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ABSTRACT

of the dissertation for the degree of Doctor of Philosophy

EFFECTS OF WATER-SOLUBLE NEUROTROPHIC PROTEIN FRACTION OF THE HYPOTHALAMUS ON RETINAL DYSTROPHY

Speciality: 2411.01 – Human and Animal Physiology

Field of science: Biology

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GENERAL DESCRIPTION OF THE WORK

Importance. Retinitis pigmentosa (RP) is a severe and, to-date, an incurable form of ophthalmic pathology. RP is the most common disease of the currently diagnosed various types of retinal dystrophies. In this type of disease, damage of the photoreceptors and retinal pigment epithelium is observed with subsequent loss of visual function. RP is characterized by functional changes and a typical fundus pattern with pigment deposits.[1]

The pathogenesis of RP was studied in animal experiments in a retinal dystrophy model. In these studies various mechanisms of the disease development have been identified. Thanks to work in the molecular biology, it was found that clinically indistinguishable forms of retinal degeneration can be a consequence of mutations of different genes and, conversely, different mutations of the same gene lead to different phenotypic manifestations.[2]

By now the most common view is that RP is an inherited disease. At the current stage of development of science it is important to know the mutation of the gene leading to the occurrence of RP, and the distribution of this mutation in the family tree. It has been established that many mutations of the rhodopsin and peripheral genes determine a number of phenotypic manifestations of RP and mechanisms of degenerative processes of photoreceptors.[3] Modern advances in human molecular genetics have made it possible to obtain new data that are important for understanding the mechanisms of morphological and functional disorders in hereditary retinal dystrophies.

Despite the large number of publications concerning the genetic

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[1] Aleman, T.S. Retinitis Pigmentosa and Allied Diseases / ed. D.Albert, J.Miller, D.Azar [et al.] // Albert and Jakobiec's Principles and Practice of Ophthalmology: Springer, Cham, – 2022. https://doi.org/10.1007/978-3-030-42634-7_1 2

[2] Шурыгина, М.Ф. Диагностика наследственных дистрофий сетчатки с позиции генной терапии / М.Ф.Шурыгина, А.М.Хотеева // Вестник офтальмологии, – 2021, т.137, №4, – с.145-151.

[3] Tebbe, L. The Interplay between Peripherin 2 Complex Formation and Degenerative Retinal Diseases / L.Tebbe, M.Kakakhel, M.S.Makia [et al.] // Cells, – 2020. 9(3); https:/doi.org/10.3390/cells9030784

and mutational points of view on the pathogenesis of this disease, according to some authors, the etiology and pathogenesis of RP are associated with a violation of the normal interaction of the hypothalamus with the retina and that the cornerstone cause of this pathology is due to the dysfunction of the hypothalamus itself and weakening of its trophic provision of cellular elements of the retina. This idea was experimentally partially confirmed by the results of studies by Prof. N.A.Hajiyeva on a model of RP in rabbits created with monoiodoacetic acid (MIAA), in which pulsed stimulation of the ventromedial nucleus of the hypothalamus promoted to a faster recovery of the amplitude of the electroretinogram (ERG) when presenting light flashes of different intensity. These results suggest the presence of a trophic influence of the hypothalamic nuclei purposed to supporting the functions of the retina, while its weakening leads to disturbances of the retinal receptor apparatus[4].

Chaperone proteins, the so-called heat shock proteins with different molecular masses and, especially, heat shock proteins with a molecular mass of 70 kDa (HSP70), ensure the maintenance of normal conformation and functioning of proteins of body tissues. It was shown that this group of proteins helps to restore the disturbed conformational structure of proteins and, due to its chaperone activity, protects body cells from such adverse factors as high temperature, hypoxia, acidic or alkaline pH values of the environment, epileptic seizures etc.[5]

At the Institute of Physiology named after academician Abdulla Garayev of the Ministry of Science and Education of Republic of Azerbaijan serotonin-modulating anticonsolidation protein (SMAP), which is in linear relations with serotonin and, probably, realizes its

[4] Гаджиева, Н.А. Исследование влияния переднего гипоталамуса на электрическую активность сетчатки / Н.А.Гаджиева, Н.М.Рзаева // Физиологический журнал СССР, – 1992. т.78, №11, – с.61-71.

[5] Belenichev, I.F. Involvement of heat shock proteins HSP70 in the mechanisms of endogenous neuroprotection: the prospect of using HSP70 modulators / I.F.Belenichev, O.G.Aliyeva, O.O.Popazova [et al.] // Front Cell Neurosci., – 2023. Apr 17; 17, 1131683. doi: 10.3389/fncel.2023.1131683.

functions at the subcellular level, was purified from the rat brains[6]. On various species of vertebrates, it has been shown that SMAP possesses antimutagenic and antitoxic activity towards unfavorable factors of chemical and bacterial nature.[7] In experiments on mice, Western blotting technique demonstrated that intraperitoneal injection of SMAP causes increased synthesis of HSP70 in the liver.

The purpose and objectives of the study. The purpose of the presented dissertation was to study the molecular pathogenic and reparative mechanisms of the retina and hypothalamus in an experimental model of retinal dystrophy in the rabbits and in the patients with RP.

In order to achieve this objective the following tasks should be realized:

1. Evaluate the changes in ERG in the rabbits with RP obtained by intravenous (i.v.) injection of MIAA in response to the presentation of light flashes of different intensities to the animal.

2. Develop an immunochemical method for the determination of rhodopsin prepared from the retina of experimental animals; to use polyclonal immunoglobulins to isolated rhodopsin after immunization of the rabbits with it as the first antibodies in ELISA test.

3. Reveal changes of the levels of rhodopsin and HSP70 in the retina by ELISA in experimental animals after injection of MIAA, and of the level of SMAP - in the hypothalamus.

4. Compare the levels of rhodopsin and HSP70 in the retina by ELISA following intravitreal (i.vtr.) injection of SMAP to rabbits after injection of MIAA.

5. Immunize rabbits with purified protein to obtain polyclonal anti-SMAP immunoglobulin and purification of polyclonal anti-SMAP

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[6] Мехтиев, А.А. Обнаружение в головном мозге крыс белка, обладающего антиконсолидационными свойствами // Бюлл. экспер.биол. мед., – 2000. т.129, №8, – с.147-150.

[7] Аллахвердиева, Т.Н. Активация серотонинергической системы способствует выживанию животных разных видов при воздействии бактериальных и химических токсинов / Т.Н.Аллахвердиева, Х.Ш.Мехтиев, А.А.Гайсина [и др.] // Журнал эволюционой биохимии и физиологии, – 2019. т.55, №1, – с.23-27.

antibodies from anti-SMAP immunoglobulin solution.

6. Investigate the effect of i.vit. injection of polyclonal antibodies to SMAP to the rabbits after injection of MIAA on the retinal rhodopsin and HSP70 levels by ELISA.

7. Investigate the immune response following immunization of the rabbits with purified SMAP on the retinal rhodopsin and HSP70 levels by ELISA.

8. Carry out a comparative assessment of the level of anti-SMAP natural autoantibodies in the serum of the patients with clinically diagnosed RP and of the healthy subjects through ELISA.

Scientific novelty of the study. The dissertation study developed an original method for determining the levels of rhodopsin based on indirect enzyme-linked immunosorbent assay. This method demonstrated reduced rhodopsin and increased HSP70 levels in the retina and increased activity serotonergic system of the hypothalamic in a rabbit model of RP.

The existence of trophic support by the serotonergic system of the hypothalamus of the retinal receptor apparatus, probably due to retrograde axonal transport, is demonstrated. The functioning of the reverse information channel through anterograde axonal transport has also been revealed, signaling the hypothalamic nuclei about the functional state of the retinal receptor apparatus and adjusting their trophic activity to its metabolic demands.

The dissertation work for the first time revealed a reduced titer of anti-SMAP natural autoantibodies in the patients with diagnosed RP, which indicates a pathogenic decrease in the activity of the serotonergic system in the tissues of these patients, including the hypothalamus.

Scientific-practical significance of the work. The results of the studies that revealed the decreased level of rhodopsin and the increased level of HSP70 in the retina, as well as the increased level of SMAP in the hypothalamus in experimental animals, suggest that the formation of RP is based on a violation of the trophic support of the retinal receptor apparatus by the serotonergic system of hypothalamic nuclei.

The dissertation demonstrated that the serotonergic system of the hypothalamus implements trophic provision of retinal receptor

cells by inducing HSP70 synthesis in them.

The detection of a reduced titer of natural autoantibodies to SMAP in the blood serum of the patients with RP indicates a reduced activity of serotonergic activity in the tissues of the patients. Based on this, the determination of the level of antibodies to SMAP in the serum of such patients can serve as an additional diagnostic criterion in the diagnosis of the severity of this disease, as well as a criterion for the correctness of the choice and effectiveness of the therapeutic measures.

Main statements to be defended:

1. The formation of RP in rabbits using MIAA causes a decrease in the amplitude of the total ERG in response to the presentation of light flashes of various intensities after 5 and 13 days.

2. The administration of SMAP into the brain lateral ventricle of the rabbits with formed RP causes a noticeable restoration of the amplitude of the total ERG caused by the presentation of light flashes of various intensities to the animal.

3. Formation of RP in rabbits induces downregulation of rhodopsin and upregulation of HSP70 in the retina, as well as upregulation of SMAP in the hypothalamus.

4. The i.vit. injection of SMAP to the animals with formed RP induces a marked upregulation of rhodopsin and HSP70 in the retina.

5. The blockade of SMAP activity in the retina of the rabbits with RP with the i.ven. injection of anti-SMAP antibodies, as well as the immunization of the intact animals with SMAP induces upregulation of HSP70 and rhodopsin in the retina.

6. The level of anti-SMAP natural autoantibodies in the blood serum of the patients with diagnosed RP is significantly lower than in the healthy subjects, which indicates the existence of trophic support of the retinal receptor apparatus from the hypothalamus on the basis of feedback.

Approbation of the dissertation. The results of the dissertation work were presented in the form of a verbal report at the following conferences: XXIII Congress of the Physiological Society named after I.P. Pavlov, Voronezh, Russiya, September 18-22, 2017; V Congress of Physiologists of Azerbaijan, Baku, October 11, 2017; II Conference

of Retinologists of Azerbaijan, Baku, May 12 2017; III Conference of Retinologists of Azerbaijan, Baku, June 01 2018; V Conference "Contemporary Problems of Systemic Regulation of Physiological Functions," Chalkidiki, Greece, 25-31 May 2019; IV International Congress of the Society of Physiologists of Georgia named after İ.S.Beritaşvili, Tbilisi, Georgia, September 23-25, 2019; International Interregional Scientific and Practical Conference of Ophthalmologists of the Southern Federal District of the Astrakhan Region and the Caspian Littoral States "Innovative Technologies in the Ophthalmic Practice of the Regions," September 22-23, 2023, Astrakhan, Russia; International Conference of Physiologists of Azerbaijan and VI Congress "Physiology of Vision and Professional Pathologies: Fundamental and Applied Aspects," October 30-31, 2023 Baku, Azerbaijan.

The name of the organization where the dissertation work was performed. Laboratory of "Molecular Basis of Integrative Activities" of the Institute of Physiology named after academician Abdulla Garayev of the Ministry of Science and Education of Republic of Azerbaijan; Department of "Eye Complications of Diabetes Mellitus and Retinal Pathology" of the National Centre of Ophthalmology named after academician Zarifa Aliyeva.

Publications. 12 scientific papers including 6 theses, 6 articles (2 of them in impact-factor journals) were published.

Structure of the dissertation. The dissertation consists of 174759 characters, is illustrated with 10 diagrams, 3 photos, 1figure and 1 table. The work consists of a table of contents (1300 characters), an introduction (15615 characters), chapters of literature review (61385 characters), materials and research methods (22893 characters), results (27111 characters), discussion (43638 characters), conclusions (1663 characters), practical recommendations (620 characters), a list of abbreviations used (534 characters) and list of references from 238 sources (2 are in Azerbaijani, 58 are in Russian, and 178 are in English).

MATERIALS AND METHODS

Studies were carried out on 65 male Chinchilla rabbits. Bovine heads (n=8) were used for purification of SMAP and bovine eyes (n=40) were used for purification of rhodopsin. Blood samples taken from the vein of 9 patients with RP and of 9 healthy persons were used in the studies. All animal studies were carried out in accordance with the requirements of the Council Directive of the European Community (86/609/EEC) and under the supervision of the local bioethics committee. Blood sampling from patients and healthy volunteer subjects was performed with their consent at the National Centre of Ophthalmology named after academician Zarifa Aliyeva.

Electrophysiological methods. The RP model was formed by injecting MIAA into the rabbits at a ratio of 26 mg per 1 kg of animal mass. After the formation of RP, ERG was recorded when stimulating the eyes of rabbits with light flashes of various intensities (0.016, 0.068, 0.45 and 1.4 J). Chemods were implanted into the left lateral ventricle of the rabbit brain according to stereotaxic coordinates to administer the SMAP solution (Ap 2.5 y 2.5 z 6.5). At the National Centre of Ophthalmology named after academician Zarifa Alieva ERG was recorded for diagnostic purposes in patients with RP according to the standards of the International Society for Clinical Electrophysiology of Vision (ISCEV).

Biochemical methods. SMAP isolation was performed from the bovine brain as previously described. In order to record RP in rabbits under the influence of test drugs, a method for determining the content of rhodopsin in the retina by ELISA was elaborated.

Immunochemical metods. Immunoglobulins for SMAP and rhodopsin were obtained from a 5-6-month immunization of rabbits with purified proteins mixed with complete Freund's adjuvant. SMAP, rhodopsin, and HSP70 levels in rabbit tissues were evaluated by indirect ELISA. Total retinal and hypothalamic proteins were used as antigens, rabbit immunoglobulins to SMAP, rhodopsin or HSP70 were used as the first antibodies, and goat anti-rabbit immunoglobulins with conjugated horseradish peroxidase were used as the second antibodies. Orthophenylene diamine was used as a substrate for peroxidase. The

reaction results were recorded at 492 nm (reference wavelength 630 nm), averaged over groups, and compared by Student's t-criterion. Polyclonal anti-SMAP antibodies were purified from immunoglobulin solution by affinity chromatography. The obtained values of the levels of studied antigens in the hypothalamus and in the retina of the eyes, as well as the level of anti-SMAP natural autoantibodies in the serum of the RP patients and the healthy persons were averaged within each group and compared according to the Student's t-criterion.

Experimental schedule. The main series of studies were performed according to the following scheme. In the 1st series of studies in rabbits (n = 8), retinal dystrophy was formed through i.v. injection of MIAA, and after 5, 13, and 27 days, the amplitude of the total ERG was recorded under presentation of the light flashes of different intensities. In this series, on the 15th day after injection of MIAA, SMAP was injected through hemod previously implanted into the left lateral ventricle in a volume of 20 μL and at a concentration of 1.5 mg/mL. ERG of the right eye contralateral to the side of injection of SMAP was recorded in the rabbits 7 days after injection of SMAP.

In the 2nd series of studies in the rabbits (n = 8) RP was formed and after 12 days the animals were euthanized, the retina and hypothalamus were extracted; the levels of rhodopsin and HSP70 in the retina and SMAP in the hypothalamus were measured by indirect ELISA.

In the 3rd series of studies 3 groups of animals were formed: 1) intact group (n = 8); 2) control group (n = 8) and 3) experimental group (n = 8). In the control group, animals were i.v. injected with MIAA and after 22 days they were euthanized and the retina was isolated. In animals of the experimental group, after 15 days since formation of RP 150 μL of SMAP was injected i.vitr. in a concentration of 1.5 mg/mL, in a sterile saline. 7 days after the injection of SMAP (22 days after the injection of MIAA), rabbits were euthanized, the retina was isolated and the level of retinal HSP70 in rabbits of three groups was measured.

In the 4th series of studies 3 groups of animals were formed: 1) the 1st control group (n = 8); 2) 2nd control group (n = 8) and 3) experimental group (n = 8). Retinal dystrophy was formed in animals of both control and experimental groups and after 15 days animals of

the 2nd control group were injected 150 μL of inactivated SMAP (30 min in a water bath at 60°C) at a concentration of 1.5 mg/mL, in a sterile saline, and the rabbits of the experimental group were similarly injected with SMAP at the same dose. After 7 days rabbits were euthanized, retina was isolated, and rhodopsin levels were determined.

In the 5th series 3 groups of animals were formed: 1) intact (n = 8); 2) control - after induction of RP i.vtr. injection of inactivated SMAP (n = 8) and 3) experimental - after induction of RP, injection of SMAP (n = 8). The drugs were administered on the 5th day after the injection of MIAA and after 7 days the level of rhodopsin and HSP70 was determined in the retina.

In the 6th series of studies 3 groups of animals were formed: 1) 1st control group (n = 8); 2) 2nd control group (n = 8); 3) experimental group (n = 8). In the animals of both control and experimental groups RP was formed and after 15 days control animals were injected intravitreally with the rabbit non-immune γ - globulins, and rabbits of the experimental group – with the rabbit anti-SMAP polyclonal antibodies. The preparations were injected in an amount of 200 μL and at a concentration of 1.8 mg/mL in a sterile saline. After 7 days the rabbits were sacrificed, the retina was isolated, and rhodopsin and HSP70 levels were measured.

In the 7th series of studies the rabbits (n = 3) were immunized with SMAP as described above for 5 months. Animals were sacrificed and retinal rhodopsin levels were measured.

In the 8th series of studies on the patients with RP (n = 9), diagnosed at the National Centre of Ophthalmology named after academician Zarifa Aliyeva and on the healthy persons (n = 9), blood samples were taken from the vein in an amount of 5 mL, serum was saved and the level of anti-SMAP natural autoantibodies was determined.

RESULTS

In the first series of studies in the registration of ERG in intact animals at the presentation of light flashes with an intensity of 0.016, 0.068, 0.45 and 1.4 J, the amplitude of ERG was, respectively, 162±10 μV, 234±12 μV, 317±8 μV and 396±15 μV. On the 5th day after the

i.v. injection of MIAA, there was no ERG response to the presentation of light flashes with an intensity of 0.016 J, and the amplitudes of ERG to the remaining intensities were 50 μV, 75 μV and 83.3 μV, respectively. On the 13th day after MIAA the amplitudes of ERG were respectively: 50 μV, 77±8 μV, 80±5 μV and 100±10 μV. On the 27th day after injection of MIAA ERG had the following amplitude values: 67±5 μV, 108±7 μV, 125±5 μV and 133 μV (Fig. 1).

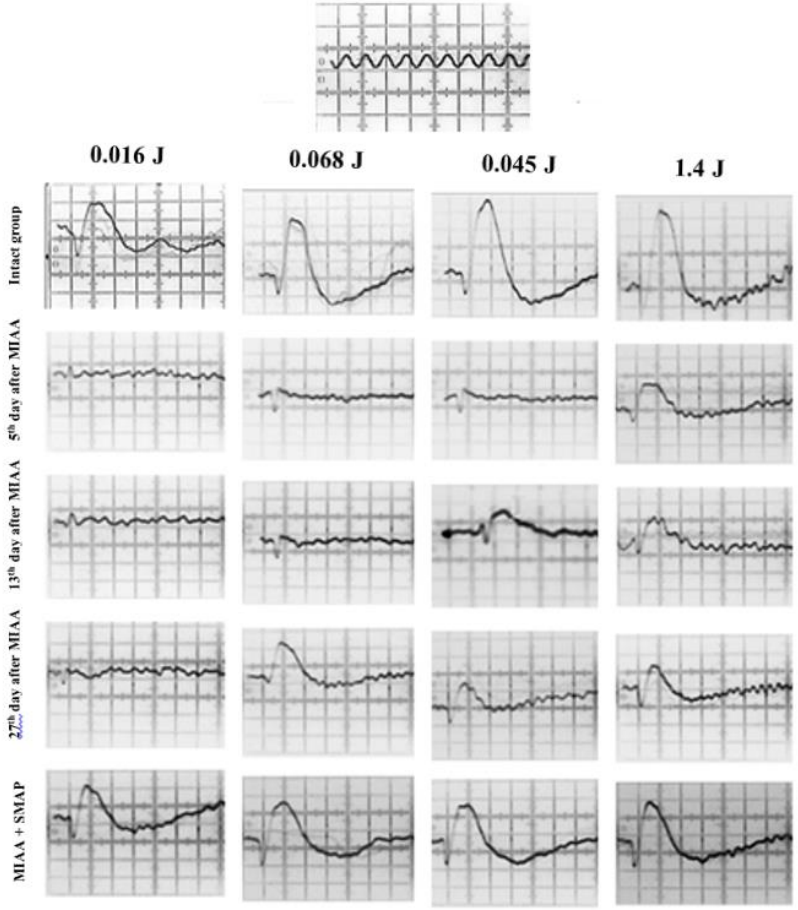

Figure 1. ERG of the rabbits exposed to light flashes of different intensities at different timeframes after i.v. injection of MIAA and intracerebral injection of SMAP.

In this series of studies, there was also analysed the effect of injection of SMAP into the lateral ventricle of the rabbit brain with RP. Intraventricular injections of drugs are used to study their effect on cells of brain structures in order to deliver them by passing the blood-brain barrier. SMAP was administered in an amount of 20 μL at a concentration of 1.5 mg/mL on the 15th day in the brain lateral ventricle of the rabbits after injection of MIAA. 7 days later this led to a significant increase of the ERG amplitude. In particular, when using 4 light flashes intensities in the rabbits, the following ERG amplitudes were recorded: 150±12 μV, 175±13 μV, 183±12 μV and 225±15 μV (Fig. 2). It should be noted that the increase of the ERG amplitude under the influence of SMAP also noticeably exceeded the ERG amplitude as a result of its spontaneous recovery, especially when presenting light flashes with the lowest and highest intensities (0.016 and 1.4 J).

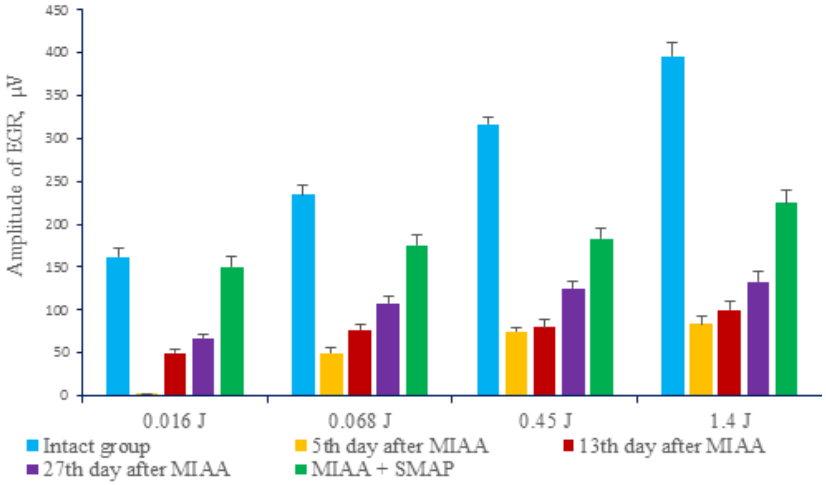

Figure 2. Changes of the amplitudes of the total ERG at different time intervals after the injection of MIAA and under the effects of SMAP.

Thus, the most pronounced decrease of ERG amplitude was observed on 5 and 13 days since injection of MIAA to the rabbits. Also in the animals with RP under the influence of SMAP, a significant increase and restoration of the ERG amplitude was observed.

For the purpose of determining the nature of the development of RP under the influence of MIAA and of studying relevantly the underlying mechanisms of this pathology an immunochemical method was developed to determine the rhodopsin level in the retina. As a result 1.8 mg of rhodopsin was purified from 40 cow eyes. 2 rabbits were immunized with this amount for 2 months and 50 mL of blood were taken from the ear vein 10 days after the 3rd and subsequent injections. Following isolation of immunoglobulins to rhodopsin, the level of immune response was determined in the ELISA-test. The results showed that in both immunized rabbits the immune response was strong: the absorption values in the wells with samples were more than 3 times higher than the absorption values in the blank wells with buffer.

In the 2nd series of studies in rabbits by i.v. injection of MIAA, RP was formed and 12 days later the animals were euthanized, and the retina and hypothalamus were isolated. Determination of rhodopsin and retinal HSP70 levels showed marked reduction in rhodopsin and increase in HSP70. In particular, if the level of rhodopsin in the intact animals was 0.275±0.011 optical units of extinction OUE, then in animals of the experimental group its level was 0.207±0.007 OUE ($p < 0.001$; Fig. 3). At the same time the level of HSP70 in the retina in intact animals was 0.094±0.004 OUE, while in experimental animals its level corresponded to 0.14±0.OUE ($p < 0.001$; Fig. 3). In addition, the determination of the level of SMAP in the hypothalamus in the intact and experimental animals revealed an increase of its level under the influence of RP. In particular, the SMAP level in animals with RP was 0.298±0.009 OUE, while in intact animals - 0.24±0.01 OUE. ($p < 0.01$; Fig. 3).

It should be stressed that the results of this series of studies undertaken with application of the ELISA-test, which revealed a decrease of the rhodopsin level in the retinas of the rabbits under RP conditions, is in line with the results of the previous series, which recorded a pronounced decrease in the amplitude of ERG in animals with RP at similar time intervals after injection of MIAA to animals.

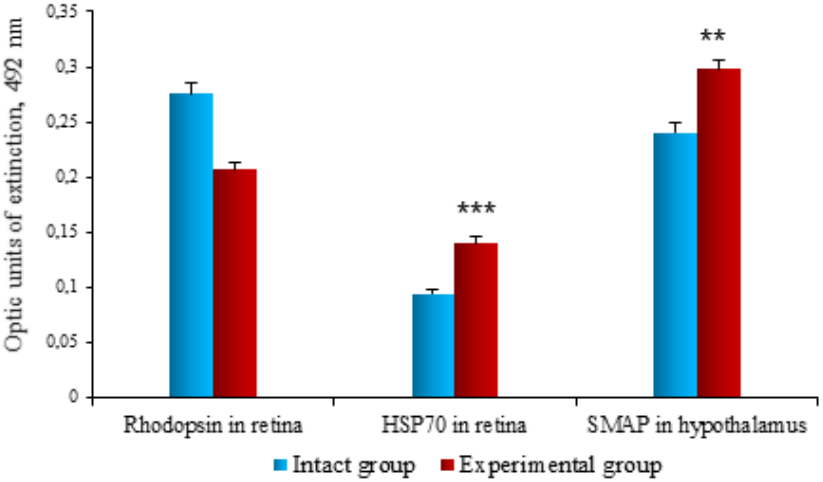

Figure 3. Changes of the levels of rhodopsin and HSP70 in the retina and SMAP in the hypothalamus of the rabbits after the i.v. injection of MIAA. **- p<0.01; *- p<0.001.**

In the 3rd series of studies to determine whether SMAP has stimulating effect on HSP70 synthesis and whether the result of a concomitant increase of the SMAP and HSP70 levels obtained in the previous series is not a simple coincidence of two events, the effect of SMAP on the level of HSP70 in the retina in rabbits with this form of pathology was studied. A sharp (23-fold) increase in HSP70 under the influence of SMAP was found compared to the control group, whose animals were also administered MIAA at a similar dose. In particular, if the level of HSP70 in the retina of intact animals was 0.367±0.04 OUE, in control animals - 0.039±0.001 OUE ($p < 0.001$), then in animals of the experimental group their level was 0.902±0.042 OUE ($p < 0.001$; Fig. 4).

Thus, bringing SMAP to the retina in animals with RP induces enhanced HSP70 synthesis in it. This fact indicates that there is indeed a cause-effect relationship between an increase in hypothalamic SMAP during RP in animals and a concurrent increase in retinal HSP70.

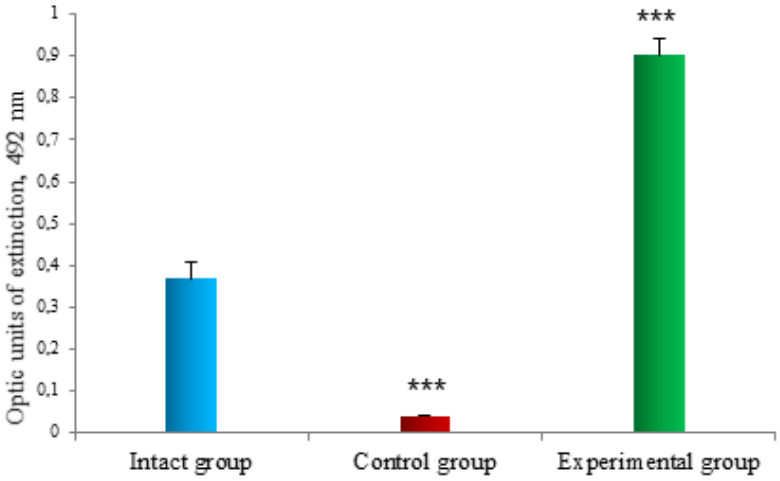

Figure 4. The effects of the i.vtr. injection of SMAP to the animals with RP on the level of HSP70 in the retinas * - $p<0.001$.**

In the next 4th series of studies, on the 15th day since i.v. injection of MIAA, heat-inactivated SMAP (2nd control group) and active SMAP (experimental group) were i.v. injected in animals. After 7 days all groups of animals were tested for rhodopsin levels. It was found that the level of rhodopsin in the retina of the animals of the experimental group did not differ from its level of the animals of the 1st control group (i.v. injection of MIAA). At the same time, in the animals of the 2nd control group, the level of rhodopsin in the retina was significantly lower than in the animals of the 1st control group. In particular, in the retina of the intact animals the level of rhodopsin was 0.255±0.01 OUE, in the animals of the 1st control group - 0.268±0.013 OUE, in the animals of the experimental group - 0.257±0.006 OUE, while in the animals of the 2nd control group - 0.2±0.009 OUE ($p < 0.01$; Fig. 5).

The results of this series indicate that by the 22nd day after i.v. injection of MIAA the level of rhodopsin in the 1st control group recovered and returned to its baseline level. This result is consistent with the results of the first series of studies in which ERG was recorded in the animals with RP. Due to self-recovery of rhodopsin over the

specified timeframe, i.vtr. injection of SMAP on the 15th day had no effect on rhodopsin levels in the experimental group. On the other hand, i.vtr. injection of inactivated SMAP prevented the natural recovery of the rhodopsin levels, apparently due to the high-affinity binding of inactivated SMAP to SMAP receptors, which prevented the interaction of endogenously produced SMAP with its receptors.

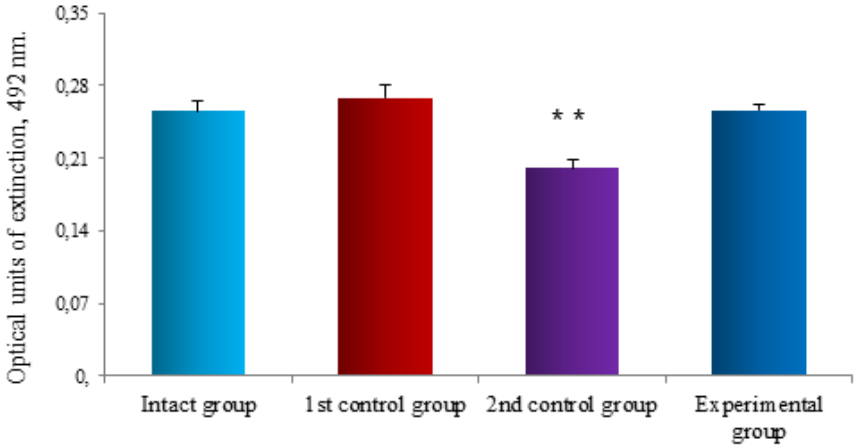

Figure 5. Effect of i.vtr. injection of SMAP and inactivated SMAP at 15th day in the rabbits with RP on the retinal rhodopsin levels at 22th day after i.v. injection of MIAA. ** - $p < 0.01$.

Due to the independent recovery of the retinal rhodopsin levels in the 1st control group 22 days since injection of MIAA to the rabbits, significantly shorter time intervals (5 days) between i.v. injection of MIAA and i.vtr. injection of the drugs were chosen in the subsequent 5th series of studies. It was found that 7 days after the i.vtr. injection of SMAP, there was a significant increase in the rhodopsin level in the retina. Specifically, in intact animals the level of rhodopsin in the retina was 0.187±0.005 OUE, in the control animals treated with inactivated SMAP injections - 0.13±0.008 OUE ($p < 0.001$), and in animals of the experimental group (SMAP) - 0.193±0.011 OUE ($p < 0.001$; Fig. 6).

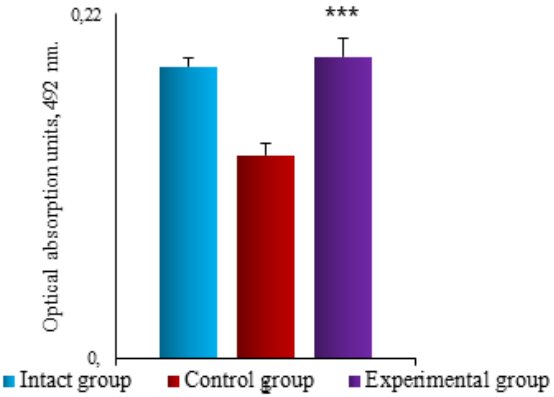

Figure 6. Effect of i.vtr. injection of SMAP 5 days since MIAA on rhodopsin levels on 12th day in the rabbit retina. * * * - p < 0.001.

However, in the same series of experiments, the level of retinal HSP70 in animals was also determined according to the above scheme. At the same time the level of HSP70 in the retina in the intact rabbits was 0.085±0.004 OUE, control - 0.102±0.004 OUE ($p < 0.05$), in the animals of the experimental group - 0.123±0.005 OUE ($p < 0.001$). Thus, under the influence of i.vtr. injection of SMAP on the 5th day after MIAA injection a concomitant increase in rhodopsin and HSP70 levels in the retina of the experimental group animals was observed (Fig. 7).

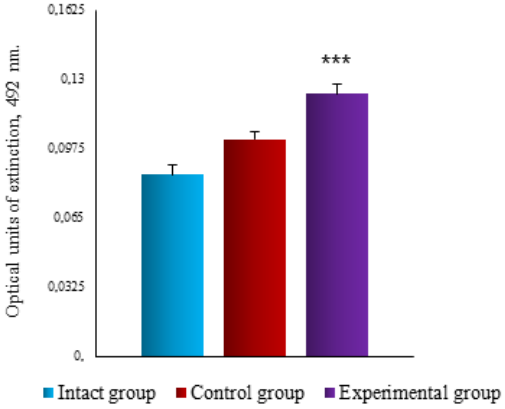

Figure 7. Effect of i.vtr. injection of SMAP 5 days after i.v. injection of MIAA on HSP70 level on day 12 in rabbit retina. * * * - p < 0.001.

In the 6th series, the objective of the study was studying the effect of antibodies-mediated blockade of SMAP on the level of rhodopsin in the retina in the rabbits with RP. As a result of the experiments it was revealed that i.vtr. injection of anti-SMAP antibodies to the RP rabbits leads to a significant increase of rhodopsin and HSP70 levels relative to their levels in the animals of the intact and 1st control groups. In particular, if in the animals of the 1st control group, the level of rhodopsin was 0.182±0.012 OUE, in the 2nd control group - 0.184±0.009 OUE, then in the animals of the experimental group its level was 0.242±0.012 OUE ($p < 0.01$; Fig. 8). At the same time in the animals of the 1st control group the HSP70 level was 0.082±0.012 OUE, in the 2nd control group - 0.109±0.004 OUE, while in the animals of the experimental group its level was 0.158±0.01 OUE ($p < 0.001$; Fig. 8). Thus, the results obtained in this series indicate that antibodies-mediated blockade of SMAP in the retinal cells on the background of RP leads to a significant increase of the HSP70 and rhodopsin level in it.

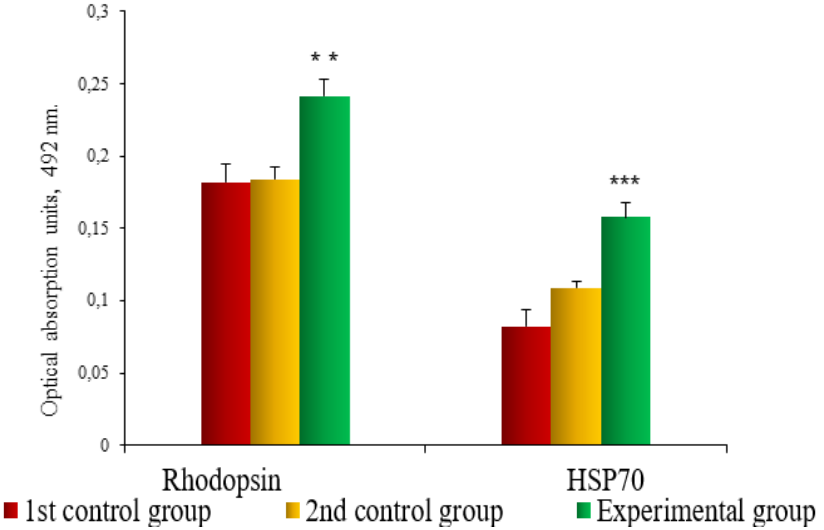

Figure 8. The effects of the i.v. injection of antibodies to SMAP on the levels of rhodopsin and HSP70 in the retinas of the rabbits. ** - $p < 0.01$, * - $p < 0.001$.**

The next 7th series of studies examined the effect of immunization with SMAP of the intact rabbits and their production of autoantibodies to SMAP on retinal HSP70 and rhodopsin levels in the absence of RP. As a result of a 5-month immunization of rabbits with SMAP, antibodies generated in the body have a systemic blocking effect on the activity of SMAP in all tissues. At the same time, antibodies to SMAP with a high titer were produced. In this series of studies the retinal HSP70 and rhodopsin levels in the rabbits immunized with SMAP were also measured. In the retina these rabbits were found to have a marked increase in HSP70 levels compared to the intact animals. In particular, if the intact animals had retinal HSP70 levels of 0.087±0.OUE, as a result of SMAP immunization its level rose to 0.176±0.01 OUE ($p < 0.001$). At the same time an increase in the level of rhodopsin in the retina was found in the animals immunized with SMAP. In the intact animals it was 0.186±0.005 OUE, in the immunized rabbits its level was 0.213±0.004 OUE ($p < 0.01$). Thus, under the influence of the rabbits immunization with SMAP and production of the anti-SMAP antibodies in their bodies, these rabbits show a sharp increase in the level of chaperone protein HSP70 (Fig. 9) and at the same time an increase in the level of rhodopsin in the retina (Fig. 10).

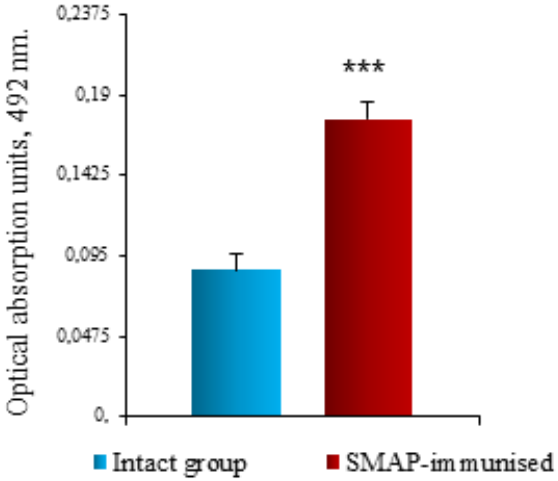

Figure 9. Effect of immunization of the rabbits with SMAP on the retinal HSP70 level. * - $p < 0.001$.**

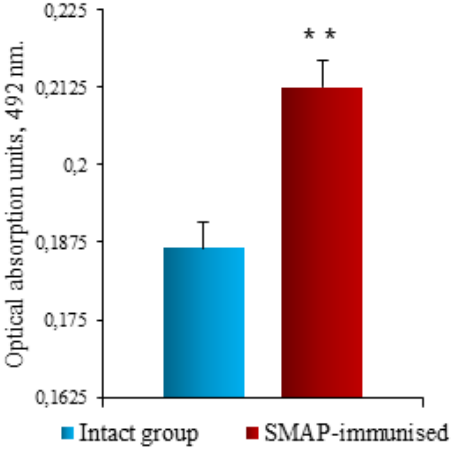

Figure 10. Effect of immunization of the rabbits with SMAP on the retinal rhodopsin level. ** - $p < 0.01$

In the 8th series of the studies the level of natural anti-SMAP autoantibodies in the blood serum of the patients with RP was measured.

The literature data indirectly indicates that natural autoantibodies to any autologous antigens in different concentrations are present in the organisms of healthy people. Consequently, on the basis of measured titers of natural autoantibodies to certain antigens of the cellular elements being equal to the level of these antigens in the organism and determined in the blood serum we can make a conclusion with high probability about the level of the studied antigens in the tissues of the organism. Obtaining such information is especially important for diagnostic purposes with regard to the measurement of the level of structural antigens in the solid tissues of the human organism.

All 9 examined patients were diagnosed with RP at the National Centre of Ophthalmology named after academician Zarifa Aliyeva using instrumental methods for studying retinal functions, namely, registration of ERG in accordance with ISCEV standards. As a result of the studies ERG was absent in patients (Fig. 11) or subnormal (Fig. 12), which indicates a serious violation of the functions of the retinal receptor apparatus.

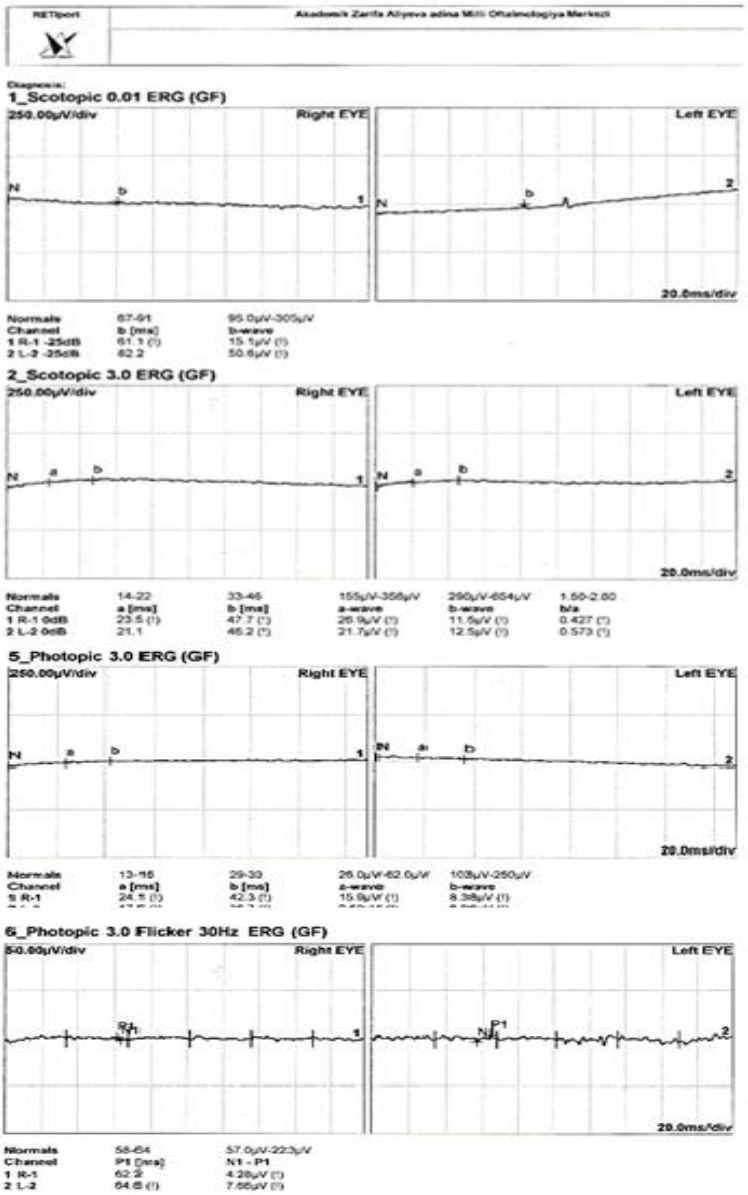

Figure 11. Absence of electroretinogram in the patient with RP.

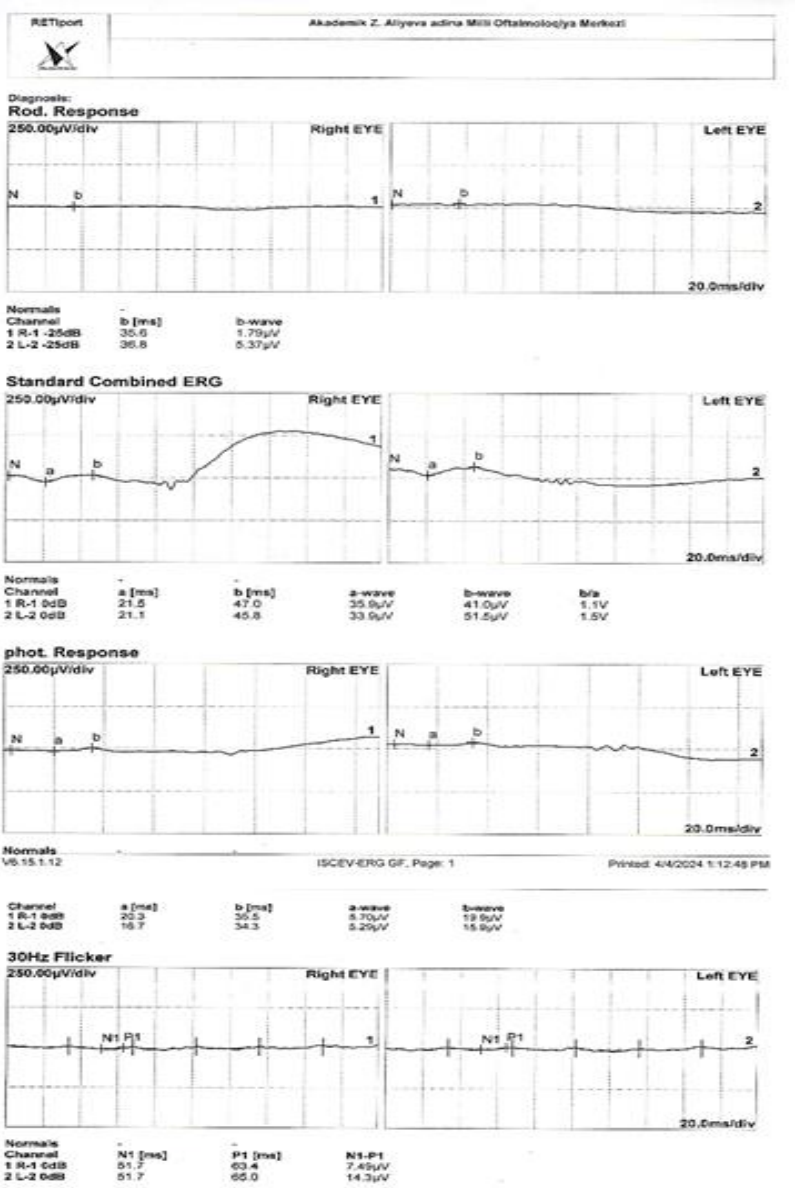

Figure 12. Subnormal electroretinogram in the patient with RP.

As a result of the studies it was found that in the blood serum of patients with RP the level of anti-SMAP natural autoantibodies was significantly lower than their level in the healthy subjects of approximately the same age group. In particular, if the level of anti-SMAP natural autoantibodies in the serum of healthy subjects was 0.106±0.008 OUE, in the RP patients their level was 0.076±0.004 OUE ($p < 0.01$; Fig. 13).

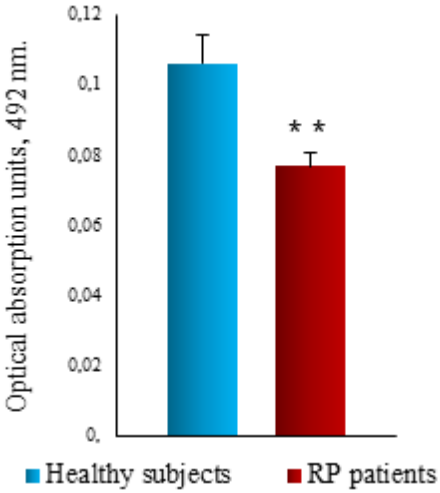

Figure 13. The change of the level of natural anti-SMAP autoantibodies in the blood serum of the patients with RP. ** - p<0.01.

Considering the mentioned above data, we can conclude that the level of SMAP is significantly decreased in the cellular elements of the tissues of the organisms including the brain subcortical structures and the retinas of the patients with RP regarding to its level in the appropriate tissues of the healthy subjects.

Summarizing the 8 series of experiments conducted on the RP model, the following conclusions can be made. Modeling RP in rabbits through i.v. injection of MYUK causes significant changes in the functioning of the retinal receptor apparatus, manifested by a sharp decrease in the amplitude of the ERG and a reduction in the rhodopsin level.

Thus, under a severe form of RP there are a number of molecular changes in the retina, apparently causing the initiation and proceeding of the reparative and regulatory processes both inside and outside of it, in particular, in the hypothalamus. Under the development of this form of pathology along with the downregulation of rhodopsin, the level of HSP70 increases in the retina as well as SMAP increases in the hypothalamus. The sequence and cause-effect relationship between these molecular events become clear when considering also the results of other series of the studies.

DISSCUSION

HSP70 proteins are the chaperone proteins that ensure the maintenance and restoration of the damaged structure of tissue proteins in the body due to various external and internal adverse effects. HSP70 proteins lack specificity regarding the nature of the adverse factor that triggered their increased synthesis.[8]

An increase in the level of HSP70 in retinal cells in animals with formed RP reflects the course of reparative processes launched in connection with the violation of the normal structure of rhodopsin and aimed to its restoration. The results of the 3rd series of studies demonstrated that injection of SMAP to animals following injection of MIAA causes a sharp increase in HSP70 levels. Therefore, enhanced retinal HSP70 synthesis is associated with an initial increase in hypothalamic SMAP.

Thus, the synthesis of SMAP in the hypothalamic nuclei and its subsequent retrograde axonal transport to retinal receptor cells contribute to the synthesis of HSP70 proteins, which, in turn, ensure the maintenance of a functionally active conformation of rhodopsin molecules. At the same time, an increase in the level of SMAP in the hypothalamus in the rabbits with RP indicates to the activation of an emergency mode of functions of the hypothalamic nuclei aimed to

8 Mayer, M.P. Recent advances in the structural and mechanistic aspects of Hsp70 molecular chaperones / M.P.Mayer, L.M. Gierasch // J Biol Chem., –2019. Feb 8; 294(6). – p.2085-2097.

restoration of the damaged rhodopsin structure.

From the literature the presence of anatomical retinal-hypothalamic nerve connections is known, which, due to retrograde axonal transport can provide transportation of neurotrophic factors similar to SMAP from the cellular elements of hypothalamic nuclei to the bodies of receptor cells of the retina. In contrast, anterograde transport is the movement of compounds from the cell body to axonal presynaptic thickening. Axonal transport supports metabolic and structural demands of the neurons, transporting mitochondria, lipids, proteins to maintain normal cell health and function.[9]

Indeed, various authors using diverse methods that allow tracking the movement of labeled compounds along axons and trans-synaptical, have described the existence of anterograde axonal transport of high-molecular compounds from the retinal cells to the hypothalamic nuclei and to other brain structures[10]. Based on the above mentioned literature, the specific mechanism of transportation of anti-SMAP polyclonal antibodies injected into the vitreous body of the rabbits with RP to the hypothalamus due to the mechanism of anterograde axonal transport becomes clear[11]. In addition, taking into account the high speed of transportation along anterograde axonal transport and the relatively short distance between the retina and the hypothalamus, anti-SMAP antibodies are able to reach their final destination in a very short time. Upon reaching the cells of hypothalamic nuclei, they bind with high specificity and affinity to SMAP molecules, leading to their inactivation.

According to Jacob and Monod's postulate, a decrease for some

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[9] Guedes-Dias, P. Axonal transport: Driving synaptic function. / P. Guedes-Dias, E.L.F. Holzbaur // Science, – 2019. Oct 11; 366(6462), – p. eaaw 9997. doi:10.1126/ science.aaw 9997.

[10] Guo, W. Axonal transport defects and neurodegeneration: Molecular mechanisms and therapeutic implications / W. Guo, K.S. Dittlau, L.V-D. Bosch// Seminars in Cell & Developmental Biology, – 2020. March; 99, –p.133-150.

[11] Liu, X-A. Pathologies of axonal transport in neurodegenerative diseases / X-A.Liu, V.Rizzo, S.V.Puthanveettil // Transl Neurosci., – 2012. Dec 1; 3(4), – p.355-372. doi: 10.2478/s13380-012-0044-7.

reason of the final reaction product in biological systems on a feedback basis causes its compensatory and enhanced formation, in terms of the amount of synthesized product, significantly exceeding its initial level. Based on this postulate, blocking SMAP molecules with antibodies and, accordingly, removing a significant number of its molecules from normal functioning should certainly lead to launching its enhanced synthesis and to increasing its number in hypothalamic cells. The newly synthesized SMAP molecules are probably transported to the retinal cells by retrograde axonal transport mechanism, which eventually leads to increase of the level of HSP70 and restoration of the conformation of the damaged rhodopsin molecules, as revealed in the 6th series of studies carried out on the animals with RP. According to the results of the 7th series of studies, immunization of intact animals with SMAP for several months caused the production of a high titer of anti-SMAP antibodies, which resulted in an increase of the HSP70 proteins levels in the retina. The reason for this was the systemic effect of anti-SMAP autoantibodies on the level of SMAP in the hypothalamus and its compensatory increase based on the above-mentioned Jacob and Monod's postulate.

The fact that anti-SMAP antibodies indeed bind specifically and with high affinity to SMAP molecules is due to the fact that they are purified from a solution of immunoglobulin G produced against SMAP molecules by immunization of rabbits with purified protein.

The existence of retrograde axonal transport of SMAP from the hypothalamus to the retina is directly indicated by the results of the 1st series of studies, in which intraventricular injection of SMAP into the animals with RP caused a very rapid recovery of ERG amplitude. The reason for this was the enhanced synthesis of HSP70 under the influence of SMAP, which led to the restoration of the activity of the damaged rhodopsin. In addition, the increase of rhodopsin level in the retina in the 1st control (i.v. injection of MIAA) and experimental groups (i.v. injection of MIAA plus i.vtr. injection of SMAP) up to the level of the intact animals with the decrease of the rhodopsin level in the 2nd control group (i.v. injection of MIAA plus i.vtr. injection of inactivated SMAP) in the 4th series of studies is also a consequence of

retrograde axonal transport of SMAP from the hypothalamus to the retina.

Thus, the phenomenon of retrograde axonal transport of SMAP from the hypothalamus to the retina, which underlies the trophic regulation by the hypothalamic nuclei of retinal receptor functioning, was observed in the experiments.

The presence of neurotrophic activity of hypothalamic nuclei in relation to retinal receptor cells is indicated by the results of the researchers' studies, who found that unilateral electrical coagulation of the suprachiasmatic nucleus of the rabbit hypothalamus leads after several days, to a sharp decrease of the ERG wave amplitudes in the eye contralateral to the side of coagulation[12]. Thus, these data allow us to conclude that there is a synthesis and retrograde axonal transport of neurotrophic factors from the suprachiasmatic nucleus of the hypothalamus to the retinal receptors, whereas its coagulation stops the subsequent synthesis and delivering of these factors to the retinal cells.[13]

Furthermore, the results of the experiments indicate the existence of feedback information communication from the retinal receptor cells by means of the mechanism of anterograde axonal transport of signal-requests to the hypothalamic cells. This makes it possible to adjust the trophic activity of hypothalamic nuclei to synthetic trophic requests of the retinal receptor cells. In particular, the increase in the SMAP level in the hypothalamus in the 2nd series of studies 12 days after i.v. injection of MIAA into animals is probably an outcome of anterograde axonal transport of molecular signal-requests from retinal receptor cells, in which the normal structure of rhodopsin was damaged by the toxin, to the hypothalamic nuclei. The phenomenon of anterograde axonal transport of signal-requests from the retinal cells to the hypothalamus can probably explain the spontaneous recovery of rhodopsin levels in the animals of the 1st

[12] Рзаева, Н.М., Дмитренко А.И. Зрительная кора и ее участие в регуляции функции сетчатки / Н.М.Рзаева, А.И.Дмитренко // Вестник офтальмологии, – 2013. №3, – с.4-9.

[13] Рзаева, Н.М. Роль гипоталамуса в регуляции функции сетчатки// Вестник офтольмологии, -2016.т.132(3),-с.32-36

control group to the normal level in the 3rd series of studies, as well as the spontaneous recovery of ERG amplitude in control animals in the 1st series of studies after a long time after i.v. injection of MIAA.

The results of the 8th series of studies are of particular interest, in which the levels of anti-SMAP natural autoantibodies in the serum of RP patients were measured. The problem of natural autoantibodies to host organism antigens is a new direction of research in modern immunological physiology.[14] In the 90s of the XX century experimental evidence of the presence in the blood of healthy individuals hundreds of types of autoantibodies directed to hormones, receptors, proteins of the cytoskeleton, DNA, histones, various enzymes, molecules of the major histocompatibility complex and other compounds were obtained. The data cited in the literature indirectly indicate that autoantibodies to any endogenous antigens are present in the body of healthy individuals and can be detected by highly sensitive immunechemical methods.[15]

CONCLUSIONS

1. A highly sensitive immunochemical method for determining the level of rhodopsin in the retina of experimental animals was developed, including preparative isolation of rhodopsin from the retina of the cow eyes, immunization of the rabbits with it and production of polyclonal immunoglobulins to the isolated rhodopsin from the serum.
2. Recording of ERG in the rabbits with formed RP in response to presentation of light flashes of different intensities revealed a marked decrease in its amplitude 5 and 13 days after MIAA injection.
3. Injection of SMAP into the lateral ventricle of the brain of the rabbits with formed RP causes a noticeable recovery of ERG amplitude

[14] Palma, J. Natural antibodies – facts known and unknown / J.Palma, B.Tokarz-Deptuła, J.Deptuła [et al.] // Central European Journal of Immunology, – 2018. 43(4), – p.466-475.

[15] Mashiko, S. Broad responses to chemical adducts shape the natural antibody repertoire in early infancy / S.Mashiko, R.R.Shihab, S.B.See [et al.] // Sci Adv., – 2023. May 12; 9(19), – 8872. doi:10.1126/sciadv.ade8872.

induced by presentation of light flashes of different intensities to the animal.

4. In the rabbits with RP a decrease in rhodopsin levels and an increase in HSP70 levels in the retina and an increase in SMAP levels in the hypothalamus were detected in experimental animals 12 days since MIAA injection.
5. I.vtr. injection of SMAP into rabbits 5 and 15 days since MIAA injection brings to increase of rhodopsin and HSP70 levels in the retina compared to the control levels.
6. I.vtr. injection of anti-SMAP polyclonal antibodies in the rabbits 15 days since MIAA injection leads to an increase in the level of rhodopsin and HSP70 in the retina.
7. Immunization of the rabbits with purified SMAP induces the production of anti-SMAP antibodies in their bodies, leading to increased levels of rhodopsin and HSP70 in the retina.
8. A decrease in the level of natural autoantibodies to SMAP was observed in the serum of the patients with diagnosed RP.
9. The results of the study indicate the existence of trophic support of the retinal receptor apparatus from the hypothalamus, as well as the existence of a reverse information channel signaling the hypothalamic nuclei about the functional state of the retinal receptor apparatus and adjusting their activity to its metabolic requirements.

PRACTICAL RECOMMENDATIONS

1. Based on the identified trophic support of the retinal receptor apparatus by the hypothalamic serotonergic system, as well as its violation in RP, it is recommended to increase the activity of the hypothalamic serotonergic system in these patients through the use of appropriate drugs.
2. Based on the detected decreased titer of anti-SMAP natural autoantibodies in the serum of patients with RP, the determination of the level of anti-SMAP antibodies in the serum of such patients can serve as an additional diagnostic criterion for the severity of this disease, as well as a criterion for the correctness of the choice and effectiveness of the therapeutic measures.

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LIST OF ABBREVIATIONS USED

ELISA – enzyme-linked immunosorbent assay

ERG – electroretinogram

HSP70 – heat shock proteins with molecular mass 70 kDa

i.v. – intravenous

i.vtr. – intravitreal

MIAA – monoiodoacetic acid

opt.un. – optical units

RP – retinitis pigmentosa.

SMAP – serotonin-modulated anti-consolidation protein

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