Рус. Eng.

UDC: 615.225:617.735-007.23 **DOI: 10.18413/2313-8971-2016-2-1-25-29**

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PHARMACOLOGICAL PRECONDITIONING BY RECOMBINANT ERYTHROPOIETIN AS THE POSSIBILITY OF INCREASING THE STABILITY OF TISSUE OF THE RETINA TO REPERFUSION ISCHEMIA IN EXPERIMENT

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Abstract. Ischemic injury was formed in laboratory rats by applying mechanical pressure (110 mm Hg) to the anterior eye chamber for 30 minutes. The experiment revealed that the recombinant erythropoietin (50 IU/kg) prevents the development of degenerative changes in retinal layers caused by ischemic injury. Intensity of the protective effect of erythropoietin on the retina of rats was assessed after 1 hour and 72 hours of reperfusion after pathology simulation. The observed protective effect of erythropoietin with the development of ischemia was confirmed by laser Doppler flowmetry, electroretinography and histomorphometry. Identification and use of pharmacological agents with the preconditioning effect may be a new approach in the correction and prevention of retinal ischemia, which is the leading element in the pathogenesis of a number of ophtalmic pathologies. The possibility of pharmacological preconditioning of ischemic lesions of the retina with erythropoietin is essential for the development of anti-ischemic agents for the treatment and prevention of ischemic ophthalmic pathologies.
Keywords: retinal ischemia-reperfusion; pharmacological preconditioning; erythropoietin; ATP-dependent potassium channels.

Introduction. Currently, vasodilators, fibrinolytics, anticoagulants, angioprotectors, biogenic stimulants, vitamins, immunomodulators, antioxidants are used in the world practice for the treatment of ischemic and reperfusion injury of retina, including diabetic and hypertensive retinopathy [1, 2].

Treatment with the above drugs is not always successful enough, that ensures the relevance of the problem of expanding the range of drugs for the treatment of ophtalmic diseases associated with ischemic conditions. The leading element in the pathogenesis of a number of fundus vascular diseases is retinal ischemia (RI). Therefore, a recombinant erythropoietin is the most promising for the study, in our view. This pharmacological agent has established itself in a previously conducted studies of the protective effect of pharmacological preconditioning conducted in models of ischemia-reperfusion injury of the brain, heart, hind limb, kidney, placenta, liver, and other organs and tissues [3-14].

Main part: Objective of this study was to investigate the effcet of distant ischemic

preconditioning (DIP) and pharmacological preconditioning of the erythropoietin on microcirculation level and histomorphometric changes in the retina upon modeling the ischemia-reperfusion of the eye.

Materials and methods. Experiments were conducted in 140 male and female mongrel rats weighing 225-275 g. Ischemia-reperfusion injury of retina was simulated under anesthesia (chloral hydrate, 300 mg/kg of animal body weight, i.p.) by applying mechanical pressure (110 mm Hg) to the anterior eye chamber for 30 minutes. The absence of ocular blood flow confirmed the formation of ischemia.

To study the protective properties of the DIP and pharmacological preconditioning with erythropoietin, we conducted 7 series of the experiment, with 10 animals in each group: 1 – intact animals; 2 – ischemia-reperfusion of the retina (control); 3 – with DIP correction; 4 – correction with recombinant erythropoietin (EPO); 5 – control + glibenclamide (ATP-dependent potassium channels blocker); 6 – DIP + glibenclamide; and 7 - EPO + glibenclamide;

DIP was performed through 10 min compression of the femoral artery by applying a tourniquet on the proximal third of the hip for 40 min prior to modeling of retinal ischemia, followed by a 30-minute reperfusion episode.

To study the preconditioning effect, the recombinant erythropoietin (Epocrin, Federal State Unitary Enterprise “Institute of Pure Biochemicals”) was administered i.p. at a dose of 50 IU/kg, 30 minutes before pathology simulation.

Glibenclamide (“Maninil” (Berlin-Chemie AG) was administered at a dose of 5 mg/kg once, 60 min before ischemia simulation.

The degree of the protective effect was evaluated by the change of the microcirculation level, electrophysiological changes in the electroretinogram (ERG) and histomorphometric changes in the retina of rats after 1 hour and 72 hours of reperfusion. For this purpose, the animals were fixed under anesthesia (chloral hydrate, 300 mg/kg of animal body weight, i.p.). The level of microcirculation in rat retina was measured by laser Doppler flowmeter Biopac-systems 150 MR-150 and acicular sensor TSD-l44 (USA). For this purpose, the anesthetized animals were fixed and microcirculation level in the retina was measured in 10 points around the sclera at a pitch of 1 mm. Microcirculation curve was recorded during 20 seconds in each point. The obtained 10 values were averaged with further recording of the average in the protocol and its use as the level of retinal microcirculation in the particular animal. The obtained 10 values were averaged with further use of the mean value as the level of retinal microcirculation in the particular group of animals.

The electroretinography principle is to register the potential of retinal cells in response to light. Assessment of retinal electrical activity was conducted with a- and b-wave amplitude of the electroretinogram. a-wave is a negative vawe reflecting the functional activity of photoreceptors, and b-wave is a positive wave reflecting the electrical activity of bipolar and Muller cells with the possible invovement of the horizontal and amacrine cells. The evoked biopotentials were run at a frequency of 1-1000 Hz, amplified, averaged and presented graphically on the screen using the Biopac-systems MP-150 with a computer program AcqKnowledge 4.2 (USA). ERG recording was carried out for 0.5 seconds in each rat in groups. To assess the severity of retinal ischemia, b- and a-wave amplitude ratio - b/a ERG index - is widely used (Neroyev V.V. et al., 2004). The 10 values obtained in each series were averaged with further recording in the protocol [5].

Then, the eyeball was subjected to enucleation. For histological examination, the eye and its immediately adjacent tissues were fixed in 10% formalin solution. After fixation, the material was completely embedded in paraffin under standard conditions. The pieces were orientated in the blocks so as to receive upon sectioning the preparations in the meridian direction strictly through the center of the eyeball. Sections for standard histological examination were stained with hematoxylin and eosin. A descriptive study of histological preparations was performed with the microscope Axio Scope A1 (Carl Zeiss Microimaging GMbH, Germany). Morphometry of retinal layers was performed with the program ImageJ 1.47 [11].

The significance of changes in absolute parameters was determined by the difference method of variation statistics with finding the average values of the shifts, the arithmetic mean, and the probability of possible error (p) by using the Student tables. Differences were considered significant at $p<0.05$.

Results and discussion. Hemodynamic impairments in retinal blood flow system after 1 h and 72 h of reperfusion after pathology simulation led to the electrophysiological and morphological changes in the retina, typical of ischemia, which confirmed the adequacy of the chosen pathology model.

After pathology simulation, the microcirculation level of the retina was measured after 1 h and 72 h of reperfusion by LDF method. The obtained results are shown in Table 1.

The level of microcirculation in the retina of intact rats was 738.9 ± 37.6 pu (perfusion units). The level of microcirculation after ischemia simulations in the control group reached 1155.0 ± 51.9 pu after 1 h of reperfusion, which was significantly higher than the value in the group of intact animals ($p<0.001$). Upon DIP correction, the level of microcirculation in the retina after 1 h of reperfusion decreases significantly up to 952.0±25.8 pu ($p<0.05$) as compared to the control group (with RI).

Table 1

Changes in the microcirculation level of the retina after 1 h and 72 h of reperfusion (M±m; n=10)

No	Experimental groups	Microcirculation level after 1 h, pu	Microcirculation level after 72 h, pu
1.	Intact	738.9±37.6^{y}	743.9±5.0^{y}
2.	Retinal ischemia (RI)	1155.0±51.9*	353.3±11.7*
3.	RI+DIP	952.0±25.8*^{y}	638.5±15.8*^{y}
4.	RI+EPO 50 IU/kg	798.5±12.3^{y}	724.0±4.1^{y}
5.	RI + glibenclamide, 5 mg/kg	1135.8±31.2*	359.4±10.3*
6.	RI + DIP + glibenclamide, 5 mg/kg	1144.7±20.7*	361.7±13.9*
7.	RI + EPO 50 IU/kg + glib., 5 mg/kg	1148.5±14.3*	372.3±13.4*

Note: *- at $p<0.05$ as compared to intact animals; ^{y}- at $p<0.05$ as compared to control animals

Upon correction of the simulated EPO pathology, the microcirculation level in the group decreases up to 798.5 ± 12.3 pu and also significantly differs from the values in the control group ($p<0.001$). Administration of glibenclamide, an ATP-sensitive potassium channels blocker, in the groups with DIP and EPO correction prevented the reduction of microcirculation level, which confirms the presence of the preconditioning effect in the recombinant EPO at a dose of 50 IU/kg in the model of retinal ischemia of rats after 1 hour of reperfusion.

The results of evaluation of microcirculation level in rat retina after 72 hours of reperfusion after RI and its DIP and EPO correction at a dose of 50 IUkg are presented in Table 1.

The level of microcirculation in the retina of intact rats was 743.9±5.0 pu. The level of microcirculation after ischemia simulations in the control group reached 353.3±11.7 pu after 72 h of reperfusion, which was significantly lower than the value in the group of intact animals ($p<0.001$), which indicates the formation of ischemia by 72 h of reperfusion. Upon DIP correction, the level of microcirculation in the retina after 72 h of reperfusion increases significantly up to 638.5±15.8 pu ($p<0.05$) as compared to the control group (Table 1).

Upon correction of the simulated EPO pathology, the microcirculation level in the group increases up to 724.0 ± 4.1 pu, which significantly differs from the values in the control group ($p<0.001$) and tends to the value in the group of intact animals.

Administration of glibenclamide in the groups with DIP and EPO correction prevented the increase in microcirculation level, which confirms the presence of the preconditioning effect in the recombinant EPO at a dose of 50 IU/kg in the model of retinal ischemia of rats after 72 hour of reperfusion.

After simulating ischemia and measuring the level of the microcirculation in the retina, the retinal electrophysiological state caused by the evoked potential was assessed. The data obtained after 1 h and 72 h of reperfusion are shown in Table 2.

Table 2

Evaluation results of electrophysiological condition of the retina after 1 h and 72 h of reperfusion (M±m; n=10)

No.	Experimental groups	b/a, ru, after 1 hour	b/a, ru, after 72 hours
1.	Intact	2.6±0.09^{y}	2.5±0.10^{y}
2.	Control	2.0±0.09*	1.2±0.04*
3.	DIP correction	2.3±0.07*^{y}	2.0±0.08^{y}
4.	EPO correction, 50 IU/kg	2.5±0.07^{y}	2.3±0.06^{y}
5.	Control + glibenclamide, 5 mg/kg	2.2±0.06*	1.2±0.05*
6.	DIP correction + glibenclamide, 5 mg/kg	2.1±0.08*	1.2±0.04*
7.	EPO correction, 50 IU/kg + glibenclamide, 5 mg/kg	2.2±0.09*	1.2±0.06*

Note: *– at $p<0.05$ as compared to intact animals; ^{y} – at $p<0.05$ as compared to control animals

The b/a index in the control group was 2.0 ± 0.09 ru, which significantly differs from the values in the group of intact rats. Increase of this indicator in the groups with the correction with recombinant EPO up to 2.5 ± 0.07 ru and DIP - up to 2.3 ± 0.07 ru indicates the preservation of retinal electrophysiological function after pathology simulation. Administration of glibenclamide in the groups of animals with EPO and DIP correction led

to a decrease in the b/a index up to values significantly different from those of the intact group, which indicates the blockade of the ATP-dependent potassium channel and confirms the presence of preconditioning properties of EPO at a dose of 50 IU/kg and DIP in the RI model after 1 h and 72 h of reperfusion.

ERG after 72 h of reperfusion was performed after measuring the level of retinal microcirculation in experimental animals. The results from these experiments are shown in Table 2.

The b/a index in the control group was 1.2±0.04 ru, which statistically significantly differs from the values in the group of intact rats. Increase of this indicator in the groups with the correction with recombinant EPO up to 2.3 ± 0.06 ru and DIP - up to 2.0 ± 0.08 ru indicates the preservation of retinal electrophysiological function after pathology simulation. Administration of glibenclamide in the groups of animals with EPO and DIP correction led to a decrease in the b/a index up to values significantly different from those of the intact group, which indicates the blockade of the ATP-dependent potassium channel and confirms the presence of preconditioning properties of EPO at a dose of 50 IU/kg and DIP in the RI model after 72 h of reperfusion.

Reduction of the b/a index in animals with simulated ischemia (control) was caused by inhibition of the positive ERG b-wave, which indicates an impaired electrophysiological function of bipolar and Muller cells with the possible involvement of the horizontal and amacrine cells. Preservation of the electrophysiological function of the photoreceptor layer is confirmed by the absence of adverse changes in the negative a-wave.

Morphological and morphometric studies confirmed the electrophysiological changes in the retina. The inner nuclear and photoreceptor layers were subjected to the morphometric assessment. The retina of the animals with EPO and DIP correction showed the preservation of the structure of inner nuclear, outer reticular, and the outer nuclear layers. The control group shows rarefication of the outer reticular layer and the separation of the photoreceptor layer from the pigment epithelium. During administration of glibenclamide, no protective effect of DIP and EPO was observed, the morphological pattern of the retinal layers in these groups did not significantly differ from the control group.

The results from this experiment are shown in Table 3.

Table 3

Morphometric indices of the retinal layers in experimental animals (M±m; n=10)

No.	Experimental groups	1 h of reperfusion		72 h of reperfusion	
		Inner nuclear layer	Photoreceptor layer	Inner nuclear layer	Photoreceptor layer
1.	Intact	23.5±0.8[y]	38.4±0.8	23.8±1.0[y]	38.1±1.2
2.	RI	25.9±0.6*	39.1±0.7	20.3±0.8*	36.9±0.9
3.	DIP	24.0±0.5[y]	38.4±0.9	21.7±0.4*	37.8±0.8
4.	EPO	23.8±0.6[y]	38.3±0.9	23.3±0.7[y]	38.0±1.0
5.	Control + glib.	26.0±0.7*	39.1±0.6	20.5±0.4*	37.1±0.8
6.	DIP + glib.	25.8±0.6*	39.2±0.6	20.6±0.6*	36.9±0.8
7.	EPO + glib.	25.7±0.6*	39.0±0.5	20.3±0.5*	37.0±0.9

Note: *– at $p<0.05$ as compared to intact animals; [y] – at $p<0.05$ as compared to control animals

During morphometric analysis it should be noted that no reliable changes in thickness of the photoreceptor layer in experimental groups is observed in any of the series, that confirms the absence of changes in the negative ERG a-wave.

The inner nuclear layer was the most sensitive to the effect of ischemia-reperfusion. Increase in thickness of the layer after 1 h of reperfusion is caused by edema, and its thinning after 72 hours is due to degenerative processes in the retina.

The group of animals treated with EPO had the inner nuclear layer thickness after 1 h and 72 h of reperfusion equal to 23.8 ± 0.62 μm and 23.7 ± 0.71 μm, respectively, which significantly differs from the values in group with the RI.

The group of animals treated with DIP had the inner nuclear layer thickness after 1 h and 72 h of reperfusion equal to 24.0±0.53 μm and 23.6±0.52 μm, respectively, which significantly differs from the values in the control group.

Pretreatment with glibenclamide contributed to the elimination of protective actions of both DIP and EPO, which confirms the presence of anti-ischemic effect in the model of retinal ischemia in rats with DIP and EPO at a dose of 50 IU/kg by virtue of the preconditioning effect.

Conclusion:

Based on these findings, we can conclude that the correction of ischemic injuries of the retina in laboratory rats is achieved by the preconditioning effect of DIP and recombinant EPO at a dose of 50 IU/kg of animal body weight, which is confirmed by LDF and ERG methods, and by the results of morphological and morphometric studies.

The absence of protective effect in groups treated with EPO and glibenclamide, and DIP and glibenclamide indicates that the retinoprotection is due to the preconditioning effect, the key role in which is played by the ATP-dependent potassium channels.

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