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BRIEF REPORT

Ex vivo thermographic analysis of the eyelid conjunctival surface during ultraviolet A-mediated photochemical crosslinking of tarsal collagen: A step in translational safety evaluation

Shuko Suzuki[1], Nadja E. Pop[2,3], Alexandra I. Manta[1,2,3,4], and Traian V. Chirila[1,2,3,5]*

[1]Queensland Eye Institute, Woolloongabba, Queensland, Australia

[2]Center for Advanced Medical and Pharmaceutical Research, George E. Palade University of Medicine, Pharmacy, Sciences and Technology, Târgu Mureș, Mureș, Romania

[3]Faculty of General Medicine, George E. Palade University of Medicine, Pharmacy, Sciences and Technology, Târgu Mureș, Mureș, Romania

[4]Faculty of Health and Behavioural Sciences, University of Queensland, St Lucia, Queensland, Australia

[5]Australian Institute of Bioengineering and Nanotechnology, University of Queensland, St Lucia, Queensland, Australia

***Corresponding author:**
Traian V. Chirila
(traian.chirila@qei.org.au)

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Abstract

Ultraviolet A (UVA)-induced collagen crosslinking can mechanically reinforce collagenous tissues and is being translated as a minimally invasive treatment for pathological eyelid laxity, yet the extent of irradiation-related heating at the conjunctival–tarsal interface remains unclear. In this study, we developed a novel procedure to treat pathological eyelid laxity. The methodology is based on the photocrosslinking of tarsal collagen, leading to mechanical reinforcement of the entire eyelid. In our previous studies, exposure to ultraviolet A (UVA) radiation at fluences below 20 J/cm^2 did not cause histopathological alterations to the eyelid tissues. Here, we further evaluated biological safety by quantifying conjunctival surface temperature increases during UVA irradiation to inform clinical translation. Using *ex vivo* sheep eyelids as a model, we used infrared thermography to measure the temperatures attained on the tarsal conjunctival surface during UVA irradiation (365 nm) at four irradiances for a maximum exposure time of 3 min, corresponding to fluences of 8.1, 13.5, 27, and 45 J/cm^2. Both the exposure time and irradiance caused an asymptotic increase in temperature, while a linear dependence was observed between fluence and temperature increase (ΔT). The recorded values for ΔT ranged from 3.3°C to 14.0°C. In summary, at clinically practicable fluences (<20 J/cm^2), such increments are not expected to raise the final temperature to the threshold for collagen denaturation and tissue damage.

Keywords: Eyelid laxity; Tarsal collagen; Photochemical crosslinking; Ultraviolet A radiation; Infrared thermography; Thermal damage

1. Introduction

Non-ionizing ultraviolet (UV) A (UVA) radiation is generally considered a non-thermal radiation. Compared to the other UV radiations (UVB and UVC), UVA has the longest wavelength (from 315 to 400 nm), corresponding to the lowest energy (3.94–3.10 eV). The effects of UVA exposure include photophysical and photochemical changes and are much less associated with the release of heat. The photophysical processes include photon absorption, localized excitation, and generation and migration of charge carriers (excited electrons and electron holes). These processes are always the same regardless of the nature of the materials. Importantly, these processes are the sources of photochemical changes, which are intricate and unfathomable, as they depend on the irradiated material's chemical structure. When absorbed by the target materials, UVA photons can excite molecules. This can trigger responses that damage biological matter, including chemical reactions such as oxidation and structural alterations to nucleic acids. Those excited electrons that do not contribute to such changes can recombine to release their energy through luminescence phenomena (fluorescence and phosphorescence) or through the production of heat.

At present, most studies have been dedicated to the complex effects of UVA and UVB radiations on the skin.[1-6] In brief, the main effects on the skin are photoaging, immunosuppression, and photocarcinogenesis. The pathways to such disorders involve radiation-induced processes, including lipid peroxidation, irreversible chemical damage to nucleic acids, chemical modification of proteins, altered intracellular signaling, and chronic inflammation. In general, these effects are mediated through UVA-photon absorption by the chromophores present in the skin and other tissues, acting as photosensitizers. Such biomolecular chromophores include lipophilic pigments, porphyrins, melanin and its precursors, flavins, pterins, aromatic amino acids, and vitamin B_6. Under certain conditions, they may protect against UV-induced damage. However, no evidence for a thermal effect of UVA on the skin has been provided or debated in the aforementioned literature.

The release of heat during UVA exposure, which may lead to an increase in the temperature of target materials, is relevant when connective tissues containing collagen are irradiated *in vivo* to induce the photochemical crosslinking of collagen. This exogenous process leads to a significant enhancement of the mechanical properties of tissues, specifically tensile strength and stiffness (Young's modulus), with remarkable therapeutic benefits in certain tissues, such as the cornea. Crosslinking of collagen has become an important method in tissue engineering and regenerative medicine, employed to generate scaffolds and implants by modifying the properties of collagenous substrates *in vitro*. The basics and practical relevance of collagen crosslinking have been comprehensively investigated,[7-19] although assigning the correct chemical structures to the resulting crosslinkages remains a challenge.[20-22]

At present, the most successful therapeutic application of UVA-induced collagen crosslinking is a treatment for keratoconus, an ectatic corneal disorder leading to severe impairment of vision. By irradiating the cornea with UVA rays in the presence of a photosensitizer, usually riboflavin (vitamin B_2), the progression of keratoconus is arrested as a consequence of the mechanical reinforcement generated through the photocrosslinking of corneal stromal collagen.[23-31] Inspired by this achievement, we have proposed[32-35] and are now developing a UVA/riboflavin strategy for treating eyelid laxity by photocrosslinking the collagen located inside the tarsal plates of the lower and upper lids. Eyelid laxity, also known as palpebral laxity, is one of the main causes for, or can be associated with, a range of pathological conditions such as floppy eyelid syndrome, involutional ptosis, ectropion, entropion, and blepharochalasis. Before validating our technique as a clinical procedure, it is crucial to assess its biological safety in terms of potential tissue damage caused by UVA radiation. Our previous investigations[32-34] have shown that at fluences (radiant exposures) lower than 20 J/cm^2, irradiation of the eyelid with UVA rays does not have any impact on the integrity of the tissues. Other investigators have confirmed our findings in *ex vivo* tarsal plate specimens of animal (rat) or human origin.[36-39]

The possibility of radiation-induced heat production at the interface with exposed eyelid tissues has never been explored. In the present study, the regional increase in temperature recorded on the surface of *ex vivo* ovine eyelids' tarsal conjunctiva resulting from its exposure to UVA (365 nm) radiation was specifically investigated at four different levels of radiant exposure. The results were compared to the temperature threshold reported for thermal denaturation of collagen. This is a significant phase in the translational cycle related to our proposed therapeutic strategy aimed at treating palpebral laxity. The study is intended to address a gap in the translational cycle associated with the proposed procedure, and the results will be of significant benefit for further clinical development.

2. Materials and methods

2.1. Materials

The eyelids were harvested on-site, postmortem, from sheep cadavers supplied by Brisbane Valley Meats Pty

Ltd., Australia, which is an abattoir unit operating under the rules of the Australian Code of Practice of the Animal Welfare Standards for Livestock Processing Establishments (2001) and the Australian Animal Welfare Standards and Guidelines for Sheep (2016). The animals were slaughtered exclusively for commercial purposes, and the eyelid tissues would have been discarded if not acquired for use in the present study.

Riboflavin 5'-phosphate (RF-5P) monosodium salt, supplied by Cayman Chemical Co. (United States of America [USA]), was dissolved in phosphate-buffered saline (PBS) to a concentration of 0.25% w/v. The solution was applied to the tissue specimens to replicate the conditions during an actual photocrosslinking procedure.

2.2. Methods

Twelve full-thickness eyelid specimens were excised postmortem from the ocular adnexa of six pairs of sheep eyes that were refrigerated for no longer than 24 h. For convenience, the eyelashes were trimmed. The specimens were soaked for 30 min at room temperature in a solution of RF-5P in PBS (0.25% w/v). After removal from the solution, each sample was placed on a Petri dish with the conjunctival side facing upward, and the excess liquid was gently blotted off with Kimwipe® paper tissues (KIMTECH Sciences, Australia).

A thermal camera model FLIR C5 (FLIR Systems Australia Pty Ltd., Australia) was used for the thermographic analysis, with a thermal sensitivity of <0.07°C. As a source of radiation, the UV curing system OmniCure® 1500 (Excelitas Technologies Corp., USA) was employed. To determine the level of irradiance at the exposure site, a radiometer (Dymax ACCU-CAL 50, Dymax Corp., USA) was used for the pre-irradiation adjustment of the distance between the UV source and the tissue surface. The experimental setting is shown in Figure 1. The samples were each exposed for 3 min to UVA radiation with a wavelength of 365 nm at irradiances of 45, 75, 150, or 250 mW/cm^2, corresponding to fluences of 8.1, 13.5, 27.0, or 45.0 J/cm^2. Three eyelid specimens were used for each irradiance level, and the data points are presented as mean values with 95% confidence intervals. Statistical analysis was performed using the Kruskal–Wallis test (n = 3 per irradiance, total n = 12) in GraphPad Prism® (Version 6; GraphPad Software, USA).

The infrared camera was mounted on a tripod and placed at a distance of ~ 20 cm from the sample. The measurement spot (the "spotmeter") on the camera screen was positioned on the surface of the tissue as centrally as possible. During each round of irradiation, images containing information regarding the temperature at the target spot were recorded every 30 s, along with baseline measurements taken before each irradiation episode. During the experiments, the ambient temperature in the laboratory was between 21 and 23°C, with a relative humidity of ~50%. The infrared camera was within its 12-month calibration interval at the time of the experiments.

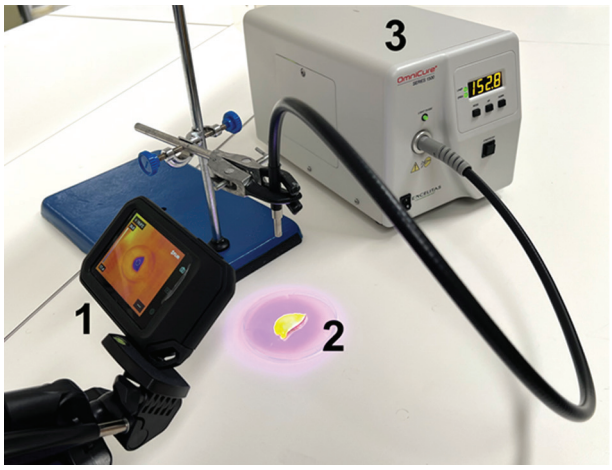


Figure 1. Experimental setting: (1) infrared camera, (2) tissue specimen in a Petri dish, and (3) ultraviolet A source

3. Results

Figure 2A shows a photograph of a sheep eyelid tissue sample that was soaked for 30 min in the photosensitizer solution, ready to be exposed to UVA radiation. Figure 2B displays the thermographic image of a tissue specimen recorded before commencing the irradiation procedure. Figure 2C and 2D show representative thermographic images recorded at the end of an exposure protocol,

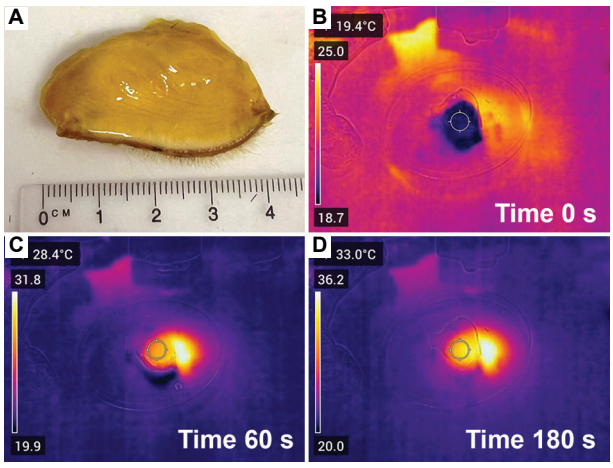


Figure 2. Photographic images of an eyelid tissue sample permeated with photosensitizer solution. (A) The eyelid tissue sample. (B–D) Thermographic images recorded (B) before and after exposure to ultraviolet A radiation (365 nm, 250 mW/cm^2) for (C) 60 s and (D) 180 s.

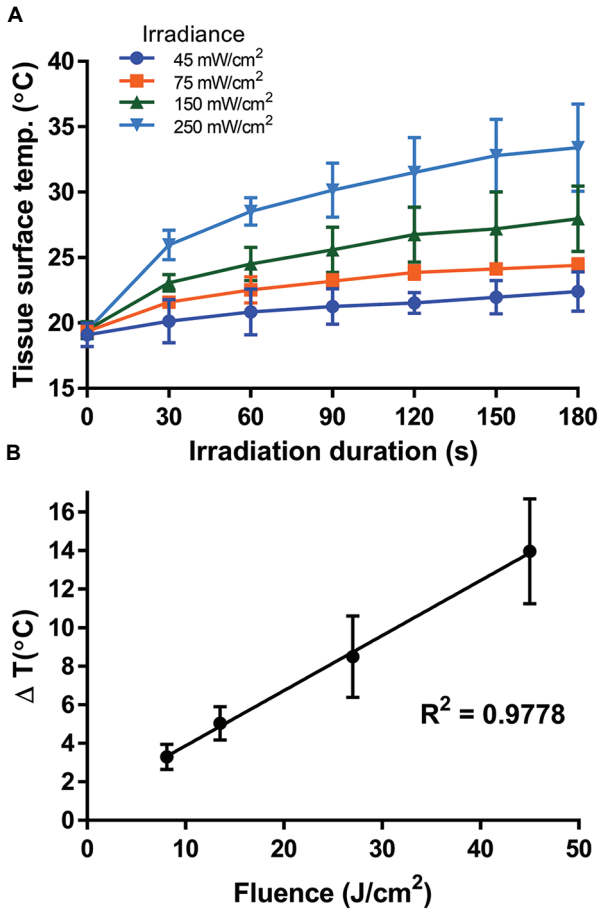


Figure 3. Thermal effects. (A) Plot of the temperature measured on the conjunctival surface as a function of exposure time at different irradiances. (B) Plot of the ΔT recorded on the conjunctival surface as a function of fluence. ΔT is defined as the difference between the temperature attained at the end of exposure time, i.e., after 3 min, and the baseline temperature recorded before commencing irradiation. Data points are presented with 95% confidence intervals.

exemplified here for an irradiance of 250 mW/cm² at two different exposure times.

As expected, the temperatures recorded on the surface of the tarsal conjunctiva increased both with the exposure time and with the irradiance (Figure 3A). Furthermore, there was a linear dependence between fluence and the temperature increase (ΔT) (Figure 3B). The baseline temperatures (before irradiation) were similar in all experiments, i.e., between 18.7 and 19.7°C, or 19.3 ± 0.3°C.

4. Discussion

When UV-induced crosslinking procedures are applied to collagenous tissues for therapeutic purposes, thermal effects associated with irradiation may induce a rise in temperature within the superficial layer of tissues or even deeper. However, as long as the temperature does not reach the threshold for thermal denaturation of collagen, which has been variably reported[40,41] between 50°C and 70°C, the procedure may be considered safe with respect to thermal tissue damage.

Reports on the thermal effect of UVA radiation on biological matter are sparse. For example, studies have been conducted[42,43] on cell cultures of human epithelial carcinoma cells (HeLa cell line), which were allowed to grow for 24 h before exposure to radiation while immersed in the growth medium. The rise in temperature recorded within the medium was highly dependent on the type of UV source. After 20 min of exposure, a BlackRay® lamp, delivering a fluence of 2.52 J/cm², produced an increase of 5.2°C, while a GreenSpot® super-pressure mercury lamp produced an increase of 1.4°C for a fluence of 7.6 J/cm². Despite apparent discrepancies, presumably due to technical variations related to the UV lamps, the recorded increases indicated minor thermal effects of UVA radiation. In a recent study[44] on UVA-based virus and bacteria inactivation, no thermal effects were mentioned for the remarkably high irradiance of 460 mW/cm², which allowed fluences of up to 800 J/cm².

More directly relevant to the present work, the effects of hyperthermia in *ex vivo* rabbit corneas, induced by ultrasonic energy delivered through the probe of a phacoemulsification machine, have been investigated using infrared thermography and histology.[45] No tissue injury was seen after 10 s of heating at 40°C. However, collagen disorganization, mild stromal edema, and cellular damage were noted at 50°C. With the same duration, massive stromal deterioration occurred at 60°C, characterized by the total disorganization of collagen fibers and endothelial detachment, which persisted after 1 week of follow-up. Another study has shown unequivocally that temperatures capable of promoting collagen denaturation can lead to significant tissue damage.[41] In a subsequent study,[46] the same research team performed thermographic analysis directly on the corneas of six patients during collagen crosslinking for treating keratoconus. During a 35-min treatment, instillations of riboflavin solution were carried out every 5 min. Irradiance and fluence were 3 mW/cm² and 5.4 J/cm², respectively. It was determined that the average temperature rise on the corneal surface during the procedure was 2.6°C. Such an increase was insufficient to raise the temperature to values characteristic of collagen thermal degradation. This possibility became even more remote due to supplementation with a solution of riboflavin, which had a temperature similar to room temperature or lower (if previously stored in a refrigerator).

We have already demonstrated histologically,[32-34] in both *ex vivo* animal and human eyelids, that UVA (365 nm) radiation

does not cause degenerative changes in the anatomical components of the eyelid (conjunctiva, tarsus, meibomian glands, other accessory glands, muscles, and skin). However, less recognizable alteration of the tarsal network of fibrous proteins might occur if the temperature induced by irradiation exceeds the threshold levels attributed to denaturation of those proteins (including collagen) essential for mammalian tissue structure and function. The temperature increments recorded in the present study, in our opinion, were insufficient to reach those levels. At the irradiance values of 45 mW/cm^2 and 75 mW/cm^2, the corresponding increases in temperature were 3.3°C and 5°C, leading to final temperatures of the conjunctival surface of 22.4°C and 24.4°C, respectively. However, when the crosslinking procedure is performed in a clinical setting involving human patients, the baseline temperature may differ. For instance, a superficial temperature of the tarsal conjunctiva of 33.0±0.8°C has been reported in human subjects,[47] a value that falls well within the normal range measured for the ocular surface in general.[48] Such temperatures may drop upon adding the photosensitizer solution to the eyelid surface. Even with these higher baseline values, it is unlikely that the final conjunctival surface temperature will exceed 40°C, which can be considered safe for maintaining the thermal stability of biological molecules.[49-53] Similar considerations regarding collagen stability and tissue integrity have been discussed in a therapeutic context involving collagen scaffolds and growth factors, where histological evaluation emphasized the importance of avoiding structural denaturation to preserve biological function.[54]

The temperature rises of 8.5°C and 14°C that we have recorded at the higher irradiances of 150 mW/cm^2 and 250 mW/cm^2 (corresponding to fluences of 27 J/cm^2 and 45 J/cm^2), respectively, can indeed increase the final temperature of the tarsal conjunctiva (admittedly starting at ~ 33°C, see above) to values in the vicinity of 40°C or higher. However, such high radiant exposures are not relevant in this context because they are not recommended for therapeutic applications to any ocular tissue, and such levels are unlikely to be employed. In a previous study,[33] when an *ex vivo* tarsal plate was exposed directly to UVA radiation, i.e., after the conjunctiva was removed, a fluence of 45 J/cm^2 (generated by applying an irradiance of 250 mW/cm^2 for 3 min) caused substantial damage to the fibrous protein network, cells, and meibomian glands residing within the tarsus. Hence, from the current study, it can also be inferred that the tarsal conjunctiva plays a protective role during the exposure of the eyelid to UVA radiation.

5. Conclusion

The exposure to UVA radiation (with a wavelength of 365 nm) leads to an increase in temperature on the surface of the tarsal conjunctiva, as evidenced in this study using the eyelid of an ovine model. The post-exposure temperature increments are remarkably low, and there is an ideal linear dependence between them and the fluence of the incident radiation. Corroborated with previously reported data, thermal effects of this magnitude are not indicative of tissue damage occurring for the ranges of irradiance (intensity) and fluence (radiant exposure) applicable in the treatment of eyelid laxity. The results of this study are important for advancing to the next translational steps, including *in vivo* animal experimentation and human clinical trials, which are both currently in the preliminary preparation stages at the authors' facilities.

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Conflict of interest

The authors declare that they have no competing interests.

Author contributions

Conceptualization: Traian V. Chirila, Shuko Suzuki
Formal analysis: Shuko Suzuki
Investigation: All authors
Methodology: All authors
Project administration: Traian V. Chirila
Writing–original draft: Traian V. Chirila
Writing–review & editing: Shuko Suzuki, Nadja E. Pop, Alexandra I. Manta

Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

Availability of data

All relevant data supporting this study are included in this article. Additional information is available from the corresponding author upon reasonable request.

Further disclosure

Australian Patent No. AU 2018201200 of February 20, 2018, and U.S. Patent No. 11,240,073 (B2) of August 23,

2022, have been granted for a method of treatment related to this research and were assigned to the Queensland Eye Institute Foundation by the authors Shuko Suzuki and Traian V. Chirila.

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