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INTEGRATED EVALUATION OF THE ENDOTHELIOPROTECTIVE ACTIVITY OF AN INNOVATIVE PEPTIDE SIMULATING THE ALPHA-HELIX OF B-ERYTHROPOETHIN IN L-NAME-INDUCED NITROGEN OXIDE DEFICIENCY AT THE LATE GESTATION PERIOD

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Abstract

Objective. To study the efficiency of using carbamylated darbepoetin, asialized erythropoietin and an 11-amino acid peptide that simulating the spatial structure of the B-erythropoietin alpha-helix in the correction of L-NAME-induced nitric oxide deficiency in the late gestation period.

Methods. The experiment was performed on 100 female white rats of Wistar strain weighing 250-300 g. Carbamylated darbepoetin (Pharmapark LLC) in a dose of 50μg/kg and 300 μg/kg was administered subcutaneously 1 time per each 7, 10, 13, 16, 19 days of pregnancy. Asialized erythropoetin in doses of 0.4μg/kg and 2.4μg/kg was also administered subcutaneously 1 time a day from the 10th to the 20th days of pregnancy. A peptide imitating the α-helix of B-erythropoietin (P-αB) was administered at a dose of 50 μg / kg ip 1 day / day from 10 to 20 days of pregnancy. On the 21st day of pregnancy, functional tests and laboratory examination were conducted.

Results. Administration of carbamylated darbepoetin, asialiated erythropoetin and peptide that imitating the α-helix of B-erythropoietin in animals causes the expressed correction of pathological changes in experimental L-NAME induced preeclampsia with the highest effect in a higher dose of the test drug. There was a significant rise in systolic and diastolic blood pressure, respectively, the improvement of microcirculation in the placenta, restoration of endothelium NO-synthesis function, proteinuria reduction. The most effective decrease in blood pressure and its maximum approximation to the target figures for SBP and DBP in intact animals was found in the group of animals treated with a peptide imitatimg the α-helix of B-erythropoietin (P-αB).

Conclusion. The results of the study indicate the promise of using a erythropoietin derivatives and peptide that imitate the α-helix of B-erythropoietin with L-NAME-induced nitric oxide deficiency in the late gestation period and justifies the feasibility of further studies in this direction.

Key words: peptide that imitate the a-helix of B-erythropoietin, erythropoietin derivatives, L-NAME induced endothelial dysfunction, rats, microcirculation, late gestation, preeclampsia.

Introduction

For the time being, preeclampsia remains one of the most serious multisystem pathological complications of pregnancy. Maternal mortality reaches 30% . Over 40 theories of preeclampsia etiology and pathogenesis exist so far. No unified theory exists that would allow covering and explaining the entire set of pathological processes occurring in a body of a pregnant woman who has this pathology. Recently, much attention in preeclampsia pathogenesis has been paid to endothelium functional condition changes. Modern studies of the endothelium functional condition at preeclampsia focus on the endothelial vasorelaxation factor represented by nitrogen monoxide (NO), which is synthesized in the body in response to the physiological need from L-arginine amino acid affected by NO-synthase (NOS) enzyme. Nitrogen monoxide is the most powerful of all endogenous vasodilators known. A normally functioning endothelium is characterized by permanent basal NO production which is required for normal basal vascular tone maintenance [1, 14]. Since NO regulates vascular tone, including that of resistive vessels, the decrease in its synthesis or bioavailability leads to vasoconstriction and the peripheral vascular resistance increase. At the same time, NO mediates dilatation of renal vessels and controls the kidney function, especially the mechanisms of sodium excretion from the body. Thus, the

weakening of these NO-dependent mechanisms contributes to hypertension development [2, 15]. NO produced under the action of eNOS doesn't only takes part in vascular tone and blood pressure regulation; it also performs the following important functions: inhibits PT adhesion, activation, secretion and aggregation, and WBC adhesion to the endothelium [3, 16], inhibits the release of mitogens from PT; regulates the formation of endothelin-1 and platelet-activating factor by endotheliocytes; inhibits migration and proliferation of vascular smooth muscle cells; regulates the angiotensin-2 biological action, etc.

For the time being, system pleiotropic protective effects of erythropoetin in the conditions of its intensified production were determined. According to reference data, EPO increases the duration of functioning of endothelium cells at oxygen starvation, has antiapoptotic effect in relation to endothelium cells, cause activation of endothelial NO-synthase and prevents vasomotor spasm, decreases NO-toxicity and has the direct antioxidant effect [4]. The EPO cytoprotective activity realization is mediated by binding with EPOR-βCR heterodimeric receptor, which, in its turn, causes the decrease of cell sensitivity to hypoxia and activation of antiapoptotic signalic pathways. The number of studies demonstrated that EPU hybrid variants, modified under amino acid residual of high- and low-affinity sites of binding with EPOR preserve their cytoprotective properties [5, 6, 14]. "Non-classic" EPO activity allows considering it as a therapeutic agent for preventing conditions associated with necrosis, apoptosis and inflammatory process in tissues, in particular, in preeclampsia therapy. However, erythropoetic activity may cause undesirable adverse effects. This contradiction led to the attempts to create modified EPO that would preserve their cytoprotective properties at the absence of blood-forming action. One of such modifications became carbamylation causing both the molecular structure and function of EPO change [5]. We used carbamylated darbepoetin (Pharmapark LLC) in our study.

Moreover, one of the directions of the "erythropoetic" defect removal is creating erythropoetin derivatives with the short life cycle. In order to activate cytoprotective effects, it is sufficient for erythropoietin to bind with heterodimeric receptor for the short period of time (5 minutes). For erythropoesis activation, it is erythropoetin should stay on the homodimeric receptor permanently for a long time. In our study, we used asialiated erythropoietin purified from EPO form sialic acids (Proteinovy Kontur LLC).

As the most promising derivative of erythropoietin with endothelial and cytoprotective activity, we selected an 11-amino acid peptide that imitate the spatial structure of the B erythropoietin alpha-helix. We obtained the specified derivative of erythropoietin under the laboratory code P-αB having the following amino acid sequence - Pyr-Glu-Gln-Leu-Glu-Arg-Ala-Leu-Asn-Ser-Ser.

The goal is to investigate the effectiveness of the use of carbamylated darbepoetin, asialized erythropoietin and an 11-amino acid peptide that imitate the spatial structure of the erythropoietin B alpha-helix in correcting L-NAME-induced nitric oxide deficiency in the late gestation period.

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Methods

The study was carried out in the Center for Clinical and Pre-Clinical Studies of FSAEI HE 'BSNRU'. The experiment was carried out in accordance with legislative acts and regulations governing the conduct of experimental studies in the Russian Federation: (Order of the Ministry of Health of Russia N199n of 01.04.2016 "On approval of the Rules of Good Laboratory Practice", GOST 33044-2014 "Principles of Good Laboratory Practice", GOST 33217-2014 "Regulations for management the management and the treatment of laboratory animals. Rules for the management and the treatment of laboratory mammals", "Regulations for the conduct of pre-clinical studies of new medicinal drugs" edited by Mironov A. N., 2012.). Ethical principles for the treatment of laboratory animals met "European Convention for the Protection of Vertebral Animals Used for Experimental and Other Scientific Purposes. CETS N170."

The experiment was performed on 140 female white rats of Wistar strain weighing 250-300 g. Pregnant rats were divided into 8 groups: Group 1 – intact; group 2 - control (L-NAME administration); group 3 - L-NAME + carbamylated darbepoetin (50 μg / kg); group 4 - L-NAME + carbamylated darbepoetin (300 μg / kg); group 5 - L-NAME + asialized erythropoietin (0.4 μg / kg); group 6 L-NAME + asialized erythropoietin (2.4 μg / kg); group 7 - L-NAME + peptide that imitate the α-helix of B-erythropoietin (P-αB) (50 μg / kg); group 8 - subcutaneous administration of erythropoietin at a dosage of 50 IU / kg..

ADMA-like agent - non-selective NO-synthase blocker of N-nitro-L-arginine methyl ester (L-NAME) was administered intraperitoneally in a dose of 25 mg/kg/daily for seven days (day 14-20 of pregnancy) [7, 8]. Carbamylated darbepoetin in a dose of 50μg/kg and 300 μg/kg was administered subcutaneously 1 time a day on 7, 10, 13, 16, 19 days of pregahncy to the animals of 3 and 4 groups. Asialiated erythropoietin was also administered subcutaneously for the period from the 10th to the 20th day of pregnancy in doses of 0.4 μg/kg and 2.4 μg/kg to 5 and 6 groups of pregnant rats, respectively. The peptide (P-αB) imitating the α-helix of B-erythropoietin (50 μg / kg) was administered intraperitoneally from 10 to 20 days of pregnancy. On the 21st day of pregnancy, a laboratory animal was anesthetized by intraperitoneal injection of chloral hydrate in a dose of 300 мг/kg from body weight, after which, functional tests followed [9].

The degree of endothelial dysfunction in experimental animals was assessed by the ratio of endothelium-dependent vasodilation and endothelium-independent vasodilation with the subsequent calculation of coefficient of endothelial dysfunction (CED) [10, 22].

The NO metabolite level was detected by colorimetric method.

In order to obtain data about the condition of microcirculation in the placenta for the 21st day of pregnancy, microcirculation level at a distance of 1 mm from the placental disc edge [11] was measured with anesthesia.

The descriptive statistics was applied for all data: data were verified for normality of distribution. The distribution type is determined by Shapiro-Wilk W test. In the case of normal distribution, the mean (M) and standard error of the mean (m) were calculated. The inter-group discrepancies were characterized by parametric (Student's t-test) or non-parametric (Mann–Whitney U test) methods, depending on the distribution type.

Study results

After the administration of L-NAME in pregnant rats, the blood pressure increase occurred: SBP was 194.8±7.88 mm Hg, DBP was 149.8±4.73 mm Hg, while in intact animals, systolic and diastolic pressure parameters were 132.30±3.46 and 92.40±3.87 mm Hg, respectively.

When a peptide (P-αB) imitating the α-helix of B-erythropoietin (50 μg / kg) was administered during the period from 10 to 20 days of pregnancy, a statistically significant ($p < 0.05$) compared to the group of "untreated" animals occurred a decrease in systolic and diastolic blood pressure to 142.80 ± 1.98 and 90.40 ± 5.21 mm Hg, respectively.

In the group of animals with the reference drug, erythropoetin, the decrease in systolic and diastolic pressure to 173.5±3.66 mm Hg and 130.9±4.38 mm Hg, respectively, was observed.

The administration of asialiated erythropoetin also led to the significant ($p < 0.05$) SBP decrease to 183.1±6.71 (in a dose of 0.4μg/kg) and 167.3±3.43 (2.4μg/kg). For diastolic BP, after asialiated erythropoetin administration, these parameters were 139.7±3.72 and 129.4±4.17 respectively. After the administration of carbamylated darbepoetin in a dose of 50μg/kg and 300 μg/kg per day, a significant decrease in

systolic BP to 189.10±6.49 mm Hg and 168.20±6.56 mm Hg, and in diastolic BP up to 144.00±3.48 mm Hg and 125.80±3.50 mm Hg, respectively, was observed (Table 1).

The administration of L-NAME in pregnant rats caused violation of vascular tone regulatory mechanisms, proved by CED increase from 1.20±0.07 to 3.17±0.22.

The introduction of erythropoietin and its derivatives led to the normalization of vascular reactions of endothelium-dependent and endothelium-independent vasodilation statistically significantly lowering the QED indicators to 1.95 ± 0.19, however, these values did not reach the target. The introduction of a peptide (P-αB) imitating the α-helix of B-erythropoietin (50 μg / kg) during the period from 10 to 20 days of pregnancy intraperitoneally led to a statistically significant ($p < 0.05$) compared with the group of "untreated" animals decrease in QED to 2.0 ± 0.06.

Course administration of carbamylated darbepoetin in doses of 50 μg/kg and 300 μg/kg to pregnant animals with ADMA-like preeclampsia reduced the CED to 2.19±0.22 and 1.44±0.18, which proves the endothelial function improvement.

In the animals with ADMA-like preeclampsia, microcirculation decrease from 465.9 ± 28.79PU to 211.8 ± 6.03 PU was observed. Administration of a peptide (P-αB) that simulates the α-helix of B-erythropoietin (50 μg / kg) during the period from 10 to 20 days of gestation intraperitoneally occurred statistically significant ($p < 0.05$) compared with the group of "untreated" animals microcirculation in the placenta up to 343.2 ± 5.98.

The administration of carbamylated darbepoetin in test doses recovered microcirculation to 347.6±15.07 PU and 415.9 ± 20.59 PU, respectively. In groups of animals with the course administration of asialiated erythropoetin, these indicators recovered to 357.3 ± 22.6 PU and 413 ± 20.0 PU, in relation to lower and higher doses. These values exceed the results of microcirculation correction with erythropoetin, for which these values were 313.4 ± 15.87 PU.

Table 1. The impact of P-αB, carbamylated darbepoetin and asialiated erythropoietin on BP, CED and microcirculation in the placenta at ADMA-like preeclampsia

		SBP(mm Hg)	DBP(mm Hg)	CED(conv.);	Microcirculation (PU)
Intact		132.3±3.46*	92.4±3.87*	1.20 ± 0.07*	465.9±28.79*
control		194.8 ± 7.88#	149.8 ± 4.73#	3.17 ± 0.22#	211.8±6.03#
Epo 50 IU/kg		173.5 ± 3.66 #*	130.9 ± 4.38#	1.95 ± 0.19 #*	313.4± 15.87 #*
(P-αB)	50μg/kg	142.80 ± 1.98*	90.40 ± 5.21*	2.0±0.06*#	343.2±5.98*#
AsEpo	0.4μg/kg	183.1±6.71*	139.7±3.72*#	2.09±0.14 *	357.3±22.6#*
	2.4μg/kg	167.3±3.43*#	129.4±4.17*#	1.67±0.12*#	413±20.0*
CEPO	50μg/kg	189.1 ± 6.49#	144.0± 3.48 #	2.19±0.22*#	347.6±15.07*#
	300μg/kg	168.2±6.56*#	125.8±3.50*#	1.44 ± 0.18*	415.9 ±20.59*

Note: From this point on - SBP, DBP – systolic and diastolic blood pressure (mm Hg); CED – the coefficient of endothelial dysfunction (conv.); PU – perfusion units; #- $p < 0,05$ compared to the group of intact animals; * – $p < 0,05$ compared to the control group

The study of the NO-synthesizing function of the endothelium was carried out on the basis of the determination of nitrite - NOx ions in blood plasma. Modeling of endothelial dysfunction led to a decrease in the content of final NOx metabolites in blood plasma. The introduction of a peptide (P-αB) that mimics the α-helix of B-erythropoietin (50 μg / kg) during the period from 10 to 20 days of pregnancy intraperitoneally led to a statistically significant ($p < 0.05$) compared with the group of "untreated" animals increasing the content of final metabolites of blood plasma to 1.59 ± 0.05 μmol / dL. After the introduction of carbamylated darbepoetin in doses of 50 μg / kg and 300 μg / kg ($p < 0.05$), the content of nitrite ions (NOx) in the blood plasma in animals with ADMA-like preeclampsia increased to 1.64 ± 0.02 μmol / dL and 1.91 ± 0.03 μmol / dL, respectively, which is close to the target values in the group of intact animals (2.31 ± 0.04 μmol / dL). With the administration of asialized erythropoietin, a similar dose-dependent result was noted, the NOx content was 1.49 ± 0.02 μmol / dL and 1.61 ± 0.02 μmol / dL.

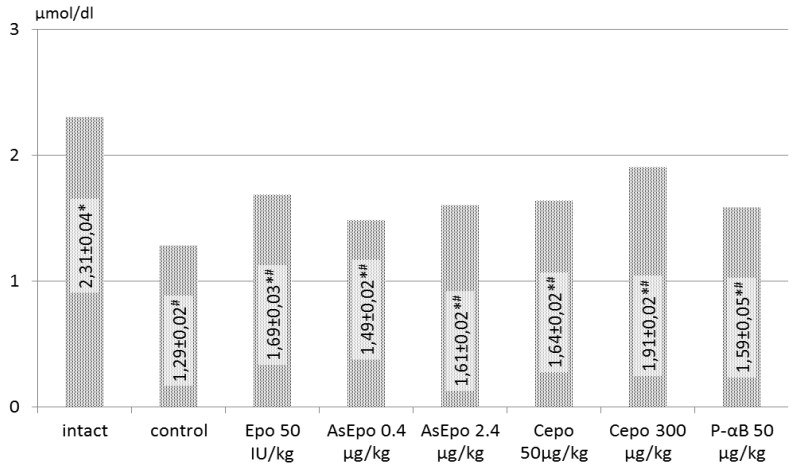

Fig. 1. The impact of erythropoietin derivatives on the level of NO metabolites at L-NAME-induced nitric oxide deficiency in the late gestation period

Note: #- p < 0,05 compared to the group of intact animals; * – p < 0,05 compared to the control group

Results and discussion

In morphological examination of the placenta in women who had preeclampsia, the researchers indicate at its formation violation. This is expressed in the fact of incomplete penetration of chorionic villi into mother's spiral arteries. At the same time, the spiral arteries retain their layers up to the muscular, causing trophoblast ischemia [13]. The response to ischemia is the ejection of a large number of humoral factors, the action of which ultimately causes endothelial dysfunction development [12, 13]. In connection with this, it would be logical to assume that anti-ischemic medicinal products can have the mediated effect on endothelial dysfunction. However, so far, the data that preeclampsia develops more frequently in women with diseases related to endothelial function violation, and a variety of its manifestation clinical variants, does not give a precise answer to the question, 'What is primary, placenta ischemia or endothelial dysfunction.' But it is obvious that endothelial dysfunction and placenta ischemia are mutually penetrating components of preeclampsia pathogenesis.

The preeclampsia model selected by us, despite the fact that it is caused by the vasoactive ADMA-like substance, has an ischemic component as well. Apparently, the placenta vessels are the most sensitive to L-NAME [21, 17], which causes their spasm and subsequent ischemia up to necrotic events [19, 20].

The results of the experiments convincingly indicate the pronounced positive effects of, erythropoetin derivatives and the peptide (P-αB) that imitate the α-helix of B-erythropoietin in the correction of endothelial dysfunction occurring in animals with ADMA-like preeclampsia. Having no erythropoesis-stimulating properties, P-αB, carbamylated darbepoetin and asialiated erythropoetin have strong antioxidant and cytoprotective properties.

Conclusion

Administration of carbamylated darbepoetin, asialiated erythropoetin and peptide that imitating the α-helix of B-erythropoietin in animals causes the expressed correction of pathological changes in experimental L-NAME induced preeclampsia with the highest effect in a higher dose of the test drug. There was a significant rise in systolic and diastolic blood pressure, respectively, the improvement of microcirculation in the placenta, restoration of endothelium NO-synthesis function, proteinuria reduction. The most effective decrease in blood pressure and its maximum approximation to the target figures for SBP and DBP in intact animals was found in the group of animals treated with a peptide imitatimg the α-helix of B-erythropoietin (P-αB).

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