DOI: 10.17816/OV1026-12

EFFECT OF CROSSLINKING WITH RIBOFLAVIN AND ULTRAVIOLET A (UVA) ON THE SCLERAL TISSUE STRUCTURE

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For citation: Ophthalmology Journal, 2017;10(2):6-12

Received: 28.12.2016
Accepted: 28.03.2017

✧ ***Aim.*** To evaluate the effect of scleral crosslinking with riboflavin and ultraviolet A (UVA) on scleral tissue structure *in vitro*. ***Material and methods.*** The study was performed on seven porcine cadaver eyes. Two parallel scleral strips were excised from each eyeball; one was subjected to the crosslinking procedure (instillation of 0.1% aqueous solution of riboflavin mononucleotide for 20 min followed by UV irradiation of 3 mW/cm^2 for 30 min), and the other was used as control. Scleral structure was evaluated using light (Van Gieson's stain) and electron microscopy. Special software was used to perform morphometric analysis of the microphotographs. ***Results.*** As a result of crosslinking, the average packing density of collagen fibers increased by 8.2%, the intermediate space decreased by 5.2%, and the average diameter of collagen fibrils increased by 12%. There were no pathological changes in the scleral structures. ***Conclusion.*** Obtained results confirm the efficacy of scleral crosslinking with riboflavin/UVA in forming additional crosslinks and the safety of the procedure for the scleral tissue.

✧ ***Keywords:*** scleral crosslinking; light microscopy; electron microscopy; morphometric analysis.

ВЛИЯНИЕ КРОССЛИНКИНГА С РИБОФЛАВИНОМ И УЛЬТРАФИОЛЕТОМ А (UVA) НА СТРУКТУРУ СКЛЕРАЛЬНОЙ ТКАНИ

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Для цитирования: Офтальмологические ведомости. – 2017. – Т. 10. – № 2. – С. 6–12

Дата поступления: 28.12.2016
Статья принята к печати: 28.03.2017

✧ ***Цель.*** Оценить влияние кросслинкинга склеры с рибофлавином и ультрафиолетом А (UVA) на структуру склеральной ткани в эксперименте *in vitro*. ***Материал и методы.*** Исследование проводили на 7 свиных глазах. Из каждого глазного яблока вырезали по два параллельных склеральных лоскута, один из которых подвергался процедуре кросслинкинга (инстилляция 0,1 % водного раствора рибофлавина мононуклеотида в течение 20 минут, облучение ультрафиолетом А мощностью 3 мВт/см^2 в течение 30 минут), второй использовался в качестве контроля. Структуру склеры оценивали с помощью световой (окраска по Ван Гизону) и электронной микроскопии. Проводили морфометрический анализ фотоснимков гистологических препаратов с использованием специального программного обеспечения. ***Результаты.*** В результате кросслинкинга склеры с рибофлавином/UVA наблюдалось увеличение плотности упаковки коллагеновых волокон в среднем на 8,2 %, уменьшение площади межуточного пространства — на 5,2 %, увеличение диаметра коллагеновых фибрилл — на 12 %. Патологических изменений структур склеры выявлено не было. ***Вывод.*** Полученные результаты подтверждают эффективность кросслинкинга склеры с рибофлавином/UVA в образовании дополнительных перекрёстных связей и безопасность процедуры для склеральной ткани.

✧ ***Ключевые слова:*** кросслинкинг склеры; световая микроскопия; электронная микроскопия; морфометрический анализ.

Myopia is one of the main causes of impaired vision, affecting nearly 1 million people worldwide [10, 11]. Myopia progression is observed in approximately half of these cases and still remains a challenge for ophthalmologists [6]. A number of authors believe that scleral crosslinking may become a new effective method for the treatment of progressive myopia, as this disease is associated with a decreased biomechanical strength of the sclera [7, 13, 16]. Crosslinking is the process of chemically joining two or more macromolecules, which generally results in strengthening of the material [1, 17]. For more than 10 years, the corneal crosslinking with riboflavin and ultraviolet A (UVA) has been successfully applied for the treatment of keratectasia. This method provides corneal tissue strengthening, thus preventing disease progression [2, 5]. Several studies have demonstrated significant improvements in biomechanical parameters of the sclera in response to photochemical crosslinking [3, 9, 14, 18]. However, very few studies have attempted to assess histological changes in the sclera that occur after the procedure [7, 8, 15]. We propose that investigation of morphological changes in the sclera after photochemical crosslinking could provide valuable information on the safety of this procedure.

This study aimed to assess the impact of riboflavin/UVA crosslinking on the structure of the scleral tissue in an *in vitro* experiment.

MATERIALS AND METHODS

Preparation of scleral tissue samples. We used seven pig eyes (no later than 3 h after slaughtering). Biomaterial was shipped to the laboratory within 30–40 min after collection in a special thermocontainer (at +4 °C) and placed in a refrigerator. The total time of storage did not exceed 6 h.

The surrounding soft tissues were completely removed from the eyeballs. Using a scalpel and surgical scissors, two 4 mm × 10 mm scleral flaps were cut in each eyeball in the sagittal direction, starting 2 mm from the limbus. One of the two scleral samples of each eye underwent crosslinking (experimental group), and the second paired sample was left intact (control group).

Crosslinking procedure. Scleral samples from the experimental group were placed on a slide with the exterior surface facing upward, there was further application of 0.1% aqueous solution of riboflavin mononucleotide, and samples were incubated for 20 min. Samples were exposed to UVA (at a wavelength of 370 nm and power of 3 mW/cm^2) using the ophthalmic device for ultraviolet (UV) crosslinking 'UValink' (Russia) for 30 min. Every 5 min, a fresh portion of photosensitizer was added to the samples.

Eight samples (from four eyeballs) were investigated using light microscopy and six (from three eyeballs) using electron microscopy.

Light microscopy. Scleral flaps were fixed in 10% neutral buffered formalin for 48 h. Then the samples were dehydrated in graded alcohols and embedded in paraffin wax. Afterward, histological sections of 5–7 μm thick were cut and stained using Van Gieson's pichro-fuchsin. Visual analysis of the samples was performed using the light microscope Axiostar Plus (Carl Zeiss) at different magnifications. Morphometric measurements were made using a computerized medical video system that included microscope (Axiostar Plus, Carl Zeiss), digital camera (Jenoptik ProgRes C10), and personal computer Pentium-IV with the software "VideoTesT Morphology." The software was used for automatic calculation of the size (in pixels) of variously stained scleral structures (collagen fibers, sclerocyte nuclei, and interstitial space). Outputs containing data on relative areas of sclera structures (in % of the total area) had particular practical value (Figure 1).

Electron microscopy. Specimens for electron microscopy were fixed in 2% glutaraldehyde in Millonig's phosphate buffer (pH, 7.2–7.4) with postfixation in 1% osmium oxide in the same buffer. Specimens were dehydrated in graded alcohols and embedded in the Epon 812 according to a standard technique [4]. Semithin and ultrathin sections were prepared using an ultratome LKB-III 8800 (Sweden), then contrasted with 2% aqueous uranyl acetate and plumbum citrate according to Reynolds, and they examined and photographed with a transmission electronic microscope Jem-1011 (JEOL Ltd., Japan) at 5,000–20,000 magnification. Diameters of collagen fibrils were compared on the basis of photographic image analysis (at 10,000 magnification) using the Olympus iTEM software for electronograms (Figure 2).

RESULTS

Light microscopy of sclera samples allowed visualization of red collagen fibers and black sclerocyte nuclei in both experimental and control samples. We found no signs of pathological changes in the samples that underwent crosslinking (Figure 3).

We performed morphometric analysis of the images to compare relative areas of collagen fibers and the interstitial space in experimental and control groups. These parameters were used to assess the

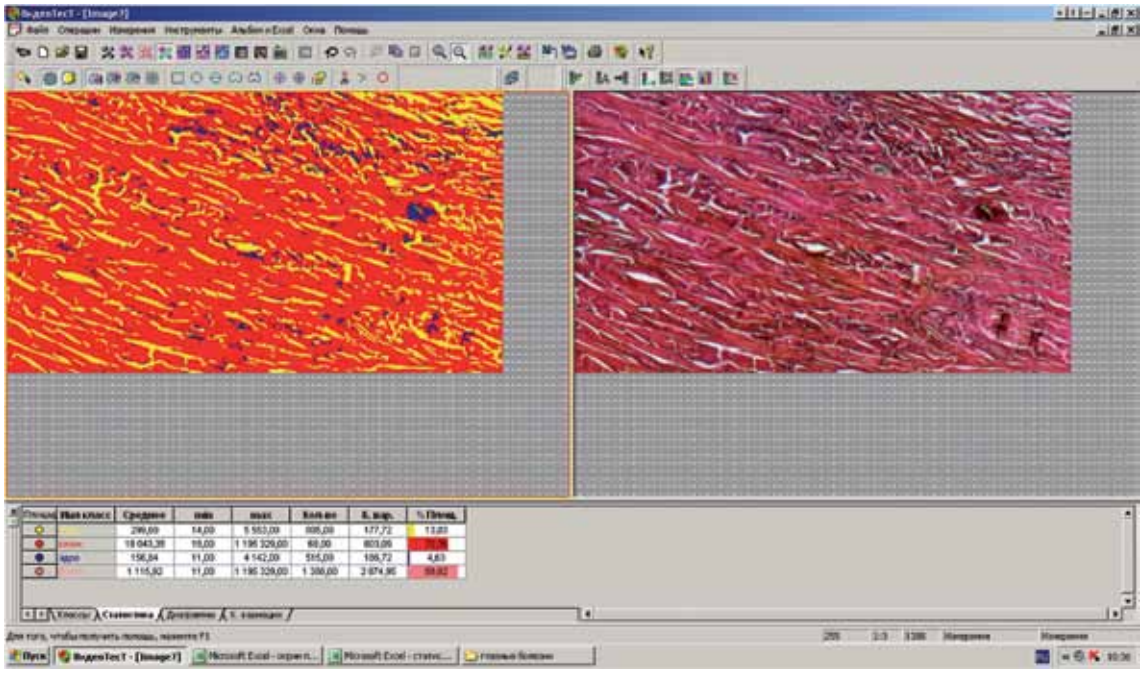

Fig. 1. Morphometric measurements of scleral histological sections photographs with the "VideoTesT Morphology" software

Рис. 1. Морфометрические измерения фотоснимков гистологических срезов склеры в программе «ВидеоТестТ Морфология»

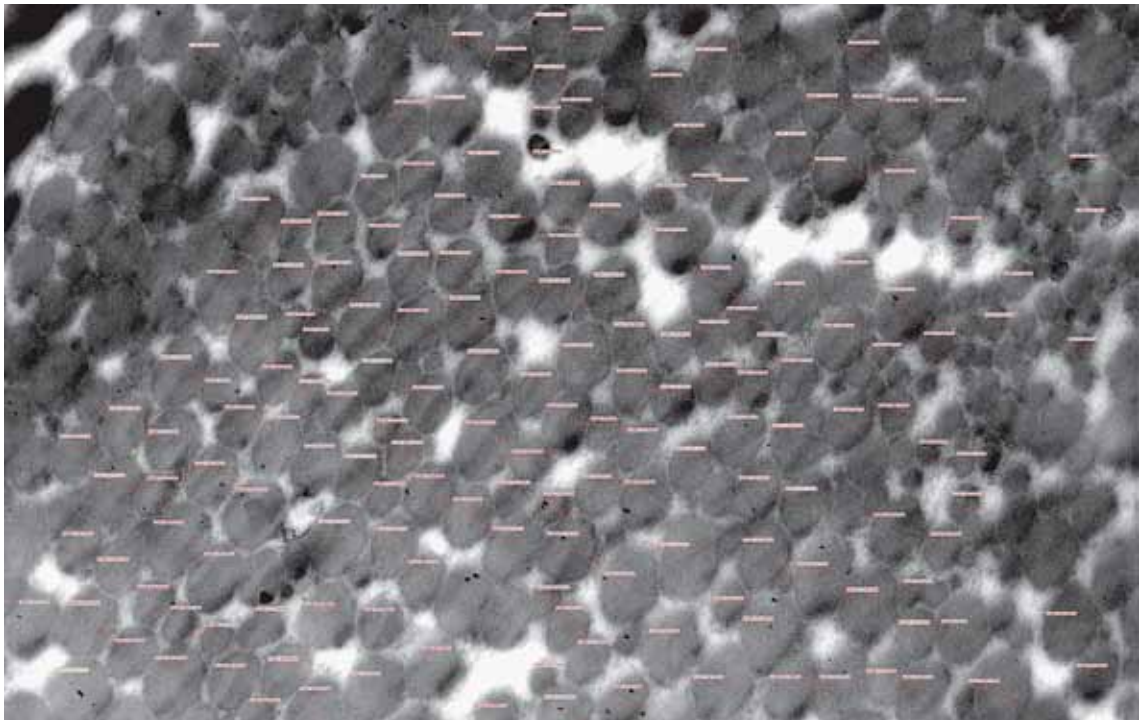

Fig. 2. Collagen fibrils diameter measurement with the Olympus iTEM software

Рис. 2. Измерение диаметра коллагеновых фибрилл в программе Olympus iTEM

impact of crosslinking on the packing density of scleral tissue (Tables 1 and 2).

We observed an 8.2% increase in the packing density of collagen fibers and a 5.2% decrease of the interstitial space in response to scleral crosslinking with riboflavin/UVA.

Regularly alternating fibrils forming collagen fibers were seen on the electron microscopic images (Figure 4). Inside the fibrils, we observed a typical cross striation with a clear regularity. We identified no pathological changes in the scleral fibers in both groups.

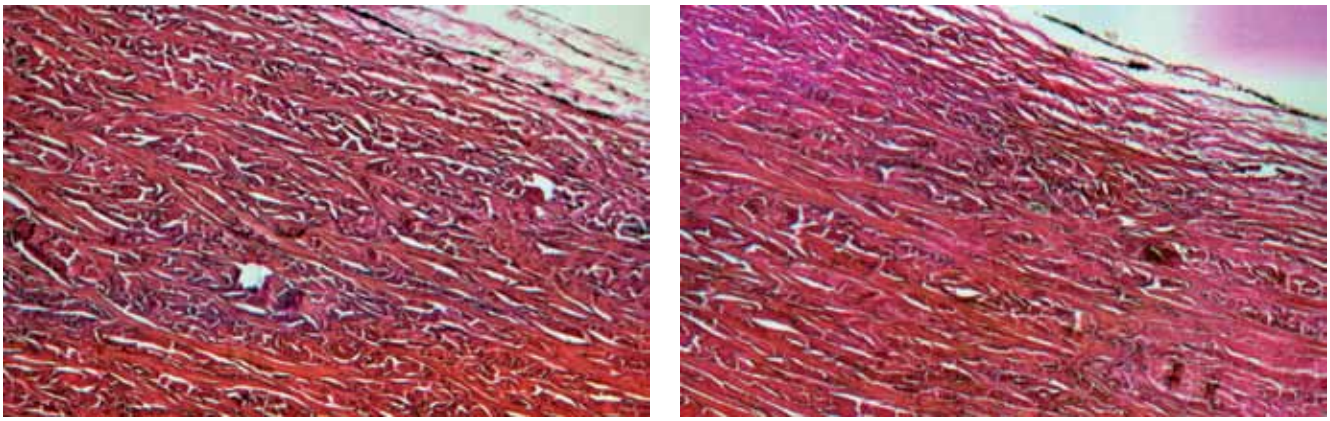

Fig. 3. Histological section of scleral samples from experimental and control groups. Van Gieson staining, magnification ×100
Рис. 3. Гистологический срез склеральных образцов опытной и контрольной групп. Окраска по Ван Гизону, увеличение ×100

We assessed the condition of sclerocytes and found that they maintained structural integrity without damages to the cell wall (Figure 5). Some sclerocytes had elongated cytoplasmic processes of irregular shape where the intact nucleus, organelles, and cytoplasm could be visualized. Quantitative analysis of electronograms showed that crosslinking with riboflavin/UVA increased the diameter of collagen fibrils by 12% on average (Table 3).

DISCUSSION

We assessed the impact of crosslinking with riboflavin/UVA on the structure of the sclera using light and electron microscopy. We found no pathological changes in the sclera of the experimental samples after crosslinking compared with control samples. On the contrary, we observed a compaction of collagen fibers and an increase in the diameter of scleral fibrils. Our data are consistent with the results of

Table 1

Comparison of the relative area of collagen fibers (% of the total area) in the experimental and control groups

Таблица 1

Сравнение относительной площади (% от общей площади) коллагеновых волокон в опытной и контрольной группах

Eye	Experiment, %	Control, %	Increase in the relative area of collagen fibers in the experimental samples compared with control samples, %
1	66.63	59.01	7.62
2	70.36	57.97	12.39
3	66.57	64.73	1.84
4	71.76	60.95	10.81
Mean value	68.83	60.67	8.17

Table 2

Comparison of the relative area of the interstitial space (% of the total area) in the experimental and control groups

Таблица 2

Сравнение относительной площади (% от общей площади) межуточного пространства в опытной и контрольной группах

Eye	Experiment, %	Control, %	Reduction in the relative area of interstitial space in the experimental samples compared with control samples, %
1	10.96	16.02	5.06
2	13.83	17.89	4.06
3	10.97	17.32	6.35
4	8.96	14.24	5.28
Mean value	11.18	16.37	5.19

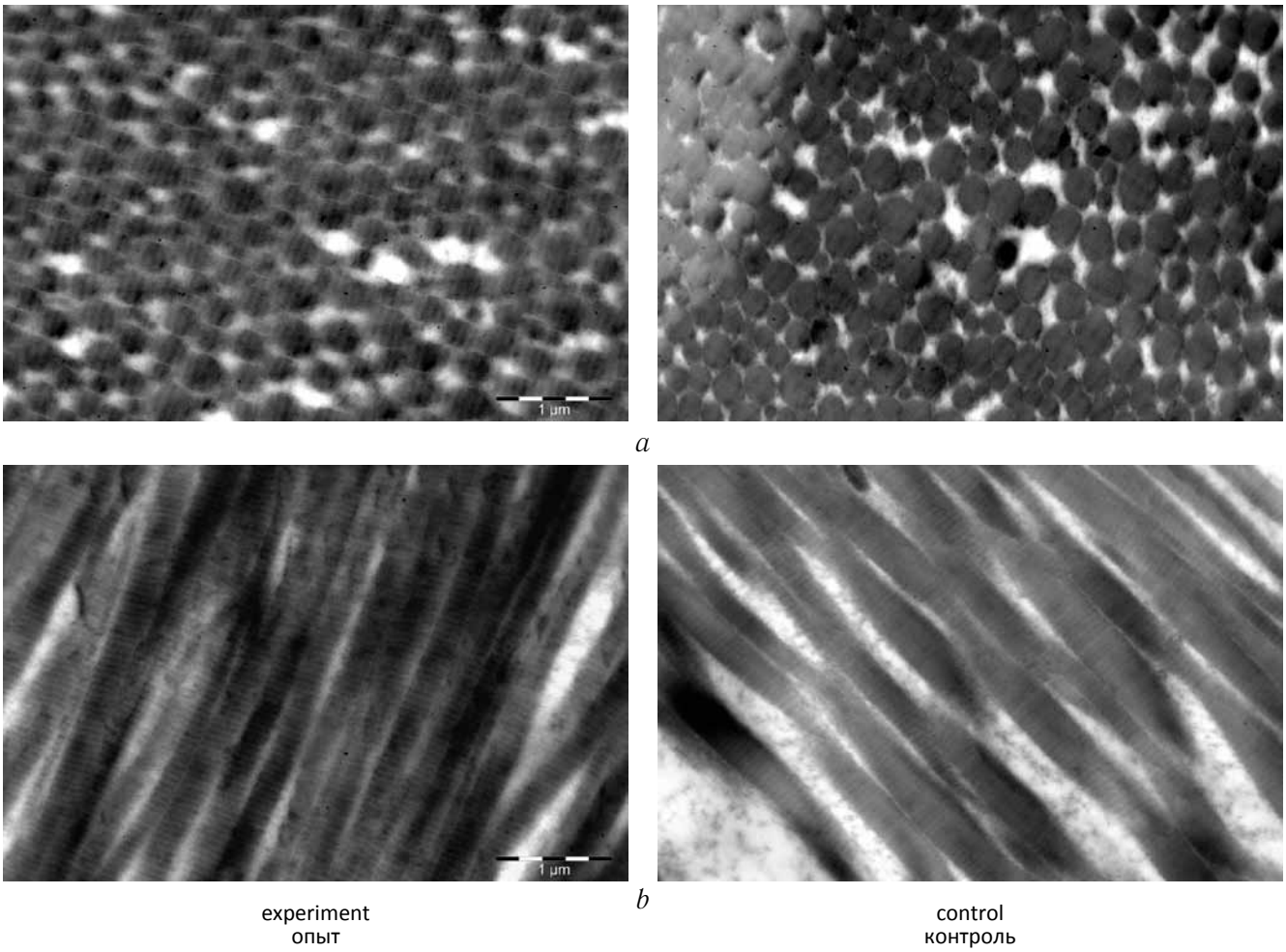

Рис. 4. Collagen fibrils bunches' transversal (*a*) and longitudinal (*b*) sections from experimental and control groups (magnification ×10,000)

Рис. 4. Пучки коллагеновых фибрилл опытной и контрольной групп при поперечном (*a*) и продольном (*b*) срезах (ув. ×10000)

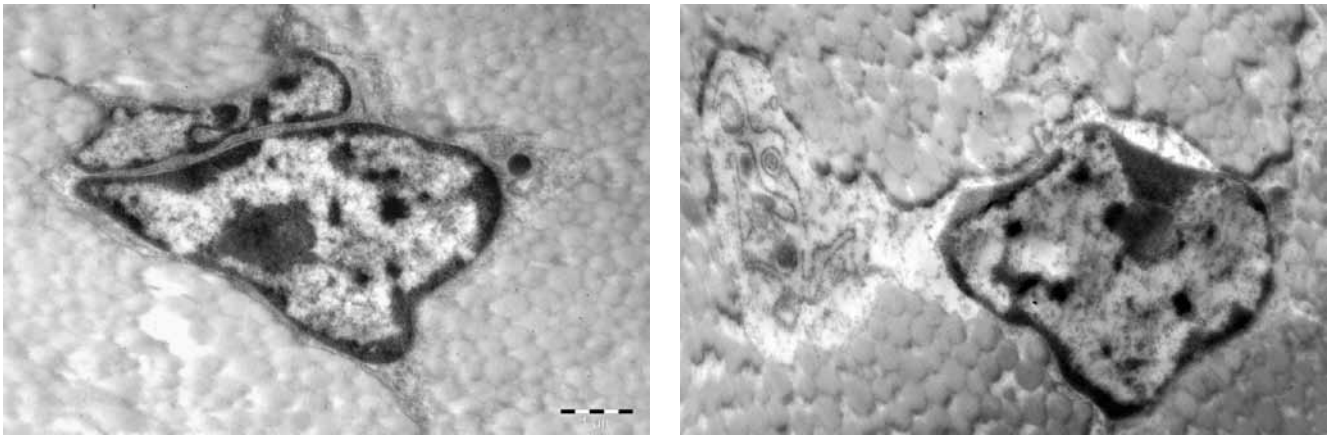

Fig. 5. Sclerocytes from experimental and control groups (magnification ×10,000)

Рис. 5. Склероциты опытной и контрольной групп (ув. ×10000)

other authors. Wollensak et al. observed only a few damaged sclerocytes after crosslinking without any pathological changes in scleral fibers, using electron microscopy. Scleral tissue was concluded to be highly resistant to harmful UV radiation [15]. Choi et al. detected a 27% increase in the diameter of collagen fibrils in the sclera after crosslinking [7]. Jung et al. observed a denser arrangement of collagen fibers after crosslinking using light microscopy. However, this study was limited to just one cadaver eye in which the density of fibers was estimated subjectively, without any morphometric analysis [8].

Table 3

Comparison of the collagen fibrils mean diameter in the scleral samples from experimental eye and contralateral control eye

Таблица 3

Сравнение среднего диаметра коллагеновых фибрилл опытных и парных контрольных образцов склеры

Eye	Experimental sample (pixel)	Control sample (pixel)
1	177.09	152.95
2	186.00	168.33
3	192.86	175.05
Mean value	185.32 (112%)	165.44 (100%)

The results of our study confirm currently available data suggesting relatively high UV stability of the scleral tissue. Increased diameter of collagen fibrils and higher density of scleral fibers after crosslinking are associated with the formation of additional crosslinks pushing the collagen molecules away from each other [8, 12].

CONCLUSION

Scleral crosslinking with riboflavin/UVA increased the packing density of collagen fibers by 8.2%, reduced the interstitial space by 5.2%, and increased the diameter of collagen fibrils by 12%. Our data confirm the formation of additional crosslinks between the macromolecules of the sclera. Exposure to UV radiation in the presence of a photosensitizer does not induce pathological changes in the cells and fibers of the sclera. Therefore, this method is safe and could be used for the sclera.

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