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Experimental realization of minimally invasive techniques of scleral collagen cross-linking

E.N. IOMDINA[1], E.P. TARUTTA[1], V.A. SEMCHISHEN[2], A.R. KORIGODSKIY[3], I.D. ZAKHAROV[3], I.P. KHOROSHILOVA-MASLOVA[1], N.YU. IGNAT'EVA[4], T.N. KISELEVA[1], A.A. SIANOSYAN[1], S.V. MILASH[1]

[1]Moscow Helmholtz Research Institute of Eye Diseases, Ministry of Health of the Russian Federation, 14/19 Sadovaya-Chernogryazskaya St., Moscow, Russian Federation, 105062; [2]Federal Research Center «Crystallography and photonics», Russian Academy of Sciencs, 59 Leninskiy prospekt, Moscow, Russian Federation, 119333; [3]HajBiTek LLC, 44-1 Mira prospekt, Moscow, Russian Federation, 129110; [4]Lomonosov Moscow State University, 1 Leninskie Gory, Moscow, Russian Federation, 119991

Aim — to realize two minimally invasive techniques of scleral collagen cross-linking (SXL) at the equator and posterior pole: 1) targeted irradiation of the region with ultraviolet A (UVA) and 2) sub-Tenon injection of Sklerateks. **Material and methods.** To perform UVA-SXL, a tool was developed that includes a UV-LED light source (370 nm, 3 mW/cm^2) and a polymer-coated silica multimode optical fiber located in one of the two channels of a detachable metal tip. The other channel is used to deliver riboflavin to the scleral surface. The study included 8 Chinchillas' eyes. Intact fellow eyes served as the controls. Scleral echodensity was measured in vivo with Voluson 730 Pro (Kretz) prior to the procedure and then 2 days and 1 month after. After enucleation, the elastic modulus and the degree of scleral cross-linking were established at the same time-points. A placebo-controlled study on the safety and effectiveness of sub-Tenon Sklerateks injections (solution of amino acid salts in the form of succinates) was conducted on 47 Chinchilla rabbits (94 eyes). Sklerateks or placebo (0.1 ml) were injected below the Tenon's capsule of either eye once a week for 1 month (4 injections; 1st series) or 3 months (12 injections; 2nd series). After the end of the course, 22 eyes were studied morphologically. In 72 eyes, scleral samples were obtained in order to evaluate the elastic modulus (Autograph AGS-H tester, SHIMADZU, Japan) and the rate of cross-linking (judging from the denaturation temperature) by differential scanning calorimetry (Phoenix DSC 204 calorimeter, Netzsch, Germany). **Results.** After UVA irradiation, the scleral echodensity increased from 86.7±5.1 to 98±4.9 dB. The elastic modulus appeared 1.5 times higher than that of the control samples. The denaturation temperature also increased indicating the rate of scleral cross-linking as high as 15–18%. Weekly Sklerateks for 1–3 months has been shown to induce neither clinical, nor morphological signs of local irritative, damaging, or toxic effect. The findings also include: a 1.8 times higher rate of scleral cross-linking, activation of cellular elements, neoformation of connective tissue on the scleral surface, and vascular growth, which together indicate a pronounced metabolic and strengthening effect of Sklerateks on the sclera. **Conclusion.** Experimental results on minimally invasive techniques of SXL allow to recommend them for further clinical investigation as a promising treatment of progressive myopia.

Keywords: *sclera, cross-links, collagen, ultraviolet A radiation, riboflavin, elastic modulus, Sklerateks.*

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As is known, patients with progressive myopia develop a dystrophic process in the scleral shell of the eye. This process is accompanied by a reduction in the level of the collagen, the main fibril forming protein, and a damage in the sclera's supporting (biomechanical) properties, which is one of the leading factors of progressing and complicated course of myopia [1, 2]. Therefore, the treatment aimed at achieving myopia stabilization must enable the trophic and the sclera strengthening effect.

For many years, bandaging scleroplastic operations or other sclera strengthening interventions carried out to stop the progression of the myopic process have been using a variety of graft materials [2, 3]. The strengthening effect of these operations is enabled by the emergence of a new biocomposite material "sclera plus transplant": its biomechanical stability is higher than the original sclera [2, 4]. However, the sclera strengthening effect achieved by such interventions is gradually declining in the late postoperative period. This is largely caused by the fact that, in the course of time, the donor plastic material is replaced by defective connective tissue of the recipient (the patient with progressive myopia) [3]. Synthetic plastic materials, especially a biologically active transplant which combines the advantages of artificial materials with stimulating properties of donor tissues are characterized by a higher biomechanical stability [5]. It must however be borne in mind that surgeries administered to children have their own limitations.

Today, there is a more promising method of sclera strengthening treatment, which is directly affecting the damaged structure of the myopic sclera: targeted increase of the level of cross linkage of its collagen structures (crosslinking). It increases the mechanical and fermentative stability due to the formation of cross links in the collagen fibers, which oppose the weakening of the sclera's supporting functions caused by the reduced number of such links in the equatorial and the posterior areas of the sclera; otherwise, this weakening becomes a key factor of irreversible elongation of the anterio-posterior axis of the eye and of myopia progression [2, 4].

For correspondence:
Iomdina Elena Naumovna
e-mail: iomdina@mail.ru

In recent years, scleral collagen crosslinking used to improve the biomechanical stability of the sclera is induced by A range ultraviolet radiation (UVA) combined with the treatment by riboflavin, the latter being the initiator of collagen photopolymerization [6, 7]. This approach proved its high clinical effectiveness in treating progressing ectasias of another connective tissue eye shell, the cornea, in particular, in treating keratoconus [4, 8, 9]. However, to achieve a sclera strengthening effect, we need to enable direct access of radiation to the equatorial and posterior pole areas of the sclera. For this purpose, work reported in [6, 7], where scleral crosslinking was described for the first time, included full-scale surgical intervention: under general and local anesthesia, a large section of the conjunctiva was carried out, the outer eye muscles were dissected and the eyeball was retracted downward with the help of two suture needles introduced intrasclerally at a distance of 2-3 mm from the limbus to allow easy access to the posterior pole area. The part of the sclera intended for crosslinking was instilled several times with 0.1% solution of riboflavin for 10 minutes, followed by UVA treatment, which lasted 30 minutes, during which 0.1% solution of riboflavin was instilled again every 5 to 7 minutes. A similar technique is used until now to perform the crosslinking of the equatorial area of rabbit sclera [10, 11].

Obviously, this method of scleral crosslinking is technically cumbersome, too traumatic and hardly justifiable for clinical practice.

In a few experiments, the use of certain chemical agents injected on the sclera surface to perform scleral crosslinking was investigated [12, 13]. However, the strategy involving an effective anti-dystrophy therapy of the myopic sclera, hardly developed. In our opinion, the creation of new effective methods of progressive myopia combining the metabolic action with scleral collagen crosslinking is a topical research and development issue.

Collagen fibrils, including those of the corneoscleral eye shell, are known to be stabilized by a whole system of cross links [14–16]. In the linking process, the limiting phase is the formation of the so-called allysine (derivative aldehyde lysine) from the lysine amino acid residues. It is caused by lysile oxidase ferment, which includes copper in its composition. Further, the Schiff base with a spatially close amino group of lysine residues belonging to a different polypeptide chain is forming spontaneously, with no participation of ferments. As a result, a strong cross linkage of collagen structures is created [15].

Preliminary studies established that polyamines – compoundsthatcontain two or more primary amino groups enable effective linkage of collagen fibrils, probably along allysine residues. The most optimal way of neutralizing these endogenous compounds is to use succinic acid (in the form of succinate salts), which takes part in the oxidizing citric acid (Krebs) cycle and satisfies the basicenergetic needs of the body. In recent years, succinates are often used to activate the process of regeneration and synthesis of proteins because they possess antioxidant, cytoprotective and antitoxic properties. Their use significantly enhances the course of tissue metabolism: cellular respiration, ion transport, protein synthesis, i.e. essentially, the regenerative processes, which was shown by experimental and clinical studies [17].

In a variety of forms, succinates are present in a number of drugs, including ophthalmological medications. As examples we can mention Catachrom eye drops [18], which contain sodium succinate and improve the energetic processes in lens tissues, or other succinate-based medications like Pirotonik or Mexidol [19, 20].

These data were taken into account in the development of a composition for medicament crosslinking of the sclera, Scleratex, which consists of primary amino acid salts in succinate forms (170 mg) and copper chloride (II) (0.5 mg). Thecompositionalsoincludesnon-activecomponents: hydroxyethyl cellulose (15 mg), preservative benzalkonium chloride (0.75 mg) and water for injection (to 5 ml).

The purpose of this work was experimental implementation of two scleral collagen crosslinking technologies 1) by low-invasive UVA radiation combined with riboflavin in the equatorial and posterior pole areas of the eye and 2) by sub-Tenon's injections of a biologically active composition, Scleratex.

Material and Methods

For UVA sclera crosslinking a device was manufactured which contains a LED source of continuous UVA radiation (wavelength 370 nm, radiation intensity 3 mW/cm^2), connected via a matching optics with multimode quartz optical fiber in a polymer shell. This fiber is located in one of the two channels of a dual removable metal ferrule; the second (hollow) channel is designed for simultaneous delivery of riboflavin solution onto the surface of the sclera (Russian Federation patent No. 161372 issued on March 29.2016). Thisdevicefacilitatesgreatlythecrosslinkingprocedure, sinceUVA radiation and riboflavin solution are delivered through a small section of the conjunctiva;therefore we need to place only one suture (**Fig. 1**). The ferrule is removable, so it is easily sterilizable. The method described was used to perform scleral crosslinking on 8 eyes of Chinchilla rabbits. The intact fellow eyes served as controls. Prior to the procedure, as well as 2 days after it and 1 month after it, acoustic density of the sclera (ADS) was measured *in vivo* using an ultrasound device, VOLUSON 730 Pro (Kretz) with a linear frequency sensor covering the range of 10-16 MHz.

A placebo-controlled study into the safety and effectiveness of sub-Tenon's capsule injections of Scleratex was performed on 47 Chinchilla rabbits (94 eyes). 0.1 ml (100 mcl) of Scleratexor placebo (a solution containing 15 mg of hydroxyethyl cellulose, 0.75 mg of benzalkonium chloride and up to 5 ml water for injection) was in-

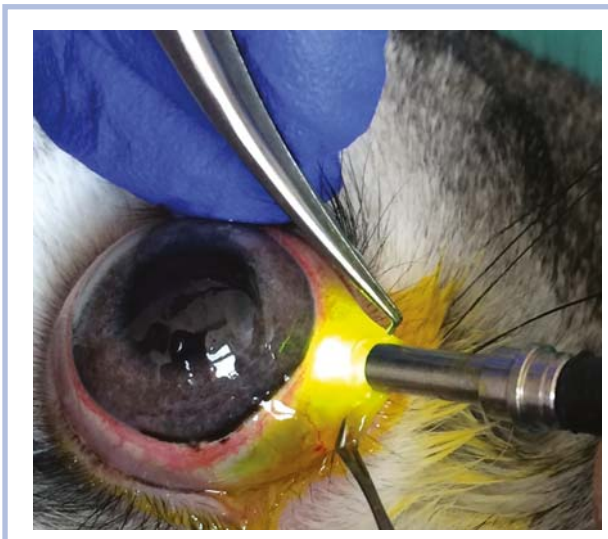

Fig.1. **Low invasive scleral collagen crosslinking procedure (UVA+riboflavin) in the equatorial and posterior pole areas of the eye.**

jected once a week under the Tenon's capsule of the experimental and the fellow eyes, respectively, after retrobulbar anesthesia with 0.4% Inocaine. The first series of experiments (4 injections) lasted 1 month, and the second series (12 injections) took 3 months.

To assess local irritation and damages, all rabbits were examined before and after the course of injections by biomicroscopic methods and with a Scheimpflug camera Galilei G2 (Ziemer Ophthalmic Systems AG 6.0.2) [21].

The safety of local application and the chronic toxicity were investigated by morphological studies of all eyeball structures using light microscopy. The material of this investigation included 18 enucleated eyes of 8 rabbits (experimental eyes and those treated with placebo) and 4 eyes of intact rabbits that served as control. All samples were fixed in 10% formalin, passed through alcohols and poured into paraffin. The preparations were stained with hematoxylin and eosin.

The sclera strengthening effect was assessed 2 days and 1 month after UVA treatment and 1 and 3 months after the completion of injection course. Scleral samples of enucleated eyes were tested on the deformation machine Autograph AGS-H, (Shiвadzu, Japan). The obtained stress-deformation correlations were used to determine the tensile strength, the elasticity modulus and the maximum longitudinal strain of scleral samples [4].

The level of collagen crosslinking of scleral samples was evaluated using differential scanning calorimetry (DSC) on the calorimeter, Phoenix DSC 204, Netzsch, Germany). DSC helps determine the heat effects of collagen structure denaturation (denaturation temperature T_d) and the heat of helix-coil conformation transfer of the protein, denaturation enthalpy (DHm), which characterize post-translational modification of collagen macromolecules, including the formation of intra- and intermolecular cross links [22–24].

The results were statistically processed using XL and Statistica 6.0 software packages.The analyzed samples were tested for normal distribution. The values having normal distribution were presented as average value (M),standard deviation (*SD*), and mean quadratic error (*m*); the significance of difference between group values with a significance level of not less than 95% was assessed using a parametric Student's t-test.

The investigations were carried out in accordance with international requirements regulating work with experimental animals (European Communities Council Directive 86/609/EEC and ARVO Statement for the Use of Animals in Ophthalmic and Vision Research) and were approved by the local ethical committee of the Moscow Helmholtz Research Institute of Eye Diseases, Russian Ministry of Health.

Results and Discussion

As a result of UVA treatment, acoustic density of the sclera (ADS) which initially was 86.7±5.1 dB, grew to 98±4.9 dB in two days. After 1 month, it practically stayed at the level achieved (**Table 1**). In contrast, ADS of the respective area of the intact fellow eye did not change compared to the initial value. The elasticity modulus (E) reached the value of 25.4±3.7 MPa two days after the treatment, which was 1.5 times higher than the respective parameter of the sclera of the intact fellow eye. 1 month after the procedure, the value of E stayed at practically the same level. T_d of the scleral samples of the experimental eyes proved significantly higher than the control; the difference corresponded to an increase of crosslinking level of scleral collagen by 15–18% (**see Table 1**). The examination carried out 1 month after the procedure revealed no clinical differences between the experimental and the intact fellow eyes. The section on the conjunctiva could not be visualized.

The obtained results show that the proposed device for scleral collagen crosslinking enables a low-invasive and minimally traumatizing UVA treatment of the equatorial and the posterior area of the sclera and in this way increase its biomechanical stability.

Biomicroscopic examinations of the experimental rabbit eyes, undertaken during the courses of sub-Tenon injections of Scleratex given during 1 month (4 injections) and 3 months (12 injections), as well as 1 and 3 months after the completion of the respective injection courses, revealed no differences in the condition of all eye structures as compared to the figures for the group of placebo and intact controls. Only upon completion of long-term (lasting 3 months) injections of the experimental (Scleratex) and the control (placebo) composition, slight desquamation of corneal epithelium was noted, which was most probably caused by regular action of the conservative (benzalkonium chloride), which was

Table 1. **Structural and biomechanical parameters of the sclera before and after UVA crosslinking (*M±m*)**

Examination times	ADS, dB	Elasticity modulus, MPa	T_d, °C
Original values	86.7±5.1	–	–
2 days after UVA crosslinking	98±4.9*	25.4±3.7*	69.1±0.7*
1 month after UVA crosslinking	103±6.0*	26.0±4.1*	69.3±0.6*
Control (intact fellow eye)	86.4±5.0	16.7±2.9	67.4±0.9

Note: Here and in Table 2: * – the difference with control values is significant, $p<0.05$.

Table 2. **Structural and biomechanical parameters of the sclera before and after a course of Scleratex injections (*M±m*)**

Examination times	Elasticity modulus, MPa	T_d, °C	Δ, °C
After 4 injections of Scleratex	27,3±4.0*	69.9±0.9*	Δ=5.8±0.17*
After 12 injections of Scleratex	29.1±3.6*	70.0±0.7*	Δ=5.9±0.19*
Control (placebo injections)	15.0±1.9	67.0±0.8	Δ=5.5±0.14

part of 0.4% inocaine applied for surface anesthesia prior to sub-Tenon injections.

Histological examinations of rabbit eyes, enucleated 1 month after the course of Scleratex injections, revealed neoplastic connective tissue with a large number of cells and newly formed vessels, which is a favorable factor contributing to the strengthening of the scleral tissue and to the improvement of its trophicity (**Fig. 2, 3**).

Experimental and placebo-controlled eyes revealed no changes in the structure of eye tissue or the condition ofocular media, which testifies to the lack of any local irritating or toxic effect of the composition discussed on inner eye structures over the period of 1 month when it was applied (4 injections).

The safety check of the sclera strengthening composition also included the evaluation of its chronic toxicity, i.e. itsinfluence on eye tissues in the case of a triple number of injections (12) during the time period which is three times longer than the targeted application course.

The morphological examination of the experimental rabbit eyes enucleated immediately after a long-term (3 months) course of Scleratex injections showed that, starting with the conjunctiva,the sclera is covered with newly formed connective tissue, which spans the surface from the anterior area to the equator. The tissue is densely merged with the sclera; it has a large quantity of cellular elements and newly formed vessels, which contributes to a strengthening of the scleral tissue and improvement of its trophicity (**Fig. 4, 5**). Collagen fibers are arranged in a more or less orderly fashion. Organoid structure is formed – a substance similar to the sclera. Proliferative activity of the cellular elements is observed; they appear to be unevenly distributed over the tissue.Proliferative activity is noted in the outer and inner layers of the sclera.

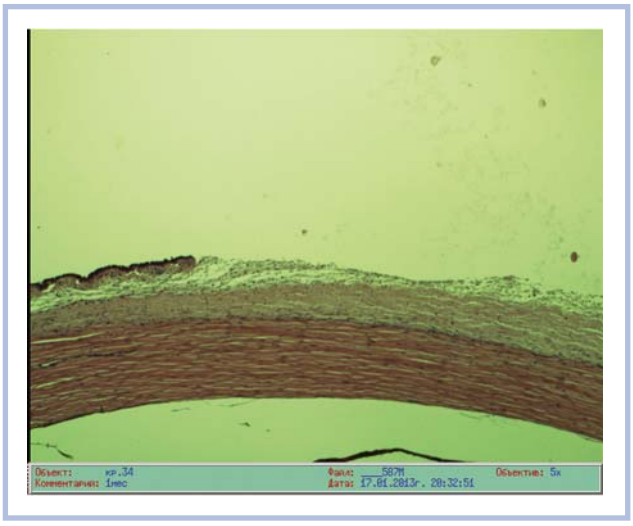

Fig.2. **Formation of new connective tissue on the scleral surface after Scleratex sub-Tenon 1 month injection course.**
Hematoxylin — eosin staining (×100).

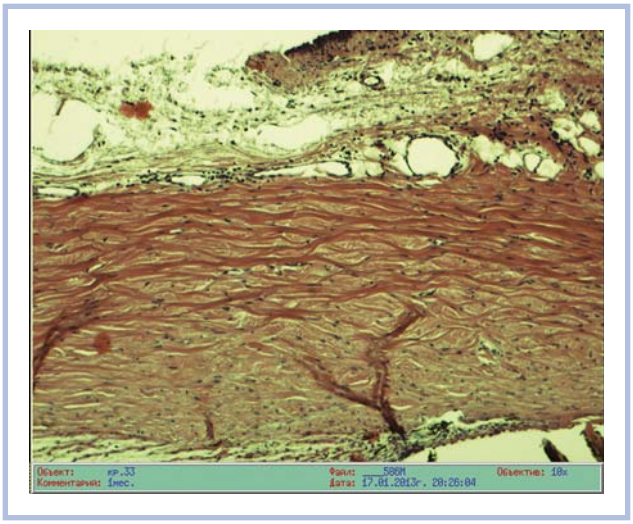

Fig.3. **Large number of cells and newly formed vessels in newly formed connective tissue after Scleratex sub-Tenon 1 month injection course.**
Hematoxylin — eosin staining (×200).

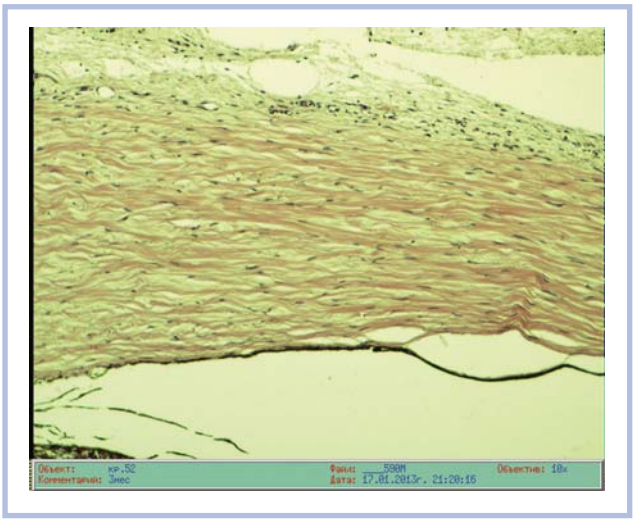

***Fig. 4*. Newly formed connective tissue after Scleratex sub-Tenon 3 month injection course is densely merged with the sclera and spans the surface from the anterior area to the equator.**

Collagen fibers are arranged in a more or less orderly fashion. Proliferative activity of the cellular elements is observed, they appear to be unevenly distributed over the tissue. Hematoxylin — eosin staining (×200).

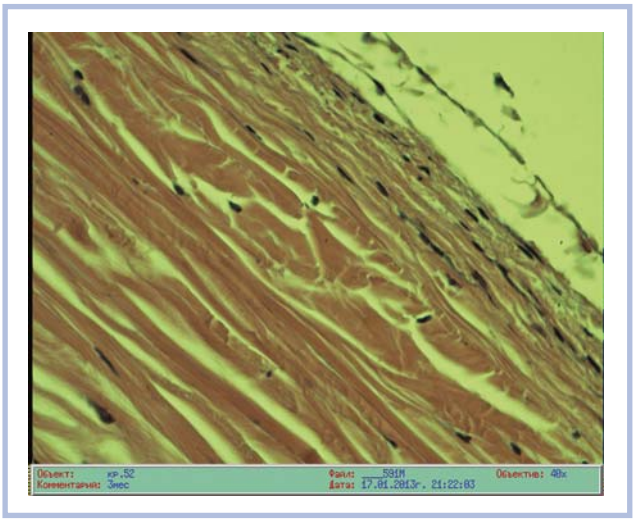

***Fig.5*. Large quantity of cellular elements and newly formed vessels in outer and inner layers of the sclera after Scleratex sub-Tenon 3 month injection course. Proliferative activity is noted.**

Hematoxylin — eosin staining (×400).

Except for desquamation of corneal epithelium, revealed in all experimental and placebo control eyes (which, as noted above, is caused by the action of preservative benzalkonium chloride present in 0.4% inocaine preparation, protractedly applied for surface anesthesia prior to sub-Tenon injections), no other pathological changes in the structure of these experimental and control eyes or in the state of ocular tissues could be revealed. This is an evidence of the absence of any toxic effect of the examined composition on inner eye structures during its long-term (3 months) application.

To assess the sclera strengthening effectiveness of Scleratex injections, we performed a contrastive examination of the level of cross links in the samples of experimental and intact eyes, as well as eyes from the placebo group, and tested the same samples biomechanically one month after the completion of the course of injections (**Table 2**).

It has been established that 1 month after the course of Scleratex injections the level of scleral crosslinking of the experimental eyes is much higher than that of the control eyes (**Fig. 6**).

While T_d of the sclera in the placebo group was 67.0 ± 0.8^0, after a series of Scleratex injections this parameter grew to 69.9 ± 0.9^0 ($p<0.02$) in one month and stayed at practically the same level after a 3-month course of injection. This is an evidence of a stable increase in scleral crosslinking level in the experimental group of eyes (**see Table 2**).

The parameter Δ (the difference between the temperature of the sample at the end of denaturation process and at its start), which is another characteristic of the quantity of stabilizing cross links in the collagen, was equal to 5.0 ± 0.14^0in the placebo and intact eyes groups, whereas in the experimental group it was found to be significantly higher: 5.9 ± 0.19^0 ($p<0.05$). We can thus assess the increase of scleral collagen crosslinking at about 15–20%.

In an earlier contrastive study, we found that the level of scleral collagen crosslinking of highly myopic eyes was, on average, 15% lower than that of emmetropic eyes [2]. This fact suggests that the crosslinking effect achieved in the present pre-clinical study is likely to be sufficient if the technology is applied in clinical practice to cure progressive myopia.

The elasticity modulus of the sclera measured one month after 4 Scleratex injections had been administered over a 1-month span, averagely grew by 1.8 times ($p<0.02$). After 12 Scleratex injections given over a 3-month span the parameter grew a bit more and was found to exceed the elasticity modulus of the sclera of the placebo group eyes (**see Table 2**). This slight additional increase (against the figure for 4 injections received within one month) is probably unrelated to the formation of additional cross links in the structure of the sclera; rather, it is caused by the formation of more mature connective tissue on the scleral surface, which is corroborated by histological studies.

The technology of scleral collagen crosslinking, which enables targeted delivery of UVA and riboflavin to the equatorial and posterior pole area of the eye through optic fiber, helps achieve a significant sclera strengthening effect comparable with that presented in [25], where the respective scleral area was treated by UFA and riboflavin directly (with the same intensity). Although the morphological examinations referred to in [25] revealed

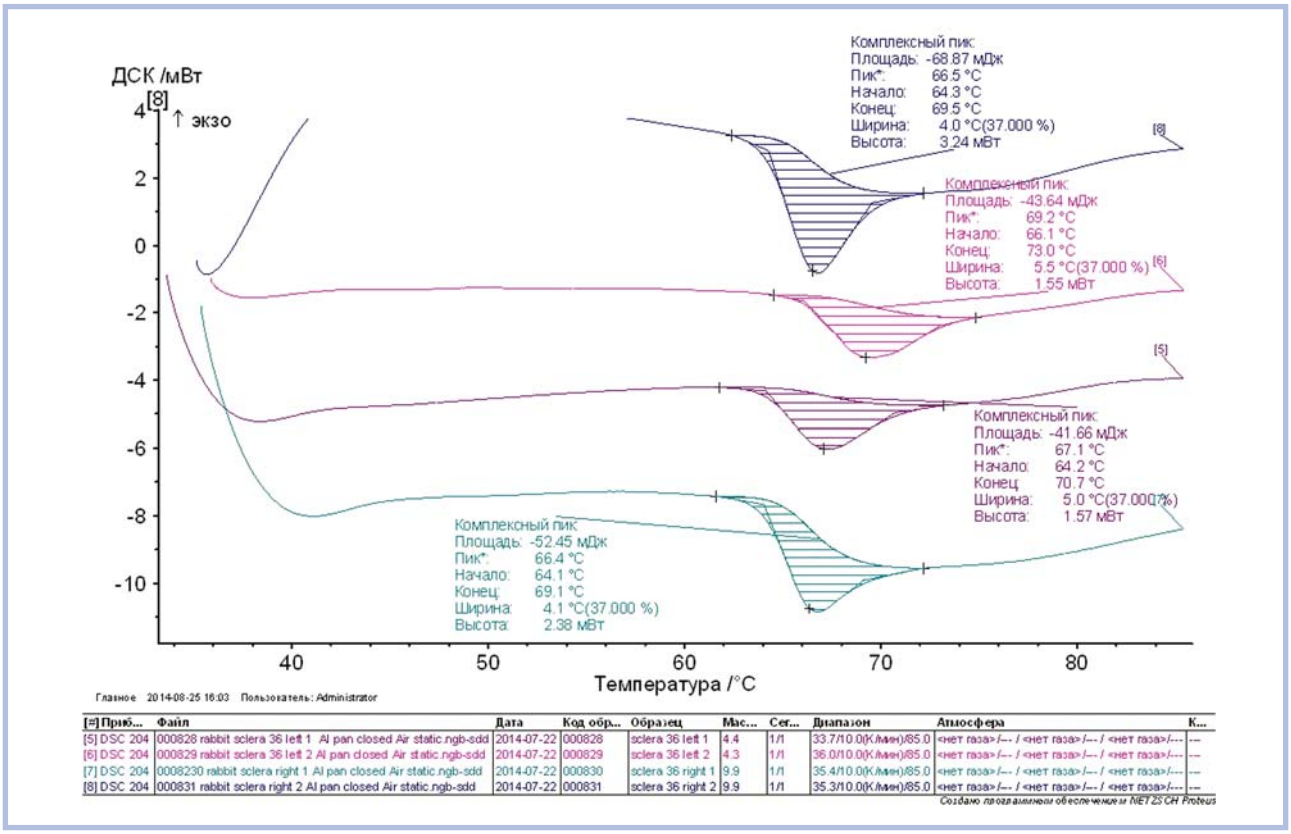

Fig.6. **DSC of experimental and control eyes sclera samples.**

Experimental eyes: 1 month after Scleratex sub-Tenon 1 month injection course: T_d°C = 69,2°, Δ=73,0°–66,1°=6,9°. Control eyes: 1 month after placebo 1 month injection course. T_d°C = 66,5°, Δ=69,5°–64,3°=5,2°.

no damaging effect of such treatment on pigment epithelium and other retinal structures of rabbits, the safety of the crosslinking technology proposed in the present paper needs to be confirmed by adequate morphological studies in future.

The other technology we are proposing, which was implemented in the pre-clinical placebo-controlled investigation of Scleratex injection solution used as a medicament crosslinking of scleral collagen, was found to have no clinically observable local irritating or toxic action when applied weekly over 1 month (in a series of 4 injections).The absence of any morphologically detectable pathological changes of ocular media and tissues testifies to the safety of applying this composition over the time span mentioned. A study into chronic toxicity, undertaken for the case when the injections are given in a similar manner for three months, showed no pathological changes of ocular media and tissues either, with the exception of desquamation of corneal epithelium that appeared in various degrees also in placebo-controlled eyes. This is caused by long-term action of bezalconium chloride preservative, present in eyedrops regularly used in sub-Tenon injections to ensure epibulbar anesthesia. Sub-Tenon injections of Scleratex ensure an essential improvement of structural and biomechanical stability of scleral tissue due to the fact that the level of crosslinking, stabilizing the collagen structures of the sclera, are increased by 15-20%, and the elasticity modulus is increased 1.8 times. Further factors are the activation of cell elements, formation of new connective tissue and additional vessels. On the whole, this testifies to the effectiveness of metabolic and sclera strengthening impact of Scleratex injection course. Prior experiments with injections of a linking agent, glyceraldehyde, also revealed a significant sclera strengthening effect and no negative impact on the retina [26], however, in contrast to the composition proposed in the present paper, no stimulating (anti-dystrophic) action was achieved.

Conclusion

The results of experimental implementation of low invasive technologies of scleral crosslinking show that these technologies should be recommended for further clinical study as promising instruments of sclera strengthening and metabolic treatment of progressive myopia.

None declared.

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