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Article

Biomechanical and Morphologic Effects of Collagen Cross-Linking in Human Tarsus

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Purpose: To investigate the feasibility of increasing the stiffness of human tarsal tissue following treatment with riboflavin and ultraviolet A (UVA) to induce cross-linking of collagen fibers.

Methods: In this case control study, 18 right and left upper eyelids were excised en bloc from 18 fresh-frozen cadavers. One side served as the control while the samples from the opposite side were cross-linked. Four 2 × 6-mm vertical strips of central tarsus were cut from the superior to inferior border of each tarsal plate. Sample tissue was irradiated with UVA at 6 mW/cm^2 for 18 minutes. A microtensile load cell and an optical coherence tomography scanner allowed calculation of stiffness (Young's modulus). Six cross-linked samples and corresponding controls were stained with hematoxylin and eosin (H&E) and Masson trichrome stains. Four controls and four cross-linked samples were also reviewed with a transmission electron microscope.

Results: Mean Young's modulus in the linear region for controls was 28 ± 9 MPa and was much higher at 138 ± 8 MPa for cross-linked samples ($P < 0.001$), yielding a 493% mean stiffness increase. Staining with H&E and Masson did not reveal any histologic changes. Transmission electron microscopy showed a decrease in average diameter of 50 randomly selected collagen fibers from 47.2 ± 1.9 nm prior to cross-linking to 34.2 ± 1.1 nm post cross-linking ($P < 0.001$). Qualitatively, the collagen fibers appeared more closely packed following cross-linking.

Conclusions: The findings of this study suggest that collagen cross-linking is a viable and effective modality for increasing the stiffness of human tarsal plates.

Translational Relevance: This work provides proof that collagen cross-linking produces stiffening of the human tarsal plate and may be used in disorders that cause eyelid laxity.

Introduction

The tarsal plates provide structural support for the eyelids and define their form and stiffness. Changes to the composition of the extracellular matrix of the tarsal plates changes their mechanical behavior. Age-related loss of collagen and elastin may contribute to entropion and ectropion.[1,2] More recently, degradation of elastin fibers within the tarsal plates has been linked to the occurrence of floppy eyelid syndrome (FES),[3] characterized by a rubbery, malleable upper tarsus with lash ptosis. Gentle upward force applied to these floppy lids everts them.[4] These features combine to produce ocular irritation that may progress to scarring and eventual loss of vision. Immunohistochemistry studies in FES have revealed upregulation of the elastin-digesting matrix metalloproteinases 2, 7, and 9.[5] Currently, the management of FES includes a conservative approach designed to control symptoms with surface lubricants and surgical

techniques designed to reduce spontaneous lid eversion. However, the surgical failure rate has been reported to be as high as 44%.[6]

Smith and colleagues[7] recently reviewed the use of photochemical cross-linking with ultraviolet A (UVA) and riboflavin (vitamin B_2) for the stiffening of ovine tarsal plates. They did not find a significant stiffening effect from this treatment. However, tarsal plates in sheep are thicker (1.5 mm) than those found in humans (0.82 mm), and therefore their results may not be generalizable to human lids. In the present study, we used UVA-riboflavin–induced cross-linking to increase the stiffness of human cadaveric tarsal tissue. The primary outcome of this study was to review the biomechanical and morphologic changes that occur due to collagen cross-linking in human tarsus.

Methods

Tissue Sources and Preparation

In conformity with legal requirements, 18 frozen cadavers were obtained from the UCLA Willed Body Program and defrosted once. Fourteen cadavers were male and four were female. Eighteen right and 18 left upper eyelids were excised en bloc from the medial to the lateral canthus. The lids were dissected to remove the skin and orbicularis from the tarsal plates, along with attachments of the levator aponeurosis and Müller's muscle, without altering the posterior conjunctiva. The tarsal plates were measured using digital calipers. Following excision, four 2 × 6-mm vertical strips of central tarsus were cut from the superior to inferior border using a no. 15 scalpel. Each strip was cut so that it contained one meibomian gland as confirmed visually.

Radiation Penetrance

To determine UVA transmission through the tarsal plate, 10 × 8-mm tarsal specimens from five eyelids were placed on a petri dish with the posterior (conjunctival) surface facing upward and illuminated at 365 nm using a commercially available corneal cross-linking system (Lightmed, San Clemente, CA). A radiometer (Edmund Optics, Barrington, NJ) with a spectral range of 335 to 380 nm was covered by the tarsal tissue to ensure detection of only light passing through the tissue. A series of measurements was then taken using UVA irradiance of 3, 9, 18, 30, and 45 mW/cm^2.

Cross-Linking Procedure

A 1% hypertonic riboflavin (Sigma-Aldrich Corp., St. Louis, MO) solution was made by dissolving in distilled water and maintained at room temperature (20°–22°C) in darkness and neutral pH as verified using a pH indicator (Whatman, Maidstone, UK). The two most central tarsal strips from each eyelid were used for this experiment. One side served as the control and was treated with riboflavin solution every 30 seconds for 18 minutes at room temperature in darkness. The samples from the opposite side were cross-linked. The tissue was placed in a petri dish and irradiated with UVA at 6 mW/cm^2 for 18 minutes. To reduce the effect of bleaching,[8] a drop of riboflavin was applied to the tissue every 30 seconds for the duration of the irradiation, after which the tissue was stored in a moist chamber for 1 to 2 minutes before mechanical testing.

Mechanical Testing

We employed a horizontally mounted microtensile load cell incorporating a linear motor (Ibex Engineering, Newbury Park, CA) having 20 nm distance resolution and a strain gauge (LSB200; FUTEK, Irvine, CA) having 5 mN force resolution. This apparatus includes a heated water bath providing physiological temperature and humidity for testing under physiological conditions (Fig. 1).[9] Specimens were measured using an industrial optical coherence tomography (OCT) scanner (Thorlabs Inc., Newton, NJ), permitting measurement of mean specimen cross-section for stress calculation. Stiffness (Young's modulus) was calculated using the method previously described by Shin and colleagues (Fig. 1).[10] Briefly, to avoid laxity, specimens were preloaded with 0.05 N stress, then elongated at 0.1 mm/s as tensile force was recorded until failure was signaled by abrupt decrease in tension and visible rupture. Specimens were assumed linearly elastic and homogeneous. A stress-strain plot was created, and Young's modulus was computed as the slope of the curve in the linear region.

Histopathology

Six cross-linked samples (to the right of the two central strips) and corresponding controls were fixed in 10% formalin and processed using a tissue processor (Excelsior AS; Thermo Fisher, Waltham, MA). The tissue was dehydrated in graded alcohol solutions before embedding in paraffin. The tissue was sectioned at 4 μm

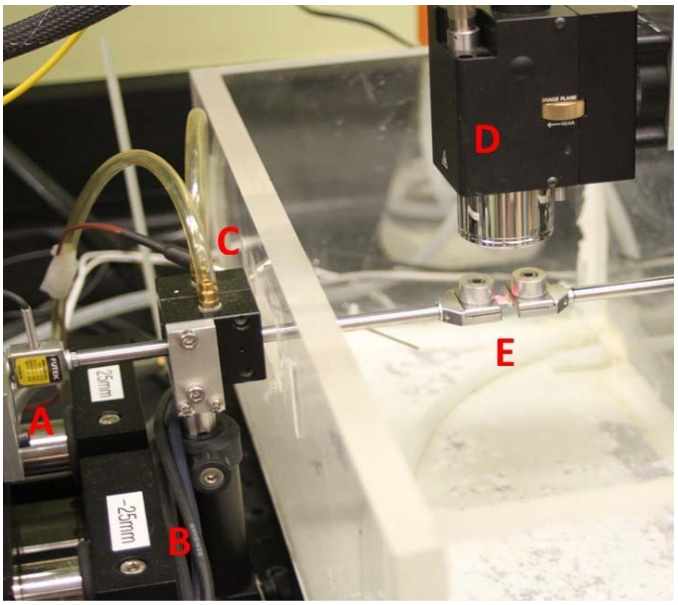

Figure 1. Microtensile load cell. A strain gauge (A) attached to a linear motor (B) was connected in the temperature- and humidity-controlled chamber to the specimen clamp (E) that was supported by a frictionless air bearing (C). An OCT scanner (D) was directed at the specimen.

in a rotary microtome (RM2235; Leica Nanosystems, Wetzler, Germany) and stained with hematoxylin and eosin (H&E), and Masson trichrome stains.[11]

Transmission Electron Microscopy

Four controls and four cross-linked samples were placed in 2.5% phosphate-buffered glutaraldehyde for 24 hours and then fixed in 1% osmium tetroxide in 0.1 mol/L for 2 hours. The specimens were dehydrated in ascending grades of alcohol. A microtome (Ultracut; Reichert, Munich, Germany) was used to cut 50-nm-thick sections. The tissue was double stained using uranyl acetate and lead citrate prior to imaging with a transmission electron microscope (100CX; JEOL, Peabody, MA). Five sections were cut for each tarsal plate, spaced at 20 μm. The widths of 10 randomly selected collagen fibers per section were measured at 100,000× magnification using software provided with the microscope.

Statistical Analysis

Because samples were taken from both eyelids from each cadaver, the generalized estimating equation method was used to compensate for possible within-donor correlations previously been validated.[12] Statistical significance was defined as $P < 0.05$.

Results

Tissue Characteristics

Prior to processing and cross-linking, tarsal plates had the mean length 19.7 ± 1.2 mm (SD) and mean thickness 0.87 ± 0.14 mm. Mean width was 1.1 ± 0.2 mm.

Radiation Penetrance

Penetrance was measured in 10 samples of mean thickness 0.81 ± 0.09 mm. Figure 2 displays the average UVA penetrance through the tarsal plates at various irradiance levels. Irradiance of 3 mW/cm^2 was sufficient to penetrate the entire tarsal plate. Progressive increase in irradiance to 45 mW/cm^2 resulted in a linear increase in UVA intensity on the opposite surface, described by the equation $y = 4.68x - 184$ (y = light intensity [Lux] and x = irradiance [mW/cm^2]).

Tensile Testing

Mean Young's modulus in the linear region for controls was 28 ± 9 MPa, but it was much higher, at 138 ± 8 MPa, for cross-linked samples ($P < 0.001$), yielding a 493% mean stiffness increase (Fig. 3). Mean Young's modulus for controls in the low toe region (<5% strain) was 1.5 ± 0.8 MPa for controls but much higher at 6.0 ± 3.1 MPa for cross-linked samples ($P < 0.001$).

Histopathology

Each of the 16 slides (eight H&E and eight Masson trichrome stained) was examined using a microscope (Nikon Eclipse E800M; Nikon, Tokyo, Japan) at 2× and 10× magnification. Staining with H&E and Masson trichrome revealed normal structures such as blood vessels, meibomian glands, nerves, and collagen bundles in both control and cross-linked samples (Fig. 4). The distribution of collagen fibers in controls did not differ from those in cross-linked samples. The structure and size of the meibomian glands and blood vessels were similar in controls and cross-linked samples.

Transmission Electron Microscopy

Transmission electron microscopy of five slides from each of four specimens and four controls at 100,000× magnification showed a decrease in average diameter of 50 randomly selected collagen fibers from 47.2 ± 1.9 nm prior to cross-linking to 34.2 ± 1.1 nm

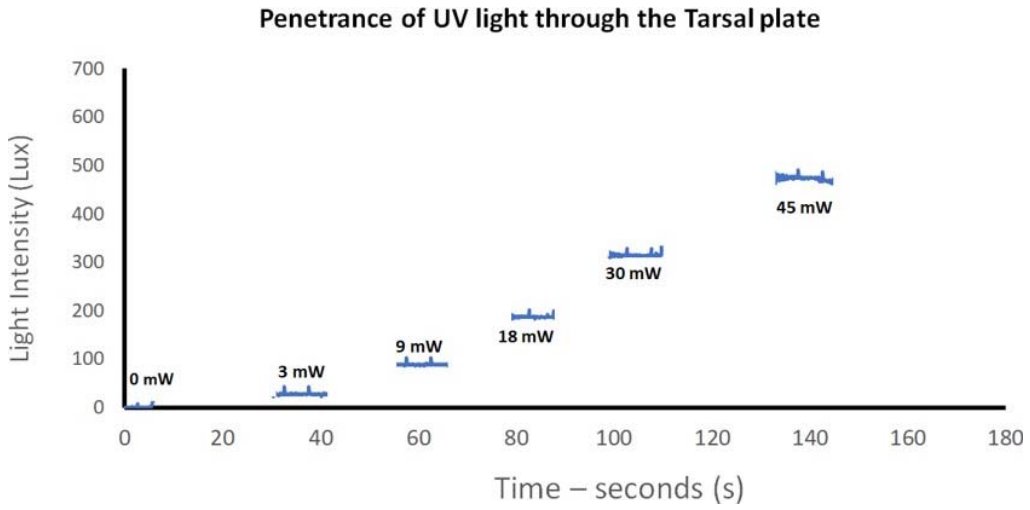

Figure 2. Intensity of UV light that has passed through a human tarsal plate at different illumination intensities.

post cross-linking ($P < 0.001$). Qualitatively, the collagen fibers appeared more closely packed following cross-linking (Fig. 5).

Discussion

In this study, collagen cross-linking with UVA and riboflavin 1% increased the Young's modulus, a measure of tensile stiffness of the human upper eyelid tarsus, about fourfold. Collagen fiber diameter was decreased by about 28% following cross-linking.

Collagen cross-linking may occur naturally as a consequence of aging[13] or diabetes.[14] Artificial induction of collagen cross-linking has previously been achieved using chemical agents,[15,16] radiation alone,[17] and radiation with photosensitizers.[18] Over the past two decades, UVA-riboflavin cross-linking has been established as a safe therapy for keratoconus.[19]

Smith and colleagues[7] reviewed the efficacy of collagen cross-linking for increasing the stiffness of the tarsal plates of sheep. They found no significant effects of low levels of irradiance (3–6 mW/cm^2) on stiffness. However, at much higher levels of irradiance (30 and 45 mW/cm^2), cross-linked samples significantly stiffened.[7] The higher levels of irradiance used by Smith and colleagues[7] cannot be used safely in humans. However, in the current human study, lower

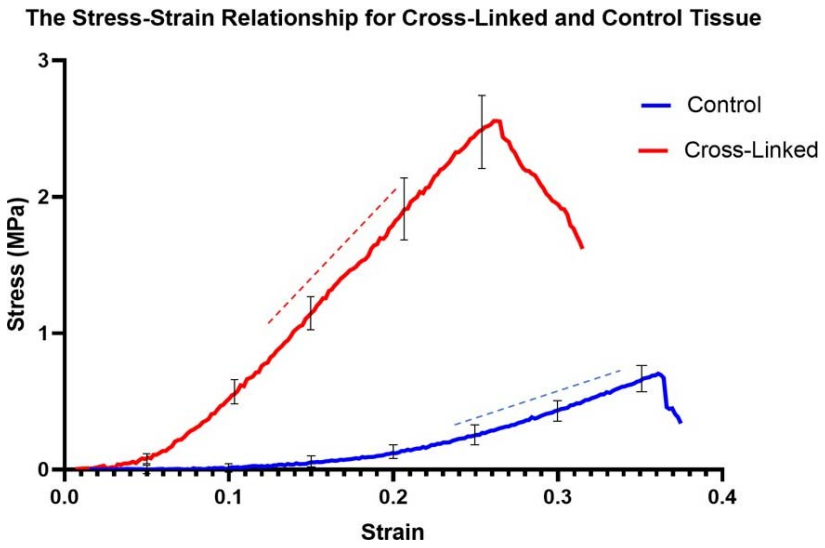

Figure 3. Stress-strain plot averaging 18 cross-linked and 18 control human tarsal specimens. The slope of the curves in the linear region (*dotted lines*) represents Young's modulus in the high-strain region.

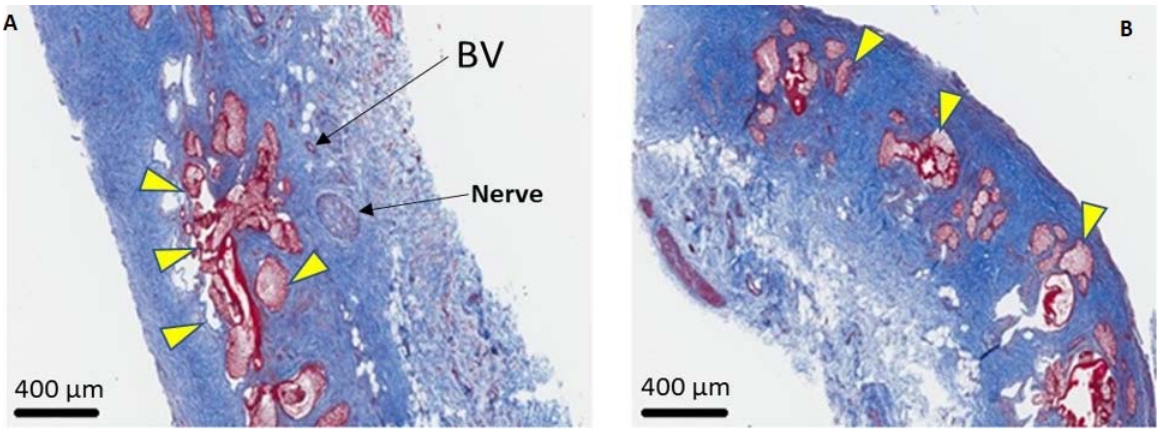

Figure 4. Masson trichrome staining of control (A) and cross-linked (B) tissue. Meibomian glands appear *red* (*yellow arrows*). Collagen appears *blue*. Blood vessels (BV) also appear *red*.

irradiance (6 mW/cm^2) significantly stiffened tarsal plates. The difference may be because ovine tarsal plates structurally differ from those in humans. For example, mean ovine upper tarsal thickness is 1.5 mm, while we found mean thickness in humans of only 0.87 mm, consistent with previous studies.[20] Furthermore, Smith and colleagues used a solution of riboflavin marketed for ophthalmic use. At present, all riboflavin preparations marketed for ophthalmic cross-linking have concentration below 0.5%. Our formulation was 1%. Although we did not test the impact of riboflavin concentration on cross-linking, variation in concentration may explain some of the efficacy differences among studies. O'Brart[21] and colleagues have previously found that the concentration of riboflavin corresponds to the efficacy of cross-linking.

We found 6 mW/cm^2 irradiance increased tarsal plate tensile stiffness almost fourfold. This level of irradiance for 18 minutes corresponds to delivery of 6.48 J/cm^2 of energy, which has proven to be safe when used on the cornea.[22] Furthermore, the UVA delivered by the Lightlink CXL (Lightmed) is reported by the manufacturer to be uniform over the exposed area, thereby reducing the occurrence of hot spots.

This study found that the diameter of collagen fibers decreased following cross-linking. This is in contrast to an earlier study that found a decrease in collagen fiber[23] diameter following cross-linking and

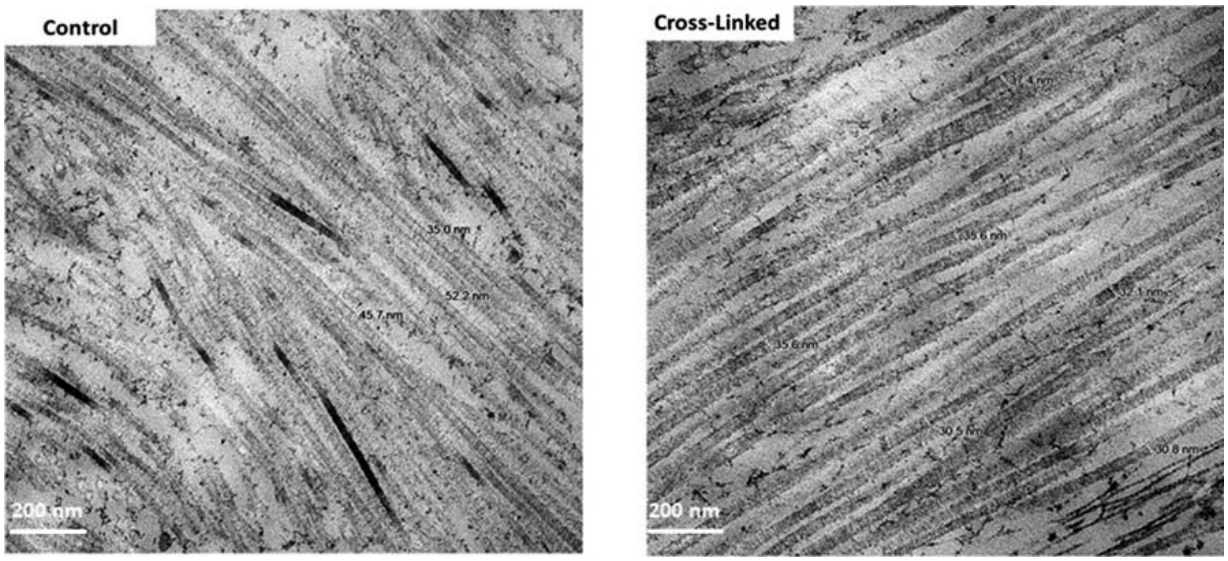

Figure 5. Transmission electron microscopy of the tarsal plates at 100,000× magnification, revealing a change in collagen organization and narrowing of collagen fibers.

also a subsequent study[24] that found no changes to fiber diameter following cross-linking. The differences between our samples and those of previously published accounts may be explained by the difference in tissue, with tarsal plates containing a greater amount of collagen type 1,[25,26] and also by the cross-linking protocol used. In this study, riboflavin was delivered to the tarsal plate every 30 seconds to reduce the impact of photobleaching, whereas in the aforementioned studies, it was delivered once every 5 minutes.

The use of frozen cadavers in our study raises the possibility of thermal degradation. We used thawed cadavers that had been frozen only once. Huang and colleagues[27] showed that when cadaveric tendons were freeze-thawed in fewer than three cycles, the Young's modulus and ultimate stress was comparable to fresh samples. Although tendons have a different macroanatomy from that of the tarsal plate, both are composed primarily of collagen fibers.

In this study, small strips of tarsal tissue were used for cross-linking. Since the tissue was dissected, the UV light and riboflavin spread to the edges of the tissue. This would not normally occur in vivo and potentially could have had an impact on the final stiffness of the tissues tested.

Histologic analysis did not reveal differences between the cross-linked and control samples. Although this is encouraging, the majority of radiation-induced effects occur in cells that are within the active phases of the cell cycle.[28] The majority of acute radiation-induced injuries manifest hours after radiation and involve disruption of the cell cycle, while more chronic effects may take months to manifest. Although few fibroblasts exist within the tarsal plates, the meibomian glands are lined with stratified squamous epithelium, much like the skin, and may be susceptible to the effects of UVA radiation. To fully appraise the potential impact of UVA radiation on tarsal tissue, in vivo models will be required.

In vivo models are also required to understand the mechanical effects of stiffening the tarsal plates in functioning lids. Although we found that uniaxial stiffness increased in cross-linked tarsal plates, further work will be required to review the interactions between a stiffer upper eyelid and the corneal surface. The current study did not characterize the bending or compressive stiffness of the tarsus, which may also be important to lid function.

This study highlights the efficacy of photochemical cross-linking with riboflavin and UVA in stiffening the human tarsus. While this approach suggests a potentially exciting opportunity for the treatment of disorders of eyelid laxity, a cautious study of its safety and suitability for use in humans will be required before therapeutic use.

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