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Birefringence and Second Harmonic Generation on Tendon Collagen Following Red Linearly Polarized Laser Irradiation

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Abstract—Regarding the importance of type I collagen in understanding the mechanical properties of a range of tissues, there is still a gap in our knowledge of how proteins perform such work. There is consensus in literature that the mechanical characteristics of a tissue are primarily determined by the organization of its molecules. The purpose of this study was to characterize the organization of non-irradiated and irradiated type I collagen. Irradiation was performed with a linearly polarized HeNe laser ($\lambda = 632.8$ nm) and characterization was undertaken using polarized light microscopy to investigate the birefringence and second harmonic generation to analyze nonlinear susceptibility. Rats received laser irradiation ($P = 6.0$ mW, $I = 21.2$ mW/cm^2, $E \approx 0.3$ J, ED = 1.0 J/cm^2) on their healthy Achilles tendons, which after were extracted to prepare the specimens. Our results show that irradiated samples present higher birefringence and greater non-linear susceptibility than non-irradiated samples. Under studied conditions, we propose that a red laser with polarization direction aligned in parallel to the tendon long axis promotes further alignment on the ordered healthy collagen fibrils towards the electric field incident. Thus, prospects for biomedical applications for laser polarized radiation on type I collagen are encouraging since it supports greater tissue organization.

Keywords—Animal model, Form birefringence, He–Ne laser, Intrinsic birefringence, Nonlinear susceptibility.

INTRODUCTION

Collagen is a vital protein that plays a major role in the extracellular matrix of connective tissue, and to a certain extent it is responsible for the physical properties of connective tissue. Type I collagen, found in the skin, tendons, bones and other connective tissues of vertebrate animals, is one of the most abundant proteins in the living organism.[19] In tendons, where collagen concentration may reach 80–90% of the dry mass, collagen fibers can be linked directly to the biomechanical function of transmitting forces from the muscle to the bone.[13]

Collagen molecules have nanometrical dimensions, are around 300 nm in length and 1.43 nm in diameter and build superstructures, such as tendons, by means of self-assembly, and in accordance with supra-molecular chemistry laws.[8] The high degree of longitudinality of the fibrils, which extend to the organizational level of the bundles, is responsible for optical anisotropies, such as birefringence and nonlinear susceptibility.[12,29] Collagen birefringence can be studied with the use of polarized light microscopy. In brief, the brightness of a birefringent object is due to refraction index difference $\Delta n = (n_e - n_o)$. The phase shift is $\delta = \left(\frac{2\pi}{\lambda}\right) \cdot L\Delta n$, where λ is the wavelength (μm), L is the sample thickness (μm), Δn is the birefringence. $L\Delta n$ is the optical retardation.

The collagen fiber, when placed between two polarizers, as occurs under a polarization microscope, shows maximum brightness when one of its axes of propagation is placed at an angle of 45° in relation to the polarizer. Thus, the polarized light emerges from the collagen in two wave fronts, after crossing the collagen. These two fronts represent a phase difference, which is related to the optical retardation. Bigger phase difference and greater optical retardation means a more organized structure around the ordinary and extraordinary axes.[12]

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Moreover, collagen is composed of molecules endowed with an anisotropic polarizability tensor, and possesses second order susceptibility which is sensitive to the structural changes suffered by molecules, thus allowing its study through Second Harmonic Generation (SHG). Since collagen is the predominant structural component in most biological tissue and the main biological source of SHG, it can be assumed that changes in the harmonic signal can be attributed to structural changes in these tissues.[17]

Thus, the study of the collagen anisotropy following linearly polarized laser irradiation can assist not only in understanding the cause of its preferential alignment in tissue, but also the optimization of dosimetric parameters of low level laser therapy,[7] since knowledge of the tissue's optical characteristics is vital to the successful use of laser radiation in life sciences.

We have showed that the electric field vector of the linearly polarized laser radiation affects collagen organization in the rat dermis.[27,33] More highly organized collagen is important in order to improve the mechanical properties of tissue, since that the functionality and performance of these tissues are controlled by their hierarchical organization ranging from the molecular up to macroscopic length scales, according some studies.[9,22] A more organized collagen matrix would be desirable mainly to overcome the loss of mineral density provoked by long-term exercise and osseous dysfunction.[2]

Based on our previous results,[27,32] in the present work we used normal healthy rat's tendon, which are composed mostly of parallel arrays of collagen fibers closely packed together, and selected the tendon long axis as a preferential direction to align it to the linear polarization of the incident electric field of a low power HeNe laser. Two innovative methods were used to investigate possible anisotropic phenomena of collagen following red irradiation, birefringence and non linear susceptibility by second harmonic generation.

MATERIALS AND METHODS

Twelve 34 day-old male Wistar rats (*Rattus norvegicus albinus*) were used. The animals were anesthetized with tribromoethanol (Avertin® 0.025 mL/g body mass), individually caged during the experiment, and maintained at a 12 h light/dark schedule at 22 °C, with access to food and water ad libitum. The skin over the left and right Achilles tendons in the hind feet was shaved. National and international laboratory animal care principles were followed.

The light source used for irradiation procedures was an HeNe laser at $\lambda = 632.8$ nm, with a power output of 12 mW (Uniphase, California, USA) mounted in a convenient arrangement. A lens system was used to obtain an expanded beam of 6 mm in diameter and a Glan–Thompson prism was inserted in the beam path to obtain a plane-polarized beam. The polarizer was held on a precision disk, which was permanently aligned at 0° with the tendon long axis. After crossing the optical system, the laser power was reduced to half its original level (i.e., to 6 mW). The laser output power was confirmed prior to irradiation, using a LM-01 power meter (Coherent, California, USA). The exposure time was 46 s and the radiation was delivered to the tissue performing a continuous up and down scanning movement. Exposure time and beam diameter were selected in order to obtain an energy density of 1 J/cm^2. The irradiance achieved under these conditions was 21.2 mW/cm^2 and the electric field generated by the HeNe laser was 200 V/m.

Right or left tendons were irradiated randomly and each experimental animal acted as its own control. Non-irradiated tendons were used as a control. The rats received only one dose of irradiation applied with the electric field vector of the incident polarized laser radiation aligned in parallel to the tendon long axis. The animals were euthanized immediately.

Following euthanasia, the calcanear tendons (irradiated and control) were dissected. The specimens were placed in 4% paraformaldehyde, dehydrated in ethanol and embedded in paraffin; eight-μm thick sections were then cut. Xylem, chloroform and methanol were used to remove paraffin. The sections were then routinely hydrated. Slides containing sections from each experimental condition were analyzed.

The measurements of collagen birefringence were made with a Zeiss polarized light microscope (Zeiss, Berlin, Germany) equipped with a 10/0.22 Pol 160/$^-$ objective, a HBO 200 W high-pressure mercury light bulb and a PIL 546 nm interference filter.

To evaluate the total birefringence (intrinsic and textural) of the samples, sections were mounted in distilled water, and these were placed between the slides. A Sénarmont compensator, which introduces a $\lambda/4$ optical retardation, was used. When the optical path difference of the sample is equal to the compensator's optical retardation, there is destructive interference between the ordinary and extraordinary rays proceeding from the sample, characterized by a dark background, which can be visualized through the ocular of the microscope. This dark background is found by varying the angle of the analyzer in relation to the light beam. Once found, the angle of the microscope, α, is registered. This angle relates to the sample's optical retardation according to the formula: $L\Delta n = \frac{\alpha}{180^\circ}\lambda$. If $\lambda = 546$ nm, this becomes: $L\Delta n = \frac{\alpha}{180^\circ}546 \Rightarrow L\Delta n = \alpha.3.03$ nm. Therefore, to determine

the sample's optical retardation ($L\Delta n$), in nm, the angle (α) must be multiplied by 3.03.

The second group of measurements was carried out with the samples immersed in pure glycerin to obtain intrinsic birefringence. Due to the values of intrinsic birefringence being smaller than the values of total birefringence a Brace–Koehler compensator, which introduces $\lambda/10$ optical retardation, was used. The formula then becomes: $L\Delta n = 47.20 \sin[2(45^\circ - \alpha)]$.

Twenty-four samples, comprising $n = 12$ that were sliced from the control tendon and $n = 12$ from the irradiated tendon were analyzed. Each area was selected after illumination of the sample in the microscope and closing the aperture to define the area in question. The diaphragm of the polarization microscope was not used with its full aperture so five areas were delimited by the diaphragm illumination and they were fitted along the tendons long axis. Thus, five areas were analyzed in each histological section. This procedure was performed evenly in the whole sample from inferior to superior extremity in five points randomly distributed totalizing 60 measures for each group.

The experimental apparatus for the generation and measurement of second harmonic from the collagen tendons consisted of a tunable femtosecond Ti:Sapphire laser (Model Mira, Coherent, California, USA, 150 fs) with a repetition rate of 76 MHz, and beam diameter $\phi = 40$ μm, $\lambda = 800$ nm and $\Delta\lambda = 10$ nm. The mean power, $P_m = 110$ mW, was confirmed prior to irradiation using a power meter (Coherent, California, USA) and obtained after passing the beam through a circular filter with changeable optical density (Newport, California, USA). The irradiance achieved by Ti:Sapphire laser was 8.75×10^7 W/m^2 and the electric field generated was 0.13 MV/m.

A zero-order quartz wave plate with $\lambda/4$ retardation (Newport, California, USA) and a Glan-Laser calcite linear polarizer (Newport, California, USA) were located in the path of the beam, which allowed it to rotate from 0° to 360°, and therefore change the direction of incident polarization. Two microscope objectives of 20× magnification and 0.35 numerical apertures (Newport, California, USA) were used to focalize and collect the incident and emergent radiation from the sample. An analyzer Glan-laser calcite linear polarizer (Newport, California, USA) was introduced after the collecting objective. A bandpass filter with a center wavelength of 400 ± 2 nm (model 10BPF10-400, Newport, California, USA) was used to ensure that only second harmonic radiation ($\lambda = 400$ nm) reached the photomultiplier tube (model R11558, gain of 1.0×10^7, Hamamatsu, Iwata, Japan) (Fig. 1). The apparatus, without the sample, was tested to verify the inexistence of harmonic generation on the optical set.

Twenty-four samples, comprising $n = 12$ that were sliced from the control tendon and $n = 12$ from the irradiated tendon were analyzed on the assumption that collagen fibril has cylindrical symmetry, and in accordance with the work of Roth and Freund.[30] In the rectangular coordinates system the z axis was chosen to represent the fibril long axis in such a way that it was possible to write the nonlinear polarization components (P) in terms of electric field (E) of Ti:Sapphire laser:

$$P_z(2\omega) = \rho E_z^2(\omega) + E_x^2(\omega) \tag{1a}$$

$$P(2\omega) = 2E_x(\omega)E_x^2(\omega) \tag{1b}$$

In Eq. (1a), ρ is the relative intensity of the hyperpolarizability tensors, specifically the tensors β_{zzz} and

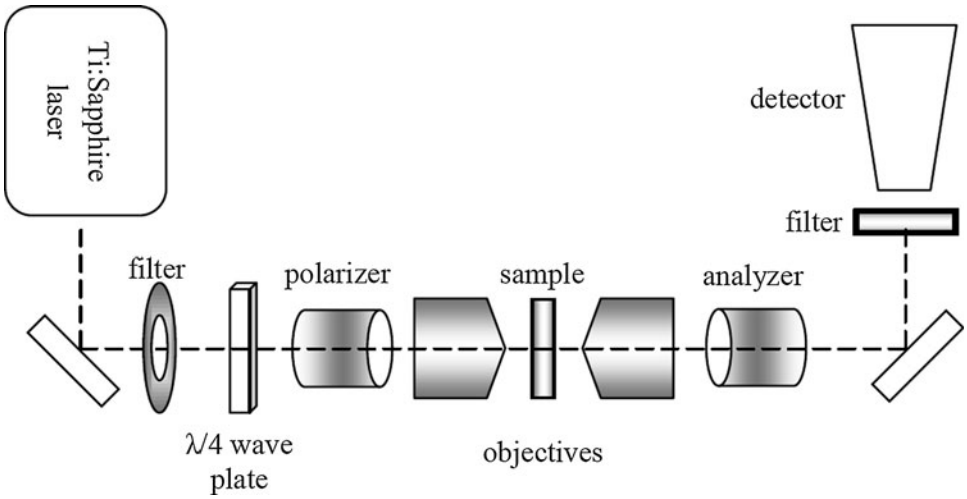

FIGURE 1. Schema of the experimental apparatus for generation and measurement of second harmonic.

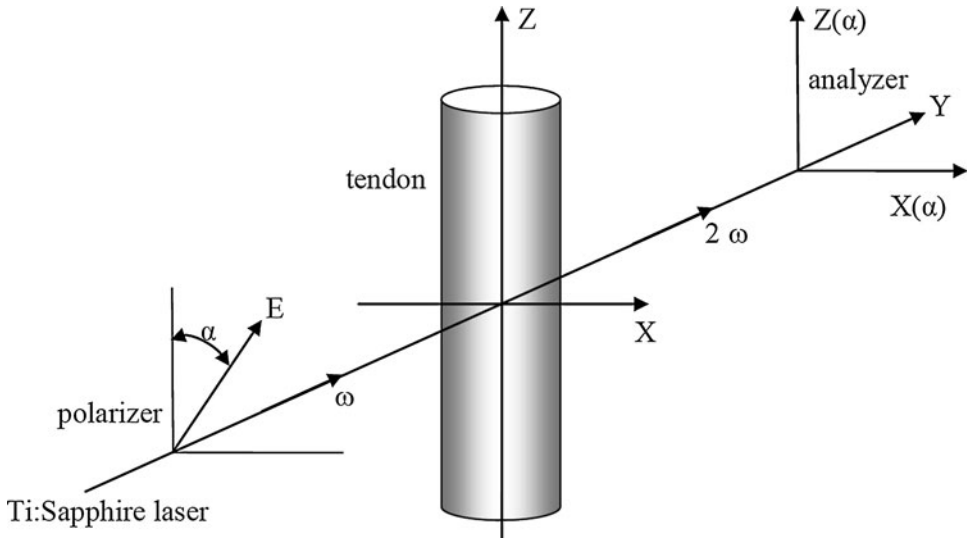

FIGURE 2. Coordinate system of SHG experimental apparatus.

β_{zxx}. Only two hyperpolarizability tensors (β_{zzz} e β_{zxx}) were considered due to Kleinman symmetry conditions.[25] The medium susceptibilities appear as a result of reactions between electrons or ions and fields in the matter. For collagen, the second order susceptibility can be written as $\chi^2 = N{\cdot}\beta$, where N is the electronic density of the medium.

In addition to the fibril coordinate system, an XYZ tendon coordinate system was introduced where Z denotes tendon long axis and direction Y the propagation of the incident beam (Fig. 2).

It was assumed that the average orientation of function distribution is symmetrical in tendon Z direction. Thus, in a hypothetical system, where fibrils are consistently aligned in parallel to the Z axis, xyz and XYZ are coincident, P_z emits second harmonic in direction Z, and P_x in direction X. The experimental apparatus consisted of introducing laser radiation whose vector electric field made an angle α relative to the Z axis, to measure the signal of second harmonic with the analyzer oriented parallel to Z (Fig. 2).

The parameter ρ was used to indicate the degree of order in the distribution function of fibrils, in relation to tendon long axis:

$$\rho = \frac{Z(0^\circ)}{Z(90^\circ)} = \frac{\beta_{zzz}}{\beta_{zxx}} \qquad (2)$$

where $Z(0^\circ)$ and $Z(90^\circ)$ are the representation of polarization at 0° and 90°, respectively, in relation to Z axis.

In accordance with Eq. (2), higher the ρ value means greater degree of order in the medium.

The sections were then immersed for at least 30 min in mineral oil, with refraction index $n = 1.48$, allowing better homogeneity between glass slide ($n = 1.50$) and samples ($n = 1.54$). A slide immersed in mineral oil was placed without sample in the experimental apparatus and this did not generate second harmonic. The samples remained permanently immersed in oil throughout the measurements.

The values that we presented in the paper are result of 10 different measurements performed in each sample. The measurements were carried out scanning the sample from the inferior to superior extremity. As the incident beam was not visible, an infrared sensitive card (model F-IRC1, Newport, USA) was used to guide and also to localize the beam in the whole sample. In addition, the signal was not collected only from the site with greater SHG. Instead, the samples were scanned and the second harmonic signal was integrated into the entire long axis of the tendons.

The optical retardation and ρ values of the tendon collagen were expressed as medians because the data were not normally distributed. To show the actual distribution of the measured data, we used box and whisker plots based on medians. Nonparametric methods were used to detect differences between groups (irradiated vs. control). Nonparametric Wilcoxon Test was used as an inference test. Null hypotheses were rejected at the 0.05 significance level. For data analysis, Origin Pro 8® software (OriginLab, Northampton, USA) was used.

RESULTS

According to our results, the values of optical retardation obtained were positive ($L\Delta n > 0$), i.e., such values show that $n_e > n_o$, as it would be expected for collagen.[44]

Moreover, in the case of the samples where the imbibing media was water, the optical retardations were higher than those analyzed in glycerin. Maximum values in water were 75.8 nm for controls and 105.4 nm for irradiated, while in glycerin maximum values were 19.9 nm for controls and 34.9 nm for irradiated. Minimum values in water were 28.9 nm for controls and 45.4 nm for irradiated, while in glycerin they were 10.1 nm for controls and 14.4 nm for irradiated, as reported in the literature.[40]

It is possible to observe from Figs. 3 and 4 that control samples presented optical retardation values lower than irradiated samples. With either, water or glycerin as the imbibing medium, the difference between irradiated and control samples at the 0.05 level was significantly different.

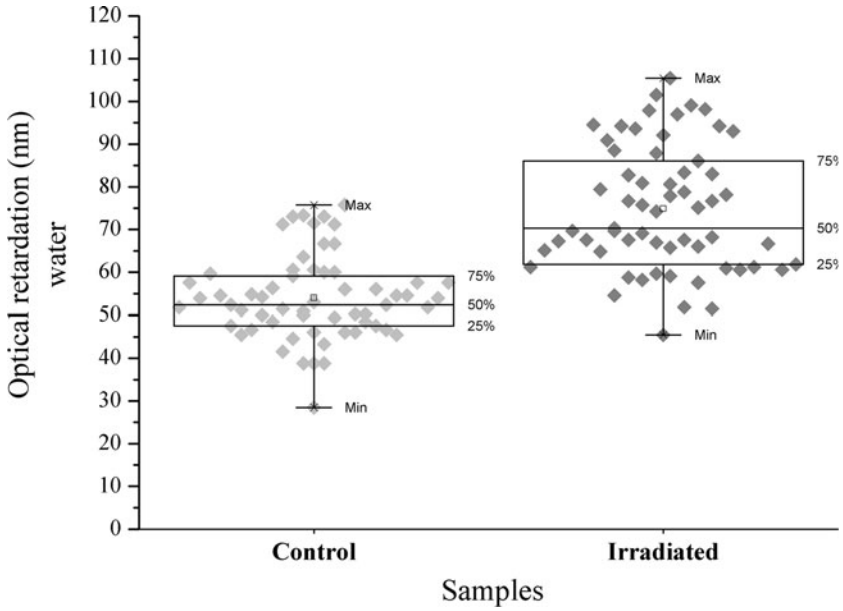

FIGURE 3. Differences of optical retardation, in nm, for control tendons (non-irradiated, $n = 12$) and irradiated ($n = 12$). All samples were immersed in water. Five measurements were performed for each sample. At the 0.05 level, the two distributions are significantly different according to Wilcoxon test.

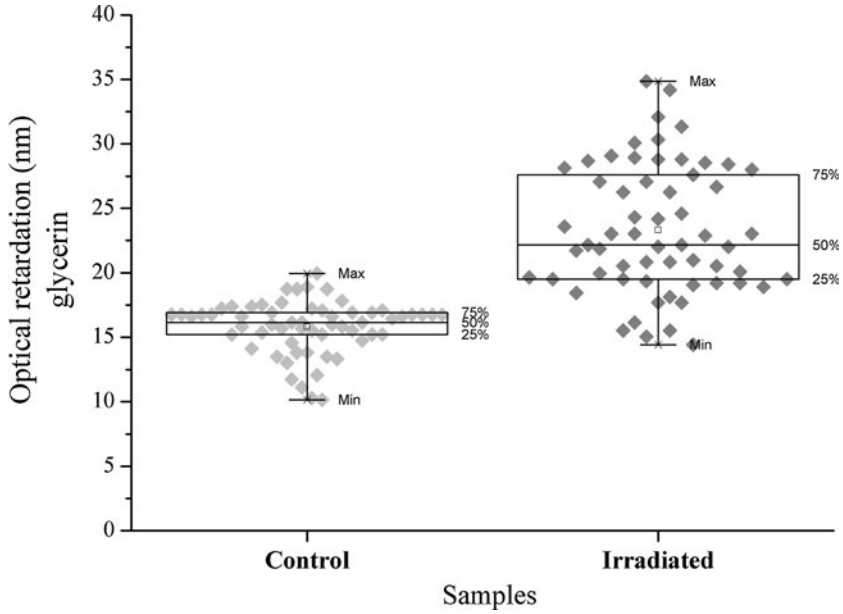

FIGURE 4. Differences of optical retardation, in nm, for control tendons (non-irradiated, $n = 12$) and irradiated ($n = 12$). All samples were immersed in glycerin. Five measurements were performed for each sample. At the 0.05 level, the two distributions are significantly different according to Wilcoxon test.

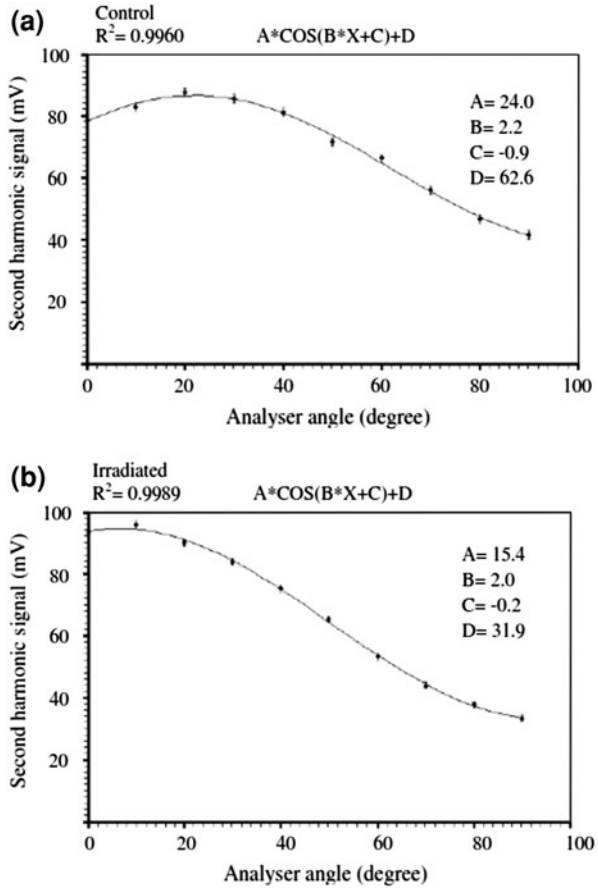

FIGURE 5. Typical signal obtained from SHG on (a) non-irradiated (control) and (b) irradiated sample.

The second harmonic signal, in mV, was measured from each sample. Figure 5 is an example of typical curves obtained from control and irradiated samples. The analysis of the signal curve was performed in accordance with Second Harmonic Generation theory,[4] which states that the signal curve of the second harmonic must obey a cosine function.

The average across all the control samples as well as all the irradiated samples was calculated. The difference between control (median 1.1) and irradiated (median 2.2) samples as a function of relative intensity of hyperpolarizability tensors, ρ, was verified. It was observed that the irradiated samples presented higher ρ compared to control samples at the 0.05 level of statistical significance (Fig. 6).

DISCUSSION

The control samples presented optical retardation values lower than irradiated samples. It is necessary to consider the effects of irradiation on collagen because, regardless of the imbibing media used, the irradiated samples always presented higher optical retardation values.

The purpose of polarizing the laser beam in parallel to the tendon long axis was to investigate the response of the medium according to incident electric field to verify whether under a preferential direction red radiation could assist the fiber arrangement. It should be highlighted that the HeNe laser operated at the lowest order mode possible with a Gaussian distribution of light across the laser beam; therefore, it kept its distribution even after the beam was focused or transmitted by the optical system.

When irradiating the tendon, the laser beam was perpendicular to the skin's surface and only a small fraction, around 4–7% of the incident radiation was expected to be reflected,[6] without considering backscattering (or diffuse reflection). The remaining radiation is transmitted and, under the conditions of our study, the nonresonant dispersion is predominant, thus favoring penetration depth. Indeed, wavelengths between 600 and 1500 nm can travel a distance of 8–10 mm when interacting with intact skin.[37] These considerations are important because radiation must pass through the skin until it reaches the tendon. Thus, we can hypothesize that the radiation reached the entire tendon because the thickness of the rat calcaneal tendon plus the skin barrier was <8 mm.

It is well known that light polarization remains unchanged through a thin layer of cells; however, in highly scattering mediums, such as living tissue, the light is depolarized after a penetration of a millimeter or so. However, the epidermis and the initial papillary dermis apparently allow the penetration of linearly polarized light with modest depolarization.[16] The light can travel a distance of 1.2 mm in normal human skin without the complete loss of the linear polarization.[36] In fact, Sankaran *et al.*[32] demonstrated the different patterns of depolarization for incident linear and circularly polarized light for different tissues. Their results indicate that for dense tissues, linearly polarized light is more preserved than circularly polarized light.

Collagen birefringence is the measure of the difference between extraordinary refraction index (n_e) and ordinary refraction index (n_o). Measurements of birefringence in collagen indicate that $n_e - n_o \sim 0.003$ for samples not fixed and not stained.[21] Birefringence values can be measured using Sénarmont's, Brace–Koehler's or Berek's compensators as a function of the section thickness. In our work we used Sénarmont's (analysis of textural birefringence) and Brace–Koehler's compensators (analysis of intrinsic birefringence). Also, we used the same thickness for all samples, i.e., 8 μm.

The collagen has two types of birefringence, intrinsic (or crystalline birefringence) and form (or textural

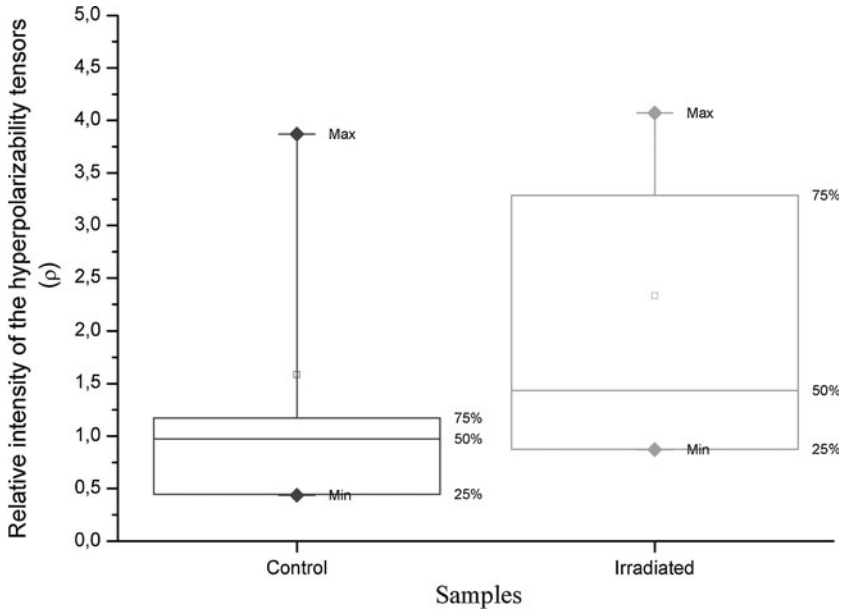

FIGURE 6. Relative intensity of the hyperpolarizability tensors for control tendons (non-irradiated, $n = 12$) and irradiated ($n = 12$). Ten measurements were performed for each sample. At the 0.05 level, the two distributions are significantly different according to Wilcoxon test.

birefringence). The intrinsic birefringence occurs from the interaction of the electromagnetic field of light with electrons of molecules and depends on their polarizabilities. On the other hand, textural birefringence is the type of anisotropy found in mixed bodies wherein asymmetrical particles of a given refractive index are dispersed with a preferential orientation in a medium of different refractive index. At least on dimension of the particles must be small relative to the wavelength of light. In the case of collagen bundles, textural birefringence depends on the partial volume (concentration), aggregation state and orientation of their components.[39]

Since we know the thickness of the sample it is possible to estimate the birefringence value by the equation$\Delta n = \frac{L\Delta n}{L}$. Then, our calculated values for linear birefringence of collagen type I were $\Delta n = 0.0017 \pm 0.0002$, which are different from values reported by other investigators because our samples were dehydrated and embedded in paraffin. In fact, Vidal and collaborators measured extraordinary refraction index of collagen I with the samples immersed in pure glycerin and observed values at about $n = 1.46$, contrary to what has been generally reported, i.e., $n = 1.53$. The finding of the smallest birefringence in paraffin sections is a constant event and is in accordance with Vidal's previous data.[41]

The variation of optical retardation was verified with respect to the variation on sections embedded in paraffin. Measurements from one tendon in a paraffin block and measurements taken at random from different tendons of different paraffin blocks were analyzed. The statistical procedure did not show significant differences between these media (data not shown). It is worth noting that the measurements taken on 8 μm thick sections do not represent significant variation or spreading, according previous study.[40]

When analyzed the textural birefringence, the optical retardations were higher than those of intrinsic birefringence, which agree with values reported by other investigators.[40] The median values of optical retardation for total birefringence were higher (52.7 nm for controls and 72.3 nm for irradiated) than those for intrinsic birefringence (16.3 nm for controls and 22.2 nm for irradiated). Vidal *et al.* used Achilles tendons from male adult guinea pigs to measure total and intrinsic birefringence and found the following results: optical retardation of 47.6 nm for total birefringence and 10.6 nm for intrinsic birefringence. These values are slightly lower than those obtained in our control samples because the animals used in our study were not adults. In fact, changes in collagen fiber molecular order and aggregation states of the collagen fibers following aging have revealed steeper birefringence curves with optical retardation values higher than in normal adult controls. These changes were related to factors associated with alterations in the collagen fiber packing structure.[24] As for the irradiated samples, undoubtedly the He–Ne laser increases the optical retardation, as seen in our results.

Therefore, an increase in birefringence may be (a) an increase of n_e or (b) a reduction of n_o. If $n = \sqrt{\kappa_E}$,

where κ_E is the dielectric constant, changes in dielectric constant take into account changes in the refraction index.[15] When the incident light is polarized, the atomic oscillators of the medium dispersed the light in specific directions. Consequently, the polarizability of the tendon long axis increased, as did the dielectric constant, which contributed to the increase of n_e and Δn. It is worthy to mention, however, that collagen reorganization following laser irradiation observed in our work may be reduced due to partial scrambling of polarization during light penetration into tendon. Thus, the observed changes in birefringence could decrease gradually with depth.

The photonic flow of the HeNe laser can be calculated by the expression $\Phi = P/hv_0$, where P is the laser output power; h is the Planck's constant, and v_0 is the laser frequency. Since the laser power was 6 mW, the photonic flow is about 1.9×10^{16} photons/s. Given that tendon does not absorb red light, the same amount of photons incident was re-emitted by the tendon. Although a portion of the incident photons have been lost due to scattering, a fraction of the light still reaches each atom of the collagen molecule, which may have generated an electric field large enough to distort the internal molecular charges distribution and generate electric dipoles that could also directly contribute to the total internal electrical field. When the dipoles oscillate along the electric field, there is an increase in the dielectric constant. Therefore, it could be suggested that the differences between control and irradiated tendons were dielectric. In fact, Miclh *et al.* showed that the increase in the mobility of ions present in polypeptidic chains modifies their dielectric constant.[25]

In the case of the collagen molecule's exterior, beyond of charged residues that are responsible for intermolecular interactions, e.g., hydrophobic and ionic effects,[27] there are also nonpolar amino acids, where the electric field is able to deform the electronic cloud, modifying its conformation relative to the nucleus and originating a nonpermanent dipole moment, which must also have been large enough to contribute to the observed effect. There is a water cylinder around the collagen triple helix and the study verified that the effect of collagen fiber alignment leads to an increase in the dipole residual separation of the water.[23]

According to Fechete and collaborators, the highest contribution of dipole residual separation was obtained when the angle between magnetic field and the long axis of fibers was 90°.[10] In an electromagnetic wave, when the magnetic field is at 90º from a reference, the electric field is 0°, as in our work. The residual water penetrates the interstitial volume of the triple helix and is fixed by hydrogen bridges due to the hydrophilic characteristic of the molecule interior. The hydrophilic interactions are forces that can drive self-assembly in longitudinal packing, which is more prevalent than the lateral packing of fibrils.[26]

In contrast to Roth and Freund's work,[29] where tissue is not irradiated before measurements are taken, the objective of our study was to investigate the tendon arrangement after irradiation, using the technique of second harmonic generation and polarization microscopy. However, the conditions assumed by Roth and Freund to validate the theory of SHG application to collagen were also assumed in our study. For example, the study assumed that the helical rotational symmetry of the molecule was surrounded by a water cylinder, with the long axis of the cylinder coincident with the long axis of the molecule. The existence of a water cylinder around the collagen molecule was, in fact, confirmed by Melacini *et al.*[23] The molecule interior was treated as polar and the exterior as nonpolar, according to the study by Walrafen *et al.*[42] This description is consistent with the symmetry of point group C_∞, which is a special case in respect of rectilinear molecules. In this way, the components of the second harmonic polarization were $P_Z(2\omega) = \beta_3 E_Z^2(\omega) + \beta_1\{E_X^2(\omega) + E_Y^2(\omega)\}$ and $P_x(2\omega) = 2\beta_1 E_x(\omega)E_z(\omega)$. The component $P_y(2\omega)$ was considered null because, according to symmetry conditions the x and y axes perpendicular to z may be chosen arbitrarily, making $E_y \equiv 0$ possible. The hyperpolarizability tensors are $\beta_3 = \beta_{zzz}$; $\beta_1 = \beta_{zxx} = \beta_{zyy} = \beta_{xzx} = \beta_{yzy}$, *etc.* These equations are in accordance with nonlinear optic tensor form, with terms simplified due to piezoelectric contraction and Kleinman's symmetry conditions. These equations describe the SHG of one isolated fibril in the understanding that $\beta_{\text{fibril}} = N\beta_{\text{molecule}}$, where N is the molecule number contained in the fibril. This last imposition allows the treatment of fibrils as supergiant molecules. This extrapolation is correct, since the geometry of the sample and the dimensions of the fibril are such that the relative phases of some optic fields do not vary greatly from one fibril to another.

As was the case in Roth and Freund's work,[29] we measured the relative intensities $\rho = (\beta_{zzz}/\beta_{zxx}) = (\beta_3/\beta_1)$ since β_3 represents polarization along the z axis, and β_1 polarization along the x axis.[30] The ratio β_3/β_1 is a convenient measurement of fibril degree of alignment because the higher the β_3, value the higher the number of entities susceptible to the electric field in direction z compared to direction x. In this way, a higher ρ index means a more aligned tendon structure around the fibril's z axis, which is also known as tendon Z axis (Fig. 2). Previously ρ had been measured by Freund *et al.* and Stoller *et al.*, which found the values 1.2–2.0 in adult tendons.[11,18] Williams *et al.* found the value 2.6.[43] When ρ was measured in immature tendons, values as little as 0.8 were obtained by Roth

and Freund.[29] We obtained the values 1.1 to controls and 2.2 for irradiated samples, remembering that our animals had immature tendons (34 day old). Our results corroborate with Roth and Freund and indicate the influence of He–Ne laser in the best order of collagen fibers.

Gusachenko *et al.* obtained $\rho = 1.40 \pm 0.03$ from experimental data collected from tendons rat-tails and showed that the raw value of ρ varies a lot with depth. It decreases from 1.40 at the surface of the tendon to 0.8 at ~90 μm depth within the tendon. These data proves that correction for diattenuation is essential for reliable measurements of ρ in thick anisotropic tissues. The authors defend that the determination of ρ deeper in the tissue would be hampered because of the low signal to noise ratio and when possible, the most reliable method is to perform polarization—resolved measurements at the sample surface to get rid of diattenuation and birefringence effects.[14] In our work we used the thickness of 8 μm, i.e., very thin so that the effects mentioned by those authors should not interfere in our results. In addition, all samples had the same thickness, whose dispersion has been discussed above.

Aït-Belkacem *et al.* analyzed the influence of the anisotropy of molecular and biological samples on polarization resolved nonlinear microscopy imaging.[1] They demonstrated that birefringence from molecular and biological samples is a detrimental factor in polarization resolved information in microscopy imaging applied to anisotropic samples. In a context where SHG microscopy constantly evolves towards the exploration of complex tissues where birefringence is present at increasing depths (the authors used samples of thickness ranging from 70 to 100 μm), this analysis is more crucial to distinguish the local nature of biomolecular assemblies.[1] In our study we did not use the SHG signal to obtain microscopic images or thick samples, then the effect of birefringence to measure second harmonic signal was discarded because all samples had the same thickness.

The analysis of the signal curve was performed in accordance with Second Harmonic Generation theory,[4] which states that the signal curve of the second harmonic must obey a cosine function (Fig. 5). It was observed that the ρ index is independent of the amplitude of the second harmonic signal, as suggested by Stoller *et al.*[34]

The curves have been constructed with the polarizer fixed at 90° and the analyzer varying from 0° to 90°. This choice was made due to the fact that collagen is a uniaxial molecule with anisotropic structure and birefringence and therefore the orientation of the field relative to the optic axis determines the speed with which the electromagnetic wave propagates. When the electric field is perpendicular to the optic axis, the light will spread with speed $v_{\perp}$ in all directions because this field is parallel to the ordinary axis.

The tendon is aligned at an angle of 90° in respect to polarizer, so the fibril bundles are tilted by 10°–20° from the tendon axis (see Fig. 5, where the maxima points are not at 0°). Because fibril bundles in an unstretched rat tendon exhibit a characteristic crimped structure, this tilt is not surprising and agrees with the observations of other authors.[1]

The observation that irradiated samples presented a ρ index as well as higher alignment in the Z direction means that irradiation caused an increase in collagen nonlinearity. One of the causes of nonlinearity increase is the electronic redistribution of the medium. The origin of this nonlinearity is the trend of molecules to become aligned with the incident electric field. The increase in nonlinearity can also be associated with an increase in molecular chirality, since only noncentrosymmetric materials are capable of generating second order nonlinearity.[38] Thus, it can be assumed that besides increasing the polarizability of the medium, the HeNe laser radiation has also increased the molecular chirality. Verbiest *et al.* demonstrated a strong enhancement of nonlinear optical properties through supramolecular chirality, determined by interaction of the electric field of light waves with molecules in a medium of propagation, which results in optical phenomena such as birefringence.[38] In this manner, birefringence and second harmonic generation are two complementary optical techniques, which depend on organizational structure of medium.

On the other hand, some papers have shown that lasers and LEDs align collagen following tendon healing.[3,5,31] In fact, collagen should be organized after cicatrization. We have performed some experiments investigating the role of polarization on burn healing,[28,33] and our results showed that there is a preferential direction on skin in which collagen is even more organized during the course of the repair following irradiation. Thus, the present study was designed to explore whether the use of an already more aligned tissue (tendon) without any lesion (i.e., control group) and the laser beam linearly polarized parallel to the tendon long axis could assist collagen alignment. According to our findings demonstrate that under conditions used in this work, healthy collagen fibrils are aligned to the electric field incident supporting a higher tissue organization.

A noteworthy remark is that heating is not responsible for the collagen ordering observed in our study. Ito *et al.*[16] reported that an infrared laser ($\lambda = 789$ nm) promotes increases in temperature <0.1 °C/mW. An extrapolation using the laser power of our work ($P = 6.0$ mW) would give values around 0.582 and 0.252 °C (surface and deep, respectively).

Also, it is important to note that we used a red laser, which promotes inferior increase in temperature compared to infrared lasers. Thus, we can assume that the heating under the studied conditions is negligible. In fact, some studies show that only at significant high temperatures (>45 °C) the SHG signal tends to decrease indicating structural changes and consequent collagen denaturation.[20,35]

As far as we are aware, this is a pioneering work that sought to investigate and contribute to the existing basis of low-power red laser interaction with collagen. Understanding the relationship between physical alterations and its effects is vital, since, as well as resonant dispersion, concomitant events should also be evaluated when working with light propagation on tissue. This work can aid the comprehension of phenomena that could be linked with the electromagnetic field polarization of laser radiation. However, a more comprehensive approach, e.g., other irradiation conditions, is still necessary to enable a fuller understanding of these photobiological phenomena.

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