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Reinforcement of Eyelids in an *Ex-Vivo* Animal Model: Photocrosslinking of Tarsal Collagen by Exposure to Blue Light and a Ruthenium-Based Photosensitizer

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Abstract

Photochemical crosslinking of the eyelid tarsal plate has been developed in our laboratories as a potential non-surgical treatment for palpebral laxity. Controlled exposure to ultraviolet A radiation (365 nm) in the presence of riboflavin as a photosensitizer was the protocol of choice for enabling mechanical reinforcement of the eyelid in order to offset the effects of laxity. In this study, tarso-conjunctival specimens excised from goat eyelids were exposed to blue light radiation (wavelength 470 nm, photon energy 2.64 eV) at different radiant exposures (0.9, 1.8, 3.6, and 6 J/cm^2). Tris(2,2'-bipyridyl)ruthenium(II) dichloride hexahydrate was used as a photosensitizer in combination with sodium persulfate. The method provided statistically significant increases in stiffness and strength in the exposed eyelids and can circumvent the need for using ultraviolet A radiation. At the highest radiant exposure (6 J/cm^2), the increase became insignificant, confirming our previous findings involving the ultraviolet A/riboflavin protocol. In conclusion, the protocol investigated in this study can be employed as an alternative procedure for reinforcing lax eyelids.

Keywords

Eyelid Laxity, Tarsal Collagen, Photochemical Crosslinking, Blue Light,

Photosensitization, Ruthenium Complex, Mechanical Properties

1. Introduction

The exogenous crosslinking of tissular collagens accomplished *in vivo* by sensitizer-assisted exposure to selected regions of electromagnetic radiation has found a major clinical application in ophthalmic medicine. Keratoconus, an ectatic corneal disorder leading to severe visual complications, is currently treated non-surgically by crosslinking the corneal stromal collagen *in vivo* upon irradiation with ultraviolet A (UVA) radiation (315 to 400 nm wavelengths) in the presence of a water-soluble derivative of riboflavin (RF) as a photosensitizer. The procedure, known by the acronym CXL, has become the routine non-surgical treatment for keratoconus [1]-[6]. As a result of subsequent investigations, the UVA/RF crosslinking system has also been proposed for preventing progressive myopia [7]-[9] and for treating eyelid laxity conditions [10]-[20].

The UVA/RF protocols appear biologically safe in CXL applications, which would be expected considering that UVA is non-ionizing radiation and the photosensitizer RF is a biocompatible chemical compound (vitamin B2), and their potentially harmful side effects have been duly investigated. Although the standard CXL procedure involves a radiant exposure (fluence) of 5.4 J/cm^2, which is higher than the limit of 1 J/cm^2 as advised by existing guidelines [21], it was shown [22] [23] that by absorbing radiation, RF actually provides a 'shield' that reduces the total exposure to levels below those recommended. Furthermore, it was found that only when UVA and RF act together can they damage *in vitro* the corneal limbal epithelial cells [24] or harm *in vivo* (mouse) the endothelial cells [25], suggesting that riboflavin alone does not affect the corneal cells. That was contradicted by an *in-vivo* study (rabbits) showing that by preventing RF solution from reaching the corneal limbus during CXL, limbal epithelial stem cells were preserved, but they were damaged in the corneas where RF solution was freely infused [26]. Likely because of such equivocal findings, alternative crosslinking systems have been suggested for CXL. Thus, the pharmacologic alternative involves using various chemical compounds as crosslinking agents, with no need for radiation [27] [28]. However, many of these agents may display cytotoxicity at certain doses, and there is little evidence so far to support their potential clinical performance.

In order to circumvent the UVA/RF protocol, UVA radiation was replaced with visible light, which allowed both lower photon energies and the utilization of photosensitizers different from RF. For instance, rose bengal dye was employed as a photosensitizer during the irradiation of animal corneas with green light radiation (532 nm). Experiments performed *ex vivo* (rabbit, lamb, pig) [29]-[32] or *in vivo* (rabbit) [33] have shown that mechanical reinforcement was equal to, or even better than, that obtained with the UVA/RF procedure. More recently re-

ported, and of relevance to our investigation, was the use of a ruthenium compound, Ru(II)$(bpy)_3Cl_2$, as a photosensitizer activated by blue light radiation for crosslinking corneal collagen in animal models. The method led to results superior to the UVA/RF procedure regarding multiple aspects (biomechanical, biochemical, optical) in both *ex-vivo* (bovine) [34] and *in-vivo* (rat) corneas [35], all at relatively low radiant exposures.

We report here the evaluation of a ruthenium-based photosensitizer associated with blue light (470 nm) as it was applied for crosslinking the tarsal collagen in order to enhance the mechanical characteristics (strength, stiffness) of the upper eyelid in *ex-vivo* animal (goat) specimens.

2. Materials and Methods

2.1. Materials

Upper eyelids were excised postmortem from domestic goat (*Capra hircus*) cadavers, aged 1 to 2 years, supplied by Brisbane Valley Meats Pty Ltd, Esk, Queensland, Australia, an abattoir unit operating under the rules of the Australian Code of Practice of the Animal Welfare Standards for Livestock Processing Establishments (Act 2001), and the Australian Industry Welfare Standards and Guidelines for Goats (Act 2020, v. 03). The eyelid tissue was retrieved from animals that had already been sacrificed for commercial purposes only, and it would have been discarded if not procured for our research. No live animals were housed, handled, subjected to experiments, or euthanized for the purpose of this study.

Tris(2,2'-bipyridyl)ruthenium(II) dichloride hexahydrate (**Figure 1**), or $Ru(bpy)_3Cl_2{\cdot}6H_2O$, was supplied by Sigma-Aldrich Co. (St Louis, MO, USA) with Cat.#224758. The same supplier provided sodium persulfate ($Na_2S_2O_8$), henceforth SPS (Cat.#S6172), and phosphate buffered saline (PBS) tablets (Cat.#P4417, Lot 0000480188). Water of high purity was employed when needed. Solutions in PBS of Ru compound (0.08% wt) and SPS (0.24% wt) were prepared for further

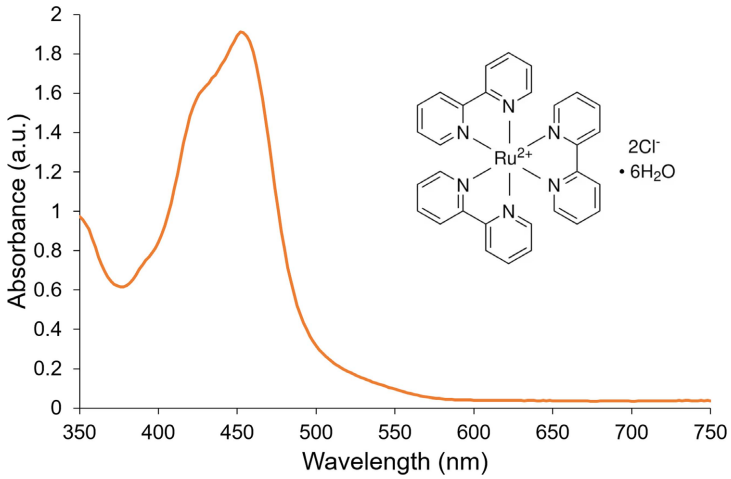


Figure 1. Absorption spectrum and chemical structure of tris(2,2'-bipyridyl)ruthenium(II) dichloride hexahydrate.

experiments. The absorption spectrum of $R(bpy)_3Cl_2{\cdot}6H_2O$ was recorded with a spectrophotometer integrated into the VANTAstar Microplate Reader (BMG Labtech, Ortenberg, Germany), using 150 μL of a solution in PBS (0.025% wt) loaded into a single well of a 96-well plate.

2.2. Tissue Preparation

Following surgical techniques detailed elsewhere [12] [16], 32 eyelids were harvested from 16 animals and individually dissected into tarso-conjunctival strips (**Figure 2(A)**) with a thickness of 1.8 ± 0.2 mm, and subsequently distributed randomly over four groups that included 8 specimens for each radiant exposure. All experiments were performed within 48 h of their excision.

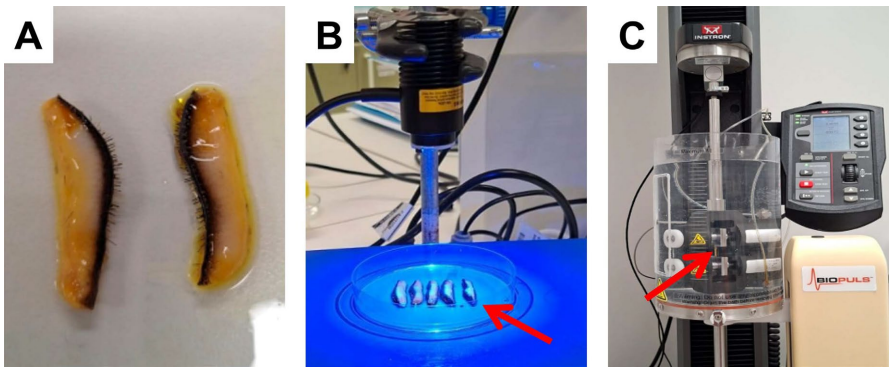


Figure 2. Images of tarso-conjunctival strips excised postmortem from animal (goat) eyelids. (A) Following soaking in the photosensitizer solution (0.08% wt $Ru(bpy)_3Cl_2{\cdot}6H_2O$ + 0.24% wt $Na_2S_2O_8$) for 30 min. The minor tissue staining is due to the presence of the Ru compound. (B) During irradiation with blue light to effect crosslinking. (C) During mechanical evaluation in the Instron® tester. The location of the tissue specimens is indicated by arrows in panels B and C.

2.3. Irradiation

The specimens were soaked for 30 min prior to irradiation in a photosensitizer solution prepared by mixing the solutions of Ru compound and SPS. A mounted light-emitting diode (LED) model M470L5 (Thorlabs, Inc., Newton, NJ, USA) was used as a radiation source in the blue light region (470 nm), controlled by a LED driver (DC40, Thorlabs, Inc.) (**Figure 2(B)**). Irradiation was performed on the conjunctival side. Three different levels of irradiance (3, 6, and 20 mW/cm^2), respectively, were applied for different durations (5 or 10 min), as measured using an Ocean Optics spectrometer equipped with a CC-3-DA cosine corrector (Ocean Optics, Inc., Orlando, FL, USA). A few drops of the photosensitizer solution were applied to the tissue every ~3 min during irradiation.

2.4. Mechanical Testing

An Instron® Materials Testing System Model #5943 (Instron, Norwood, MA, USA), equipped with a 50-N load cell, was employed to record the stress-strain plots in the uniaxial mode. The protocol was the same as previously published

[16]. In brief, the tarsal strips were loaded at a gauge distance of 15 mm and submerged into PBS (at ~37˚C) in a BioPuls™ unit for the duration of measurement (Figure 2(C)). A tensile preloading of 100 mN was applied at the elongation rate of 0.01 mm/s. Fifteen consecutive identical cyclic tensile loading/unloading tests were performed for 10% displacement (strain) at the rate of one percent per second. The last cycle (15th) was used to calculate Young's modulus (M_Y), which is a measure of stiffness, from the final region of the stress-strain plot. The specimens were then removed, exposed to irradiation to effect crosslinking, soaked in PBS for at least 30 min, and re-evaluated in the tester. Note: whenever used, the term "strength" refers to the stress recorded at a strain of 10%.

2.5. Statistical Analysis

The results of mechanical testing were expressed as mean values ± s.e.m. of 8 specimens. For their statistical comparisons, the GraphPad® Prism software (version 6.0) was used to carry out the paired 2-tailed *t*-test for each set of specimens before and after irradiation.

3. Results

As seen in Table 1, at radiant exposures of 0.9, 1.8, and 3.6 J/cm^2, respectively, there were statistically significant increases in both M_Y (stiffness) and strength. However, at the highest exposure tested (6 J/cm^2), involving an irradiance of 20 mW/cm^2, the mechanical enhancement was very small and not statistically significant.

Table 1. Effects of ruthenium-assisted irradiation with blue light on mechanical properties of the eyelid in an *ex-vivo* goat model.

Radiant Exposure[A] (J/cm^2)	Irradiance (mW/cm^2)	Exposure duration (min)	Young's modulus (M_Y)[B] (MPa)			Strength[B,C] (MPa)		
			Native	Irradiated	p-value	Native	Irradiated	p-value
0.9	3	5	6.62 ± 0.36	8.21 ± 0.41	0.008	0.24 ± 0.02	0.30 ± 0.02	0.008
1.8	3	10	7.02 ± 0.56	8.84 ± 0.57	0.016	0.23 ± 0.01	0.31 ± 0.01	0.008
3.6	6	10	8.38 ± 0.61	11.11 ± 1.37	0.008	0.30 ± 0.03	0.39 ± 0.06	0.055
6	20	5	8.08 ± 0.69	8.34 ± 0.64	0.641	0.29 ± 0.03	0.30 ± 0.02	0.742

[A]Regulated name for "fluence". [B]$n = 8$. [C]Stress recorded at a strain of 10%.

For a more suggestive evaluation, a comparison between the percentage increases against native untreated samples of M_Y and strength following the radiation-induced crosslinking is illustrated as bar graphs in Figure 3. Although the increases as such are not statistically significant between them, this representation confirms the enhancing effect of crosslinking and its marked decline at the highest radiant exposure applied.

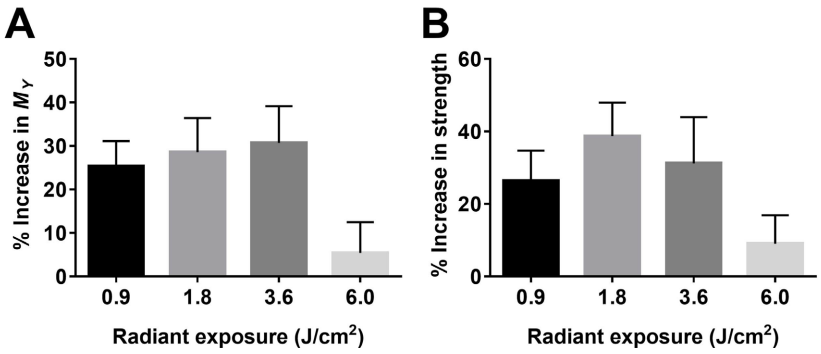


Figure 3. Graphic representation of the percentage increase in (A) stiffness (M_Y) and (B) strength following sensitizer-assisted radiation-induced crosslinking.

4. Discussion

A method to reduce the pathological laxity of eyelids has been developed in our laboratories [10]-[17], and its conceptual validity and potential efficacy have been confirmed by other investigators [18]-[20]. The method is based on the use of a UVA/RF crosslinking protocol. Our goal in the current study was to evaluate a ruthenium compound as a photosensitizer during exposure of the palpebral tissue to blue light radiation (470 nm) to achieve the crosslinking of the eyelid's tarsal collagen.

Photochemistry of the complexes with bipyridine ligands of ruthenium (and other metals) has experienced spectacular progress over the past decades, as described in several seminal reviews [36]-[39]. The prototype coordination divalent cation, $[Ru(bpy)_3]^{2+}$, displays a distinctive combination of chemical and photochemical properties that play important roles in photochemistry, photophysics, and related fields, and generated research works reported in about 9000 publications between 1985 and 2020 [39]. It is no surprise that this cation has been deservedly qualified as "one of the most influential molecules for the development of chemistry in the last five decades" [39], and effusive reviews have been recently dedicated to it [40]-[42].

Drawing upon previous research on the oxidative crosslinking of proteins [43]-[47] and the need to find rapid and facile methods for crosslinking proteins as a tool to investigate protein-protein interactions in multiprotein complexes, the first use of $Ru(bpy)_3Cl_2$ as a photosensitizer in such processes was reported in 1999 [48]. A number of selected proteins were crosslinked upon irradiation with light generated by a xenon lamp over very short exposure durations. According to the mechanism proposed [48]-[50], and generally accepted, the photochemical reactions also require the "sacrificial" presence of the persulfate anion $\left(S_2O_8^{2-}\right)$ as an electron acceptor that contributes to the formation of the $[Ru(bpy)_3]^{3+}$ trication by the transfer of an electron from the excited dication, alongside a sulfate anion and a sulfate anion radical. It was found [49] that in the absence of a persulfate anion, the yields of crosslinked reactions were very low. The $[Ru(bpy)_3]^{3+}$ trication is a strong oxidant able to subtract electrons from amino acids such as collagen's

tyrosine residues, thus generating tyrosine radicals that produce dityrosine crosslinks, while the $[Ru(bpy)_3]^{2+}$ dication is recovered into its ground state. The rapid crosslinking of proteins assisted by ruthenium coordination compounds in association with electron acceptor molecules (e.g., persulfate anions) is included in the category of "photo-induced crosslinking of unmodified proteins" (PICUP). In the field of analytical crosslinking for encoding information related to the supramolecular structure of noncovalent multiprotein complexes in living tissues, PICUP constitutes one of the main approaches [51] [52]. In a distinct approach, aiming at creating novel biomaterials such as tissue adhesives or sealants, or templates for tissue engineering applications, the $Ru(bpy)_3Cl_2$/persulfate/blue light protocol was employed for crosslinking proteins containing tyrosine residues, such as resilin, fibrinogen, or gelatin [53]-[58], where the purpose was different but the Ru complex played the same role of a photosensitizer.

As mentioned, Gulzar *et al.* [34] [35] were the first to apply, both *ex vivo* and *in vivo*, the $Ru(bpy)_3Cl_2$/persulfate/blue light system to an ocular tissular component (the deepithelialized cornea) in animal models. The aim was to find a safer alternative for implementing CXL that would be associated with fewer complications and less clinical failure [34]. Radiant exposures of 7.2 J/cm^2 (irradiance 3 mW/cm^2 for a 40-min exposure time) for *ex-vivo* experiments, and 0.9 or 1.8 J/cm^2 (3 mW/cm^2 for 5 or 10 min) for *in-vivo* animal (rat) trials were used by investigators [34] [35]. They determined in cell cultures of three different cell lines that a concentration of 1 mM (~0.08% wt) of ruthenium compound was biologically safe. These investigators' conclusive finding was that the new protocol generated results superior to those produced by the UVA/RF protocol regarding safety, biocompatibility, and functionality [35].

In our present study, the $Ru(bpy)_3Cl_2$/persulfate/blue light protocol was applied to achieve the crosslinking of collagen(s) in the tarsal plate of an *ex-vivo* animal eyelid, upon activation with blue light (wavelength 470 nm). By using radiant exposures (fluences) between 0.9 and 3.6 J/cm^2, generated through irradiances of 3 or 6 mW/cm^2 for exposure durations of 5 or 10 min, the expected mechanical reinforcement occurred with statistical significance (**Table 1**). In the context of our previous results on the sheep upper eyelids using the UVA/RF protocol (at 7.2 or 9 J/cm^2) [10] [11], both final stiffness and strength are numerically higher in the current study, and they were recorded at lower radiant exposure. Importantly, based on the results recorded at 6 J/cm^2, our findings confirm that the mechanical enhancement declines with increasing radiant exposures, as previously reported in sheep eyelids [10] [11].

Based on rather scant reporting, there may be a difference between the outcomes of crosslinking the corneal stromal collagen and crosslinking the tarsal collagen. Relatively high radiant exposures have been used on the human cornea with the UVA/RF protocol (12 J/cm^2) [59] and on the animal cornea with the $Ru(bpy)_3Cl_2$/persulfate/blue light protocol (7.2 J/cm^2) [34], with acceptable biomechanical enhancement. However, we have recorded a decline in this trend

with both the UVA/RF protocol at ~9 J/cm^2 in sheep eyelids [11] and at ~6 J/cm^2 in goat eyelids with the $Ru(bpy)_3Cl_2$/persulfate/blue light protocol as shown in the present study. Hypothetically, this can be a result of the unique composition and properties of the tarsal plate (known also as the palpebral tarsus), which is composed of networks of several collagen types that surround the meibomian glands. In a detailed summary [13] of the current knowledge on this eyelid component, we have suggested that its formal inclusion within the fibrocartilage category might be wrong, as it does not contain collagen type II (typical of cartilages [60]), but contains collagen type VI, which is found in reduced amounts in other cartilages where is mainly located within the pericelluar matrix [60]-[63]. It is reasonable to assume that the tarsal plate is also substantially different from the corneal stroma.

5. Conclusion

Based on the findings presented here, the $Ru(bpy)_3Cl_2$/persulfate/blue light protocol is able to induce the crosslinking of the eyelid's tarsal collagen at relatively low radiant exposure levels and short exposure durations, resulting in the mechanical reinforcement of the eyelid. Accordingly, this procedure can be applied as a potential non-surgical treatment for eyelid laxity conditions, also offering some advantages over UVA/RF, our previously employed protocol.

Ethics Approval and Consent to Participate

None applicable. Neither experiments with live animals nor trials on human subjects were involved in this study.

Author Contributions

T.V.C. initiated the project, suggested the methodology, conducted the literature search, and wrote the first draft. S.S. designed, coordinated, and supervised the experimental protocols. M.M. carried out the experiments. All authors were involved in the interpretation of results. S.S. and M.M. contributed to and reviewed the final draft, and approved its submission. The authors assume full responsibility for the veracity of the results reported in this article.

Availability of Data

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

Conflicts of Interest

The authors declare that the experiments carried out and the preparation of the manuscript were conducted in the absence of any commercial or personal relationships or restrictions that could be construed as conflicts of interest.

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Symbols and Abbreviations

CXL	corneal collagen crosslinking
LED	light-emitting diode
M_Y	Young's modulus
PICUP	photo-induced crosslinking of unmodified proteins
RF	riboflavin
Ru(II)$(bpy)_3$	tris(2,2'-bipyridyl)ruthenium(II)
s.e.m.	standard error of the mean
SPS	sodium persulfate
UV	ultraviolet radiation
UVA	region A of ultraviolet radiation (315 to 400 nm)