Further Investigations on the Crosslinking of Tarsal Collagen as a Treatment for Eyelid Laxity: Optimizing the Procedure in Animal Tissue

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ORIGINAL INVESTIGATION

Further Investigations on the Crosslinking of Tarsal Collagen as a Treatment for Eyelid Laxity: Optimizing the Procedure in Animal Tissue

Tai M. Smith, B.Sc., M.B.B.S., F.R.A.N.Z.C.O., Shuko Suzuki, M.Sc., Ph.D.*, Nestor Sabat, B.Sc.*, Cassie L. Rayner, B.App.Sc.*, Damien G. Harkin, B.Sc., Ph.D.*†‡, and Traian V. Chirila, B.Eng., Ph.D., D.h.c., F.R.A.C.I., C.Chem., F.R.S.C.*§‖¶#*

**Queensland Eye Institute, South Brisbane, Queensland, Australia; †Queensland University of Technology, Faculty of Health, Brisbane, Queensland, Australia; ‡Institute of Health and Biomedical Innovation, Kelvin Grove, Queensland, Australia; §Queensland University of Technology, Science and Engineering Faculty, Brisbane, Queensland, Australia; ‖Australian Institute for Bioengineering and Nanotechnology, St Lucia, Queensland, Australia; ¶University of Queensland, Faculty of Medicine, Herston, Queensland, Australia; and #University of Western Australia, Faculty of Science, Crawley, Western Australia, Australia*

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Conflict of Interest Disclosure: Australian Patent Application No. 2018201200, filed on Feb. 20, 2018, and U.S. Patent Application Pub. No. US 2018/0236261 A1, Aug. 23, 2018, have been both assigned to the Queensland Eye Institute Foundation, by the authors Tai M. Smith, Shuko Suzuki and Traian V. Chirila as inventors. The remaining authors have no financial or conflicts of interest to disclose.

Address correspondence and reprint requests to Professor Traian V. Chirila, B.Eng., Ph.D., D.h.c., F.R.A.C.I., C.Chem., F.R.S.C., Queensland Eye Institute, 140 Melbourne Street, South Brisbane, QLD 4101, Australia. Telephone: 61-7-3239-5024. E-mail: traian.chirila@qei.org.au

Running head: *Optimizing the Conditions for Tarsal Collagen Crosslinking*

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Précis

Safe irradiation conditions are established for the exposure of ex vivo ovine tarsus to ultraviolet-A radiation as a potentially effective treatment for eyelid laxity in human patients.

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Structured Abstract

Purpose: A follow-up experimental study on the exposure of animal tarsal plate to ultraviolet-A radiation aimed at establishing an optimum range for safe irradiation conditions.

Methods: Sheep tarsus specimens were excised post-mortem and then subjected to irradiation with ultraviolet-A rays (wavelength 365 nm) at higher irradiances than those reported in an initial study, using a laboratory radiation source. The mechanical properties (tensile strength and Young's modulus) of irradiated and non-irradiated samples were evaluated in a mechanical tester. The test and control specimens were examined histologically with an aim to assess the effects of radiation upon the meibomian glands and tarsal collagen networks, and to establish a safe range for the exposure irradiance level.

Results: As expected, irradiation induced both stiffening and strengthening of the tarsal plate specimens. At an irradiance of 50 mW/cm^2 for 3-min exposure, these effects were at their maximum level, after which a decline in mechanical characteristics were observed. No destruction of the tarsal connective tissue or the meibomian glands were noticed up to an irradiance of 125 mW/cm^2 for 3-min exposure, corresponding to a fluence of 22.5 J/cm^2. Histology revealed that the collagen network surrounding the glands were packed more compactly following irradiation. At a fluence of 45 J/cm^2, massive destruction of periglandular collagen-rich network and meibocytes were demonstrated histologically.

Conclusions: The study indicates that irradiation of tarsal collagen leading to tissue stiffening shall be carried out at levels of fluence between 10 and 15 J/cm^2, a region that is deemed safe. The exposure time can be adjusted according to the surgeon's decision.

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Laxity of the eyelids is caused by either reduction or loss of the inherent rigidity and firmness of the tissue, and is a prominent etiologic feature in most of the abnormalities of the eyelid including the floppy eyelid syndrome (FES).[1-3] This acquired disorder is chiefly attributed to structural and organizational alterations in collagen and elastin, the major elastic proteins of the tarsal plate, where they are arranged into fibrous systems.[1,2,4,5]

In spite of the current availability of a relatively large number of conservative and surgical strategies for treating FES, and of their satisfactory rates of clinical success that have been generally reported, the recurrence of the symptoms over time is rather common.[6]

Considering that the eyelid's tarsus has the characteristics of the fibrocartilage type of connective tissue where collagen imparts strength and rigidity while elastin assures elasticity, the authors have previously proposed the photochemically induced crosslinking of tarsal collagen by exposure to ultraviolet (UV)-A radiation as a process to restore or enhance its rigidity, and have assessed its validity ex vivo in ovine eyelids.[7] A rationale for the proposal was that the accumulation of collagen I and collagen III fibers noticed in the lax eyelids and judged as an adaptive response[2] seems to be insufficient in compensating for an increased elasticity. Further, it is unlikely that irradiation would have any effect on the elastin component, based upon the following reasons: first, the histologically demonstrated decrease in elastin fibers[2,8,9] is not consistent with the increased elasticity clinically observed in FES; second, elastin is already a crosslinked material as a result of the enzyme-induced natural elastogenesis,[10] and further irradiation would trigger alternative chemical processes, such as degradation, rather than crosslinking.[11,12]

In this follow-up study, the experiments are aimed at establishing and recommending a safe window for the irradiation dose delivered ex vivo to the ovine tarsus samples, expressed by the radiation fluence and based on its intrinsic relation with irradiance and exposure time.

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METHODS

The ovine tarsal plates were harvested post-mortem on site from the cadavers of animals that were supplied by Highchester Meats Pty Ltd, Gleneagle, Queensland, Australia, an abattoir unit operating under the Code of Practice of the Animal Welfare Standards for Livestock Processing Establishments, Australia. The animals were sacrificed for commercial purposes, and the eyelid tissue would have been discarded if not used in our study.

Sample Preparation. Ovine (sheep) upper and lower eyelids were isolated according to a procedure reported in detail elsewhere.[7] Fifteen cadaveric sheep (Merino breed, 1 to 2 years old) were used in this study, providing a total number of 60 eyelids. The lids were individually fashioned into tarsal strips, which subsequently were distributed for tensile measurements and histologic analysis. The irradiation of these strips was performed within 5 to 10 hours from their excision.

Irradiation Procedure. The tarsal strips, with an average thickness around 1.5 mm, were soaked for 30 min in a solution of riboflavin 5'-phosphate monosodium salt (as a photosensitizer), supplied as VibeX™ Rapid 0.1% solution (Medio-Haus Medizinprodukte GmbH, Kiel, Germany). The soaked tarsal strips were then exposed to a beam of UV-A radiation with a wavelength of 365 nm, which was generated by a UV Curing System OmniCure® 1500 (Excelitas Technologies Corp., Waltham, MA). A radiometer (Dymax ACCU-CAL™ 50, Dymax Corp., Torrington, CT) was employed to measure the irradiance at the site of exposure on the surface of specimens, while the distance was adjusted in order to assure the required value for the irradiance. The detailed parameters of the irradiation procedure are presented in Table 1. In a separate experiment, a tarsal strip was exposed to very high irradiance (250 mW/cm^2) for the same duration (3 min), and then examined histologically.

Insert TABLE 1

Mechanical Testing. The tensile properties of non-irradiated and irradiated tarsal strips were evaluated in an Instron Materials Testing System, Model 5943 (Instron, Norwood, MA) equipped with a 50-N load cell, as previously described.[7] For each specimen, 4 to 6 measurements were carried out in accordance to the established parameters. From the recorded stress-strain plots, Young's moduli were computed in the linear region. The results were statistically processed by the one-way analysis of

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variance (ANOVA) in conjunction with Tukey-Kramer multiple comparisons, using the GraphPad® Prism software (Version 6.0).

Histology. The tissue samples, all excised from upper eyelids, were immersed in molds containing optimal cutting temperature (OCT) medium (Tissu-Tek®, ProSciTech, Queensland, Australia), then snap-frozen after freezing in dry ice, and stored at −80 °C. During freezing in OCT, tissue specimens were orientated to enable sectioning along the longitudinal axis of each tarsal plate. Sections measuring approximately 3 μm in thickness were cut using a cryostat, transferred to coated slides (Superfrost™, Thermo Fisher Scientific Australia Pty Ltd, Victoria, Australia) and stored at −20°C. The sections were subsequently stained using a modified Van Gieson protocol designed for demonstration of collagen-rich extracellular matrix components. In brief, sections were brought to room temperature over 5 min, rinsed three times for 5 min in deionized water, and treated for 1 min in Van Gieson solution (a saturated aqueous solution of picric acid, supplemented with 0.1% w/v acid fuchsin). The stained sections were carefully blotted using filter paper, dehydrated by two brief rinses in absolute ethanol, cleared in two changes of xylene, and finally mounted beneath a glass coverslip in plastic mounting medium (Entellan®, ProSciTech). The slides were subsequently masked to hide their treatment group, and examined by two independent observers for discernible differences in morphology, at magnifications ranging from 4X to 40X. Examples of notable morphology were photographed using an Olympus BX41 (Olympus Australia, Victoria, Australia) microscope equipped with a Nikon Ri1 digital camera. Image montages were stitched together from low-power (4X) microscopic fields using the Adobe Photoshop® photoediting software.

RESULTS

Figure 1 summarizes the mechanical properties as measured for the sheep eyelid strips, prior to and after exposure to UV-A radiation at 4 different levels of irradiance.

Insert FIG. 1

Following irradiation, the highest stiffness (reflected by Young's modulus) and strength (equivalent to tensile stress) were displayed by the samples exposed for 3 min to 50 mW/cm^2, followed by those exposed to 75 mW/cm^2 for an identical duration. A decline in the values of these parameters is evident at the higher irradiances (fluences) employed in this study. As a general observation, it appears

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again[7] that the lower eyelids are stiffer and stronger that the upper eyelids, both prior to and after irradiation.

Staining with Van Gieson solution demonstrated the presence of collagen-rich connective tissue (red), in sharp contrast to other tissue components such as meibomian glands, hair follicles, epidermis, and occasional fragments of skeletal muscle, all predominantly yellow (Figure 2).

Insert FIG. 2

In particular, the montages created from low magnification micrographs revealed striking columns of connective tissue separating the meibomian glands.

No discernible differences in the architecture of the meibomian glands were observed following exposure to irradiance levels between 50 and 125 mW/cm^2, equivalent to radiation fluences from 9 to 22.5 J/cm^2. Interestingly, the connective tissue surrounding the glands appeared better preserved after irradiation (Figures 2B and 3B). Exposure to a much higher irradiance (250 mW/cm^2) for the same duration, however, resulted in substantial destruction of the glands, including loss of cell nuclei and disruption of the surrounding connective tissue (Figure 3C).

Insert FIG. 3

DISCUSSION

In the authors' previous study,[7] radiation fluences (i.e. the energy delivered per surface area unit) between 5.4 and 7.2 J/cm^2 were achieved for irradiances (i.e. the power incident per surface area unit) of 30 and 45 mW/cm^2 employing variable exposure times. Higher fluences were also achieved for lower irradiances (3 or 6 mW/cm^2) by extending significantly (10-fold) the exposure time. However, overlong irradiation treatments would be impractical for the surgeon and inconvenient for the patients, therefore to employ low irradiances appears irrelevant to clinical applications if extrapolated to human patients.

There has been no attempt so far to assess the irradiation threshold after which the improved mechanical properties induced by irradiation start to regress, neither to find when

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the radiation-induced destruction of the tarsal tissue and/or meibomian glands begins. The present study aimed at elucidating such issues. The results of mechanical testing, as detailed in Figure 1, show that the optimum stiffness and strength was achieved at an irradiance of 50 mW/cm^2 for an exposure time of 3 min, equivalent to a fluence of 9 J/cm^2. At higher irradiances, the values for Young's modulus and tensile stress declined gradually. In the range of irradiance investigated here (50 to 125 mW/cm^2), the histologic examination could not detect any radiation-induced degradation of tarsus or meibomian glands. On the contrary, the post-irradiation more compact fine structure of the collagen-rich connective tissue network surrounding each meibomian gland (Figures 2B and 3B) indicates a better preservation of the tissue, which is consistent with the collagen having been crosslinked. However, at a very high irradiance (250 mW/cm^2), equivalent to a fluence of 45 J/cm^2, massive destruction of the connective tissue and the degeneration of acini, with loss of meibocytes' nuclei, were observed. In an earlier study[13] on tarsal tissue retrieved upon the eye exenteration in patients who were previously subjected to irradiation with X rays for treating sinus malignancies, the predominant effects have included involutional atrophy of the glands, in some cases with total loss of glands and ducts, and cystic dilatation of the ducts. The present findings cannot be discussed, however, in the same context considering the much higher energy of the X rays (10^2—10^5 eV compared with 3—4 eV for the UV-A rays) and the fact that irradiation was performed in vivo in human patients. At odds with both these findings, however, are those reported[14] on the irradiation with γ rays (^{60}Co) of tissue-bank human tarsal plates prior to implantation in a primate model for reconstruction of full-thickness eyelid defects. Although the energy output of the γ ray photons generated by the decay of radioactive ^{60}Co nuclei is over 10^6 eV, there was no histologically observable damage to the meibomian glands.

The present results are also different from those of the histologic examination of tissue retrieved from aging eyes. In a large series of human tarsal plates obtained post-mortem, the main age-induced changes have included cystic dilatation of acini and/or ducts, acinar atrophy, granulation tissue, and lipogranulomatous inflammation.[15] In more recent studies[16] in a murine

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animal model, it was suggested that the atrophy of acini/glands may be the major cause of age-related meibomian gland dysfunction, rather than hyperkeratinization leading to ductal obstruction. No acinar atrophy or ductal keratinization were noticed in the present study; however, the significant damage induced by exposure to UV-A radiation at excessive irradiances (fluences), as shown in Figure 3C, is clearly incompatible with a normal function of the meibomian glands. Therefore, adequate irradiation parameters must be selected for the clinical applications of the method to make sure that the procedure does not cause meibomian gland dysfunction.

The authors' results suggest an irradiance between 50 and 75 mW/cm^2 (corresponding to fluences between 9 and 13.5 J/cm^2) as optimum, leading to lack of histologically detectable damage to the tissue and to the best stiffening and strengthening effects. Generally, the authors recommend a range of fluence between 10 and 15 J/cm^2. Any choice in this range can be associated with a conveniently short exposure time, based on the fact that—by definition—the irradiance equals fluence divided by irradiation time. For instance, if the surgeon decides to employ a fluence of 12 J/cm^2, and an irradiation time of 4 min, this will correspond to an irradiance of 50 mW/cm^2. For the same fluence and an exposure of 3 min, the irradiance will increase to about 67 mW/cm^2, while for an exposure of 5 min it will decrease to 40 mW/cm^2. Clearly, the choices are practically unlimited, provided that the fluence is maintained within the limits recommended here. Overlong exposure is not recommended as it leads to high fluences surpassing the threshold.

Although fluences beyond 15 J/cm^2 did not cause internal tissue damage, the present study shows that increasing fluence reduces the stiffening and strengthening induced by crosslinking, therefore to irradiate at fluences over this threshold is rather pointless.

The results discussed here refer to the sheep tarsal tissue. Although it is expected that their extrapolation to human treatment would be straightforward, an evaluation in human tissue is mandatory.

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CONCLUSIONS

For the recently proposed treatment of eyelid laxity, based on the photochemical crosslinking of tarsal collagen by exposure to UV-A radiation, an irradiation fluence between 10 and 15 J/cm^2 is recommended in association with short exposure durations at the surgeon's choice. This shall provide irradiance values that enhance the stiffness and strength of the tarsus without causing degenerative complications involving the meibomian glands and surrounding connective tissue.

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LEGENDS TO FIGURES

FIG. 1. Comparative bar graphs of the measured Young's modulus (**A**) and tensile stress (**B**) in ovine upper and lower tarsal strips, prior and after irradiation with UV-A (365 nm) rays for 3 minutes each. **a**, Control samples. **b**, Irradiance 50 mW/cm^2, fluence 9.0 J/cm^2. **c**, Irradiance 75 mW/cm^2, fluence 13.5 J/cm^2. **d**, Irradiance 100 mW/cm^2, fluence 18.0 J/cm^2. **e**, Irradiance 125 mW/cm^2, fluence 22.5 J/cm^2. Statistically significant differences: *$p < 0.05$, ** $p < 0.05$. UV, ultraviolet.

FIG. 2. Montages created from multiple low magnification (4X) images of: **A**, control (non-irradiated) tissue, and **B,** treated tissue (irradiance 75 mW/cm^2, fluence 13.5 J/ cm^2), after cryosectioning and staining with Van Gieson solution. Regions containing collagenous connective tissue are stained red, while the Meibomian glands, hair follicles, epidermis and skeletal muscle are stained yellow. Breaks within large columns of connective tissue were occasionally seen in both control and treated tissue samples. Scale bar: 500 µm.

FIG.3. Examination of tissue sections at higher magnification (20X), revealed marked differences between treatments. The fine networks of connective tissue surrounding Meibomian glands were better preserved in tissue that have been irradiated at doses between 50 and 125 mW/cm^2, but a higher dose of 250 mW/cm^2 caused marked destruction of tissue components including the Meibomian glands. **A**, Control (non-irradiated) tissue. **B**, Tissue irradiated at an irradiance of 75 mW/cm^2. **C**, Tissue irradiated at an irradiance of 250 mW/cm^2. Scale bar: 100 µm.

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348 **TABLE 1.** Experimental conditions for the irradiation of tarsal strips*

		Number of samples (N)	
Irradiance (mW/cm^2)	**Fluence (J/cm^2)**	**Upper eyelid**	**Lower eyelid**
0	0	4	4
50	9	4	6
75	13.5	5	5
100	18.0	5	5
125	22.5	5	5

349 * Exposure time was 180 seconds for all irradiated samples.

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Figure 1

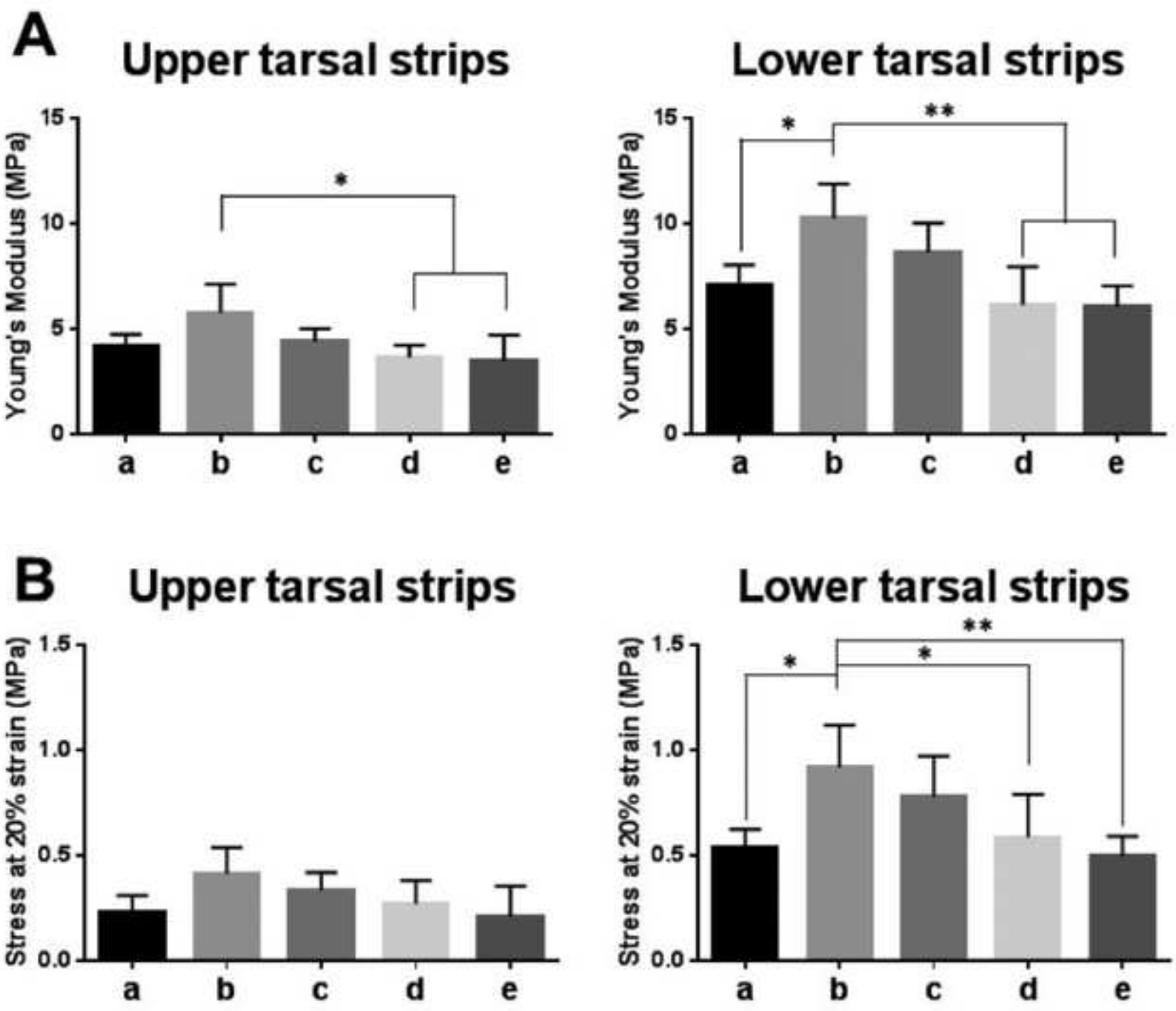

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Figure 2

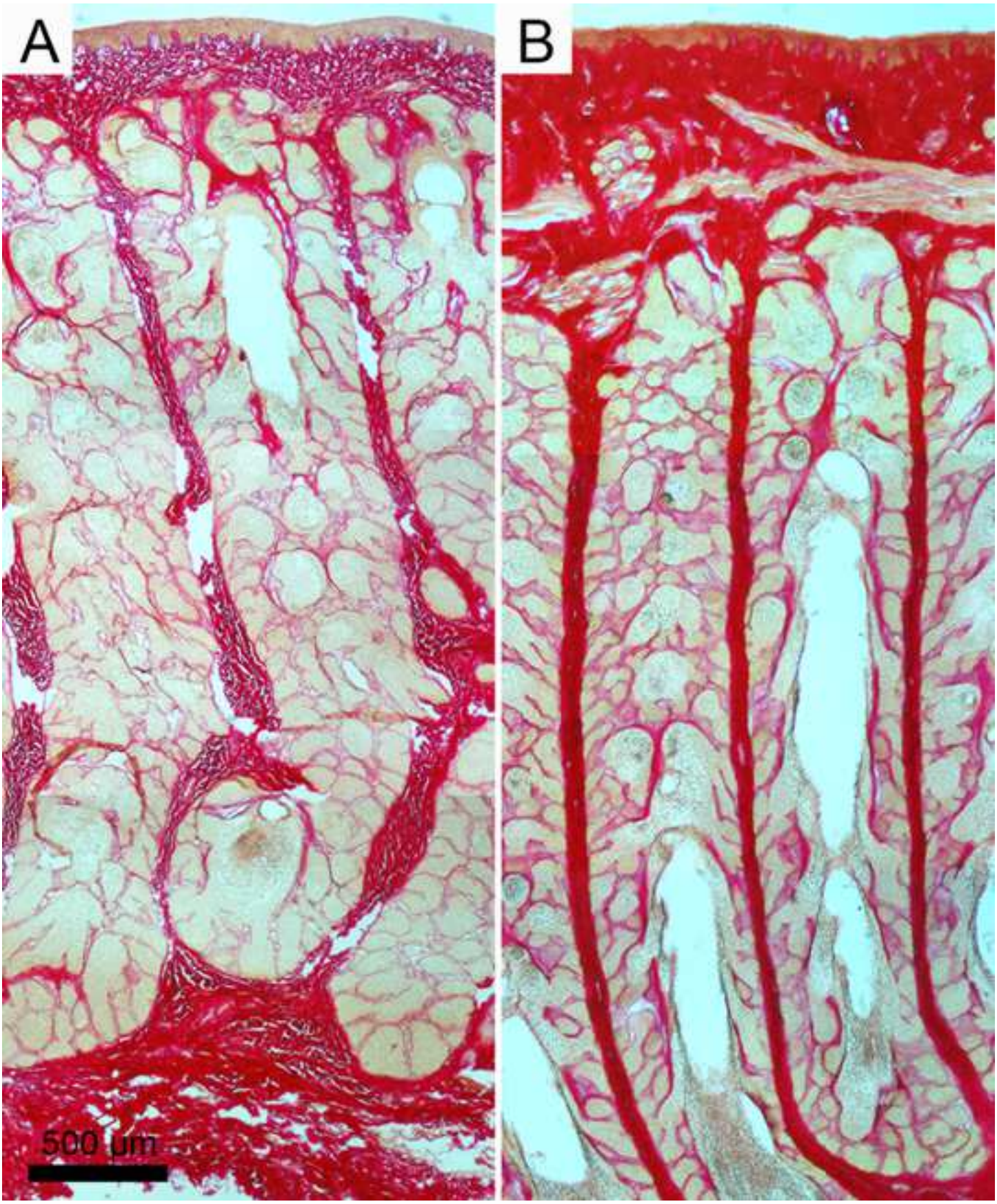

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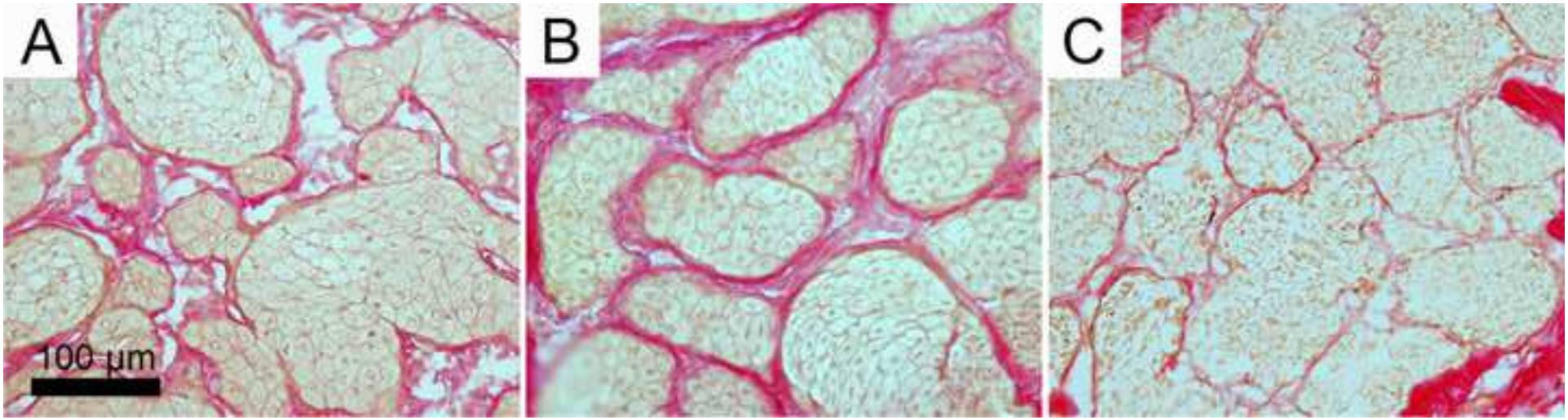
Figure 3



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125	22.5	5	5

* Exposure time was 180 seconds for all irradiated samples.

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