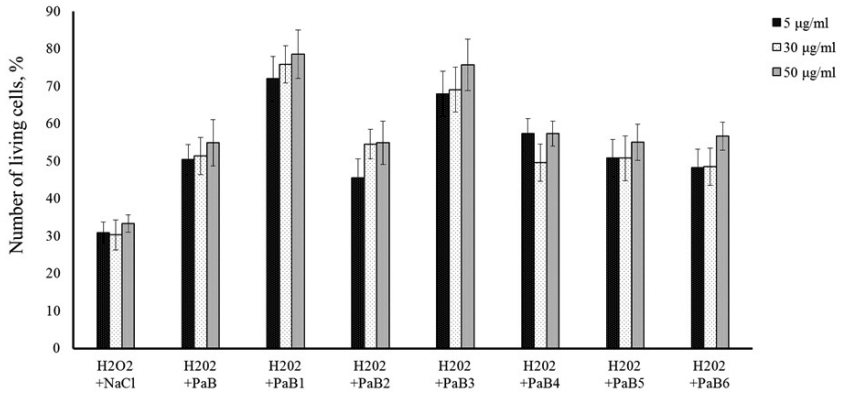

Figure 1. Effect of innovative peptides mimicking the α-helix B of erythropoietin on the viability of HUVEC cell culture in the simulated oxidative stress. **Note:** * – $p < 0.05$ compared to the control group.

Table 2. The effect of innovative peptides mimicking the α-helix B of erythropoietin on functional parameters in ADMA-like Preeclampsia (M ± m, n = 10).

Group	Indicator						
	SBP, mm Hg	DBP, mm Hg	CED, relative units.	Microcirculation, PU	Proteinuria, g/L	NOx, µmol/DL	Edema of the greater omentum, %
Intact	128.8±2.61^{y}	80.8±3.27^{y}	1.25±0.04^{y}	541±23.4^{y}	0.29±0.10^{y}	2.22±0.04^{y}	44.74±1.16^{y}
L-NAME	202.8±6.86*	138.4±2.95*	3.11±0.20*	218±6.4*	2.57±0.11*	1.36±0.05*	54.12±1.31*
P-αB (50 µg/kg)	142.8±1.98^{*y}	90.4±5.21*	2.0±0.06^{*y}	343±6.0^{*y}	1.88±0.18^{*y}	1.59±0.05^{*y}	49.07±1.16^{*y}
P-αB1 (50 µg/kg)	143.1±5.18*	100.6±3.80*	1.80±0.15^{*y}	378±9.45^{*y}	0.58±0.18^{y}	1.83±0.04^{*y}	48.67±0.74^{*y}
P-αB2 (50 µg/kg)	172.8±5.06^{*y}	135.2±3.54^{*y}	2.25±0.16^{*y}	351±10.04^{*y}	1.21±0.21^{*y}	1.60±0.08^{*y}	49.25±0.74^{*y}
P-αB3 (50 µg/kg)	144.8±4.49^{*y}	104.3±3.36^{*y}	1.98±0.19^{*y}	370±9.2^{*y}	0.66±0.16^{y}	1.71±0.07^{*y}	48.14±0.88^{*y}
P-αB4 (50 µg/kg)	138.3±5.44^{y}	94.0±6.59^{y}	1.80±0.22^{*y}	337±8.7^{*y}	0.51±0.14^{y}	1.80±0.06^{*y}	46.33±0.53^{*y}
P-αB5 (50 µg/kg)	168.7±5.68^{*y}	124.5±5.72^{*y}	2.19±0.16^{*y}	322±6.8^{*y}	2.00±0.12^{*y}	1.46±0.04*	49.64±0.70^{*y}
P-αB6 (50 µg/kg)	174.6±6.58^{*y}	129.7±5.75^{*y}	2.67±0.28^{*y}	303±6.4^{*y}	2.46±0.19*	1.50±0.05*	52.40±1.10^{*y}

Note: SBP and DBP – systolic and diastolic blood pressure; CED – coefficient of endothelial dysfunction; PU – perfusion units; NOx – terminal NO metabolites; * – $p < 0.05$ in comparison with the control group; ^{y} – $p < 0.05$ in comparison with the L-NAME group.

When administering the studied innovative peptides mimicking the α-helix B of erythropoietin, it was found that the greatest endothelial protective effect was observed in P-αB1, P-αB3, and P-αB4 (Table 2). The coefficient of endothelial dysfunction decreased to 1.80 ± 0.15, 1.98 ± 0.19, and 1.80 ± 0.22, respectively. Peptides P-αB2 and P-αB5 also decreased the CED, but much less. In the group of animals administered with P-αB6, the CED was lower than in the control group of animals, but there was no statistically significant difference.

The most pronounced improvement in the placental microcirculation occurred after P-αB1 and P-αB3 administration. Its values rose to 378 ± 9.45 PU and 370 ± 9.2 PU, respectively, but it did not reach the target level (Table 2). Administration of the other studied peptides also improved the placental microcirculation, but the effects were significantly less pronounced.

The study of the NO-synthesizing function of the endothelium was carried out on the basis of the determination of NOx nitrite ions in blood plasma. Simulated ADMA-like preeclampsia led to a decrease in the content of terminal NOx metabolites in blood plasma (Table 2). Intraperitoneal administration of the peptide (P-αB) mimicking the α-helix of B erythropoietin (50 µg/kg) from the 10th to 20th days of pregnancy resulted in a statistically significant ($p < 0.05$) increase in terminal NOx metabolites compared to the group of the "untreated" animals.

After the administration of the studied innovative peptides mimicking the α-helix B of erythropoietin at the dose of 50 µg/kg, the greatest increase in the concentration of nitrite ions (NOx) in blood plasma in the animals with ADMA-like preeclampsia ($p < 0.05$) occurred under the influence of P-αB1, P-αB3, and P-αB4 and amounted to 1.83 ± 0.04 mmol/DL, 1.71±0.07 mmol/DL and 1.80 ± 0.06 mmol/DL, respectively, but the target level (groups of intact animals (2.22 ± 0.04 mmol/DL)) were not achieved. When P-αB2 was administered, there was also an increase in the concentration of NOx terminal metabolites in blood plasma, but this effect was less pronounced.

The administration of the studied innovative peptides mimicking the α-helix B of erythropoietin in the animals with experimental preeclampsia led to a decrease in proteinuria. The most pronounced effect was observed in the groups administered with P-αB1, P-αB3, and P-αB4. The level of proteinuria did not differ statistically from that of the intact animals. There was no statistically significant decline in the group administered with P- αB6 in relation to the control group of animals. The values of proteinuria

in the groups administered with P-αB2 and P-αB5 were in between (Table 2).

The study of the content of fluid in the greater omentum tissues, which is a reflection of the edema severity, showed its increase in the group of animals with experimental preeclampsia (Table 2). The most pronounced positive effect is observed when administering P-αB4. The level of fluid content after its administration reduced to the level of no statistically significant difference compared to the group of intact animals. The administration of P-αB1, P-αB2, P-αB3 and P-αB5 also decreased the swelling of the greater omentum tissues, with a less pronounced effect. There was no statistically significant effect when P-αB6 was administered.

In the histological study of the placenta, the most pronounced positive effect was observed in the group of animals administered with P-αB4. This is evidenced by the results of morphometry. When correcting the cell pool in the maternal and fetal parts of the placenta, the most pronounced effect is observed for P-αB4. But the target level is not reached. The same pronounced effect is observed with the administration of P-αB1, P-αB2, and P-αB3. When P-αB5 or P-αB6 is administered, the effect is absent or minimal. With the administration of P-αB1, P-αB2, P-αB3, and P-αB4, the diameter of the villi reaches the level of the intact animals.

The same pattern can be observed when estimating the size of the maternal and fetal parts of the placenta (Figure 2A). The most pronounced effect was observed when administering the innovative peptides mimicking the α-helix B of erythropoietin under laboratory codes P-αB1, P-αB3, and P-αB4. The minimal effect was observed when using P-αB5 and P-αB6. When administering peptide P-αB3, the pronouncement of the effect was intermediate.

The analysis of fetal height indicators in the animals with ADMA-like preeclampsia revealed the following pattern in the effects of the innovative peptides mimicking the α-helix B of erythropoietin. All the studied peptides did not significantly affect the height (length) of the fetus (Fig. 2B). When administering all the studied peptides, an increase in fetus weight was observed in comparison with the group of control animals' fetuses. However, it should be noted that a more sensitive indicator (the ratio of height and weight) did not reach a statistically significant level compared to that in the control group of animals when P-αB6 was administered.

Thus, on the basis of the results of the experiment, it is possible to identify the following patterns. The base compound with the laboratory code P-αB having the amino acid sequence (U/Q)EQLERALNSS at a dose of 50 μg/kg shows pronounced protective properties in the simulated ADMA-like preeclampsia, but the target level is not reached. The addition of the tripeptide motifs RGD, KGD leads to an increase in protective effects of the innovative peptides mimicking the α-helix B of erythropoietin. However, it should be noted that the level of some indicators reaches the target level. The most pronounced effects are shown for the compound P-αB4, obtained by the addition the KGD motif to the base peptide. This compound we chose for the next series of experiments.

In the treatment of any pathology, a pharmacotherapy based on a comprehensive approach occupies a privileged position. By affecting various pathogenetic links, more opportunities are created for achieving the desired result or relieving a severe condition. Based on this, it is self-evident that the new drugs will be used in combination with the drugs already included in the treatment standards or recommended for treatment. These circumstances predetermined the final series of the experiments for this stage of research aimed at studying the effectiveness of an innovative peptide that mimics the alpha-helix B of erythropoietin P-αB4 in combination with a drug included in the standards for the treatment of hypertensive conditions in pregnant women in the correction of morphofunctional disorders in ADMA-like preeclampsia.

The effect of P-αB4 peptide in combination with methyldopa on the development of morphofunctional disorders in ADMA-like preeclampsia

The combined administration of the peptide mimicking the α-helix B of erythropoietin P-αB4 and the drug included in the standard therapy of hypertensive conditions in pregnant women during the correction of morphofunctional disorders in experimental preeclampsia led to mutual potency of their effects (Table 3). This resulted in a more pronounced decrease in blood pressure. The potentiation of the effects was especially pronounced after using P-αB4 at a dose of 10 μg/kg, with blood pressure almost reaching the level of that in the group of intact animals (Table 3). In both groups administered with P-αB4 at a dose of 50 μg/kg, arterial pressure was not statistically distinguishable from that in the group of intact animals, so a statistically reliable pattern of lowering blood pressure is debatable.

In addition, there was an improvement in endothelial function, which is evidenced by a decrease in the coefficient of endothelial dysfunction. In the case of administering the combination P-αB4 at a dose of 50 μg/kg with methyldopa, the coefficient of endothelial dysfunction reached a level not statistically distinguishable from that in the group of intact animals (Table 3). In the group of animals administered with P-αB4 at a dose of 10 μg/kg with methyldopa, the coefficient of endothelial dysfunction also decreased, but did not reach the level of the intact animals.

In both groups with the combined administration of P-αB4 and methyldopa, there was a marked improvement in placental microcirculation. However, it should be noted that the target level of the intact animals could not be reached (Table 3).

The study of NO-producing function in the animals with ADMA-like preeclampsia treated with the combination of the peptide mimicking the α-helix B of erythropoietin P-αB4 at the doses of 10 μg/kg and 50 μg/kg with methyldopa showed the most pronounced effect at a higher dose (Table 3). However, it should be noted that in

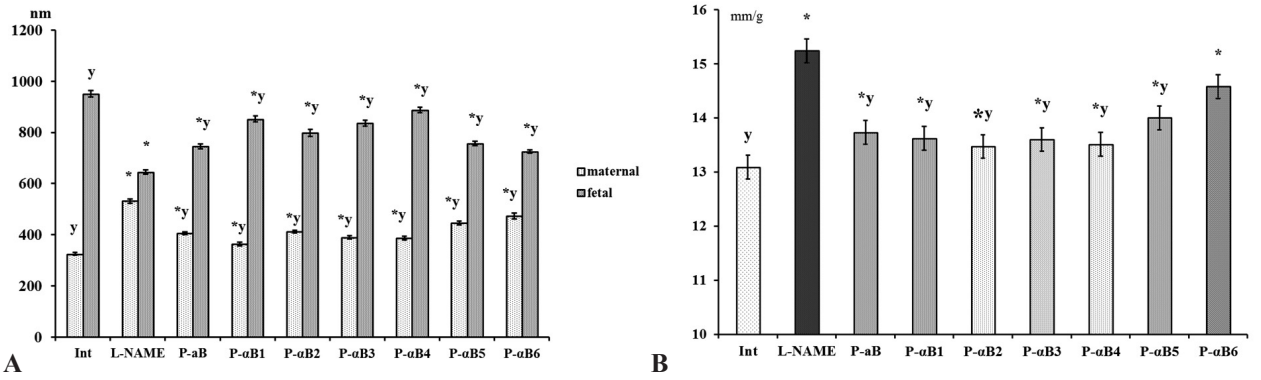

Figure 2. The effect of innovative peptides mimicking the α -helix B of erythropoietin on the size of the fetal and maternal parts of the placenta (A) and the height weight ratio of the fetuses (B) in ADMA-like preeclampsia. **Note:** * – $p < 0.05$ in comparison with the group of intact animals; ^{y} – $p < 0.05$ in comparison with the L-NAME group.

Table 3. The Effect of Innovative Peptide Mimicking the α-helix B of Erythropoietin P – αB4 on Functional Parameters in ADMA-like Preeclampsia (M ± m; n = 10).

Group	Indicator						
	SBP, mm Hg	DBP, mm Hg	CED, relative units	Microcirculation, PU	Proteinuria, g/L	NOx, µmol/DL	Edema of the greater omentum, %
Intact	128.8±2.61^{y}	80.8±3.27^{y}	1.25±0.04^{y}	541±23.4^{y}	0.29±0.10^{y}	2.22±0.04^{y}	44.74±1.16^{y}
L-NAME	202.8±6.86*	138.4±2.95*	3.11±0.20*	218±6.4*	2.57±0.11*	1.36±0.05*	54.12±1.31*
Methyldopa (0.086 g/kg)	159.1±3.53^{*y}	111.2±4.05^{*y}	2.31±0.11^{*y}	243±7.2^{*y}	1.71±0.15^{*y}	1.64±0.03^{*y}	49.97±0.90^{*y}
P-αB4 (10 µg/kg)	172.4±2.67^{*y}	123.0±2.29^{*y}	2.22±0.12^{*y}	300±9.1^{*y}	2.14±0.07^{*y}	1.71±0.04^{*y}	49.24±0.87^{*y}
P-αB4 (50 µg/kg)	138.3±5.44^{y}	94.0±6.59^{y}	1.80±0.22^{*y}	337±8.7^{*y}	0.51±0.14^{y}	1.80±0.06^{*y}	46.33±0.53^{*y}
P-αB4 (10 µg/kg) + Methyldopa (0.086 g/kg)	144.5±3.51^{*y}	98.7±3.27^{*y}	1.8±0.09^{*y}	438±15.1^{*y}	1.12±0.10^{*y}	1.88±0.05^{*y}	47.06±0.91^{y}
P-αB4 (50 µg/kg) + Methyldopa (0.086 g/kg)	129.2±2.33^{y}	85.1±2.55^{y}	1.4±0.09^{y}	489.1±9^{y}	0.39±0.10^{y}	2.13±0.05^{y}	45.51±1.00^{y}

Note: SBP and DBP – systolic and diastolic blood pressure; CED – coefficient of endothelial dysfunction; PU – perfusion units; NOx – NO terminal metabolites; * – $p < 0.05$ in comparison with the intact group; ^{y} – $p < 0.05$ in comparison with the L-NAME group.

the group of animals administered with P-αB4 at a dose of 10 µg/kg, there was also a potentiation of the effects.

In both groups administered with P- αB4 at a dose of 50 µg/kg, the severity of proteinuria was at the level of the intact animals. Therefore, the potentiation of the effects of the studied pharmacological agents is observed when adding methyldopa to P- αB4 at a dose of 10 µg/kg (Table 3). However, it should be noted that the target level of proteinuria in this group of animals was not reached.

In the animals with experimental preeclampsia, the administration of the studied pharmacological agents showed a decrease in the fluid content in the greater omentum tissues. In the group treated with the peptide mimicking the α-helix B erythropoietin P-αB4 at a dose of 50 µg/kg, the level of edema reached that of the intact animals. When a peptide mimicking the α-helix B of erythropoietin P-αB4 at a dose of 10 µg/kg or methyldopa at a dose of 0.086 g/kg was administered, a pronounced positive effect was also observed, but it did not reach the target level (Table 3). When adding methyldopa to the studied peptide in the group with P-αB4 at a dose of 50 µg/kg, the level of fluid content remained at the level of that in the intact animals. The addition of methyldopa to the studied peptide in the group with P-αB4 at a dose of 10 µg/kg allowed achieving the level of fluid content in the intact animals.

In the histological examination, morphometry showed that the most pronounced effect for the correction of the cell pool in the maternal and fetal parts of the placenta was observed in combination of P-αB4 at a dose of 50 µg/kg and methyldopa. The same pattern can be observed when estimating the size of the maternal and fetal parts of the placenta (Fig. 3A). The most pronounced effect was observed for the innovative peptide mimicking the α-helix B of erythropoietin P-αB4 at a dose of 50 µg/kg in combination with methyldopa. The minimal effect was observed for methyldopa in the studied dose. In the other experimental groups administered with the studied pharmacological agents in other modes of administration, the pronouncement of the effect was at the intermediate level.

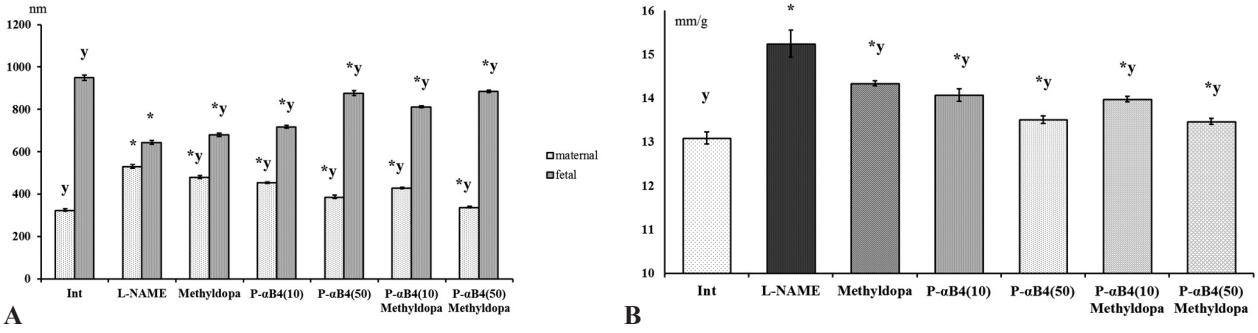

Figure 3. The effect of the innovative peptide mimicking the α-helix B of erythropoietin P- αB4 in combination with methyldopa on the size of the maternal and fetal parts of the placenta (A) and the height weight ratio of fetuses (B) in experimental preeclampsia. **Note**: * – $p < 0.05$ in comparison with the intact group; y – $p < 0.05$ in comparison with the L-NAME group.

When analyzing fetal growth indicators in the animals with ADMA-like preeclampsia, the most pronounced effect was observed in the group with a combined use of the peptide mimicking the α-helix B of erythropoietin P-αB4 at a dose of 50 μg/kg (Fig. 3B). However, it should be noted that the target level, not statistically different from that in the group of intact animals, was not achieved.

Thus, the results of the final series of experiments suggest the presence of a potentiating effect of the innovative peptide mimicking the α-helix B of erythropoietin P-αB4 in combination with methyldopa on the correction of morphofunctonal disorders in ADMA-like preeclampsia.

Preeclampsia is a complex multi-factorial disease and is traditionally characterized by a combined manifestation of edema, hypertension, and proteinuria. According to various authors, it affects from 2% to 10% of all pregnancies (Messerli et al. 2019; Tomimatsu et al. 2019; un Nisa et al. 2019).

The explanation of pathogenetic events in the modern view is provided in more than 30 theories concerning the origin and development of preeclampsia. One of the triggers is incorrect placentation as a result of impaired trophoblast invasion during pregnancy, which leads to placental ischemia (Seamon et al. 2020). Placental ischemia causes the release of a large number of humoral factors that provoke endothelial dysfunction of the mother's systemic vessels (Granger et al. 2018). In addition, in the pathogenesis of preeclampsia, a significant part is played by a disruption of platelet function, as evidenced by a decrease in the number of platelets, an increase in the average volume of platelets, and an increase in plasma concentrations of beta-thromboglobulin and platelet factor 4 (Jakobsen et al. 2019). Progression of thrombocytopenia and increased platelet activation are the early laboratory predictors of preeclampsia (Jakobsen et al. 2019).

Based on the main features of preeclampsia, one of the promising directions for creating new drugs for the treatment and prevention of preeclampsia is the use of drugs with anti-ischemic and cytoprotective activities. Such drugs are erythropoietin medications, which are allowed for use in pregnant women with other indications. The previous studies have shown high protective activity of erythropoietin in various simulated pathologies and in many pathological conditions (Robertson et al. 2012; Lin et al. 2017; Huang et al. 2018). In the experimental studies, recombinant erythropoietin has demonstrated pronounced effects on correction of morphofunctional disorders in experimental preeclampsia (Gureev et al. 2012; Gureev 2016; Lokteva et al. 2020). However, the presence of an undesirable erythropoietic effect and pro-aggregational properties in ischemic events limits the variation of its doses (Kittur et al. 2013; Yanagawa et al. 2013; Kapitsinou et al. 2015).

Particular interest in erythropoietin is caused by published studies, which showed the possibility of separating the erythropoietic and pleotropic effects of erythropoietin (Robertson et al. 2012; Collino et al. 2015). The bone marrow megakaryocytes have a homodimeric receptor (EPOR/EPOR) (Lin et al. 2017; Huang et al. 2018). In other tissues, there is a heterodimeric receptor (EPOR-βCR), so-called the innate repair receptor (IRR) (Culver et al. 2017; Lin et al. 2017; Wu et al. 2017; Yan et al. 2018).

A logical continuation of this trend was an experimental study of recombinant erythropoietin derivatives that do not have an erythropoietic activity. Asialo-erythropoietin and carbamylated darbepoetin were used as such derivatives. In experimental preeclampsia, both pharmacological agents improved the endothelial function, increased placental microcirculation, and reduced placental ischemic damage (Weber-Schoendorfer et al. 2015). However, it must be acknowledged that these derivatives also have a large mass, which is not an optimal property given the passage of the molecule through tissue barriers.

A fundamentally new approach is to search for low-molecular-weight peptide derivatives of EPO that have anti-ischemic and cytoprotective activities. The first such derivative is 11-amino acid peptide pHBSP (PubChem CID: 91810664), which is an amino acid chain (Pyr/Gln)-Glu-Gln-Leu-Glu-Arg-Ala-Leu-Asn-Ser-Ser and has a selective affinity for heterodimer complex EPOR/CD131 (Hache et al. 2016). When developing this peptide, the authors drew on the amino acid sequence of the α-helix B of erythropoietin, since this is the only structural part of the molecule that does not participate in spatial interaction with the EPOR/EPOR homodimeric complex. A number of studies have shown that pHBSP has greater antiapoptic properties compared to the base molecule, demonstrates

an analgesic activity, but does not have a hematopoietic effect of EPO (Hache et al. 2016).

A short peptide chain determines not only more favorable properties in terms of passing through tissue barriers, but also creates the possibility of adding short peptide motifs with predetermined properties to in order to provide new derivatives with them. Taking into account the fact that the malfunction of the hemostasis system, in particular, the disruption of the aggregation ability of platelets plays a special part in the pathogenesis of preeclampsia and its complications, interesting polypeptide motifs are represented by RGD (Arg-Gly-Asp), KGD (Lys-Gly-Asp), and PGP (Pro-Gly-Pro). which are known for their anti-aggregation properties (Pastorova et al. 2001; Lyapina et al. 2007; Liapina et al. 2010; Kuo et al. 2019). The resulting derivatives were the object of the study described in the present paper.

Based on the results of the presented experiment, the following patterns can be identified. The derivatives obtained by adding tripeptide motifs to pHBSP peptide retain their cytoprotective activity in simulated oxidative stress in HUVEC cell culture at the doses of 5 mcg/ml, 30 mcg/ml, and 50 mcg/ml. The compounds with laboratory codes PaB1 and PaB3 had the most pronounced cytoprotective activity.

The base compound with the laboratory code P-aB having the amino acid sequence (U/Q)EQLERALNSS at a dose of 50 μg/kg shows pronounced protective properties in simulated ADMA-like preeclampsia, but the target level is not reached. The addition of tripeptide motifs RGD, KGD leads to an increase in the protective effects of the innovative peptides mimicking the α-helix B of erythropoietin. And it should be emphasized that the level of some indicators reaches the target level. The most pronounced effects are shown for P-aB4 obtained by adding the KGD motif to the base peptide.

The obtained results can be explained by the ability of the peptides selectively bind to EPOR-βCR. In this case, pleotropic effects specific to recombinant erythropoietin are realized: anti-ischemic, anti-apoptotic, and anti-inflammatory (Zhang et al. 2017a; Zhang et al. 2017b; Tan et al. 2018). This contributes to cytoprotective effects in the fetoplacental complex, reducing the formation of humoral factors that cause endothelial dysfunction and eNOS activation. In addition, the effectiveness of the initial peptide mimicking the α-helix B of erythropoietin can be enhanced by addition of the polypeptide motifs with antiplatelet properties to it (Kuo et al. 2019). In preeclampsia, the interaction of activated platelets with the endothelium contributes to the aggravation of its dysfunction, which creates a vicious circle. The endothelium with dysfunction of the vascular system loses its antiplatelet properties, thus creating conditions for more intensive platelet activation. A logical explanation for the potentiation of the protective properties of the innovative peptides in the correction of morphofunctional disorders in experimental preeclampsia is the mechanism that leads to the interruption of the pathogenetic events described above.

The results of the final series of the experiments revealed the presence of the potentiating effect of the innovative peptide mimicking the α-helix B of erythropoietin P-αB4 in combination with methyldopa in the correction of morphofunctonal disorders in ADMA-like preeclampsia. This effect is explained by the ability to affect different application sites, using multiple pharmacological agents. Since a complex therapy plays a leading role in the treatment of many pathological conditions, including preeclampsia, the presence of a potentiating effect also indicates the prospects of the chosen direction in the creation of drugs aimed at the use of modified short-chain peptides mimicking the α-helix B of erythropoietin.

Conclusion

1. The innovative peptides mimicking the spatial structure of the α-helix B of erythropoietin obtained by addition of tripeptide motifs to the pHBSP peptide retain their cytoprotective activity in simulated oxidative stress in HUVEC cell culture at the doses of 5 mcg/ml, 30 mcg/ml, and 50 mcg/ml. The compounds with laboratory codes PaB1 and PaB3 had the most pronounced cytoprotective activity.
2. The innovative peptides mimicking the spatial structure of the α-helix B of erythropoietin at a dose of 50 μg/kg cause a pronounced correction of morphofunctional disorders in ADMA-like preeclampsia. The most pronounced protective properties are shown for the derivative under the laboratory code P-aB4. This is evidenced by a decrease in systolic and diastolic pressure to the levels statistically indistinguishable from those in the group of intact animals, a decrease in the coefficient of endothelial dysfunction by 1.73 times, 1.55-time improvement in microcirculation, a 32.4% increase in the concentration of the terminal metabolites of NO in plasma, a decrease in proteinuria and edema of the greater omentum tissue to the levels of those in the intact animals, the restoration of the diameter of villi to the level of that in the intact animals and improving other morphometric and histological indicators of the fetoplacental complex and the fetuses.
3. A synergy of effects is observed for a concomitant use of the leader compound, an innovative peptide mimicking the spatial structure of the α-helix B of erythropoietin under laboratory code P-aB4, with a drug included in the standards for the treatment of hypertensive conditions in pregnant women – methyldopa. This is evidenced by the positive dynamics of morphofunctional indicators with greater pronouncement at a dose of 50 mg/kg: a decrease in the coefficient of endothelial dysfunction and an increase in the concentration of the terminal metabolites of NO in plasma to the level of that in the intact animals, as well as a statistically significant increase in microcirculation in the placenta and correction of morphometric parameters of the fetoplacental com-

plex compared to the monotherapy groups treated with the studied pharmacological agents.

Conflict of interest

The authors declare no conflict of interest.

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