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EXPERIMENTAL ARTICLES

Glutamate Excitotoxicity and Oxidative Stress Induced by Experimental Thrombosis of Retinal Vessels

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Abstract—We studied the effect of thrombosis of retina vessels on the development of glutamate excitotoxicity in chinchilla rabbits. Thrombosis was induced by administration of Bengal rose dye at a dose of 40 mg/kg into the auricular vein of the animals followed by focal lighting with white light. Lighting was performed for 10 min on average at the temporal vascular arcade in the location of the angulation of vessels over the border of the disk of the optic nerve using a binocular ophtalmoscope and a condensing +14.0 D lens. We found that experimental thrombosis induced the accumulation of glutamate, aspartate, glycine, and GABA, as well as oxidative stress, which was associated with decreased activity of superoxide dismutase, glutathione peroxidase, and glutathione-S-transferase.

Keywords: thrombosis of retina vessels, glutamate excitotoxicity, oxidative stress, ischemia, modeling

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INTRODUCTION

Visual acuity is one of most socially important problems of medicine. According to recent data, approximately 285 million people suffer from poor vision worldwide. Thirty-nine million of these are the group of people with a 10–20% weakening of vision as compared to the optimal level [1]. Pathologies of a vascular origin compose a substantial part of all eye diseases. Specifically, thrombosis of retinal veins may be found on average in 0.93 cases per 1000 patients at the age before 64 and in 5.36 cases per 1000 patients at the age after 65 [2]. In Russia, there is a trend to juvenation of this pathology; thus, in 2000 25% of the total number of patients with eye diseases of a vascular origin were observed in the group of 41–60-year-old people, whereas in 2009, such patients were observed at a rate of 39% in the same age group [3].

In order to study the pathogenesis of this disease in detail and to develop new approaches to their pharmacological treatment, it is important to use appropriate experimental models of retinal ischemia. Impaired functioning of neurotransmitter and neuromodulatory systems, specifically, the release of the excitatory transmitter glutamate and so-called "hyperexcitation death" or "excitotoxicity phenomenon" is considered as a leading mechanism of ischemic damage to the retina and the brain. Under ischemia conditions, neurons release glutamate into the intercellular space. An excessive accumulation of glutamate activates ionotropic NMDA- and AMPA-subtypes of receptors and induces a massive influx of Ca^{2+} ions into the cytoplasm of postsynaptic neurons. In its turn, calcium triggers several processes, such as activation of the respiratory chain in mitochondria with increased leakage of superoxide anion-radical and hydroxyl radicals, activation of $NADPH_2$-oxidase followed by the accumulation of the superoxide anion radical, activation of NO-synthase and accumulation of NO, and activation of hemoxygenase, which transforms Fe^{3+} to Fe^{2+}. All these processes enhance lipid peroxidation and activate the antioxidant system of cellular defense. Long-term hypoxia results in exhaustion of the antioxidant system, induction of oxidative stress and death of neurons via apoptosis or necrosis that depends on the extent of cell damage [4].

Several approaches to ischemia modeling are presently known, including intravascular injections of cobra toxin [5], atherosclerotic material from the human aorta [6] or platelet aggregates or mixtures of platelets and leukocytes [7], air embolism of the central retinal artery induced by injection of 0.6 mL of air [8], occlusion of vessels such as the common carotid artery, the central retinal artery or posterior ciliar arteries [9], and laser coagulation of vessels [10]. The

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limitations of these methods are the high injury rate, high risk of complications, and the necessity for additional techniques, materials, and devices.

One of the most promising models of local ischemia is photoinduced vascular thrombosis, in which a photosensitizer is administered intravenously and then a proposed ischemic locus is irradiated. Initially, this approach was proposed by Watson et al. [11] for modeling thrombosis in the femoral, common carotid, and middle cerebral arteries. Later, Romanova et al. [12] modified this technique for the modeling of cortical thrombosis. The use of an argon laser for irradiation hinders experimental modeling of thrombosis. We suggest using visible light for the irradiation of a photosensitizer in a model of thrombosis of retinal vessels.

The aim of the present study was to examine the amino-acid composition and expression of oxidative stress in the retina after experimental vascular thrombosis.

MATERIALS AND METHODS

Thirty-nine mature chinchilla male rabbits, weighing on average 2500–3000 g, were used for experimental modeling of acute retina ischemia (77 eyes). All experiments with animals were performed in accordance with the rules of laboratory practice (Order no. 708n, August 23, 2010, of the Ministry of Health and Social Development of the Russian Federation).

For modeling of the experimental pathology, the animals were anesthetized with an intramuscular injection of xylasine hydrochloride (Rometar, Bioveta) at a dose of 4.0–6.0 mg/kg and 20 min later, with intramuscular injections of Zoletyl-50 (Virbac) at a dose of 5–10 mg/kg near both eyes. The pupils were dilated by triple application of tropicamide (Mydriacyl, Alcon-Couvreur) and cyclopentolate (Cyclomed, Promed Exports). A photosensitizing Bengal rose dye with the wavelength of induction of $\lambda = 560$ nm was used for thrombosis of retinal vessels. A photosensitizer was injected into the auricular vein at a dose of 40 mg/kg of body weight. Immediately after intravenous Bengal rose administration, transpupilar focal lighting of the temporal vascular arcade in place of angulation of vessels over the border of disk of optic nerve using a binocular ophtalmoscope and a condensing +14.0 D lens was performed. Lighting was performed for 10 min on average and occlusion of retinal vessels was controlled visually. In order to prevent corneal drying, it was washed with 0.9% NaCl. The animals were examined using ophtalmoscopy and only those animals were included in the study in which thrombosis was recanalized 1 day after induction.

Pathogenesis of experimental thrombosis was studied in two groups of animals: Group 1 ($n = 36$) was used for the assessment of the free radical state of the retina and Group 2 ($n = 41$) was used for assessment of the amino-acid composition of the retina at various time points after the thrombosis of retinal vessels. Group 1 consisted of the animals from five experimental series, including intact animals and animals that were sacrificed 1 h, 3, 5–7, and 14 days after thrombosis of retinal vessels; Group 2 consisted of the animals from four experimental series, including intact animals and animals that were sacrificed 3, 5–7, and 14 days after the thrombosis of retinal vessels.

The animals of both groups were subjected to binocular ophtalmoscopy, photographs of the eye ground, and electroretinography.

At the end of the experiment all animals were euthanized by air embolism. The retina was sampled from the pathological locus for the study.

Retina samples from the animals of Group 1 were homogenized in a phosphate buffer. The homogenates were used for measuring TBA-reactive products in a reaction with thiobarbituric acid [13], free sulfhydryl (SH) groups in the reaction with disulfide 5,5-dithiobis-2-nitrobenzoate [14], the activity of glutathione peroxidase (G-per) in the reaction of oxidation of reduced glutathione with tert-buthyl hydroperoxide conjugated with the reaction of the reduction of formed oxidized glutathione with $NADPH_2$ [15], activity of glutathione-S-transferase (G-tr) in the reaction of the conjugation of glutathione with 1-chloro-2,4-dinitrobenzene catalyzed by this enzyme [16], and the activity of superoxide dismutase (SOD) using the reaction of the inhibition of quercetin autooxidation [17].

Retina samples from the animals of Group 2 were homogenized in 20 volumes of 0.1N $HClO_4$. The contents of aspartate, glutamate, glycine, taurine, and GABA were assayed using the modified HPLC method [18] with fluorescent detection. An Agilent 1100 chromatograph (United States) equipped with a 4.6 × 250 mm, 5 μm HYPERSIL ODS column was used. The wavelengths of excitation and emission were 230 and 392 nm, respectively.

Statistical analysis was performed using StatSoft Statistica 7.0 software. The significance of the differences of the data on the indices of the free-radical state and amino-acid content in the retina was estimated using the one-way analysis of the variances (ANOVA) and electroretinography data were estimated using ANOVA with repeated measures. The Newman-Keuls test was used for post hoc comparisons. The data in the tables are presented as the mean and standard deviation ($M \pm SD$).

RESULTS AND DISCUSSION

The experimental model of thrombosis of retinal vessels is based on the ability of the Bengal rose photosensitizer to generate active oxygen species under the action of light at $\lambda = 560$ nm, which results in damage to the vascular wall and vascular thrombosis. The advantages of this model are selectivity, targeted action, and a low injury rate. In this model, cells of the

Table 1. Indices of a dark (scotopic) electroretinogram at various time points after ischemia (mean ± standard deviation)

Group	Amplitude of a-wave, mV	Latency of a-wave, ms	Amplitude of b-wave, mV	Latency of b-wave, ms
Control ($n = 16$)	4.24 ± 1.81	16.04 ± 1.31	116.47 ± 30.12	41.78 ± 2.39
1 h ($n = 13$)	2.59 ± 1.63	16.29 ± 1.43	78.65 ± 24.47 ($p = 0.031$)*	39.97 ± 1.93
1 day ($n = 14$)	4.63 ± 3.07	16.31 ± 0.89	129.78 ± 35.49	42.58 ± 2.71
3 days ($n = 14$)	5.29 ± 3.76	15.51 ± 0.97	134.75 ± 35.44	40.92 ± 3.19
5–7 days ($n = 6$)	4.75 ± 3.20	16.10 ± 1.34	115.87 ± 53.58	42.80 ± 2.53
14 days ($n = 6$)	1.58 ± 0.73	17.30 ± 1.94	54.70 ± 38.07* ($p = 0.007$)	41.20 ± 0.98

*$p < 0.05$, significant differences as compared to intact animals.

Table 2. Contents of amino acids in the retina at various time points after ischemia (mean ± standard deviation), μmol/g of tissue weight

Group	Asp	Glu	Gly	Tau	GABA
Control ($n = 9$)	1.07 ± 1.55	6.66 ± 6.54	2.36 ± 1.03	73.52 ± 33.08	2.13 ± 2.92
3 days ($n = 8$)	2.76 ± 2.80	14.46 ± 15.22	3.03 ± 1.94	75.61 ± 42.96	4.76 ± 4.66
5–7 days ($n = 14$)	4.09 ± 2.71*	30.51 ± 18.14*	6.96 ± 3.81*	115.7 ± 49.11	10.03 ± 5.82*
14 days ($n = 10$)	3.08 ± 2.59	24.98 ± 19.87*	4.85 ± 2.82	104.93 ± 54.33	7.25 ± 5.55

Asp, aspartate; Glu, glutamate; Gly, glycine; Tau, taurine; GABA, gamma-aminobutiric acid. * $p < 0.05$, significant differences as compared to intact animals.

vascular endothelium are selectively damaged with minimal effect on the surrounding tissues.

At 1 day after retinal vascular thrombosis, vein dilation and tortuosity, constriction of arteries, a small number of vascular breaks, hemorrhages along the vascular arcade, edema of myelinated fibres, strong edema of the retina along the impaired vessels and in the central part, and edema of the optic nerve disk were observed. At 14 days after thrombosis, edematous retina, dilated plethorical veins, occluded small vessels, spiral form and irregularity of vascular calibers, light hyperemia and edema of the optic nerve disk, and edema of myelinated vessels were still observed.

General (maximum) electroretinography revealed that the amplitudes of a- and b-waves and their latencies were similar in the experimental and intact animals.

Dark phase (scotopic) electroretinography revealed a significant ($p < 0.05$) decrease in the amplitude of b-waves 1 h and 14 days after thrombosis by 32.5 and 53.0%, respectively, as compared to the normal value (Table 1). The other indices of an electroretinogram were not substantially changed ($p > 0.05$). In accordance with classical retinal physiology, the amplitude of b-waves reflects the activity of bipolar cells, Muller's cells, and probably ganglion cells of the retina [19]. The decreased amplitude of b-wave indicates impaired functioning of these cells in the model of pathology that was used.

Data on the contents of neurotransmitters in the retina are presented in Table 2. At 3 days after thrombosis, the contents of the studied neurotransmitters were similar to those found in intact animals ($p > 0.05$). On days 5–7, we observed increased levels of aspartate (Asp), glutamate (Glu), glycine (Gly), and GABA by 282.2, 358.1, 194.9, and 370.9%, respectively. At 14 days after thrombosis, the Asp, Gly, and GABA contents recovered, whereas the Glu level increased by 275.5% ($p < 0.05$). The taurine (Tau) level did not change during the experiment.

An excess of glutamate is one of the main pathological signs of ischemia. Glutamate is a principal excitatory neurotransmitter in the retina. It is released by photoreceptors, as well as bipolar and ganglion cells [20]. If the glutamate content remains at an increased level for a relatively long period it may result in the death of excited neurons. This mechanism of cell death is considered as excitotoxicity.

Retinal ischemia is also associated with extracellular accumulation of neurotransmitters, including GABA, glycine, dopamine, and acetylcholine. An increase in the levels of inhibitory transmitters is

Table 3. The free-radical state of the retina at various time points after ischemia (mean ± standard deviation)

Group	TBA-reactive products, nmol/mg of protein	SH-groups, μmol/mg of protein	SOD, UA/mg of protein	G-per, nmol NADPH/min mg of protein	G-tr, μmol CDNB/ min mg of protein
Control ($n = 16$)	10.23 ± 1.09	15.48 ± 0.88	3.72 ± 0.39	15.58 ± 0.61	0.43 ± 0.04
1 h ($n = 13$)	11.03 ± 0.85	15.98 ± 0.88	4.02 ± 0.61	16.13 ± 0.66	0.46 ± 0.08
1 day ($n = 14$)	11.87 ± 1.30	13.73 ± 0.84*	3.08 ± 0.32	14.08 ± 0.98*	0.277 ± 0.06*
3 days ($n = 14$)	14.97 ± 1.07*	12.72 ± 1.65*	3.17 ± 0.54*	13.7 ± 0.89*	0.31 ± 0.059*
5–7 days ($n = 6$)	13.78 ± 1.23*	13.41 ± 1.69*	4.13 ± 0.50	14.62 ± 0.74	0.34 ± 0.09
14 days ($n = 6$)	11.63 ± 0.74	13.22 ± 1.39*	4.20 ± 0.29	16.03 ± 1.03	0.39 ± 0.052

SOD, superoxide dismutase; G-per, glutathione peroxidase; G-tr, glutathione-S-transferase. * $p < 0.05$, significant differences as compared to intact animals.

thought to play a protective role and to attenuate the effect of glutamate due to the prevention of depolarization [21].

Experimental thrombosis of retinal vessels was accompanied by the development of oxidative stress; data on biochemical changes are presented in Table 3. The content of TBA-reactive products increased 1 day after ischemia–reperfusion. The maximum level, i.e., 46.3% higher as compared to the level in intact animals ($p < 0.05$), was found 3 days after thrombosis; it then gradually decreased. At 14 days after the treatment, it was similar to the norm. The content of nonprotein SH-groups decreased 1 day and remained lower up to 14 days after thrombosis. The minimum level, i.e., 17.8% lower than in the norm ($p < 0.05$), was observed 3 days after thrombosis. SOD activity decreased by 14.8% ($p < 0.05$) 3 days after thrombosis as compared to intact animals. The activity of G-per and G-tr was decreased on the first day and third days after thrombosis by 9.6 and 35.6% ($p < 0.05$), respectively, and by 12.1 and 27.9% ($p < 0.05$), respectively.

We believe that substantial and relatively rapid generation of free radicals may inhibit normal cellular antioxidant protective mechanisms, which results in oxidative stress and various tissue lesions [22]; our data support this conclusion.

Thus, in this study we demonstrated that an experimental model of photoinduced thrombosis of retinal vessels reproduces the main stages of this pathology, such as metabolic dysfunction of retinal neurons, accumulation of glutamate, and development of oxidative stress.

CONCLUSIONS

1. Intravenous administration of Bengal rose and focused irradiation of retinal vessels with white light allowed us to produce a typical picture of thrombosis of retinal vessels.

2. This experimental model reproduces the main stages of this pathology, such as metabolic dysfunction of retinal neurons, accumulation of glutamate, and development of oxidative stress.

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