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Research Article

Assessment of the correction of neuroretinal changes using 2-ethyl-6-methyl-3-hydroxypyridinium *N*-acetyltaurinate in the modeling of POAG

Svetlana V. Efimenko[1], Mikhail V. Korokin[1], Oleg S. Gudyrev[1], Ludmila M. Danilenko[1], Lilia V. Korokina[1], Aleksandr A. Dolzhikov[1], Tatyana G. Pokrovskaya[1], Anastasia A. Ulyanova[1], Nikita S. Zhunusov[1], Yana S. Kochergina[1], Anton P. Danilenko[1], Anna A. Peresypkina[1]

1. Belgorod State National Research University, 85 Pobedy St., Belgorod 308015 Russia

Corresponding author: Anna A. Peresypkina (peresypkina_a@bsu.edu.ru)

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Abstract

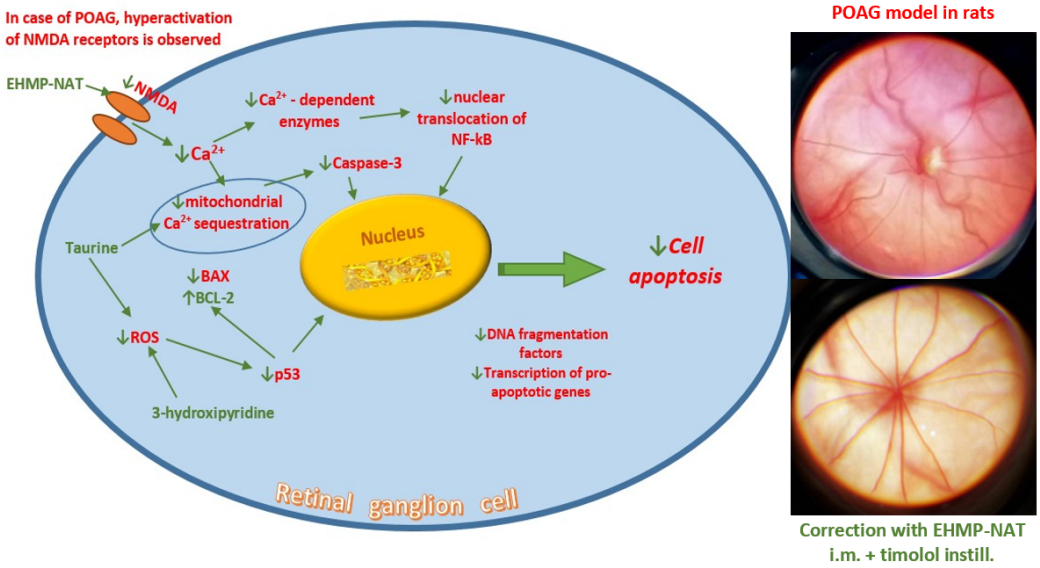
Introduction: Elevated intraocular pressure (IOP) has been identified as a major risk factor for the progression of primary open-angle glaucoma (POAG). However, the continued progression of POAG in some patients, despite the successful IOP reduction, indicates that IOP-independent mechanisms contribute to the development of optical neuropathy. Thus, it is relevant to further determine the molecular mechanisms of retinal ganglion cells (RGCs) death in order to develop therapeutic approaches independent of IOP in order to stop the progression of the disease. **The aim:** to research the correction possibility of experimental POAG induced by intracameral (i.c.) administration of hyaluronic acid using 2-ethyl-6-methyl-3-hydroxypyridinium *N*-acetyltaurinate.

Material and Methods: The study of the correction of neuroretinal changes on a model of POAG was carried out on Wistar rats. In order to correct POAG, 2-ethyl-6-methyl-3-hydroxypyridinium *N*-acetyltaurinate (EHMP-NAT) was used at doses of 27.5 mg/kg intramuscularly (i.m.) and 0.5 mg/kg instillationally. Further, the effectiveness of the combination of timolol eye drops with i.m. EHMP-NAT was studied. The effectiveness of the studied agents was evaluated on the 67th day of the experiment using ophthalmoscopic semi-quantitative assessment of the fundus condition, ocular tonometry, estimation of oxidative stress markers and markers involved in retinal apoptosis pathway using enzyme-linked immunosorbent assay (ELISA).

Results: On the model of POAG, it was shown that 2-ethyl-6-methyl-3-hydroxypyridinium *N*-acetyltaurinate with the laboratory code EHMP-NAT has a pronounced neuro-, retinoprotective action based on the data of ophthalmoscopy, semi-quantitative assessment of the fundus condition, ocular tonometry, estimation of oxidative stress markers (GSH, CAT, SOD) and markers involved in retinal apoptosis (BAX, BCL-2, Caspase-3). On the 67th day of the experiment, the results of a comprehensive analysis revealed that the combination of EHMP-NAT 27.5 mg/kg i.m. + timolol 0.04 mg/kg instillationally has a more pronounced protective effect than EHMP-NAT 27.5 mg/kg i.m. in monotherapy in the POAG model.

Conclusion: The data obtained indicate the need for the combined use of drugs with a hypotensive effect to normalize IOP with neuroretinoprotective agents in the correction of POAG.

Graphical abstract



Keywords

2-ethyl-6-methyl-3-hydroxypyridinium *N*-acetyltaurinate, primary open-angle glaucoma, retinoprotection, neuroprotection

Introduction

Life expectancy has been increasing in all countries in recent decades. There is an increase in both the number and the proportion of elderly people. According to a report by the World Health Organization, between 2015 and 2050, the proportion of the world's population over the age of 60 will almost double from 12% to 22% (Cordeiro et al. 2024). The aging of the population is accompanied by an increase in the prevalence of diseases that threaten vision, including glaucoma, age-related macular degeneration, diabetes and diabetic retinopathy, as well as cataracts (GBD 2019 Blindness and Vision Impairment Collaborators 2021).

Glaucoma is a heterogeneous group of diseases characterized by damage to the optic nerve disc and visual field defects due to the loss of retinal ganglion cells (RGCs) (Efimenko et al. 2023). According to some authors, the expected prevalence of glaucoma in the Russian Federation by 2025 will amount to 1,408.5 thousand per 100,000 adult population (Movsisyan et al. 2022). The total number of cases of primary open-angle glaucoma (POAG) in the world was about 68.6 million in 2020, and by 2040, it is expected to grow to 111.8 million (Zhang et al. 2021).

Glaucoma is the most common cause of irreversible blindness worldwide. This disease is divided into primary and secondary. Primary glaucoma refers to the presence of glaucoma in the absence of any other eye diseases. It includes POAG, primary closed-angle shape and primary congenital glaucoma. In case of POAG, the diagnosis is based on the exclusion of other types of glaucoma. Currently, POAG accounts for more than half of glaucoma cases worldwide (Ju et al. 2023). It is the most common form of glaucoma. POAG affects approximately 45 million people worldwide (Lu et al. 2017).

Increased IOP has been identified as the main risk factor for the development and progression of POAG; therefore, drug treatment of glaucoma is aimed at reducing IOP in order to stop the progression of the disease (Alekseev et al. 2022). In addition, POAG therapy has a pathogenetic orientation; treatment is focused not only on the level of tolerant IOP, but also on the correction of hypoxia, neuroprotection, and correction of metabolic disorders. It is also important to treat concomitant diseases that adversely affect the course of the glaucomatous process (Getmanova et al. 2020).

Derivatives of 3-hydroxypyridine are known to have neuroprotective, antioxidant effects in the correction of ischemic eye damage (Peresypkina et al. 2020). It has been shown that after ischemic reperfusion injury, treatment with antiapoptotic agents was effective for preserving cellular populations in the retina (Ulbrich et al. 2016; Jiang et al. 2020). Caspases are the main regulators of RGCs degeneration. Caspase-3 and caspase-9 are involved in RGCs degeneration after axotomy (Weishaupt et al. 2003). It is believed that transcription factors, NF-kB, p53, and AP-1, contribute to the generation of apoptotic cascades in RGCs damage. The proapoptotic protein Bax is activated in both NF-kB and p53-mediated apoptosis after axon damage; p53-mediated apoptosis of RGCs is accompanied by an increase in Bax and Bid levels and activation of the caspase-3-dependent signaling

cascade (Lambuk et al. 2021). Prolonged activation of NMDA receptors activates the proapoptotic signaling pathway, leading to the death of RGCs in glaucoma and retinal ischemia. This is confirmed by activation of apoptosis in NMDA-induced excitotoxicity in experimental rats, mice, rabbits and primates (Sakamoto et al. 2017).

Based on the review of the existing glaucoma models and taking into account the nature of the course and pathogenesis of POAG, we chose for the experiment a model with the administration of hyaluronic acid into the anterior chamber of the eye. The values of IOP in the eyes with the administration of hyaluronic acid remain high and stable from the seventh to the tenth week of the study (Benozzi et al. 2002). This method is accompanied by a prolonged and moderate increase in IOP and is easy to perform.

The object of this study was a new compound – 2-ethyl-6-methyl-3-hydroxypyridinium *N*-acetyltaurinate with the laboratory code EHMP-NAT. As is known, 3-hydroxypyridine has an antioxidant effect and contributes to the correction of glutamate excitotoxicity (Voronina 2012). The level of taurine in the retina is a crucial factor in preventing loss of RGCs. Taurine can prevent RGCs degeneration *in vitro* and *in vivo*, which has been shown in rats with glaucoma and in a retinitis pigmentosa model (P23H rats) (Froger et al. 2013). In addition, taurine is able to partially prevent NMDA-induced excitotoxicity (Froger et al. 2012). In connection with the above, the study of the neuro-, retinoprotective effect of EHMP-NAT in the correction of POAG in the experiment was relevant.

The aim of the current study was to research the correction possibility of experimental POAG induced by intracameral (i.c.) administration of hyaluronic acid using EHMP-NAT.

Materials and Methods

POAG modeling

Experimental studies were approved by the Local Ethics Committee of Belgorod State National Research University (Minutes No. 6/22 of March 21st, 2022). The study was conducted on male Wistar rats aged 4 months weighing 225-275 g. Ethical principles of handling laboratory rats were observed in accordance with the European Convention for the Protection of Vertebrate Animals Used for Experimental and Other Scientific Purposes and Directive 2010/63/EU.

Experimental POAG was induced in rats using a series of i.c. injections of hyaluronic acid, one injection per week for 9 consecutive weeks (Kalatanova et al. 2021). To increase and maintain IOP levels throughout the study period, we used the glaucoma model described by Benozzi et al. (2002). Using a Hamilton syringe (Hamilton GASLIGHT Syringes, 1700 Series; Sigma-Aldrich Corp., USA) with a 30-gauge needle, a corneal puncture was performed with the needle beveled upwards closer to the scleral-corneal limb. Twenty-five µL of viscoelastic substance, hyaluronic acid, was slowly injected into the anterior chamber of both eyes of rats at a dose of 10 mg/mL. Before performing all painful manipulations, the rats were injected with chloral hydrate at a dose of 300 mg/kg. After manipulation, the antibiotic tobramycin was injected topically into both eyes in the form of 0.3% eye drops (Tobrex® ALCON-COUVREUR N.V., S.A., Belgium) twice a day.

Experiment design

There were 10 animals in each group. The experiment included the following groups:

1 – intact;
2 – control (saline, i.c.);
3 – POAG simulation;
4 – POAG + EHMP-NAT 27.5 mg/kg intramuscularly (i.m.);
5 – POAG + EHMP-NAT 0.5 mg/kg instillationally;
6 – POAG + EHMP-NAT 27.5 mg/kg i.m. + timolol 0.04 mg/kg instillationally.

From the 63rd day of the experiment, pharmacological agents were administered to the rats daily for 4 days. In order to correct POAG, EHMP-NAT was used at a dose of 27.5 mg/kg i.m. (Efimenko et al. 2023) and at a dose of 0.5 mg/kg instillationally. In the control group, an equivalent volume of 25 µL of saline was administered in a series of i.c. injections, one injection per week for 9 consecutive weeks. Timolol was administered at a dose of 0.04 mg/kg in the form of eye drops 0.5% (S.C. ROMPHARM Company, S.R.L., Romania) in combined therapy with i.m. EHMP-NAT. The doses and modes of administration of pharmacological agents are based on efficacy in experimental studies, or obtained from therapeutic doses for humans, followed by recalculation for rats using interspecific coefficients.

The effectiveness of the studied agents in the POAG model was evaluated on the 67th day of the experiment using ophthalmoscopic semi-quantitative assessment of the fundus condition, ocular tonometry, estimation of oxidative stress markers and markers involved in retinal apoptosis pathway using enzyme-linked immunosorbent assay (ELISA).

Ophthalmoscopic semi-quantitative assessment of the fundus condition

On the 67th day of the experiment, the rats were anesthetized and ophthalmoscopy was performed using a V78C lens (Volk Optical, USA) followed by a semi-quantitative assessment of fundus changes. For mydriasis, phenylephrine was used conjunctivally in eye drops Neosinephrine-POS® 50 mg/mL (Ursafarm Artsnaimittel GmbH, Germany). A solution of eye drops was injected into the conjunctival cavity using a micropipette in terms of body mass of rats. The estimated dose of Neosinephrine-POS® was 0.004 mL/kg.

For further data processing, the degree of fundus changes in modeling and correction of POAG was estimated in points (Table 1).

Ocular tonometry

On the 63rd day of the experiment (before the start of treatment), a lifetime control of the formation of pathology was performed; the level of IOP was determined. By the method of rebound tonometry using the ophthalmological veterinary tonometer Icare TonoVet (Finland), IOP was measured in conscious rats without sedation, according to the manufacturer's instructions. IOP measurements were performed on the right eye of all rats at the same time (8:00-9:00 a.m.) to prevent circadian oscillations. Six IOP values were obtained for each eye in close contact with the cornea. The average value of these readings was recorded as IOP for this eye. The obtained average value was used to calculate the average IOP value for the groups. On the 67th day of the experiment, the rats underwent IOP registration (Efimenko et al. 2023).

Table 1. The method for ophthalmoscopic semi-quantitative assessment of the fundus changes in the modeling and correction of POAG

Glaucoma Stages	Points
Norm. The optic disk is round or oval, pale pink. It has clear boundaries, lies in the plane of the retina. The central retinal vessels come out of the middle of the optic disk. The veins and arteries are straight, their caliber is uniform, no tortuosity. The retina is pink.	0
The initial stage. The optic disk is round or oval and pale pink. It has clear boundaries, lies in the plane of the retina. The optic disk excavation is expanded, but does not reach the edge of the disk. The outlet of the central retinal vessels (CRV) is shifted (nasal excavation). The veins and arteries are straight, their caliber is uniform, no tortuosity. The retina is pink.	1
The advanced stage. The optic disk is round or oval, stands out against the background of the retina in a grayish color. It has clear boundaries, lies in the plane of the retina. The optic disk excavation is expanded, in some departments it reaches the edge of the disc. The outlet of the CRV is shifted significantly. There is a tortuosity of the vessels, a change in caliber. The retina is pink.	2
The far-advanced stage. The optic disk is round or oval, stands out against the background of the retina in gray. It has clear boundaries, lies in the plane of retina. The optic disk excavation is expanded, the marginal subtotal, reaches its edge. The output of the CRV is shifted significantly. There is a tortuosity of the vessels, a change in caliber. The retina is pale pink.	3
The terminal stage (the POAG model). The optic disk is round or oval, gray. The disk has fuzzy borders. The neuronal girdle is pale, almost undefined. Displacement and exposure of the vascular bundle (total excavation). Hemorrhages are observed on the surface of the disc. The retina is grayish-pale, thinning of the retinal nerve fiber layer.	4

Estimation of oxidative stress markers and markers involved in retinal apoptosis pathway

Reduced Glutathione (GSH)

Retinal concentration of GSH was determined by an enzymatic recycling method in which glutathione reductase is used for the quantification of GSH. In this assay kit, the sulfhydryl group from GSH reacts with 5,5'-dithio-bis-2- nitrobenzoic acid and Ellman's reagent to produce a yellow coloured 5-thio-2- nitrobenzoic acid which then produces a mixed disulfide, which is then reduced by glutathione reductase to recycle the production of GSH. The production of 5-thio-2-nitrobenzoic acid is directly proportional to the concentration of GSH in the sample. The solution was mixed using a vortex mixer. Then, the mixture was centrifuged at 2000 xg for 2 minutes at 4°C, and the supernatant was collected carefully. Then, 50 µL of 4 M triethanolamine reagent was added to supernatant and immediately mixed using vortex mixer. 50 µL of sample mixtures and standards were pipetted into designated wells and then 150 µД of freshly prepared assay cocktail containing MES buffer, reconstituted cofactor mixture, reconstituted Enzyme Mixture, and reconstituted 5,5'-dithio-bis-2- nitrobenzoic acid were added to each well. The well was incubated in a dark room, on an orbital shaker for 25 minutes at room temperature. The absorbance was measured at 405 nm after incubation using microplate reader.

Catalase (CAT)

The activity of CAT in this study was determined by utilizing the peroxidative activity based on the reaction between CAT with methanol in H_2O_2 which produces formaldehyde. Formaldehyde reacts with 4-amino-3-hydrazino-5-mercapto-1, 2, 4-triazole to form a bicyclic heterocycle. Upon oxidation, 4-amino-3-hydrazino-5-mercapto changes from colourless to purple in colour. CAT activity was measured based on the amount of formaldehyde concentration produced from the samples. One unit of enzyme activity was defined as the amount of CAT that causes formation of 1.0 µmol of formaldehyde per minute at 25ºC. For the assay, retinal tissues were homogenized in 50 mM cold potassium phosphate pH 7.0 containing EDTA by sonication for 1 minute in a 2 mL vial on ice prior to centrifugation at 10,000 xg for 15 minutes at 4°C. Twenty µL of the collected supernatants and standard were added to each well. Then, 100 µL of diluted assay buffer and 30 µL of methanol were added. To initiate the reactions, 20 µL of diluted hydrogen peroxide was added, and the well plate was incubated on a shaker for 20 minutes at room temperature. The reaction was terminated by adding 20 µL of diluted potassium hydroxide followed by 30 µL of CAT purpald. Next, the well plate was incubated for 10 minutes on a shaker at room temperature. Finally, 10 µL of CAT potassium periodate was added and incubated for 5 minutes at room temperature. The absorbance was read at 540 nm using a plate reader.

Superoxide dismutase (SOD)

The SOD assay kit used in this study utilizes tetrazolium salt for the detection of superoxide free radicals generated by xanthine oxidase and hypoxanthine. This assay kit measures all types of SOD. For the assay, retinal tissues were homogenized in cold 20 mM HEPES buffer, pH 7.2 containing 1 mM EGTA, 210 mM mannitol, and 70 mM sucrose, by sonication for 1 minute in a 2 mL vial on ice. The homogenized tissue was then centrifuged at 1,500 xg for 5 minutes at 4°C. Ten µL of collected supernatants and standards and 200 µL of diluted radical detector were added into the wells. Then, 20 µL of diluted xanthine oxidase was added in order to initiate the reaction. The plate was placed on a shaker for a few seconds to mix all the reagents well. The plate was then incubated on a shaker for 30 minutes at room temperature. The absorbance was read at 450 nm using a microplate reader.

BAX, BCL-2 and Caspase-3

Retinal BAX, BCL-2 and Caspase-3 levels were measured using commercially available ELISA kits. Dissected retinal tissues were rinsed in ice cold PBS (0.01 M, pH7.4) followed by homogenization in PBS. The homogenates were then centrifuged for 5 min at 5000 xg and supernatant was collected. One hundred µL of standard working solution containing 0.04 % Proclin 300 was added to each well and incubated at 37°C for 90 min. One hundred µL of Biotinylated Detection Ab working

solution containing antibody specific for rat BAX/BCL-2/Caspase-3 were added to each well and mixed up gently followed by incubation at 37°C for 1 hour. Three hundred fifty µL of wash buffer containing 0.05 M Tris, 0.138 M NaCl, 0.0027 M KCl, pH 8.0, with 0.05% Tween 20 was added to each well and soaked for 2 min before being aspirated. This process was repeated three times. Horseradish peroxidase conjugate working solution containing enzyme that catalyzes the oxidation of diaminobenzidine was added and incubated at 37°C for 30 min. Next, the washing process with wash buffer was repeated five times. Ninety µL of Substrate Reagent containing 3,3',5,5'-tetramethylbenzidine was added to each well and incubated at 37°C for 15 minutes. Finally, 50 µL of Stop Solution containing 9.8% sulfuric acid to react with ELISA substrate 3,3',5,5' – tetramethylbenzidine (TMB) was added. In the presence of horseradish peroxidase enzyme conjugates, TMB and peroxide react to produce a blue byproduct having maximum absorbance at 605 nm. The absorbance was read at 450 nm using microplate reader.

Statistical data processing

The data were checked for type of distribution. In normal distribution, the average value (M) and standard error of the mean (m) were calculated. In abnormal distribution, the median (Me) and the quartile range (QR) were calculated. Between-group differences were analyzed by parametric (t-Student criterion) or non-parametric (Mann-Whitney U-test) methods. The differences were determined at a 0.05 significance level. The statistical analyses were performed using Statistica 10.0 software (StatSoft, USA).

Results

Results of ophthalmoscopic examination and semi-quantitative assessment of the fundus condition

On the 67th day of the experiment, all groups of rats were anesthetized for ophthalmoscopy. The general view of the eye fundus of animals from groups 1 and 2 did not differ and is shown in Figure 1 A. The picture has the following form: optic disk of a round shape, pale pink; the boundaries are clear. It does not penetrate into the vitreous body; the center of the retina and the periphery are pink, not thinned, and tightly adheres to the choroid. The vascular bundle exits from the center of the optic disc.

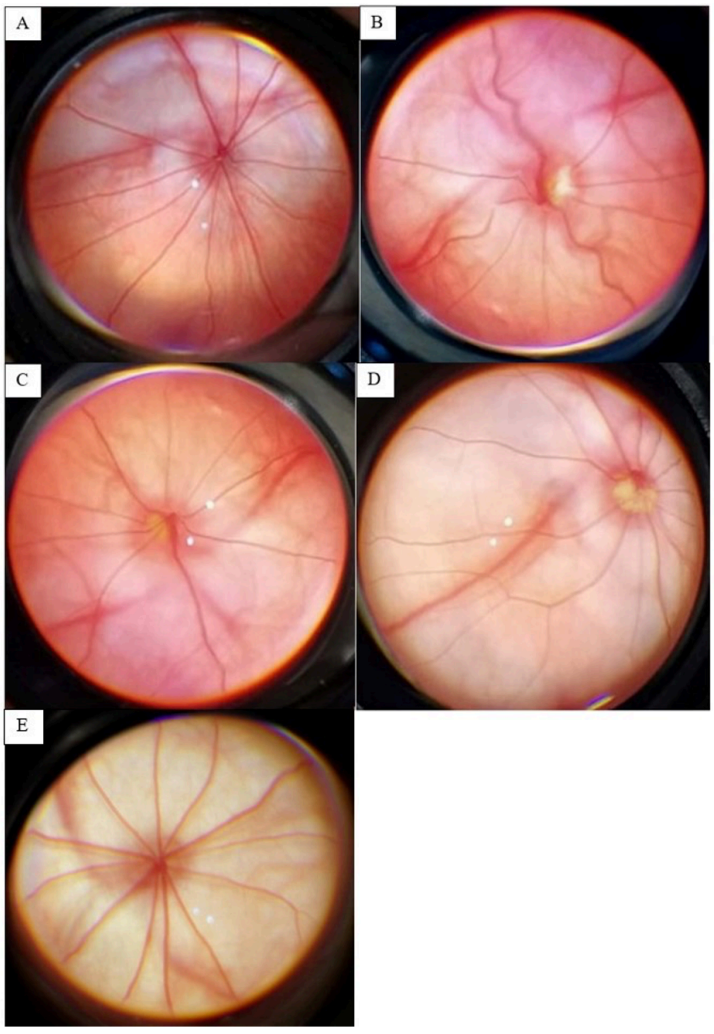


Figure 1. Eye fundus of Wistar rats from: **A** – control group; **B** – POAG simulation group; **C** – group with POAG + EHMP-NAT 27.5 mg/kg i.m.; **D** – group with POAG + EHMP-NAT 0.5 mg/kg instillationally; **E** – group with POAG + EHMP-NAT 27.5 mg/kg i.m. + timolol 0.04 mg/kg instillationally.

The picture of the eye fundus of a rat when modeling the POAG is shown in Figure 1 B. The picture has the following form: optic disc is pale; the boundaries are indistinct; the vascular bundle is shifted. The arteries are narrowed; the veins are dilated. The retina is pale in some parts.

The eye fundus during correction by EHMP-NAT at a dose of 27.5 mg/kg i.m. has the following description: optic disc is round or oval, pale pink; it has clear boundaries and lies in the plane of the retina. The excavation of the disc is expanded, but does not reach its edge. The output of the CRV is shifted. The veins and arteries are straight; the caliber is uniform; there is no tortuosity. The general background of the retina is pink (Fig. 1 C).

With instillation administration of EHMP-NAT at a dose of 0.5 mg/kg, the eye fundus did not differ from the POAG model (Fig. 1 D).

The general view of the eye fundus of the rat from group with a combination of EHMP-NAT and timolol is as follows: optic disc is round or oval, pale pink; the boundaries are clear. It does not penetrate into the vitreous body; the retina is pink, not thinned, and fits snugly to the choroid. The vascular bundle exits from the center of the optic disc (Fig. 1 E).

The results of ophthalmoscopic semi-quantitative assessment of the fundus condition in the experimental groups are presented in Table 2.

In the groups with instillation administration of EHMP-NAT, no significant differences were found from the group with the POAG simulation, probably due to the lack of this agent reaching the posterior segment of the eye. There was an improvement in the eye fundus condition in the groups with i.m. administration of EHMP-NAT. The observed improvement is probably related to the antioxidant potential of EHMP-NAT in retinal and optic nerve tissues and inhibition of retinal excitotoxicity (Efimenko et al. 2023).

Table 2. Ophthalmoscopic semi-quantitative assessment of the fundus condition in the experimental groups (M ± m; n = 10).

Groups	Points
Intact	0.2 ± 0.1^{z}
Control	0.3 ± 0.1^{z}
POAG simulation	3.9 ± 0.2^{xy}
POAG + EHMP-NAT 27.5 mg/kg i.m.	1.1 ± 0.1^{xyz}
POAG + EHMP-NAT 0.5 mg/kg instillationally	3.7 ± 0.3^{xy}
POAG + EHMP-NAT 27.5 mg/kg i.m. + timolol 0.04 mg/kg instillationally	$0.4 \pm 0.1^{z\varepsilon}$

Note: ^{x} – $p < 0.05$, compared to intact group; ^{y} – $p < 0.05$, compared to control group; ^{z} – $p < 0.05$, compared to POAG simulation group; $^{\varepsilon}$ – $p < 0.05$, compared to group with POAG + EHMP-NAT 27.5 mg/kg i.m.

Results of ocular tonometry

The results of the IOP measurement in the experimental groups are shown in Figure 2. On day 63 of the experiment, the average IOP value was significantly higher in the eyes with the introduction of hyaluronic acid than in the eyes of intact animals ($p < 0.01$) and than in control eyes ($p < 0.05$).

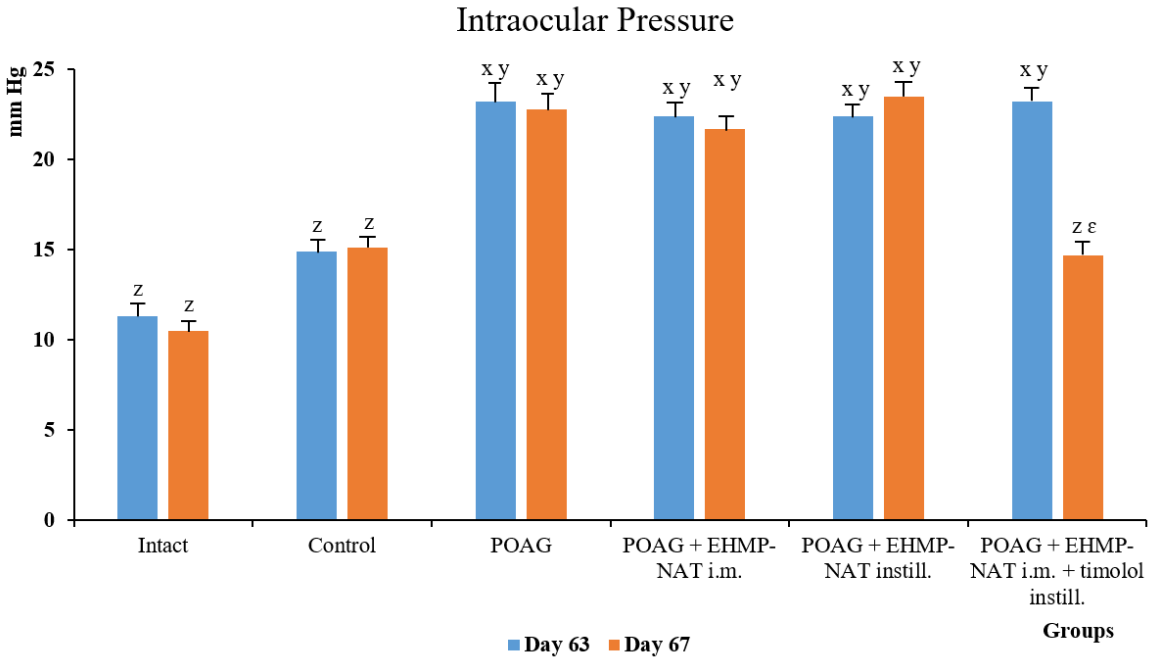


Figure 2. The effect of the combination of EHMP-NAT and timolol in comparison with EHMP-NAT monotherapy on IOP during POAG correction. *Note*: ^{x} – $p < 0.05$, compared to intact group; ^{y} – $p < 0.05$, compared to control group; ^{z} – $p < 0.05$, compared to POAG simulation group; $^{\varepsilon}$ – $p < 0.05$, compared to group with POAG + EHMP-NAT 27.5 mg/kg i.m.

On day 67, the differences between the above-mentioned groups were similar, which indicates a steady increase in IOP over time. At these dates, IOP was slightly higher in the control group than in the group of intact animals. In the groups with EHMP-NAT administration, there was no significant decrease in IOP compared to the group with the POAG model, which indicates the absence of a hypotensive effect in this agent. In the group with a combination of EHMP-NAT and timolol, after 4 days of treatment, there was a significant decrease in IOP in comparison with the group with the POAG model by 35.5% ($p < 0.05$) and by 32.3% ($p < 0.05$) in comparison with the group with monotherapy by EHMP-NAT. In this group, the target values of IOP were achieved after the treatment.

Results of level estimation of oxidative stress markers and markers involved in retinal apoptosis pathway

The retinal level of GSH was lower in POAG group by 4.4 folds ($p < 0.01$) and 4.5 folds ($p < 0.01$) compared to intact and control groups, respectively. Similarly, POAG + EHMP-NAT instill. group showed a lower GSH level compared to intact and control groups by 3.7 folds ($p < 0.01$) and 3.8 folds ($p < 0.01$), respectively. The retinal level of GSH was higher in POAG + EHMP-NAT i.m. + timolol instill. group by 2.4 folds ($p < 0.05$) compared to POAG group. No significant difference was observed in the retinal GSH level between POAG + EHMP-NAT i.m. and POAG + EHMP-NAT i.m. + timolol instill. groups (Fig. 3 A).

Activity of CAT in POAG group was lower compared to intact and control groups by 3.7 folds ($p < 0.01$) and 3.3 folds ($p < 0.01$), respectively. POAG + EHMP-NAT instill. group showed a lower CAT level by 2.8 folds ($p < 0.05$) compared to intact group, but the difference was not significant when compared to POAG group. The CAT level in POAG + EHMP-NAT i.m. + timolol instill. group was higher than in POAG group by 2.5 folds ($p < 0.05$). Again no significant difference was observed in the retinal CAT level between POAG + EHMP-NAT i.m. and POAG + EHMP-NAT i.m. + timolol instill. groups (Fig. 3 B).

The retinal SOD level in POAG group was significantly lower compared to intact group by 1.8 folds ($p < 0.05$) and control group by 1.7 folds ($p < 0.05$). POAG + EHMP-NAT i.m. + timolol instill. group demonstrated a higher SOD level by 1.6 folds ($p < 0.05$) compared to POAG group. POAG + EHMP-NAT i.m. group also showed a higher retinal SOD level compared to POAG group by 1.4 folds ($p < 0.05$) (Fig. 3 C).

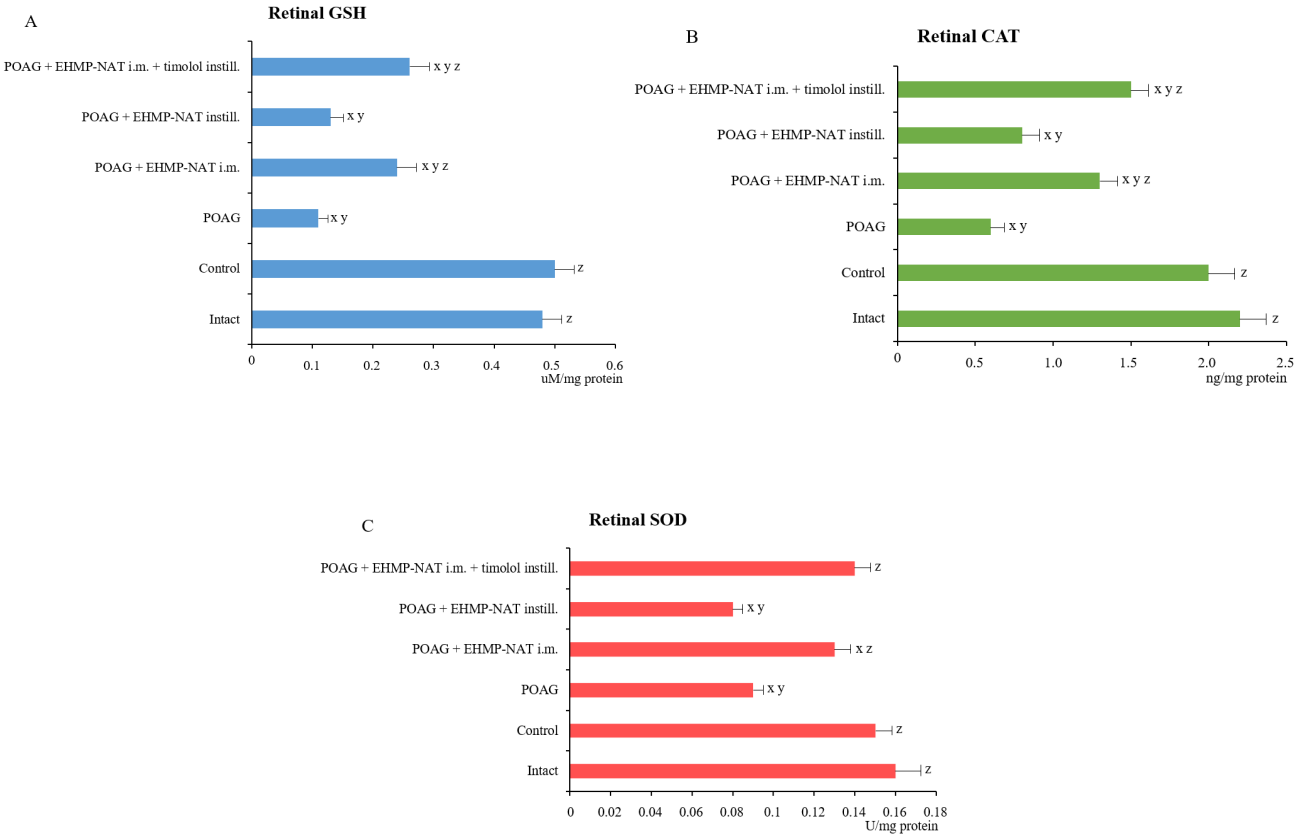


Figure 3. The effect of the combination of EHMP-NAT and timolol in comparison with EHMP-NAT monotherapy against POAG-induced changes of GSH, CAT and SOD levels. *Note*: ^{x} – $p < 0.05$, compared to intact group; ^{y} – $p < 0.05$, compared to control group; ^{z} – $p < 0.05$, compared to POAG simulation group; ^{e} – $p < 0.05$, compared to group with POAG + EHMP-NAT 27.5 mg/kg i.m.

The most prominent downregulation of anti-apoptotic protein BCL-2 level was observed in POAG group compared to both intact and control groups by 3.0 folds ($p < 0.01$). Its downregulation is associated with an increased level of apoptosis (Semeleva et al. 2020). POAG + EHMP-NAT i.m. + timolol instill. group showed a higher BCL-2 level by 2.7 folds ($p < 0.01$) compared to POAG group. POAG + EHMP-NAT i.m. group showed a higher BCL-2 level as compared with POAG and POAG + EHMP-NAT instill. groups by 2.3 folds ($p < 0.01$) and 1.8 folds ($p < 0.05$), respectively. No significant difference was observed in the retinal BCL-2 level between POAG + EHMP-NAT i.m. and POAG + EHMP-NAT i.m. + timolol instill. groups (Fig. 4).

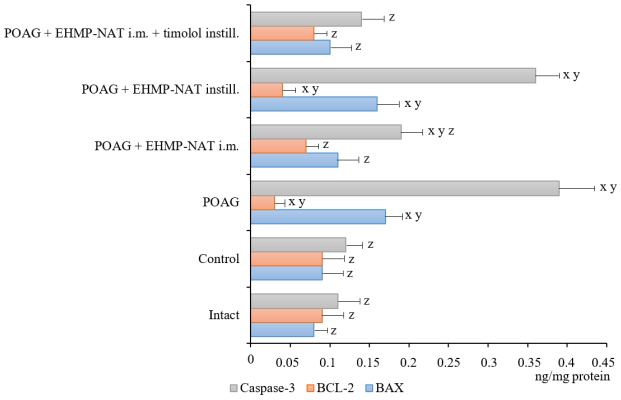


Figure 4. The effect of the combination of EHMP-NAT and timolol in comparison with EHMP-NAT monotherapy against POAG-induced changes of Caspase-3, BCL-2 and BAX levels. ***Note*:** ^{x} – $p < 0.05$, compared to intact group; ^{y} – $p < 0.05$, compared to control group; ^{z} – $p < 0.05$, compared to POAG simulation group; $^{\varepsilon}$ – $p < 0.05$, compared to group with POAG + EHMP-NAT 27.5 mg/kg i.m.

In contrast to the BCL-2 protein level, the expression of the pro-apoptotic BAX protein was the highest in the POAG group with a difference of 2.1 folds ($p < 0.01$) and 1.9 folds ($p < 0.01$) and 1.7 folds ($p < 0.05$) in comparison with intact, control and POAG + EHMP-NAT i.m. + timolol instill. groups, respectively. POAG + EHMP-NAT instill. also demonstrated a higher BAX level compared to intact group by 2.0 folds ($p < 0.01$) and to POAG + EHMP-NAT i.m. + timolol instill. group – by 1.6 folds ($p < 0.05$) (Fig. 4).

Caspase-3 is one of the most important mediators of cellular apoptosis (Porter et al. 1999). Retinal caspase-3 expression in the POAG group was significantly higher than in the intact group by 3.5 folds ($p < 0.001$), in control group – by 3.3 folds ($p < 0.001$) and in EHMP-NAT i.m. + timolol instill. group – by 2.8 folds ($p < 0.01$). It was also observed that POAG + EHMP-NAT i.m. group showed a lower caspase-3 level by 2.1 folds ($p < 0.01$) in comparison to POAG group. No significant difference was observed in the retinal caspase-3 level between POAG + EHMP-NAT i.m. and POAG + EHMP-NAT i.m. + timolol instill. groups (Fig. 4).

Discussion

In our study, we studied the correction possibility of neuroretinal changes using 2-ethyl-6-methyl-3-hydroxypyridinium *N*-acetyltaurinate and its combination with timolol in the modeling of POAG in rats using ophthalmoscopic semi-quantitative assessment of the fundus condition, ocular tonometry, estimation of oxidative stress markers and markers involved in retinal apoptosis pathway using ELISA. On the 67th day of the experiment, the results of a comprehensive analysis revealed that the combination of EHMP-NAT 27.5 mg/kg i.m. + timolol 0.04 mg/kg instill. has a more pronounced protective effect than EHMP-NAT 27.5 mg/kg i.m. in monotherapy in the POAG model. The data obtained indicate the need for the combined use of drugs with a hypotensive effect to normalize IOP with neuroretinoprotective agents in the correction of POAG.

Several studies have shown that in glaucoma, an increase in extracellular glutamate activates NMDA-receptors, causing an influx of Ca^{2+} into the RGCs. Then, under the action of NO synthase, NO is produced in the mitochondria, which leads to the formation of $ONOO^{-}$ (Lambuk et al. 2017). Stimulation of the NMDA-receptor activates NF-kB and plays a role in ROS-induced apoptosis of RGCs. It has been shown that NF-kB p65 is activated in NMDA-mediated excitotoxicity in the retina, as well as in other neurodegenerative pathologies (McInnis et al. 2002). Our previous study demonstrated that a series of i.c. injections of hyaluronic acid to Wistar rats for 9 weeks increases the expression of the NF-kB p65 gene in the retina on day 67 of the experiment. It is likely that the observed changes in NF-kB expression during this time period are associated with a reaction to NMDA activation in glial and RGCs. Correction of EHMP-NAT significantly reduced the expression of the NF-kB p65 and p53 genes in the POAG model (Efimenko et al. 2023). Overexpression of BAX is accompanied by p53 upregulated modulator of apoptosis (PUMA), a member of the BCL-2 family as a pro-apoptotic protein. PUMA plays a crucial role in p53-mediated apoptosis. *In vitro* overexpression of PUMA corresponds to BAX expression, a change in BAX conformation, translocation into mitochondria, release of cytochrome C and a decrease in the membrane potential of mitochondria. NOXA is another member of the Bcl-2 family involved in pro-apoptosis as a mediator of p53-induced apoptosis. Studies show that the activation of caspase-9 is associated with the localization of NOXA in mitochondria, therefore inhibiting the anti-apoptotic effects of members of the BCL-2 family (Oda et al. 2000).

Apart from the excessive influx of Ca^{2+}, another potent inductor of cell apoptosis is oxidative stress that occurs when there is an excess production of ROS and RNS, compared to the number of antioxidants available to neutralise them. In excess, ROS and RNS cause direct damaging effects or activate the mitochondrial pathway of apoptosis by activating p53 and/or JNK signalling, subsequently stimulating the BAX proteins that may inhibit the anti-apoptotic BCL-2 proteins (Yu et al. 2023). In our study, POAG-induced retinal damage was associated with a low level of antioxidants GSH, CAT and SOD, suggestive of an increase in retinal oxidative stress. In the current study, treatment with the combination of EHMP-NAT i.m. and timolol instill. and EHMP-NAT i.m. in monotherapy significantly improved the retinal antioxidant enzymes levels.

Despite the absence of a hypotensive effect of EHMP-NAT, we found its retinoprotective effects in the POAG model, which confirms the need in the correction of addition to reducing IOP. We propose that the detected

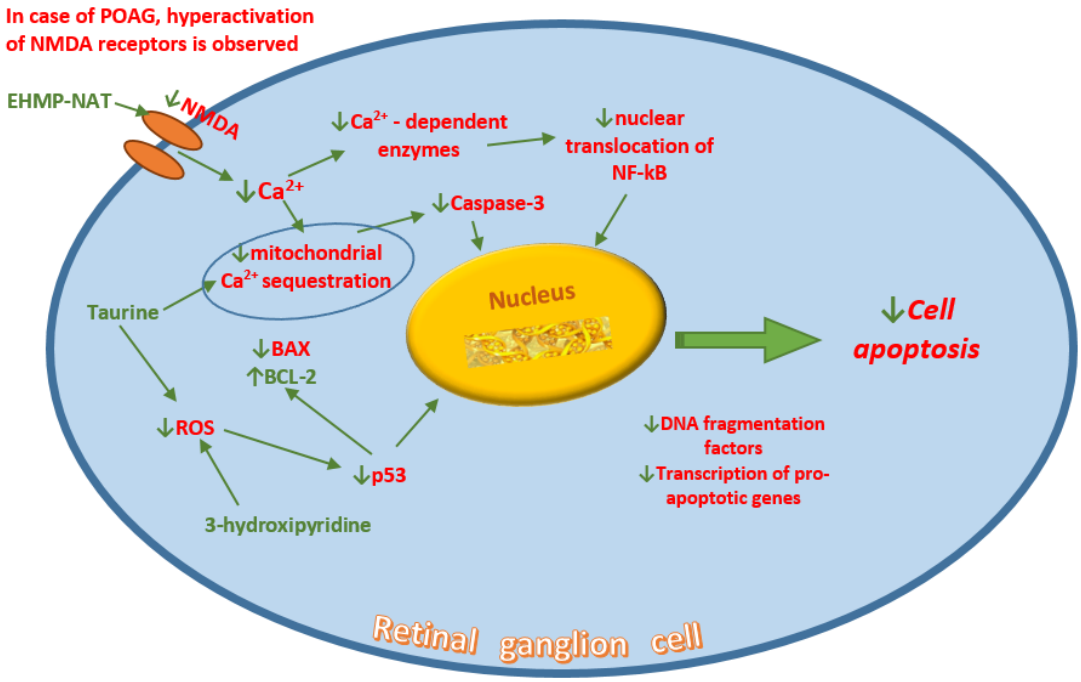


Figure 5. The implementation mechanism of neuro-; retinoprotective action by EHMP-NAT in POAG. *Note:* BAX – BCL-2 - associated X protein, BCL-2 – B-cell lymphoma 2, DNA – deoxyribonucleic acid, EHMP-NAT – 2-ethyl-6-methyl-3-hydroxypyridinium *N*-acetyltaurinate, NF-kB – nuclear factor kappa B, NMDA – N-methyl-D-aspartate, POAG – primary open-angle glaucoma, ROS – reactive oxygen species.

neuro-, retinoprotective effect of EHMP-NAT in the correction of experimental POAG may be related to the following: 3-hydroxypyridine is an "absorber" of peroxides and superoxides of fatty acids, which has antioxidant and anticytotoxic effects in the retina (Peresypkina et al. 2020); taurine, an antioxidant and the predominant free amino acid in the retina, which maintains the structural integrity of membranes, regulates Ca^{2+} binding and transport, and inhibits excitotoxic damage to the retina (Schaffer et al. 2018). The hypothetical mechanism of implementation of neuro-, retinoprotective action by EHMP-NAT in POAG is shown in Figure 5.

Conclusion

On the model of POAG, it was shown that 2-ethyl-6-methyl-3-hydroxypyridinium *N*-acetyltaurinate with the laboratory code EHMP-NAT has a pronounced neuro-, retinoprotective action based on the data of ophthalmoscopy, semi-quantitative assessment of the fundus condition, ocular tonometry, estimation of oxidative stress markers (GSH, CAT, SOD) and markers involved in retinal apoptosis (BAX, BCL-2, Caspase-3). On the 67th day of the experiment, the results of a comprehensive analysis revealed that the combination of EHMP-NAT 27.5 mg/kg i.m. + timolol 0.04 mg/kg instillationally has a more pronounced protective effect than EHMP-NAT 27.5 mg/kg i.m. in monotherapy in the POAG model. The data obtained indicate the need for the combined use of drugs with a hypotensive effect to normalize IOP with neuroretinoprotective agents in the correction of POAG.

Conflict of interests

Authors declare no conflict of interests.

Data availability

All of the data that support the findings of this study are available in the main text

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Author contributions

- **Svetlana V. Efimenko**, MD, Postgraduate student of the Department of Pharmacology and Clinical Pharmacology, Belgorod State National Research University, Belgorod, Russia; e-mail: spotapova96@mail.ru; **ORCID ID** https://orcid.org/0000-0002-1041-9978. The author took part in conceptualization and direction development of the research, generation of key aims and objectives conducting experimental work, analysing the material, and writing the text of the article.

- **Mikhail V. Korokin,** Doct. Sci. (Medicine), Professor of the Department of Pharmacology and Clinical Pharmacology, Belgorod State National Research University, Belgorod, Russia; e-mail: mkorokin@mail.ru, **ORCID ID** https://orcid.org/0000-0001-5402-0697. The author took part in direction development of the research and in the analysis of the material.

- **Oleg S. Gudyrev**, Cand. Sci. (Medicine), Associate Professor of the Department of Pharmacology and Clinical

Pharmacology, Belgorod State National Research University, Belgorod, Russia; e-mail: gudyrev@mail.ru; **ORCID ID** https://orcid.org/0000-0003-0097-000X. The author took part in processing the results obtained.

- **Lyudmila M. Danilenko**, Doct. Sci. (Pharmacy), Professor of the Department of Pharmacology and Clinical Pharmacology, Belgorod State National Research University, Belgorod, Russia; e-mail: Danilenko_L@mail.ru; **ORCID ID** https://orcid.org/0000-0001-6101-8712. The author was engaged in collection and interpretation of the data for the paper.

- **Liliya V. Korokina**, Cand. Sci. (Medicine), Associate Professor of the Department of Pharmacology and Clinical Pharmacology, Belgorod State National Research University, Belgorod, Russia; e-mail: korokina@bsu.edu.ru; **ORCID ID** https://orcid.org/0000-0002-4115-1564. The author was engaged in conducting the literature analysis.

- **Aleksandr A. Dolzhikov**, Doct. Sci. (Medicine), Professor, Professor of the Department of Human Anatomy and Histology, Belgorod State National Research University, Belgorod, Russia; e-mail: dolzhikov@bsu.edu.ru, **ORCID ID** https://orcid.org/0000-0001-7425-8416. The author took part in conceptualization and direction development of the research, and interpretation of the data for the paper.

- **Tatyana G. Pokrovskaya**, Doct. Sci. (Medicine), Professor of the Department of Pharmacology and Clinical Pharmacology, Belgorod State National Research University, Belgorod, Russia; e-mail: pokrovskaia-tg@mail.ru, **ORCID ID** https://orcid.org/0000-0001-6802-5368. The author took part in writing the article and processing the results obtained.

- **Anastasia A. Ulyanova**, postgraduate student of the Department of Pharmacology and Clinical Pharmacology, Belgorod State National Research University, Belgorod, Russia; e-mail: ul-nastya@mail.ru; **ORCID ID** https://orcid.org/0009-0007-8345-4209. The author took part in enzyme-linked immunosorbent assay.

- **Nikita S. Zhunusov**, Teaching Assistant of the Department of Pharmacology and Clinical Pharmacology, Belgorod State National Research University, Belgorod, Russia; e-mail: zhunusov@bsu.edu.ru, **ORCID ID** https://orcid.org/0000-0002-1969-3615. The author participated in ocular tonometry and ophthalmoscopy conducting.

- **Yana S. Kochergina**, forensic medical expert, Belgorod Bureau of Forensic Medicine, Belgorod State National Research University, Belgorod, Russia; e-mail: yana5568@mail.ru, **ORCID ID** https://orcid.org/0009-0003-8167-5666. The author participated in the article writing.

- **Anton P. Danilenko**, postgraduate student of the Department of Pharmacology and Clinical Pharmacology, Belgorod State National Research University, Belgorod, Russia; e-mail: danilenko_a@bsu.edu.ru, **ORCID ID** https://orcid.org/0009-0001-2419-8109. The author took part in the experimental work.

- **Anna A. Peresypkina**, Doct. Sci. (Biology), Professor of the Department of Pharmacology and Clinical Pharmacology, Belgorod State National Research University, Belgorod, Russia; e-mail: peresypkina_a@bsu.edu.ru; **ORCID ID** https://orcid.org/0000-0003-2829-9860. The author consulted on the research idea and design of the study, and edited the text of the article.