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Analysis of laser-induced modification of collagen structure using nonlinear optical microscopy

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Abstract. **Nonlinear optical microscopy is used to monitor changes in the collagen structure of patellar ligament after IR laser heating up to a temperature of 60 °C. Pre-denaturation modification of the tissue matrix exhibits specific properties and consists in splitting of initially parallel fibre bundles into array of divergent fibres. Image processing employing Fast Fourier transform analysis is used for characterising images of the fibre structure and determining the collagen orientation index. An increase in manifestation of laser-induced disordering with an increase in IR laser radiation power from 0.3 to 2 W is accompanied by a decrease in the collagen orientation index. The specific disorganisation of the collagen tissue structure during laser heating is supposed to be due to fast speed and local action of the laser heating, which leads to mechanical distortions of the structural elements of tissue matrix.**

Keywords: *laser heating, collagen structure disorganisation, nonlinear optical microscopy, Fourier transform.*

1. Introduction

Laser exposure of moderate intensity is increasingly used in sub-ablative procedures for thermotherapy of connective tissues [1, 2]. This stimulates researchers to experimentally study the conversion of collagen, which is the main structural protein of connective tissue, during laser heating. It is well known that when a collagenous material is heated to a critical temperature (usually above 60 °C), collagen molecules pass from the triple helix conformation to a random coil. As a result of this transition, a highly organised quasi-crystalline structure is transformed into an amorphous one [3]. The complete conversion process is called collagen denaturation. Since the primary effect of moderate intensity IR laser exposure is due to the conversion of the absorbed radiation energy into heat, the direct response of the collagen matrix of the connective tissue to such irradiation is usually limited to the possible thermal denaturation of collagen [1, 2].

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Recent papers have demonstrated differences in the behaviour of the collagen matrix during laser and traditional heating [4–8]. It has been established, in particular, that laser-induced disorganisation of the collagen fibrous structure has a specific character [6–8]. The most informative images of modified collagenous samples were obtained using nonlinear optical microscopy in second harmonic generation (SHG) detection mode [3, 6, 8]. Indeed, SHG microscopy is successfully employed to analyse changes in the architecture of connective tissues, highly ordered at all hierarchical levels (from non-centrosymmetric collagen molecules to bundles) [3, 9, 10]. Approaches to the quantitative analysis of SHG images of collagen structures of connective tissues are described in detail in review paper [9]. Among these approaches, a special place is occupied by the fast Fourier transform (FFT), which allows one to obtain a quantitative characteristic of the organisation of structural elements of a two-dimensional image containing, for example, a set of co-directional fibres. The cross section of the two-dimensional power spectrum of the initial two-dimensional SHG image obtained by FFT has the shape of an ellipse elongated in the direction orthogonal to the direction of the fibres in the image. As a numerical score of the degree of orientation of fibrous structures, it is convenient to use either the ellipse aspect ratio (AR) [9, 8], or the collagen orientation index $N = 1 - \mathrm{AR}$ [11], which equals zero for ideally isotropic tissue.

The aim of this study was to identify the features of laser-induced pre-denaturation modification of the collagen matrix. As an object of study, a patellar ligament was chosen, which is a suitable model for revealing fine effects of collagen changes under various impacts. The matrix of this tissue is highly ordered: All fibrous structures from macromolecules to bundles are oriented parallel to the long axis of the ligament [12]. The tissue was subjected to laser and hydrothermal heating to a temperature of 60 °C, which is below the denaturation limit [6]. To estimate the number of intact three-helix molecules, we used differential scanning calorimetry (DSC) [13]. Changes in the organisation of the collagen matrix were evaluated on the basis of SHG microscopy images of intact and heated tissue samples. The collagen orientation index was determined from these images using FFT analysis.

2. Materials and methods

Patellar ligaments were obtained from adult rabbits no later than 5 hours post mortem. The patellar ligament was mechanically separated from adjacent tissues, stored in a 0.15 M NaCl solution at 4 °C and used in the experiment no later than 36 hours after collection.

2.1. Laser and hydrothermal treatment

A 1.56 μm erbium-doped quartz fibre laser LS-1.56-5 (IRE-Polus, Russia) emitting continuously with a maximum power of 5 W was employed to perform laser-induced modification of the collagen matrix. Pulse duration can be set automatically in the range from 0.01 to 10 s using a laser control unit. The laser radiation was delivered remotely through a quartz optical fibre with a core diameter of 600 μm (numerical aperture 0.22), which was oriented perpendicular to the surface of the sample. The distance between the fibre end and the irradiated surface was 5 mm, while the diameter of the irradiated zone amounted 1.6 ± 0.1 mm. For laser treatment the following 4 power values were chosen: 0.3, 0.5, 1, and 2 W, corresponding to the L1-L4 modes, respectively.

The exposure time to achieve a target temperature of 60 °C with the selected power value was determined in each experiment. To this end, the dynamics of temperature fields on the tissue surface was recorded using a FLIRA655*sc infrared scanner (FLIR Systems, Inc, USA) with a frame acquisition rate of up to 200 Hz and with a FOL25 lens. Thermogram processing was performed using FLIR Research IR Max software.

When performing laser-induced modification of the collagen matrix, for each selected laser power (L1 – L4 modes) the dynamics of the temperature field on the tissue surface was obtained.

The hydrothermal treatment of the samples was performed in a temperature-controlled 0.15 M saline bath at 60 and 85 °C (T1 and T2 modes, respectively) for 2 min. A preliminary experiment using a needle thermocouple showed that within 1.5 minutes the temperature inside the heated sample reached the temperature of the bath.

As a result, 7 groups of samples were obtained and studied (Table 1): intact tissue, tissue after laser treatment (L1 – L4) and tissue after hydrothermal treatment (T1, T2).

Table 1. Changes in collagen orientation index N in ligament tissue after different exposures.

Mode	Exposure	Exposure parameters	N
Intact	–	–	0.58 ± 0.01
L1	IR laser	0 .3 W, 15 s	0.52 ± 0.01*,
L2	IR laser	0.5 W, 4 s	0.54 ± 0.01*,**
L3	IR laser	1 W, 1 s	0.38 ± 0.01*,***
L4	IR laser	2 W, 0.4 s	0.34 ± 0.01*,***
T1	Hydrothermal	60 °C, 120 s	0.48 ± 0.01*
T2	Hydrothermal	85 °C, 120 s	0.15 ± 0.01*

The values are presented as mean ±SD;
* significantly different from intact samples ($p < 0.05$);
, * no significant difference.

2.2. Analysis of collagen matrix transformation

Laser irradiation of the ligament was performed at five different locations for each exposure mode. After each treatment, the irradiated zone with a characteristic size of 1.6 × 1.6 mm and a thickness of ~1 mm was excised for analysis. Two of five samples were subjected to thermal analysis immediately after exposure and three samples were fixed in a 4% neutral formaldehyde solution for further optical inspection. Each hydrothermal treatment mode was applied to three ligament samples: One of them was further subjected to thermal analysis, while the two others undergone optical inspection.

Thermal analysis of the samples was performed with a Phoenix DSC 204 differential scanning calorimeter (Netzsch, Germany) in hermetically sealed pans in the temperature range from 20 to 90 °C with a heating rate of 10 K min^{-1}. For intact samples, a peak appeared in the DSC thermograms in the temperature range of 62 – 76 °C with a maximum at 67.0 ± 0.5 °C, indicating denaturation of collagen. The peak area corresponded to the collagen denaturation enthalpy and was $\Delta H_d = 63 \pm 3$ J g^{-1} of collagen. For the samples subjected to treatment, the preservation of ΔH_d indicated preservation of the triple helix structure of the collagen macromolecules, and the disappearance of the peak approved the complete denaturation of macromolecules as a result of the treatment.

SHG visualisation of the samples was performed with an LSM 510 META laser scanning microscope (Carl Zeiss, Germany). The excitation was carried out by pulsed femtosecond radiation from a Ti:sapphire laser (MaiTai HP, Spectra Physics, USA) at a wavelength of 800 nm with a pulse duration of 100 fs and a pulse repetition rate of 80 MHz. The radiation power of the IR laser was below 7 mW; this corresponds to a peak intensity in the focal region of about 0.1 TW cm^{-2}, which is several times lower than the typical intensity of pulsed fs radiation causing cell damage [14]. The SHG signal was separated using a dichroic visible radiation filter (KP650, Carl Zeiss) and a narrow-band filter (λ = 400 nm, $\Delta\lambda$ = 10 nm). For visualisation, tissue samples were cut along the fibres' direction, and ~500 μm thick sections were placed between coverslips. The SHG image of the slice containing 1024 × 1024 pixels was formed using an EC-Plan-Neofluar (Carl Zeiss, Germany) lens with a magnification of 40×/1.3 Oil DIC M27 resulting in a field of view of 225 × 225 μm. Each image line was averaged over 8 scans to improve the signal-to-noise ratio.

Processing of SHG images by the two-dimensional (2D) FFT was performed with a software developed in the MATLAB environment [15]. The original SHG image was divided into 16 square areas. For each square, FFT conversion was performed, and for the resulting two-dimensional brightness spectrum of the SHG image, the aspect ratio of the ellipse (AR) and the collagen orientation index $N = 1 - AR$ were calculated. The average value of the parameters AR and N was obtained based on the processing of 5 original SHG images. To compare the mean values of two independent samples, Student t-test was used with a significance level of 0.05. Different groups of samples were compared in pairs (Table 1).

3. Results and discussion

Figure 1 shows examples of temperature dynamics in the centre of a laser spot for powers of 0.5 and 1 W. The final times of reaching the target temperature of 60 °C were determined based on such temperature dependences for all considered modes and are presented in Table 1.

Figure 2 shows SHG images of intact ligament tissue (Figs 2a and 2b). The images clearly identify parallel collagen fibres stacked in a bundle. Such a highly ordered collagen organisation is characterised by a rather high value of the collagen orientation index $N = 0.58 \pm 0.01$. Note that this value is close to $N = 0.59 \pm 0.08$ obtained for normal dermis [11, 16]; moreover, the authors of these works performed FFT analysis of SHG images of 60 × 60 μm in size, where part of only

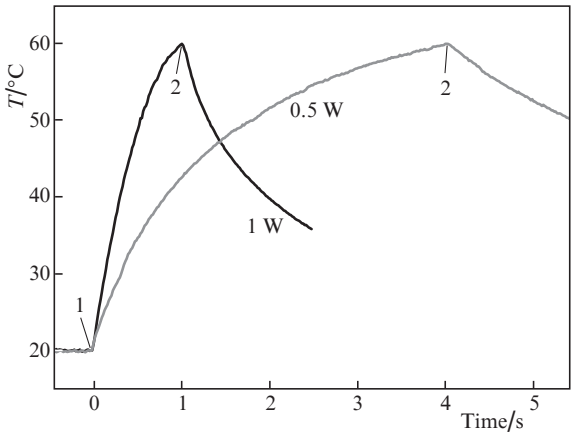

Figure 1. Examples of temperature dynamics in the laser spot centre for powers of 0.5 and 1 W: (*1*) beginning and (*2*) end of the exposure.

one collagen bundle with parallel fibres is presented . The value of N for corneal tissue, where collagen fibrils are organised into lamellae, does not exceed 0.4 due to the wavy shape of fibril groups [8]. In certain tissue ligaments, collagen fibres form wave-like foldings (crimps), characteristic of this type of tissue, with a typical size varying from 70 μm to 120 μm. In different SHG images, the number and length of the crimps may vary; however, the average values of the collagen orientation index over the frame were quite close. For example, for visually different images shown in Figs 2a and 2b, N values were 0.59 ± 0.01 and 0.57 ± 0.01, respectively. We believe that this is due to the fact that N is determined for a smaller image, within which the packaging of collagen fibres remains parallel.

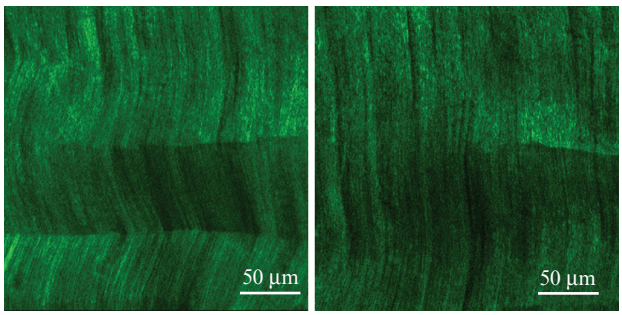

Figure 2. SHG images of intact ligament tissue at N = (a) 0.59 ± 0.01 and (b) 0.57 ± 0.01.

For samples after heat treatment at 85 °C, thermal analysis indicated that all molecules underwent a helix–coil transition. The SHG signal in these samples dropped critically, the fibre structure in the SHG images disappeared, and the tissue became isotropic (Fig. 3a). The collagen orientation index was close to zero and amounted to $N = 0.15 \pm 0.01$, which almost corresponds with the same value for amorphised corneal tissue [8].

After heating the tissue to 60 °C, both by radiation and under hydrothermal conditions, according to thermal analysis, the collagen macromolecules remained intact. Collagen fibres were visualised in SHG images, although their relative

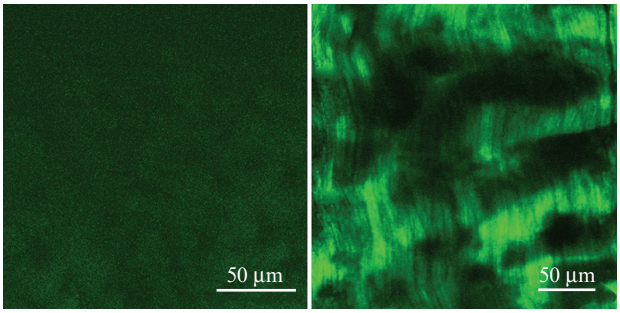

Figure 3. SHG images of ligament tissue after hydrothermal heating up to (a) 85 °C and (b) 60 °C.

position changed as compared to the intact ligament. Figure 4 shows SHG images of the central region of laser irradiation area for samples of groups L1–L4 (Figs 4a–4d). For comparison, an example of a SHG image of a bundle subjected to hydrothermal treatment is given (Fig. 3b). Calculated N values are shown in Table 1.

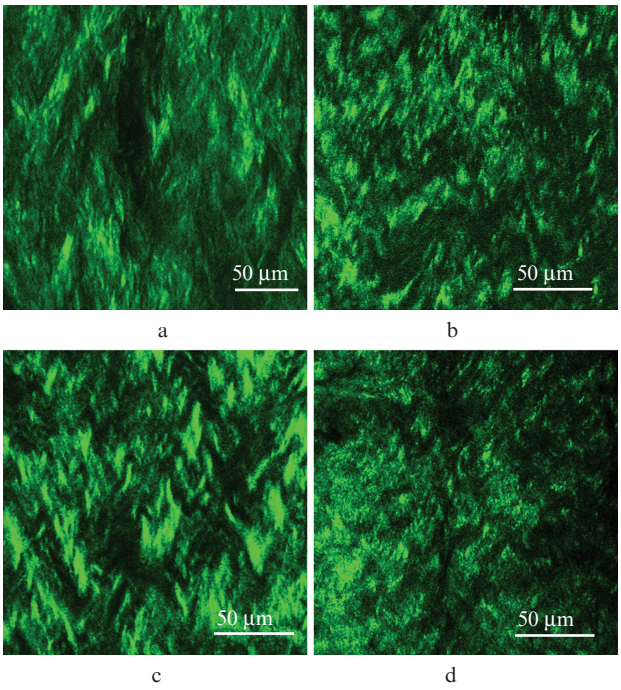

Figure 4. SHG images of ligament tissue in the laser spot centre for samples of groups (a) L1, (b) L2, (c) L3, and (d) L4.

Disorganisation of the ligament collagen network after laser irradiation had specific features. While the fibres were preserved as structural units, the fibre bundle was divided into groups. These groups of fibres diverged and intersected, changing orientation from right to left and from top to bottom. The collagen orientation index decreased as the laser radiation power increased (Table 1); however, its value remained larger than 0.3, which confirmed the preservation of a predominant orientation of the fibres. The observations correlate with the results of paper [8], where SHG images of the cornea subjected to IR laser heating to temperatures of ~50 and 60 °C were presented and analysed. The authors of [8]

demonstrated that the disorganisation of the fibrillar structures of corneal collagen consists in increase of multidirectonality of the fibril groups orientation and decrease of the N value with increasing temperature.

Collagen network distortion after hydrothermal heating demonstrated a different nature. The fibres as a whole continued to be parallel to each other; however, the folding of the structure significantly increased, i.e., the direction of all fibres in the bundle changed simultaneously (Fig. 3b). Such a change in orientation led to a decrease in N down to 0.48 ± 0.01. Note that the authors of paper [3] observed smooth distortions and an increase in the wave-like character of the orientation of collagen fibril groups during hydrothermal heating of the cornea.

Unlike quasi-equilibrium hydrothermal heating, laser heating is inhomogeneous in space and nonstationary in time [1, 2]. Nonequilibrium heating in addition to the heat flow initiates the flow of substances, including the outflow of interstitial fluid from the exposure zone. Presumably, a combination of inhomogeneous heating of a bundle of parallel fibres, water outflow, and resistance of a dense collagen package in tissue to this flow leads to local stresses in the fibre bundle and, as a consequence, to deformation and displacements of fibre groups in different directions. Such an effect of laser heating can attributed as photothermomechanical one. The intensity of the process depends on the temperature gradient and heating dynamics, which, in turn, are determined by the radiation power. According to the values of N, two ensembles can be distinguished: groups L1 – L2 and groups L3 – L4. In each ensemble, the values of N are close in each ensemble; however, the difference between the ensembles is significant. We associate the difference with a change in the dynamics of heating at powers of 0.3 – 0.5 W (L1 – L2) and 1 – 2 W (L3 – L4).

The time of heating to the target temperature was significantly longer at lower powers; nevertheless, the dependence of this time on power is not linear (Table 1). This is due to the relaxation of heat in the course of laser heating. The thermal relaxation rate $\tau_{rel} = L^2/4\kappa$, where L is the linear size of the heated zone and $\kappa \sim 1.4 \times 10^{-7}$ m^2 s^{-1} is the thermal diffusivity [1, 2]. Under considered experimental conditions (the radius of the heated zone of 1.5 mm and typical radiation (λ = 1.56 μm) penetration depth of ~0.8 mm [4], respectively), the temperature relaxation occurs in time from ~4 s (in lateral direction) to ~1 s (in axial direction). We suppose that the most significant changes caused by the photothermomechanical effect occur within 1 s. For groups L1 – L2, the exposure time exceeds τ_{rel}, and a temperature field that is close to stationary is established in the system (Fig. 1). The temperature gradient for a time exceeding 1 s is not so significant; as a result, the role of nonequilibrium effects decreases. In the L3 – L4 groups, the exposure time appeared to be shorter than the thermal relaxation time τ_{rel}; the large difference in temperature at the centre of the spot and at the boundary of the exposure zone is achieved faster, and the deviation from equilibrium increases, and the photothermomechanical effect is more pronounced, which leads to more significant changes. Thus, a decrease in the index of collagen orientation with increasing power is an objective characteristic of the intensity of laser exposure and quantitatively corresponds to the degree of distortion of the collagen matrix and the degree of process deviation from equilibrium.

The distortion of collagen packing under conditions of traditional hydrothermal heating consists in an increase in folding within the fibre bundle, which also leads to a decrease in the collagen orientation index. Nevertheless, it seems incorrect to use N values as the only characteristic of collagen fibre ordering when comparing different exposure modes, leading to a specific disorganisation of the tissue collagen matrix.

4. Conclusions

In this paper, the nature of the deformations of the connective tissue collagen matrix during laser heating is studied. As a result of laser heating, the ordered uniaxial packing of fibrils, fibres and bundles of collagen in the ligament tissue was transformed into separate and dispersed groups of interconnected fibrous structures, which retained the general direction of the fibres. The degree of laser-induced disorganisation is reflected in a decrease in the orientation index N of collagen calculated based on two-dimensional FFT of SHG images. It is likely that the specific disorganisation of the tissue collagen matrix during laser heating is due to its speed and localisation in a small volume, i.e., the nonequilibrium nature of the heating, which leads to mechanical distortions of the structural elements of the tissue matrix. Thus, when developing and optimising the characteristics of laser exposure to collagen-containing tissues, to achieve the target effect, it is necessary to take into account the features of laser-induced changes in the matrix. In addition, it is impossible to unambiguously match the results of laser and hydrothermal heating when modelling a clinical procedure using laser radiation due to the significantly different nature of the transformation of the collagen matrix.

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