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Study of the possibility of increasing the intensity of photochemical processes of riboflavin/UV photocrosslinking of scleral collagen by means of tissue immersion clearing

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Abstract. **The possibility of increasing the efficiency of light action during scleral collagen riboflavin/UV photocrosslinking by means of immersion clearing of tissue before UV irradiation is studied. The effectiveness of photo-action is assessed by a decrease in the relative content of the photocrosslinking sensitizer, riboflavin, which is determined from the decay times of tissue fluorescence at different stages of the photocrosslinking process. The fluorescence decay times are measured using a multiphoton tomograph in the mode of time-resolved two-photon fluorescence recording. Studies are performed *in vitro* on porcine sclera samples. An 88 % aqueous solution of glycerol is used as an immersion clearing agent. Optical clearing is shown to make it possible to increase the intensity of photoinduced conversion of riboflavin into non-fluorescent leukoforms during photochemical reactions preceding collagen crosslinking. According to the experimental data obtained for cases of photocrosslinking without optical clearing and with preliminary optical clearing of tissue under the same irradiation conditions, the content of riboflavin that has not undergone photoconversion in tissue, at depths from 40 to 75 μm, after UV irradiation in the first case is approximately 5.6 times (average over 15 regions of two samples) higher than in the second case.**

Keywords: *riboflavin/UV photocrosslinking, sclera, time-resolved two-photon fluorescence, optical immersion clearing.*

1. Introduction

Riboflavin/UV photocrosslinking is a method of purposeful enhancement of crosslinking in the collagen structure of biological tissue by UV irradiation in the presence of riboflavin as a photosensitizer [1–12]. One of the results of this treatment is an increase in the mechanical strength of tissue [2, 3, 6, 8–11]. Riboflavin/UV photocrosslinking is already used in ophthalmology to strengthen the cornea in keratectasia [2–5]. Currently, in experiments in animals and scleral tissues *ex vivo*, the possibilities of using this method for modifying the properties of the sclera are studied [6–13]. The implementation of riboflavin/UV photocrosslinking of scleral collagen is associated with certain difficulties [11, 14]. One of them is due to the fact that in the native sclera, in contrast to the cornea, the strong light scattering leads to a sharp drop in the intensity of UV radiation and, as a consequence, to a decrease in the rate of photochemical reactions deep in tissue. A possible way to increase the penetration depth of UV radiation into tissue during scleral photocrosslinking is optical immersion clearing of the sclera before UV irradiation, since it is proved that such clearing of the sclera can significantly increase its transparency, including the UV spectral region [14–18].

The aim of this work is to compare the intensity of photochemical processes of riboflavin/UV photocrosslinking of scleral collagen without immersion clearing and with immersion clearing based on monitoring data on changes in the relative content of riboflavin in tissue caused by photochemical processes associated with photocrosslinking.

Diffusion of riboflavin, fluorescent upon UV excitation [19, 20], into tissue and its photochemical transformations that occur during riboflavin/UV photocrosslinking lead to significant changes in tissue fluorescence intensity [21–25]. In the case of the cornea, this is used to assess both the average concentration of riboflavin in the tissue and the distribution of riboflavin over its thickness at different stages of photocrosslinking [22–25]. The local concentration of riboflavin in the cornea in [22, 23, 25] was assessed using two-photon fluorescence confocal microscopy. To estimate the riboflavin content, they used the measured depth dependences of the time-average intensity of the recorded fluorescence signal. In the case of the sclera, strong light scattering in the tissue does not allow this approach. In this work, the contribution of the fluorescence of exogenous fluorophores (riboflavin and fluorescent products of its photoinduced conversion) at different stages of riboflavin/UV photocrosslinking was estimated from the mean fluorescence decay time in the probed tissue volume, determined from fluorescence lifetime imaging microscopy (FLIM) data.

2. Experimental technique. Samples

Experiments were performed with porcine sclera samples. Before the experiments, the sclera was thoroughly cleaned from the retina and cut in the sagittal direction into 0.5 × 1.3-cm strips. Tissue blocks taken from the equatorial region of the eyeball were used. Before and between measurements, the samples were stored in saline (0.9 % NaCl aqueous solution) at 4 °C.

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At the first stage of treatment, scleral samples were soaked in 0.1% riboflavin solution for 20 min in accordance with the photocrosslinking technique described in [8–11]. Some of the samples were subjected to immersion clearing. As an immersion agent, we used an 88% aqueous solution of glycerol, in which the samples were kept for 20 min. Preliminary experiments have shown that during this time the volume of scleral samples becomes minimal [26]. Then all the samples were irradiated for 30 min with a UV LED with the radiation maximum at a wavelength of 370 nm; the sample irradiance was 3 mW cm^{-2}. After the UV treatment, the samples were kept in saline for a day.

To characterise the samples, we used a JenLab MPTflex multiphoton tomograph (Germany) with a tunable near-IR femtosecond Ti:sapphire laser. The instrument records the fluorescence signals from the sample, excited due to two-photon absorption of laser radiation. This allows the measurement of the fluorescence decay time of the samples and the use of FLIM technique. The samples were exposed to radiation with a pulse duration of 200 fs and a pulse repetition rate of 80 MHz. The centre wavelength of the laser was 760 nm, and the radiation power was 50 mW. The objective numerical aperture NA = 1.3 ensured a spatial resolution of 0.5 μm in the lateral direction and 2 μm in the axial direction. The recorded fluorescence signal is integral over the spectral range from 409 to 680 nm.

In the case of photocrosslinking without optical clearing of the samples, FLIM measurements were carried out in the following stages: 1) after keeping the sample in a riboflavin solution; 2) immediately after UV treatment; and 3) after keeping the samples in saline for a long time (one day) (to wash out riboflavin and photolysis products from the sample). In the case of photocrosslinking with optical clearing, FLIM measurements were carried out: 1) after keeping the sample in a riboflavin solution; 2) after holding in a glycerol solution (optical clearing); 3) immediately after UV treatment; 4) after keeping the samples in saline for a day.

Figure 1 shows FLIM images of scleral samples in the native state and at different stages of riboflavin/UV photocrosslinking without optical clearing (Fig. 1a) and with optical clearing (Fig. 1b) at different depths. These images allow visualisation of the mean fluorescence decay time τ_i (see below).

At the first stage (after keeping the sample in the riboflavin solution), the fluorescence of the samples is determined mainly by the fluorescence of endogenous fluorophores and riboflavin. In the course of optical clearing, a certain amount of water leaves the tissue, and a small amount of glycerol (not fluorescent when excited at a wavelength of λ = 380 nm) penetrates into the tissue. During UV treatment with an intensive course of photochemical reactions associated with photocrosslinking, the

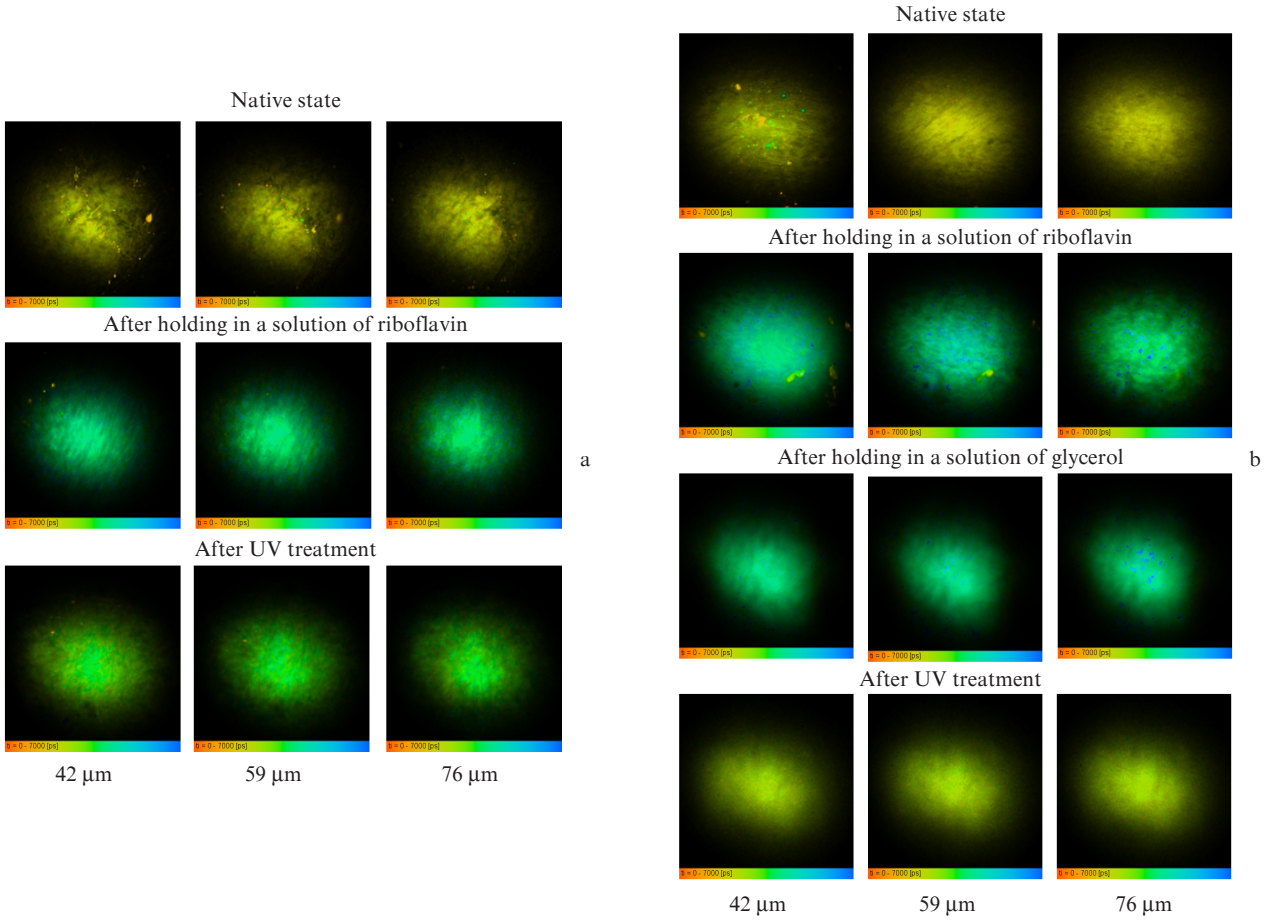

Figure 1. (Colour online) FLIM images of the sclera in the native state and at different stages of riboflavin/UV photocrosslinking (a) without optical clearing and (b) with optical clearing at depths of 42, 59, and 76 μm.

contribution of riboflavin fluorescence to the total fluorescence should decrease significantly. It is well known that under the action of UV radiation, riboflavin solutions can partially discolour as a result of the conversion of some portion of riboflavin into leukoflavin (1.5-dihydroriboflavin) [19–21]. Leukoflavin is colourless, has a significantly lower extinction coefficient at $\lambda = 380$ nm than riboflavin [19, 21], and does not fluoresce [20, 27]. The latter causes the fact that the partial conversion of riboflavin to leukoflavin decreases the fluorescence of the solution. This decrease is the greater, the more riboflavin is converted into leukoflavin and the greater is the current concentration of leukoflavin in the solution: leukoflavin, interacting with molecular oxygen, can be converted into fluorescent substances such as formylmethylflavin, lumiflavin, and lumichrome [20, 21, 27–29], as well as back into riboflavin [28]. Formylmethylflavin and lumiflavin have absorption and fluorescence properties similar to riboflavin [30–34]. Lumichrome under our conditions of excitation, in contrast to riboflavin, maximally fluoresces not in the green, but in the blue region of the spectrum [27, 31, 33, 35, 36]. Experiments on photocrosslinking of the cornea show that at standard concentrations of the riboflavin solution and standard density of UV irradiation, the fluorescence intensity of tissue impregnated with riboflavin decreases significantly in the region of 500–600 nm during UV treatment [22–24]. The absorption by tissue in the near-UV region also significantly decreases [37]. For instance, in the experiment described in [37], immediately after the cornea was impregnated with riboflavin and 5 and 30 min after the start of UV treatment, the measured values of the sample transmittance at a wavelength of 370 nm were 24%, 75%, and 94%, respectively, relative to its initial values (before soaking with riboflavin). Such changes in the transmittance of the sample indicate a sharp increase in the concentration of leukoflavin in the exposed area due to the conversion of riboflavin into leukoflavin and a small contribution to the optical properties of the sample at a time interval of 5–30 min from the beginning of UV treatment of other riboflavin derivatives due to their low concentration (at $\lambda = 380$ nm, the extinction coefficients of formylmethylflavin, lumiflavin, and lumichrome are comparable to the extinction coefficient of riboflavin). This suggests that a significant decrease in the contribution (intensity) of the fluorescence of exogenous fluorophores in the tissue during its UV treatment can be considered as an indicator of the intensive photochemical reactions preceding collagen crosslinking.

As noted above, in the experiment under consideration, the contribution of the fluorescence of exogenous fluorophores at different stages was estimated from the mean fluorescence decay time determined from the FLIM data. Measurements showed that the mean decay time of the stroma fluorescence of the native sclera upon excitation at a wavelength of 380 nm is about 2.03±0.24 (±SD) ns. The average decay time of riboflavin fluorescence under such excitation conditions is much longer and amounts to about 4 ns (according to our measurements, it is 4.03±0.02 ns). For sclera samples impregnated with riboflavin, the average decay time of fluorescence was 3.98± 0.15 ns. Based on these data, it can be expected that in case of an intense course of photochemical reactions, the average decay time of the fluorescence of the sample will significantly decrease, approaching its value for the native state. Otherwise, after UV treatment of the sample, it should change little.

The average fluorescence decay time for scleral samples at different stages of the experiment was estimated using the approximation of the experimental local fluorescence decay data by a two-exponential function [38]

$$\tilde{F}(t) = a_1 e^{-t/\tau_1} + a_2 e^{-t/\tau_2}, \quad (1)$$

where τ_1 and τ_2 are the characteristic decay times of fluorescence of the components of a two-component system; and a_1 and a_2 are the fluorescence intensities of the components at the initial moment of time. The local value of the mean fluorescence decay time was calculated using the formula [38]

$$\tau_i = \frac{a_1\tau_1^2 + a_2\tau_2^2}{a_1\tau_1 + a_2\tau_2}. \quad (2)$$

Calculations were carried out using the SPCImage 6.4 data processing software. A region of approximately 0.023 mm^2 (~28000 pixels) was chosen in each FLIM image, for which the area-average value of τ_i was calculated. The ultimate mean value $\langle\tau_i\rangle$ was calculated by averaging the area-averaged τ_i values obtained in this way over the whole region of interest of the sample and different samples. The values of $\langle\tau_i\rangle$ presented below were obtained as averages for a depth range of 40–75 μm.

3. Experimental results

Figure 2a shows the obtained values of $\langle\tau_i\rangle$ (averaging was performed over 15 regions of two samples) of tissue at different stages of the photocrosslinking process without optical clearing. It can be seen that UV treatment of the samples led to a slight decrease in the average fluorescence decay time $\langle\tau_i\rangle$ from 3.97±0.35 ns to 3.48±0.02 ns, which indicates a slight decrease in the contribution of exogenous fluorophores to the total fluorescence of the sample. After keeping these samples in saline for a day, $\langle\tau_i\rangle$ significantly approached its average value for the native state (2.03±0.24 ns) and amounted to 2.48±0.02 ns. The difference in these times indicates the presence of a residual amount of exogenous fluorophores (riboflavin and its fluorescent derivatives) in the tissue after keeping it in saline.

Figure 2b shows the average values of $\langle\tau_i\rangle$ (over 15 regions of two samples) obtained at different stages of the photocrosslinking process with optical clearing. Immersion clearing of the sample in a glycerol solution led to a slight decrease in $\langle\tau_i\rangle$, to 3.86±0.03 ns. Most likely, this is due to partial washout of riboflavin during tissue dehydration under the action of the immersion agent. UV treatment of samples subjected to immersion clearing led to a significant decrease in the average decay time of fluorescence, to 2.67±0.05 ns, much greater than in the case when no clearing was used (3.48±0.02 ns). This indicates that during UV treatment of cleared samples, the photochemical reactions preceding collagen crosslinking proceed more intensively.

Based on the data presented, we estimated the ratio of the amount of riboflavin in the tissue after UV treatment of non-cleared samples to the amount of riboflavin in the tissue after UV treatment of the cleared samples. Let the mean fluorescence decay times for two tissue states differing in riboflavin content (state 1 with riboflavincontent Q_1 and state 2 with riboflavin content Q_2) be known and denoted by $\langle\tau_i^{(ct1)}\rangle$ and $\langle\tau_i^{(ct2)}\rangle$. Then, assuming invariable fluorescence decay times of endogenous components and riboflavin in

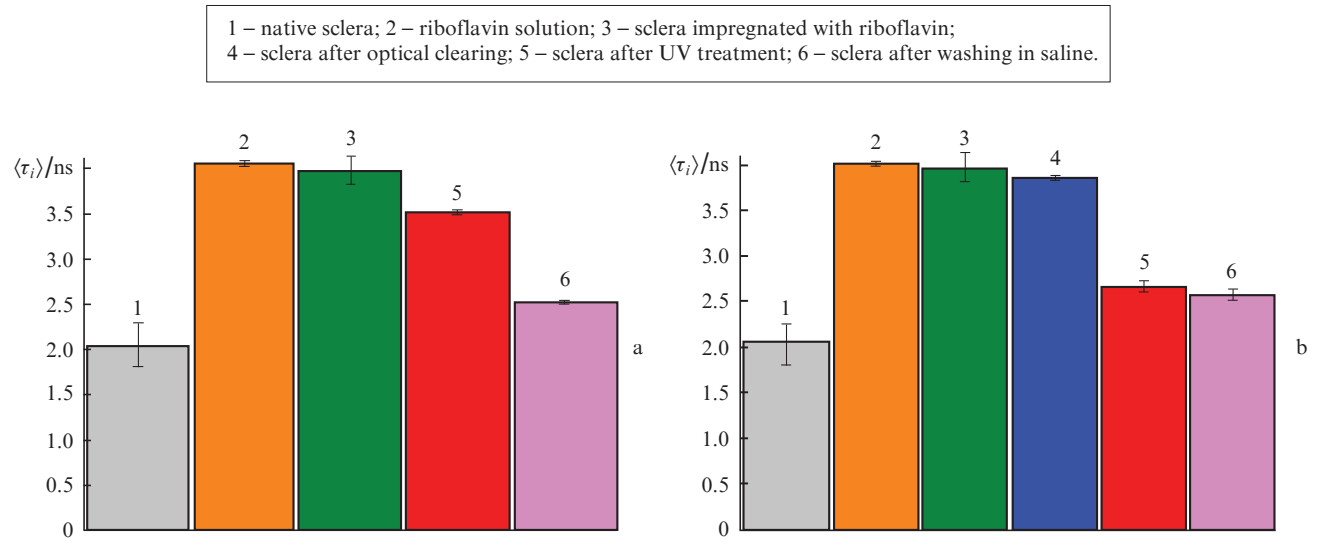

Figure 2. Average decay time of fluorescence in scleral samples at different stages of the riboflavin/UV photocrosslinking process, performed (a) without immersion clearing and (b) with immersion clearing.

tissue, the ratio Q_2/Q_1 can be approximately estimated using the formula

$$\frac{Q_2}{Q_1} = \frac{\left(\left\langle \tau_i^{(\mathrm{ct}2)}\right\rangle - \left\langle \tau_i^{(\mathrm{nt})}\right\rangle\right)\left(\left\langle \tau_i^{(\mathrm{r})}\right\rangle - \left\langle \tau_i^{(\mathrm{ct}1)}\right\rangle\right)}{\left(\left\langle \tau_i^{(\mathrm{r})}\right\rangle - \left\langle \tau_i^{(\mathrm{ct}2)}\right\rangle\right)\left(\left\langle \tau_i^{(\mathrm{ct}1)}\right\rangle - \left\langle \tau_i^{(\mathrm{nt})}\right\rangle\right)}, \qquad (3)$$

where $\left\langle \tau_i^{(\mathrm{nt})}\right\rangle$ is the average decay time of fluorescence for tissue in the native state (endogenous fluorophores); and $\left\langle \tau_i^{(\mathrm{r})}\right\rangle$ is average decay time of riboflavin fluorescence. Unfortunately, in our case, when considering the states before UV treatment (immediately after keeping in a solution of riboflavin) and after it, Eqn (3) does not allow correct estimation of the change for riboflavin in tissue. This is due to the very small difference in the values of $\langle \tau_i \rangle$ for the riboflavin solution and for the riboflavin-impregnated tissue, which is of the same order of magnitude as the error in the experimental estimate of these times. However, Eqn (3) can be used to compare the riboflavin content in the tissue after UV treatment in the case of photocrosslinking without preliminary optical clearing of tissue and with it. It follows from (3) that, in the first case, the riboflavin content in the tissue after UV treatment is approximately 5.6 times (average over 15 regions of two samples) higher than in the second case.

4. Conclusions

Thus, on porcine sclera samples, using FLIM technique, we have shown experimentally that in riboflavin/UV photocrosslinking of the sclera, the use of immersion clearing before UV irradiation makes it possible to enhance photochemical processes associated with collagen photocrosslinking inside the tissue. Thereby, the principal possibility of increasing the efficiency of riboflavin/UV photocrosslinking of the sclera by tissue immersion clearing has been demonstrated. We propose an *in vivo*-applicable technique for FLIM monitoring of the riboflavin content in tissue after UV treatment. Evaluation of the real potential of introducing the stage of immersion clearing into the riboflavin/UV human sclera strengthening protocol requires many additional studies, primarily related to safety issues, in particular, ensuring the safety of the retina under UV irradiation under clearing conditions. The latter seems to make admissible only a small degree of optical clearing of the sclera, which can be achieved using less concentrated glycerol solutions than that used in this work. Generally speaking, the practical application of highly concentrated glycerol solutions for clearing the sclera *in vivo* is hardly possible due to many negative side effects, such as dehydration of the vascular network [39], decreased blood flow rate, and partial dissociation of tissue collagen [40, 41]. The relative safety of using glycerol solutions of moderate concentration (no more than 30 %) for optical clearing of tissue *in vivo* was experimentally demonstrated in [42] on rat skin. Also promising for clearing the sclera is the OmnipaqueTM (it is widely used in medical practice as an X-ray contrast agent), which can act as a biocompatible immersion clearing agent [43].

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