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Morphological Changes in the Retina Under Conditions of Experimental *In Vivo* Regional Ischemia/Reperfusion

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Two models of retinal ischemia/reperfusion were developed in an experiment on rats and structural changes in eye tissues in the early and late postischemic periods were studied. Ischemia/reperfusion was modeled by elevation of intraocular pressure to 110 mm Hg for 30 min with air injection into the anterior chamber of the eye with a special device or subconjunctival administration of 0.2 ml 4×10^{-6} M endothelin-1. Morphological studies of the retina of enucleated eyes were performed in 3, 7, and 30 days after ischemia/reperfusion. In 3 days, signs of retinal ischemia were seen (retinal edema and ganglion cell damage). In the late post-ischemic period (30 days), atrophy of the outer nuclear and outer plexiform layer of the retina was observed in animals with retinal ischemia/reperfusion caused by intraocular pressure elevation and complete destruction of neuronal cells was found after administration of endothelin-1.

Key Words: *ischemia/reperfusion of the retina; experimental model; intraocular pressure; endothelin-1; morphological studies*

Retinal ischemia is a common cause of vision impairment and loss that develop against the background of vascular pathologies of the eyes (optic neuropathy, occlusion of the retinal vessels, diabetic retinopathy, glaucoma, *etc*.). In light of this, it is interesting to study morphological features of ischemia/reperfusion damage to the retina in experiments on animals for profound understanding of the pathogenesis and the choice of treatment strategy in this pathology. In many ways, the success of treatment depends on our understanding of changes in hemocirculation and metabolic and morphological disorders during retinal ischemia/reperfusion (RIR) [12,14].

It is known that transient circulatory disorders of the eye include two periods: no-flow (ischemia) and its restoration (reperfusion), which are part of the same pathological process — ischemia/reperfusion. During the reperfusion period, ROS are formed, inflammatory interleukins are released, which lead to cell membrane damage, migration of leukocytes to the inflammatory focus with increased damage of tissue [5].

There are a number of experimental models of transient retinal ischemia, including occlusion of the cerebral artery, ligation of the carotid arteries, photocoagulation of the retinal vessels and administration of endothelin-1 [1,2,7,13,14]. In the articles [8,9] the modeling of ischemic damage, based on the increase in intraocular pressure (IOP) above the level of systemic blood pressure, is presented. Induction of regional ischemia in the tissues of the eye by the increase of IOP is fairly easily reproducible, but has a significant limitation, since high IOP causes a combined compression-ischemic trauma typical of the glaucomatous mechanism of damage. Single publications are devoted

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to the method of modeling of RIR in animals with the introduction of endothelin-1 (ET-1) under the conjunctiva, intravitreal or intravenously [6,10,11]. The model of RIR with the introduction of ET-1 is easily reproduced, does not require operating room and special equipment. However, the disadvantages include the need to use a high dose of ET-1 and the probability of its penetration into the systemic circulation. Therefore, a search is still underway for the most adequate experimental RIR model, which simultaneously meets the requirements of high efficiency, reproducibility and efficiency, which makes it possible to investigate hemodynamic, neurotrophic and structural changes in the retina at the postischemia.

The goal of this study was to create two different RIR models in an experiment on rats and to study the structural changes in the eye in the early and late postischemic periods.

MATERIALS AND METHODS

The work was conducted on Wistar male rats (n=34) with an average weight of 220±30 g at the age of 130±30 days. The basic rules for keeping and caring complied with the Rules for Work with Experimental Animals and the Sanitary Rules for Keeping Experimental Biological Clinics (Vivariums), approved by the USSR Ministry of Health (July 6, 1973), Order No. 755 of the Ministry of Health of the USSR (August 12, 1977), and the European Convention on Vertebrate Animals used for Experiments or other Scientific Purposes (Strasbourg, 1986). The study was approved by the local ethics committee (protocol No. 23/3; February 26, 2014).

All invasive interventions were performed with a combination of general anesthesia in accordance with the standard scheme (based on intravenous dose of a substance by body weight, adopted in laboratory practice for working with rats) and local anesthesia with a 0.4% solution of oxybuprocaine (inocaine).

The animals were divided into 3 groups. Twelve rats (12 eyes) were included in group 1: RIR modeling by IOP elevation to 110 mm Hg for 30 min with injection of air into the anterior chamber of the eye using a special device [3]. Twelve rats (12 eyes) comprised group 2: subconjunctival administration of 0.2 ml of ET-1 solution (4×10^{-6} M) in 0.05 M phosphate buffer (pH 7.4) [4]. Control group 3 consisted of 10 intact rats (20 eyes).

Instrumental examination included inspection under a binocular lens to assess the state of the anterior segment of the eye and direct ophthalmoscopy to confirm retinal ischemia. The severity of keratopathy and intensity of the reaction of the anterior eye segment

TABLE 1. Criteria of the Severity of Damage to the Anterior Segment of the Eye in Rats

Sign	Severity	Score
Corneal edema	None	0
	Mild (uneven partial edema, mostly epithelial)	1
	Moderate (uniform edema of the entire cornea, mostly epithelial)	2
	Pronounced — «frosted glass» (diffuse with involvement of stroma)	3
	Strongly pronounced — «porcelain glass»	4
Iris edema	None	0
	Weakly expressed (smoothed relief, slight dilation of the vessels)	1
	Moderate (very flattened relief, dilation of blood vessels)	2
	Pronounced (the relief is almost absent; vessels are significantly widened)	3
Blood in the anterior chamber	None	0
	≤$^1/_3$ volume of front camera	1
	≤$^2/_3$ volume of front camera	2
	All front camera	3
Photophobia and blepharospasm	None	0
	Present	1
Corneal neovascularization	None	0
	Moderate (superficial or deep neovascularization less than $^3/_4$ of the cornea area)	1
	Pronounced (superficial and/or deep more than $^3/_4$ of the cornea area)	2

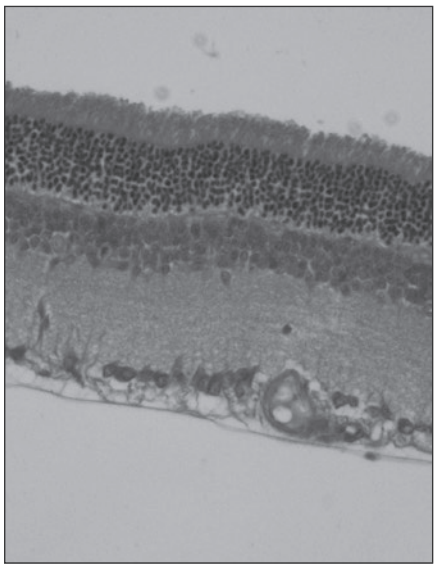

Fig. 1. Histological picture of the retina of a healthy rat, group 3 (control). Staining with hematoxylin and eosin, ×40.

were scored using an original scale that included 5 parameters (Table 1). Exclusion criteria were retinal detachment, endophthalmitis, hypopyon, and ciliochoroidal detachment in the post-ischemic period.

Histopathological examination was conducted at the Department of Pathological Histology and Anatomy, Helmholtz Moscow Research Institute of Eye Diseases. The animals of each experimental group were sacrificed in 3 (3 eyes), 7 (3 eyes), and 30 days (6 eyes) after RIR. After decapitation, eyeballs were enucleated, fixed in Bouin fixative, subjected to macroscopic examination, and cut into 3 flaps. The central flap was subjected to standard histological processing and embedded in paraffin. Paraffin blocks were used to prepare 3-5-μ sections that were stained with hematoxylin and eosin (Fig. 1). Microscopic examination of histological specimens was conducted under a Leica DM2500 light microscope with a DFC 7000T digital camera.

Statistical analysis of the results was performed using Statistica 6.0 (StatSoft, Inc.). Student's *t* test and Mann—Whitney test were used to assess statistical significance. The differences were significant at $p \leq 0.05$.

RESULTS

In the post-ischemic period immediately after introduction of air into the anterior chamber and pressure elevation to 110 mm Hg, the conjunctiva and the fundus of the eye became pale in all animals of group 1. These changes were confirmed by visual examination and ophthalmoscopy. The red reflex of the fundus was restored immediately after IOP normalization.

In 3 days, damage of the anterior segment of the eye were observed in group 1: photophobia, blepharospasm, edema of cornea, iris and hyphema. Examination of the fundus in 5 (42%) of 12 rats was difficult due to moderate corneal edema and hyphema. The severity of damage to the anterior segment of the eye was 5.4 points. In 7 days, 77% animals showed cornea and iris edema, blepharospasm and photophobia persisted, and partial resorption of blood occurred at hyphema. The mean score of anterior segment damage severity was 5.2. In 30 days, blepharospasm and photophobia, hyphema and edema persisted in 4 (66%) of 6 rats of the group 1, and 2 (33%) of the rats showed marked edema and neovascularization of the cornea. The mean score of damage to the anterior segment of the eye decreased to 4.3.

After RIR simulation by subconjunctival administration of ET-1, the conjunctiva and fundus became pale in all the animals of group 2 immediately after the injection vasospasm, which was confirmed by external objective examination and ophthalmoscopy. Normal color of the conjunctiva was restored in 1-1.5 h. In 3 days, the clinical picture of ischemic damage was characterized by the following symptoms: mild corneal edema, keratopathy, and photophobia. Five (42%) of

TABLE 2. Morphometric Parameters of the Thickness of the Retinal Layers (nm) 1 Month after the Simulation of RIR by Increase of IOP

Retinal layers	Control	No. 1	No. 2	No. 3	No. 4	No. 5	No. 6	*M*±*m*
NFL+GCL	11.4	11.2	11	10.9	11.4	11.2	11.3	11.16±0.18
IPL	35.3	35.1	32.2	35.3	35	35.2	35.2	34.67±1.21
INL	24	24	23.9	23.7	23.8	23.9	23.8	23.85±0.1
OPL	4.7	0	0	0	0	0	0	0
ONL	44.5	7.3	7.2	7.7	7.9	7.2	7.6	7.48±0.29
PL	27.5	12	11.4	13.3	9.5	12.8	14.4	12.23±1.69

Note. Nos. 1-6, eye number. NFL is the nerve fiber layer, GCL is the ganglion cell layer, IPL is the inner plexiform layer, INL is the inner nuclear layer, OPL is the outer plexiform layer, ONL is the outer nuclear layer, PL is the photoreceptor layer.

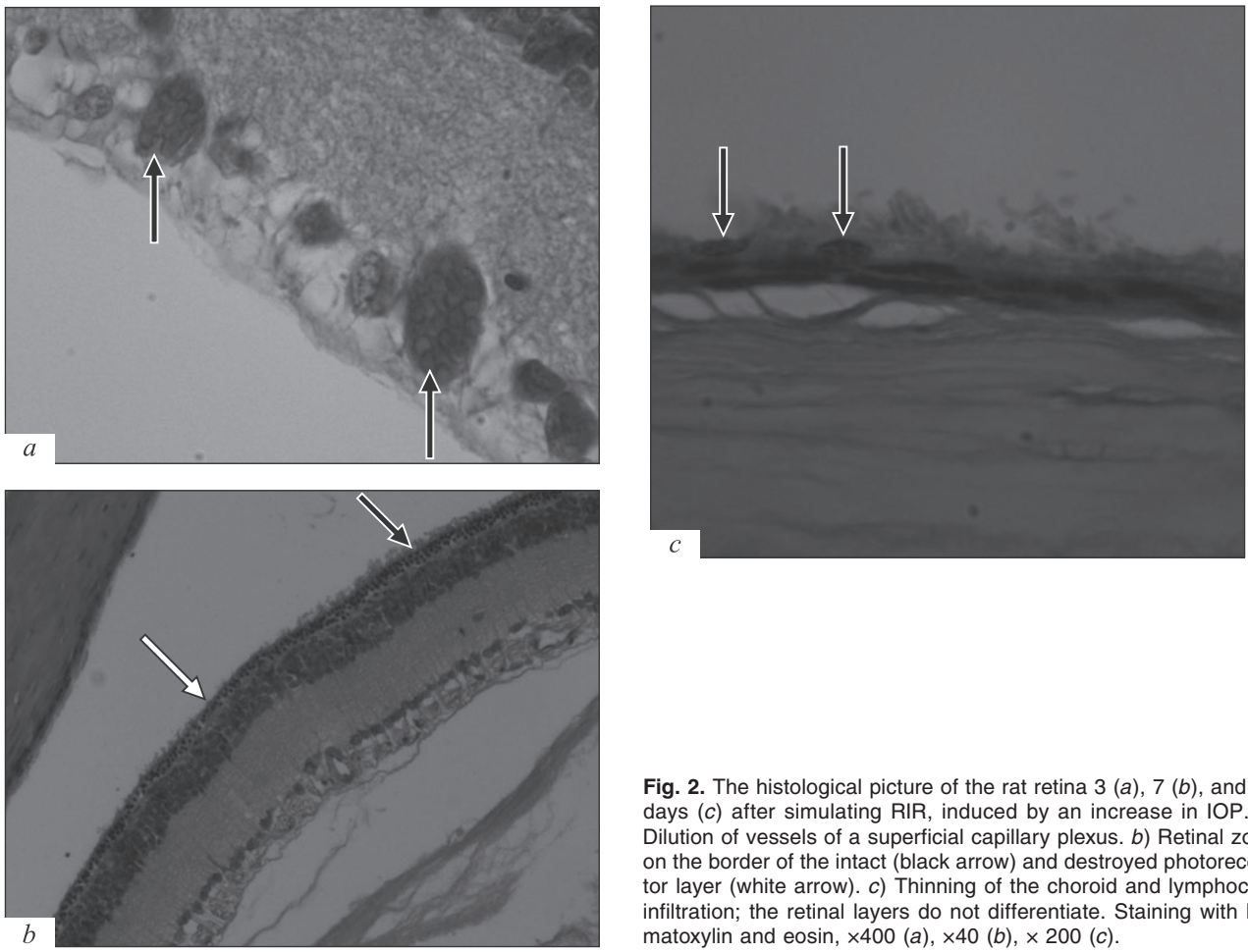

Fig. 2. The histological picture of the rat retina 3 (*a*), 7 (*b*), and 30 days (*c*) after simulating RIR, induced by an increase in IOP. *a*) Dilution of vessels of a superficial capillary plexus. *b*) Retinal zone on the border of the intact (black arrow) and destroyed photoreceptor layer (white arrow). *c*) Thinning of the choroid and lymphocyte infiltration; the retinal layers do not differentiate. Staining with hematoxylin and eosin, ×400 (*a*), ×40 (*b*), × 200 (*c*).

12 rats had hyphema. The mean severity of damage to the anterior segment of the eye was 3. In 7 days, both groups showed a decrease in the level of hyphema and corneal edema, the disappearance of photophobia in 7 (77%) of 9 rats, which corresponded to 1.8 points averagely. In 30 days, moderate corneal edema was observed in 4 (66%) of 6 rats with almost complete resorption of hyphema in the anterior chamber, and in 2 (33%) rats — single newly formed vessels of the cornea (2.6 points on average).

The results of the examination of the anterior segment showed a large trauma of cornea and the risk of retinal detachment at simulation of RIR caused by increase of IOP. Blepharospasm and photophobia, observed after 3 days, were caused not only by ischemic, but also compression damage to the cornea.

The morphological picture of the eye membranes in animals of the group 1 in the early post-ischemic period (after 3 days) was characterized by corneal edema with thinning and degeneration sites, lymphocytic infiltration of the cornea, iris and ciliary body, retinal edema, mainly due to the internal plexiform and the nerve fiber layers. The partial destruction of the photoreceptor layer, the presence of atrophy of ganglion cell attracted attention, while the outer and inner nuclear layer maintained normal thickness (6-8 rows and 8-10 rows of cells, respectively). Numerous vessels of the superficial capillary plexus were significantly dilated, had thinned walls (Fig. 2, *a*).

Seven days after RIR, an increase in the area of the destroyed photoreceptor layer and a decrease in the thickness of the outer nuclear layer (5-6 rows of cells) were observed with persistent retinal edema (Fig. 2, *b*).

After 30 days, in all animals of this group, corneal vascularization, pronounced fibrous changes in the form of thin film formation in the angle of anterior chamber and at the base of the ciliary body, and iris atrophy were noted. In addition, infiltration of the choroid by lymphocytes and its thinning (Fig. 2, *c*), as well as pronounced changes of retina in the form of thinning of the photoreceptor layer by 44%, the outer nuclear layer by 83.2% of the normal thickness, and the disappearance of the outer plexiform layer were recorded (Table 2).

Analysis of the results of histopathological examination of the eyes in 12 rats with subconjunctival administration of ET-1 showed that after 3 days the size of the eyeball was within the normal range, corneal

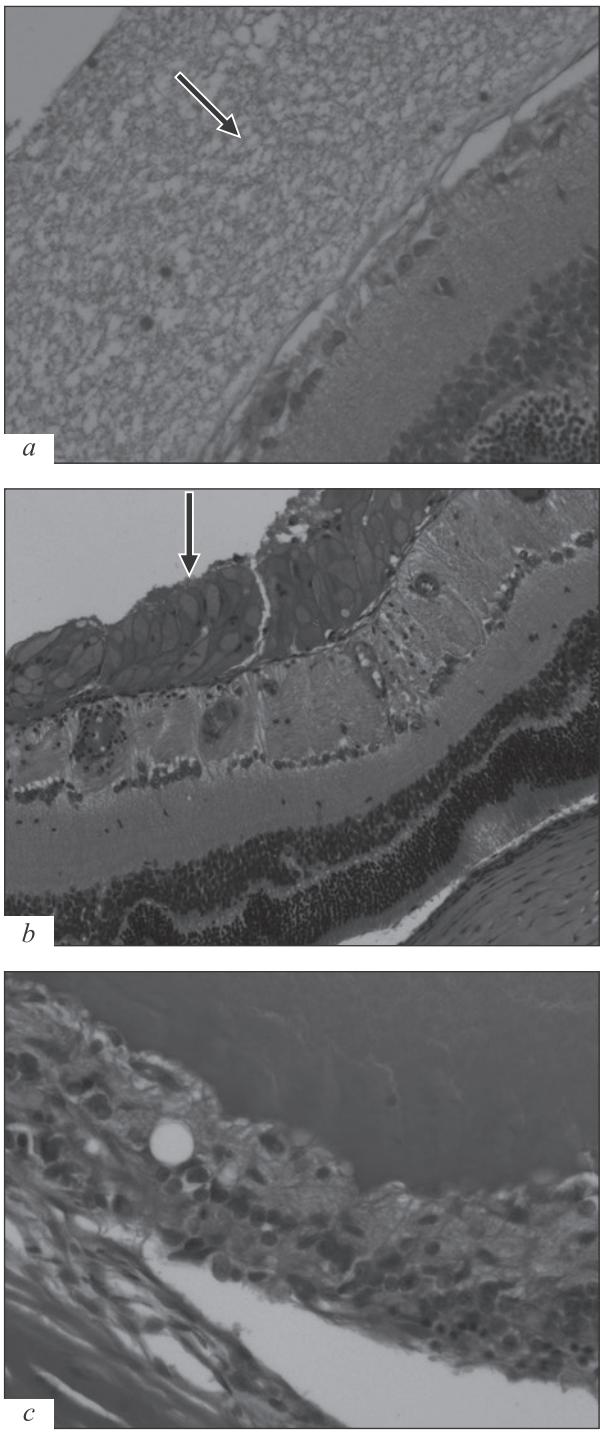

Fig. 3. The histological picture of the rat retina 3 (*a*), 7 (*b*), and 30 days (*c*) after RIR induced by ET-1. *a*) Preretinal hemorrhage (arrow); retinal edema. *b*) Preretinal hemorrhage in the resorption stage (arrow), a multitude of macrophages with vacuoles; swelling and folding of the retina. *c*) No layer of ganglion cells, the structure of the inner and outer nuclear layer is disturbed. Staining with hematoxylin and eosin, ×100 (*a*), ×40 (*b*), ×200 (*c*).

edema was observed in all animals, in 66% of cases the cornea was infiltrated by lymphocytes and blood leakage to the anterior chamber.

The preretinal hemorrhage in all animals, retinal edema mainly due to the internal plexiform layer and the layer of nerve fibers, its folding (Fig. 3, *a*) attracted attention. The signs of death of ganglion cells without damage to other layers of the retina were determined, a spasm of the walls of the central artery of the retina with preservation of its lumen and occlusion of small diameter vessels was visualized.

Seven days after RIR, corneal edema and infiltration with lymphocytes persisted. The features of the histopathological picture included the initial signs of hemophthalmum resorption on the retinal surface, which was manifested by the accumulation of detritus from destroyed hemolyzed erythrocytes and multiple hemosiderophages. There was marked edema of the retina, especially the inner plexiform layer. Changes in ganglion cells were received in the form of the formation of vacuoles from cell nuclei and the concentration of chromatin along the edge of the nuclear membrane. In addition, areas of a decrease in the number of cells in the inner nuclear layer to 7-8 rows and a partial destruction of a layer of rods and cones were determined.

An analysis of the results of histopathological studies conducted after 30 days showed that animals of the group 2 had vascularization of the cornea and the lack of clear boundaries between its layers, isolated lymphocytes in the stroma of the cornea, and numerous folds of the Descemet's membrane, between of which isolated red blood cells were visualized. The infiltration of the iris and ciliary body with lymphocytes and erythrocytes, thickening of the sclera and episclera due to vascular reaction and cellular infiltration with lymphocytes, thickening of the choroid and the presence of a large number of proliferating fibroblasts and many newly formed vessels were determined. A morphological study of the retina with a morphometric assessment received a thinning of the nerve fiber layer and the ganglion layer by 38.6%, the inner plexiform layer by 48.9%, the outer plexiform layer by 77.4% and the outer nuclear layer by 26.8% (Fig. 3, *c*) of the values in the control group (Table 3). In 4 of 6 rats, it was impossible to differentiate the layers of the retina due to its infiltration with lymphocytes, erythrocytes and replacement of the retina with glial tissue with the loss of neuronal structures (Fig. 3, *c*).

Data on histopathological changes in eye membranes in the early period after modeling of retinal RIR under the influence of high IOP, characterized by retinal edema and atrophy of ganglion cells [15], are consistent with the results of our study. We have identified histopathological changes of the retina in the distant post-ischemic period (30 day) — damage to predominantly the outer layers of the retina, probably associated with compression-ischemic injury of

TABLE 3. Morphometric Parameters of the Thickness of the Retina Layers (nm) 1 Month after the Simulation of RIR by Introduction of ET-1

Retinal layers	Control	No. 1	No. 2	No. 3	No. 4	No. 5	No. 6	*M±m*
NFL+GCL	11.4	6.2	7	7.1	6.9	7.3	7.5	7.00±0.44
IPL	35.3	17.5	18.6	18.9	18.2	17.3	17.7	18.03±0.63
INL	24	23.8	23.7	24	22.9	23.5	23.7	23.60±0.37
OPL	4.7	2.1	2.3	2	0	0	0	1.06±1.17
ONL	44.5	31.5	33.2	32.4	33	31.8	32	32.32±0.67
PL	27.5	25	24.8	24.6	26.7	26.5	25.4	25.50±0.89

Note. Nos. 1-6, eye number. NFL is the nerve fiber layer, GCL is the ganglion cell layer, IPL is the inner plexiform layer, INL is the inner nuclear layer, OPL is the outer plexiform layer, ONL is the outer nuclear layer, PL is the photoreceptor layer.

the choroid that leads to its thinning and infiltration by lymphocytes.

In contrast to the method of simulation of retinal RIR [10] with subconjunctival administration of 0.3 ml 4×10^{-5} M ET-1 to rats, our proposed method suggests reduce of the ET-1 concentration by 10 times and the volume of the injected drug by 30%. This reduces the risk of a possible systemic effect of ET-1 and makes the study more cost-effective while maintaining the effectiveness of the RIR model. This model is accompanied by damage not only to the outer layers of the retina (outer nuclear and plexiform layers), but also to pronounced structural changes in the inner layers in the late postischemic period (after 30 days), which is associated with microcirculatory disorders in the system of retinal vessels.

Thus, we identified ischemic changes in the tissues of the anterior segment of the eye (edema and lymphocyte infiltration of the cornea and iris), retinal edema and degenerative changes in ganglion cells in the early post-isthmic period and a decrease in the number of rows of both nuclear layers of the retina with atrophy of the photoreceptor layer in the late post-ischemic period indicates the reproducibility of the presented models of regional RIR and the possibility of their use to study the mechanism of damage of retinal cells, as well as the assessment of the effectiveness of methods for correction of post-ischemic changes.

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