Photocrosslinking of Tarsal Collagen for Treating Eyelid Laxity: Applying the Exposure Reciprocity Law to Establish an Optimal Irradiant Profile

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Abstract. The aim of this study was to develop an irradiant exposure strategy able to control validity of the exposure reciprocity law upon exposing tarso-conjunctival tissue to ultraviolet A (UVA) radiation as a treatment for palpebral laxity, and thus to establish a research-based criterion for implementing optimal irradiation levels in clinical settings. Tarso-conjunctival specimens were harvested postmortem from ovine and caprine eyelids and exposed to UVA radiation (365 nm) in specified conditions. Thermographic infrared analysis was applied to monitor the photothermal response. By performing irradiation in the presence of a photosensitizer (riboflavin) a photochemomechanical response was also determined based on changes in mechanical properties as evaluated in a mechanical tester. A graphical method was employed to define the failure zones of reciprocity law when applied to radiation-induced photoresponses. Discontinuities in law's validity were found and confirmed by descending values of Schwarzschild's *p*-coefficients. The photothermal response showed that law's failure zone centered on an irradiance of 30 mW/cm^2 in both sheep and goat tissues, for radiant exposures between 6 and 18 J/cm^2. The photochemomechanical response indicated a failure onset around 60 mW/cm^2. Anticipating the concept of safest application route, an irradiance of 30 mW/cm^2 appeared an appropriate upper limit for the validity zone of reciprocity law, as indicated by the photothermal response, at exposure durations assuring a radiant exposure between 5 and 20 J/cm^2.

Keywords: eyelid laxity; collagen; ultraviolet A radiation; photochemical crosslinking; Bunsen-Roscoe law; Schwarzschild's coefficients.

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1 Introduction

A method has been developed in our laboratories aimed at reinforcing mechanically the tarsal plate in the eyes afflicted by palpebral laxity, which is associated with a range of conditions such as floppy eyelid syndrome, entropion, ectropion and ptosis, and may affect the performance of meibomian glands. There is evidence [1–6] that tarsal laxity is linked to deficient biosynthesis of elastin and collagen, and to disarrangement of their networks.

Surgical procedures are frequently employed for treating eyelid laxity. According to the principle of our proposed method, the exposure of the tissue to ultraviolet radiation A (henceforth, UVA) in the presence of photosensitizers, such as riboflavin, can lead to an increase in both tensile strength and stiffness of the tarsus. Such mechanical enhancement is a consequence of the photochemical crosslinking of tarsal collagen, and could result in reversing the excessive palpebral laxity or in forestalling its post-surgical recurrence. We have confirmed augmentative mechanical effects both in *ex vivo* animal tissue (sheep) [7, 8] and in human cadaveric tissue [9], and other investigators have confirmed our findings in *ex vivo* tarsal plate specimens of animal (rat) or human origin [10–13]. The biological safety of tarsal collagen crosslinking procedure (henceforth, TXL) is critical for its therapeutic applications pertaining introduction to clinical practice. UVA radiation (conventionally between the wavelengths 315 and 400 nm) is less energetic than other UV regions, and is regarded as a non-ionizing and non-thermal radiation; however, it can still be deleterious to cells and tissues. Effects of UV radiation on skin [14–18] and ocular tissues [19–22] have been extensively investigated, but the scope was confined mainly to natural radiation as generated by the sun.

The effect of artificially sourced UVA radiation in non-cutaneous medical applications has been sparingly reported. In fact, most information on the subject comes from ophthalmology, where a therapeutic application of UVA-induced corneal stromal collagen crosslinking (henceforth, CXL) proved to be a successful treatment for keratoconus, an ectatic corneal disorder associated with severe visual disability. Using established UVA/riboflavin crosslinking protocols, progression of keratoconus can be effectively arrested due to mechanical reinforcement of the cornea that is induced through the photocrosslinking of stromal collagen [23–30]. Based on its additional germicidal and anti-inflammatory effects, UVA radiation was subsequently applied to treat other cornea disorders including pellucid marginal degeneration, postsurgical ectasia, refractive myopia, infectious keratitis, and bullous keratopathy [23, 31, 32] with some improvements in visual acuity and keratometric parameters.

When applying therapeutically the UVA radiation to various segments of the eye, it is essential to avoid injury to the target tissue and adjacent tissues caused by exposure to radiation, a process entirely dependent on the radiant exposure (i.e., the energy of radiation delivered and absorbed by the target), which in turn is determined by both irradiance (i.e., the power of radiation delivered to the target) and exposure time, in agreement with an inverse proportionality relationship, as discussed later in depth. While for CXL a number of protocols have been developed which proved to be safe when applied in the clinic, aspects of the biological safety of TXL have been much less investigated [7–13, 33, 34]. Our study aims primarily at providing a scientific foundation for a rational selection and implementation of safe irradiation boundaries.

2 Methods and Materials

2.1 Materials

Eyelids were dissected postmortem and collected on site from domestic sheep (*Ovis aries*) and goat (*Capra hircus*) cadavers supplied by Brisbane Valley Meats Pty Ltd, Esk, Queensland, Australia. The supplier is an abattoir unit operating under the rules of the Australian Code of Practice of the Animal Welfare Standards for Livestock Processing Establishments (2001), the Australian Animal Welfare Standards and Guidelines for Sheep (2016), and the Australian Industry Welfare Standards and Guidelines for Goats (v. 03, 2020). The animals were slaughtered solely for commercial purposes, and the eyelid tissue would have been discarded if not acquired by us for the present study. Animals were between 1 and 2 years old at the time of sacrifice.

Riboflavin 5´-phosphate monosodium salt (RF-5P) was supplied by Cayman Chemical Co. (Ann Arbor, MI, USA). A solution of RF-5P in phosphate-buffered saline (PBS), at a concentration of 0.25% w/v, was applied only to the tissue specimens designated to undergo photocrosslinking.

2.2 Infrared Thermographic Analysis

Fifty full-thickness eyelid specimens were excised postmortem from the ocular adnexa of sheep and goat eyes that were refrigerated for less than 24 h. Eyelashes were trimmed, and the specimens were soaked for 30 min at room temperature in PBS solution. Each sample was then removed from solution and placed on a Petri dish with the conjunctival side facing upwards. Any excess of liquid was carefully blotted out with Kimwipe® paper tissues.

A thermal camera model FLIR C5 (FLIR Systems Australia Pty Ltd, Mulgrave, Victoria, Australia) with a thermal sensitivity of less than 0.07 °C was used for thermographic analysis following instrument calibration. As a source of radiation, the UV Curing System OmniCure® 1500 (Excelitas Technologies Corp., Waltham, MA, USA) was employed. To determine the level of irradiance at the exposure site, a radiometer Dymax ACCU-CAL 50 (Dymax Corp., Torrington, CT, USA) was used for the pre-irradiation adjustment of the distance between the UV source and tissue surface.

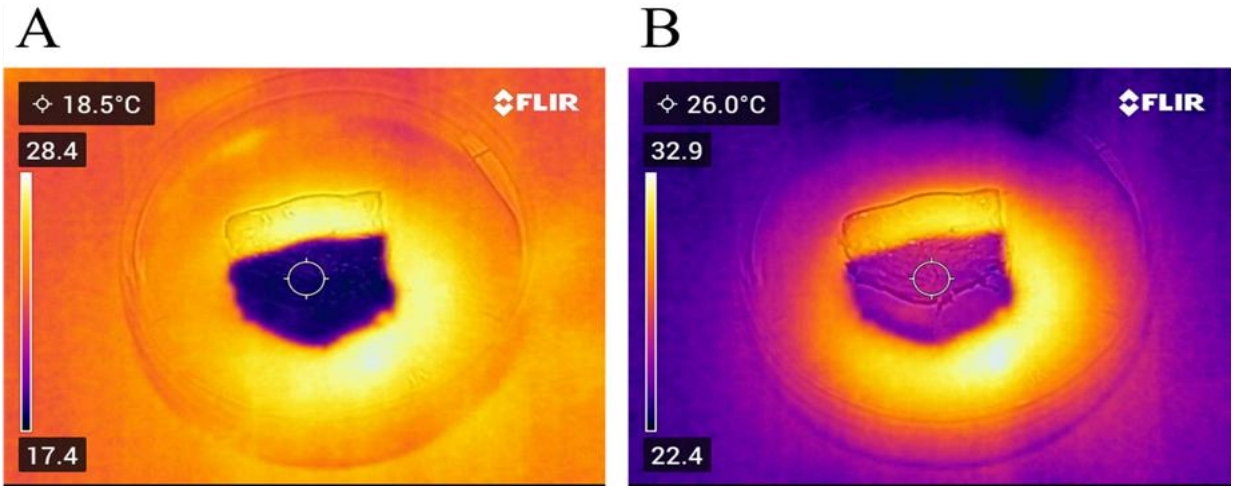

Fig. 1 Thermographic images recorded (A) before and (B) after the exposure to UVA radiation (365 nm) of a tarso-conjunctival specimen (goat eyelid) at an irradiance of 75 mW/cm^2 for an exposure time of 240 s. Total radiant exposure: 18 J/cm^2.

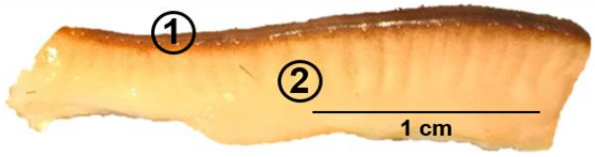

Fig. 2 Photographic image of a tarso-conjunctival specimen excised from goat eyelid: 1 – lid margin; 2 – palpebral conjunctiva.

The samples were each exposed for variable durations to UVA radiation (365-nm wavelength), at 10 different irradiances from 5 to 150 mW/cm^2, assuring radiant exposures of 6, 13.5, and 18 J/cm^2, respectively. Five specimens were used at each irradiance. The data were presented as mean values ± s.e.m.

The infrared camera mounted on a tripod was placed at a distance of *ca* 20 cm from the sample. The measurement spot (the 'spotmeter') on the camera screen was positioned onto the surface of the conjunctiva as centrally as possible. During each irradiation episode, the thermal images were recorded at the temperature on the target spot for a period corresponding to the target radiant exposure, and the baseline measurement prior to irradiation was also included. During experiments, the ambient temperature in laboratory was between 21 and 23 °C. The baseline temperature, i.e., the temperature of the conjunctival surface prior o irradiation, was comparable in all experiments (20.0±1.4 °C). Fig. 1 shows a typical thermographic image recorded during measurements before and after exposure at a radiant exposure of 18 J/cm^2, resulting from an irradiance of 75 mW/cm^2 applied for 240 s.

2.3 Photocrosslinking of Tarso-Conjunctival Tissue

Eighty tarso-conjunctival specimens were harvested postmortem from the upper eyelids of goats following a surgical technique we have previously described in detail [9] for the dissection of equivalent human cadaveric tissue. Fig. 2 shows a tarso-conjunctival specimen as excised from the eyelid of a goat before its exposure to UVA radiation.

Each specimen was soaked in the RF-5P solution (0.25%) for 30 min, and then exposed to UVA (365-nm wavelength) radiation on the conjunctival side of the eyelid, using the protocol described in the previous section. After irradiation, all samples were stored in PBS until further processing and evaluation.

2.4 Biomechanical Analysis

Each tissue specimen was subjected to mechanical testing, both prior and after crosslinking. The Instron® Materials Testing System Model #5943 (Instron, Norwood, MA, USA), equipped with a 50-N load cell, was used to obtain stress-strain plots under uniaxial loading. Tarsal strips were secured using pneumatic grips set to a gauge length of 14 mm and submerged in PBS buffer at 21.5 ± 1 °C in a BioPuls™ unit for the duration of testing. A tensile preloading of 30 mN was applied at the elongation rate of 0.01 mm/s. Subsequently, 15 cyclic tensile loading-unloading tests were performed to 10% displacement (strain) at a rate of 1%/s. The final cycle (#15) was used to determine the stress (σ) at 10% strain and to compute Young's modulus (MY). The specimens were then removed from the mechanical tester, exposed to irradiation in order to accomplish crosslinking, and re-evaluated in the tester after soaking in PBS for at least 30 min.

3 Results

Fig. 3 shows the plots of the maximum temperature increase (ΔT) at the conjunctival surface as a function of irradiance (E_e), at three different levels of radiant exposure (H_e) in two different animal eyelids, sheep (Fig. 3A) and goat (Fig. 3B).

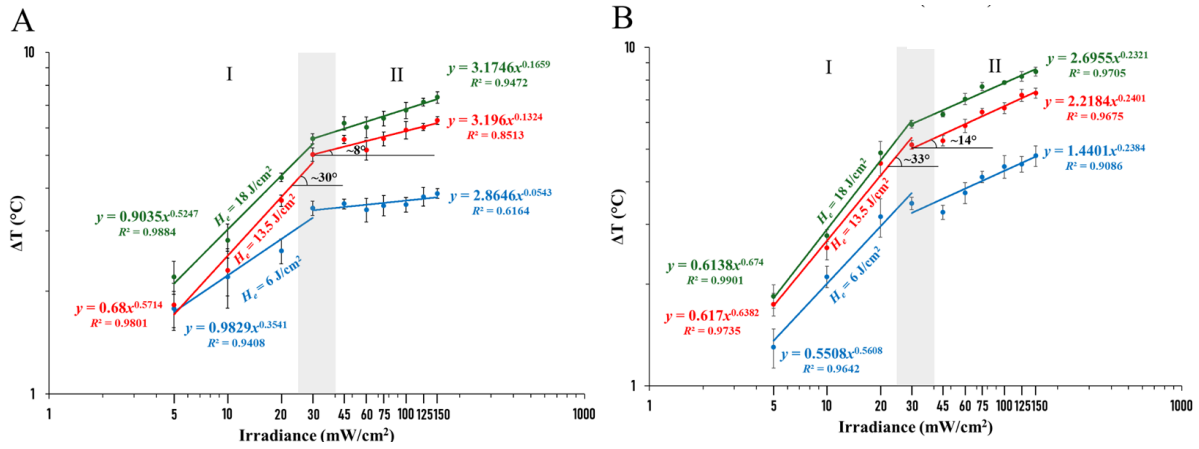

Fig. 3 Graphical method to assess the validity of Bunsen-Roscoe law and to quantify the value of Schwarzschild's *p*-coefficients based on the photothermal response (ΔT) in irradiated tarso-conjunctival specimens excised from (A) sheep and (B) goat eyelids, and exposed to UVA radiation (365 nm). Thermographic analysis was conducted at three different levels of radiant exposure (6, 13.5, and 18 J/cm^2). Each data point value is an average of 5 measurements ± s.e.m. Zone I – validity zone; zone II – failure zone. The inclination angle (θ) is indicated for each plotted line.

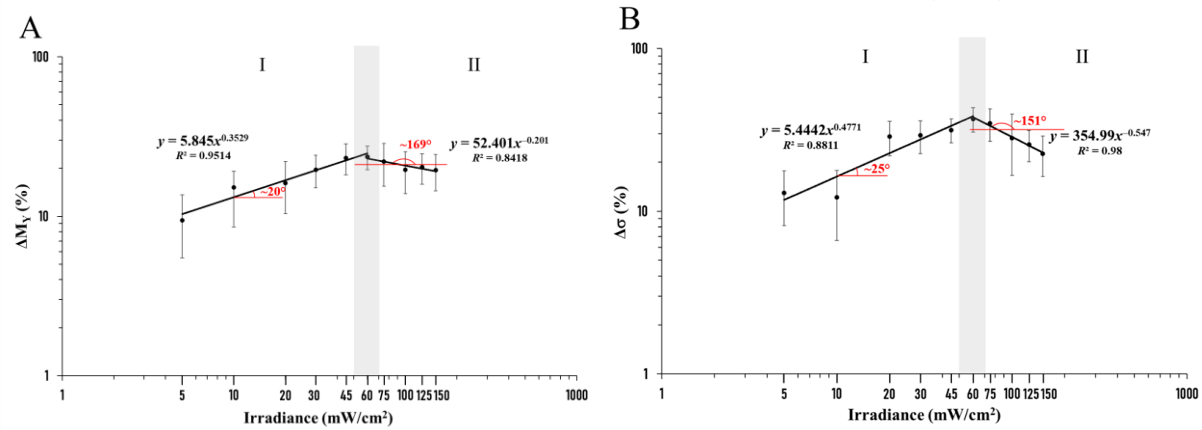

Fig. 4 Graphical method to assess the validity of Bunsen-Roscoe law and to quantify the value of Schwarzschild's *p*-coefficients based on the photochemomechanical responses as defined by increases of (A) stiffness (ΔM_Y) and (B) strength ($\Delta\sigma$) in the irradiated tarso-conjunctival specimens exposed to UVA radiation (365 nm). Tissue specimens were excised from goat eyelid. The measurements were completed at a radiant exposure of 13.5 J/cm^2. Each data point value is an average of 8 measurements ± s.e.m. Zone I – validity zone; zone II – failure zone. The inclination angle (θ) is indicated for each plotted line.

When Young's modulus (M_Y) and stress (σ), measured in MPa units, were selected as the target photoresponses, the resulting plots are shown in Fig. 4, where the calculated percent increase of either M_Y (Fig. 4A) or σ (Fig. 4B) were plotted against irradiance at a radiant exposure of 13.5 J/cm^2.

4 Discussion

To deal with a rather confusing amalgam of terms in current use for the quantities employed in radiometry, photometry, and astrophotography, we followed here the radiometric terminology and symbols recommended by the International Standard ISO 80000–7:2019 [35]. Terms such as 'fluence' and 'energy dose' for *radiant exposure* (H_e, J/m^2), or 'intensity', 'flux', 'flux density', 'fluence rate' and 'illuminance' for *irradiance* (E_e, W/m^2) are no longer recommended and should be avoided or used only in specified circumstances. In fairness, when perusing photochemical or photobiological publications, it seems unlikely that those terms will be abandoned soon [36].

4.1 Significance of Bunsen-Roscoe/ Schwarzschild Law

Radiation-induced photochemical and photobiological processes and their physicochemical effects on matter are due entirely to the total amount of electromagnetic radiant energy absorbed per unit area by the material target, i.e., the radiant exposure [37–39]. A photoresponse (symbolized by $\mathcal{P}$) is dependent on the

product of the two individual quantities that actually define the radiant exposure itself, namely the irradiance (E_e) and the duration of exposure (t). In principle, this can be expressed by Eq. (1).

$$\mathcal{P} = f(xy), where\ x = E_e\ and\ y = t. \quad (1)$$

Furthermore, the same response will be observed when the target receives the same total radiant exposure, irrespective of how the energy was delivered; it is the total absorbed energy that actually matters, not the method of exposure. By either applying a higher irradiance for a shorter duration, or a lower irradiance for a longer duration, the same effect of the irradiation shall be achieved. The law can be expressed mathematically through any of the inverse variation relationships in Eq. (2a) or (2b). In other words, when targeting a certain photoresponse in an irradiated system, one variable quantity, either E_e or t, can be expeditiously changed while modifying the other one in order to maintain constant the product of their multiplication.

$$E_e \propto 1/t, \quad (2a)$$

$$E_{ei} \times t_i = const, \forall i \in \{1,2,\dots,n\}. \quad (2b)$$

In essence, as long as the multiplication product of irradiance and exposure time is the same, the photoresponse shall be the same. This law is known as Bunsen-Roscoe law (henceforth, BRL), acknowledging the two 19th century scientists who demonstrated it experimentally using photographic films [40, 41], and is alternatively known as the 'exposure reciprocity law'. The law also stipulates that a certain radiant exposure will remain the same regardless whether the duration of exposure is continuous or interrupted within the same experiment [42, 43]. While BRL is seldom regarded as one of the fundamental laws of photochemistry [44, 45], alongside Grotthuss-Draper law and photochemical equivalence law (known also as Stark-Einstein law) [37, 39], it can, however, be interpreted as a quantitative expression of the former [38]. BRL is also applied in vision science for investigating how the eye perceives very short flashes of light, where is known as Bloch's law, named after the scientist who was the first to relate the concept to vision [46], apparently without being aware of the existence of BRL. The role and limitations of BRL and Bloch's law in eye perception have been investigated to some extent [47–51].

The above statements, as well as the relationships in Eqs. (2a) and (2b) themselves, are only valid as long as the experimental data comply with the law. However, failures of BRL have been noted in the past [52–62], and are still reported [43, 63–67]. In general, the reciprocity law holds for photochemical processes, with deviations noted usually at extreme levels of irradiance. In irradiated biological systems, the failure of BRL is expected to be more significant, considering that the responses of cells and tissues involve complex interacting mechanisms, photoreactivation repair processes, and involvement of photosensitizing biomolecules present in tissues [37, 65].

To rationalize the failure of BRL, Schwarzschild developed an empirical model based on his work in astrophotography [53]. The model was further improved [54–60, 62] to match closer certain experimental findings. In its new form, known also as Schwarzschild law, BRL can be generally expressed by the Eqs. (3), where p or q are known as Schwarzschild's coefficients (or exponents), and $p \times q = 1$.

$$E_e \times t^p = const, \quad (3a)$$

$$E_e{}^q \times t = const. \quad (3b)$$

If p (or q) = 1, the two laws are identical, and therefore Schwarzschild law can be considered a generalization of BRL, and it is adequate for monitoring the reciprocity. Other proposed models display additional mathematical complexity and most of them are related to the photographic field [43, 60, 63]. Closer to our times, the interest in RBL failure has expanded to other fields such as dermatology, biology, photomedicine, photocatalysis, and photodegradation of materials. In a seminal review [43], a survey of the literature on RBL failure, including estimated values for Schwarzschild's coefficients (where available), indicated that the reported cases of failure in biology and medicine were actually less than those in other fields. The quantum band theory [68, 69] has been episodically employed as a theoretical model able to explain the variation of Schwarzschild's p-coefficients in certain photoprocesses, but is limited mostly to photoconductance and photography studies, and is based entirely on photophysical rather than photochemical processes [43, 60, 70–74].

In the biomedical applications of electromagnetic radiation, it is important to understand the limits to validity of BRL, which will allow suitable irradiant strategies. In practice, formulae such as in Eqs. (4) are frequently employed in order to calculate the radiant exposure that results from a certain irradiance applied over a given exposure time; nevertheless, they yield workable values only if used within the confines of BRL's validity, i.e., outside the failure zones.

$$H_e\left[\frac{J}{m^2}\right] = E_e\left[\frac{W}{m^2}\right] \times t[s], \quad (4a)$$

$$H_e\left[\frac{J}{cm^2}\right] = E_e\left[\frac{mW}{cm^2}\right] \times t[s] \times 10^{-3}, \quad (4b)$$

$$H_e\left[\frac{J}{cm^2}\right] = E_e\left[\frac{mW}{cm^2}\right] \times t[min] \times 0.06. \quad (4c)$$

When selecting a preferred irradiation regime in a clinical setting, the ophthalmic surgeon may choose to apply irradiances (E_e) and/or exposure times (t) falling outside the law's validity zone, either on purpose or unintentionally. While such a choice does not necessarily put at risk the safety of the procedure, values of the radiant exposure (H_e) estimated with Eqs. (4) will be

different from the actual values, therefore outside surgeon's cognizance or control.

In proposing our procedure as a potential treatment for eyelid laxity conditions, we took as a model the UVA-induced CXL, an established treatment for keratoconus that has been the subject of over 2000 studies published by the year 2020 [75]. Foreseeably, CXL was the application that aroused an interest in BRL's role in ophthalmology. That was mainly a consequence of certain innovative developments in the procedure, including accelerated protocols [25, 32, 76–84], the use of high radiant exposures (fluences) [25, 77, 78, 85–88], and supplementation with oxygen during procedure [25, 89–93]. Regarding the radiant exposure, 20 J/cm^2 was the highest reported value in *ex vivo* animal corneas [87], and 12 J/cm^2 for treating human patients [85]. The most complete study on the relation between irradiation parameters and validity range of BRL has been performed [78] on excised porcine corneas, where the tensile elasticity modulus (or Young's modulus, a quantitative measure of stiffness) was monitored as a photoresponse. Ten different pairs of E_e and t, each generating the same radiant exposure (H_e = 5.4 J/cm^2), were applied starting with E_e = 3 mW/cm^2 and t = 30 min and ending with E_e = 90 mW/cm^2 and t = 1 min. It was found [78] that BRL maintains validity only for irradiances around 45 mW/cm^2 and lower, and at exposure durations longer than 2 min, as indicated by measuring the changes in stiffness. Regarding the role of oxygen during irradiation, based on the mechanism and kinetics of UVA/riboflavin-induced CXL [89], its presence appears to be beneficial to the photochemical reactions leading to formation of crosslinks. While hyperoxic conditions resulted indeed in more efficient crosslinking in *ex vivo* animal corneas [90, 92, 93], the absence of any effect has been also reported [91]. Predictably, the role of oxygen shall be relevant in CXL procedures, and its participation 'may be the missing piece to understand the invalidity of Bunsen-Roscoe's law' [93].

4.2 Validation of BRL Law Based on Photoresponses

Failure of reciprocity law has been extensively investigated in the photographic field, where graphical methods were developed to identify the failure region [43, 55–58, 60, 63]. Such methods are generally based on monitoring the irradiance (E_e) and its product with the duration of exposure ($E_e \times t$) for a fixed photoresponse, i.e., for a given state of transformation induced by irradiation. Experimentally, this is easy to achieve in photographic research, as it implies no more than a facile darkening of photosensitive films or plates. Outside the photographic field, however, it is rather difficult to find a photoresponse amenable to being maintained at a fixed value while the other parameters (irradiance, time) shall be varied and monitored until they attain the values needed to generate that particular photoresponse. In our present study, we have used a graphical method whereby a measurable photoresponse was plotted against irradiance for a chosen radiant exposure. The plot is on a logarithmic scale, allowing data points to fall on the same line at each exposure value [43]. Although less complicated, the method has been only episodically employed to investigate BRL failure, for instance in studies involving target photoresponses such as intensity of photocurrents generated in dyes by exposure to light [94], photovoltage generated by exposure to UV radiation of titanium dioxide films [95], or rate of isopropanol UV-induced photooxidation [96].

According to this method, a change in the slope of the logarithmically linearized plot $\log \mathcal{P} = f(\log E_e)$ indicates a discontinuity in the validity of BRL, i.e., where the law breaks down. In the linearized power function $y = ax^m$, the exponent m represents the slope. Furthermore, the slope is the equivalent of Schwarzschild's p-coefficient [43], and the closer its value is to 1 (for an inclination angle θ = 45°), the lower is the level of failure.

The target tissue in our study was the tarsal conjunctiva of the eyelid in two bovid species (sheep and goat), which we have exposed to UVA radiation (365 nm). Two different photoresponses were considered upon exposure to selected levels of irradiance: (a) the increase of the local temperature on the conjunctiva surface at the site whereupon radiation impinges and its energy is transferred to the target; and (b) the irradiation-induced increase of stiffness (expressed through the values of Young's modulus) or increase of strength (expressed by the measured stress), both recorded during experimental stretching of the tarso-conjunctival substrate, prior to and after crosslinking.

Fig. 3 shows that at any of the tested levels of exposure based on the photothermal response, a drop in the slopes (i.e., the Schwarzschild's p-coefficients) occurred within an irradiance region approximately between 25 and 40 mW/cm^2 (shaded areas) in each of the two animal tissues, with 30 mW/cm^2 being a suitable and safe estimation for a practicable limiting value. This region divided the plot into two zones, validity zone (I) and failure zone (II). A variation in the values of p-coefficients shall be interpreted as a change in the law's validity, in this case due to thermal effects, and can be regarded as the commencement of law's failure in zone II. Table 1 summarizes the actual values of p-coefficients in each tissue at three different radiant exposures, as extracted from the equations plotted in Fig. 3. The difference between coefficients in the two zones is compelling, clearly suggesting breakdowns in BRL's validity.

Fig. 4 shows the plots of crosslinking-induced mechanical augmentation as reflected by increases of Young's modulus and stress, i.e., stiffness and strength, respectively. Since the results involving three different exposure levels of two different tissues showed very similar patterns (as seen in Fig. 3), we selected only one level (H_e = 13.5 J/cm^2) for investigating the functional relationship between E_e and M_Y or between E_e and σ, and investigated only the goat tarso-conjunctival tissue.

Table 1 Values of Schwarzschild's *p*-coefficients for the photothermal responses.

Eyelid Tissue	Sheep		Goat	
Zone-specific plots[a]	I	II	I	II
Radiant exposure (H_e, J/cm^2)	*p*-Coefficients[b]			
6	0.3541	0.0543	0.5608	0.2384
13.5	0.5714	0.1324	0.6382	0.2401
18	0.5247	0.1659	0.6740	0.2321

[a] Linear zones characterized by two different slopes as shown in Fig. 3A and 3B. Zone II represents the law's failure zone.

[b] Schwarzschild's *p*-coefficients are identical with the slopes m in the linearized power functions ($y=ax^m$) plotted in Fig. 3A and 3B.

A dramatic decrease of the *p*-coefficients occurs in the region approximately between 50 and 70 mW/cm^2, furthermore attaining negative values in zone II. An acceptable limiting value of 60 mW/cm^2 for the validity zone seems appropriate. It can be seen that the effects of UVA on mechanical properties of the tarsus led to higher irradiance levels that marked the BRL failure as compared to those indicated by thermal response.

A question arises as to why the discontinuity in the slope, marking the failure of BRL, was more pronounced when the monitored photoresponse was the enhancement of either the mechanical stiffness (ΔM_Y) or of the built-up force ($\Delta\sigma$) during stretching of the tissue, i.e., a photochemomechanical response, as compared to the irradiation-induced temperature increase at the surface of the tissue, i.e., a photothermal response. To attempt an explanation we should briefly address what is known about the effects of exposing materials to UVA radiation, which is considered a non-ionizing and non-thermal radiation. The photoresponses in multiparticulate systems are the result of time-directed, cause-and-effect processes, where the UVA radiation is the causal agent. Like with any electromagnetic radiation, such processes induce photophysical and photochemical changes. The former are single-particle events that include photon absorption, localized excitation, as well as generation and migration of charge carriers (e.g., excited electrons), and they are always the same irrespective of the nature of irradiated material substrate. Importantly, the photophysical processes are precursors to photochemical changes. The latter are intricate and unpredictable, as they depend on both chemical structure and homogeneity of the irradiated substrates. Upon their absorption by the target, the UVA photons possess sufficient energy to excite molecules, which are then able to trigger chemical processes causing significant transformations in biological matter including lipid oxidation, structural alteration of nucleic acids, and crosslinking of native proteins. The excited electrons that did not contribute to such changes can release their energy through luminescence phenomena (fluorescence, phosphorescence), or through production of heat. Notably, the amount of heat generated by UVA radiation is generally low. Although some of the released heat can also be a result of exothermic chemical reactions activated by the radiation itself, such contribution was minor in our experiments because no photosensitizer was present to initiate chemical reactions during evaluation of the photothermal response. That was clearly illustrated by our measured ΔT values indicating a weak response. Moreover, we have found in a previous study [34] that even in the presence of a photosensitizer the thermal effect of UVA on tarso-conjunctival tissue was minor at irradiances lower than 75 mW/cm^2. If we further consider a baseline temperature higher than that employed in the present study, such as that reported for human tarsal conjunctiva (*ca* 33°C) [97], it is still unlikely that UVA radiation could induce a surface terminal temperature above 40°C, the well-known safe limit for the thermal stability of biological macromolecules [98–101].

The photochemomechanical response, ultimately manifested in changes of modulus M_Y and stress σ, was the result of a photochemical reaction (crosslinking of collagen) concomitantly followed by a process of mechanical augmentation of the tarsal tissue. Crosslinking-induced enhancement of collagen's mechanical properties is a complex process. Molecular models have been developed [102–105] in order to understand the relation between crosslink density and deformation mechanisms. A crucial role of crosslinks is to assure interconnectivity between fibrillary collagen molecules, essential for enhancing the network's strength and toughness. Insufficient crosslinks cause intermolecular sliding resulting in weak structural networks. Chemistry of the covalent crosslinkages is also relevant: for the same crosslink density, the networks based on trivalent crosslinks are stronger than those based on divalent crosslinks, due to different interconnectivity levels. The fully crosslinked collagen networks undergo deformation in synergy with relatively minor intermolecular sliding and macromolecular uncoiling but can stand up to external forces that otherwise would cause the mechanical failure of uncrosslinked networks. At a certain degree of deformation, however, the crosslinks may start to break down; in principle, additional crosslinking may mitigate the process if given the opportunity.

We suggest that due to an intrinsic complexity of the underlying mechanisms leading to reinforcement of the tissue, the photochemomechanical response is delayed, leading to an apparent extension of BRL's validity zone, at the same time causing a dramatically more significant discontinuity in the slope (Fig. 4) than that seen when a localized surface-level temperature increase was the intended photoresponse (Fig. 3).

It remains to consider which of the two investigated photoresponses shall be selected as a criterion for defining the BRL's failure zone and for recommending practical values for the irradiation parameters aimed at preventing the use of an irradiant profile different from the actual profile. Although the photochemomechanical response is associated with major structural changes in the biological substrate and would therefore appear to be more relevant as a marker for the onset of BRL's failure, we propose the photothermal response as a criterion, notwithstanding that the thermal effects induced by UVA radiation on the tarsal conjunctiva are minor. Our reasons are mainly of a biological safety nature. Thus, the principle of the safest application route was a reason for recommending limiting irradiances around 30 mW/cm^2 instead 60 mW/cm^2, as less power delivered to the tissue means less potential damage. Any accidental thermal damage will be more devastating and faster than that produced by the photochemical crosslinkling. Another reason is based on the vast published experience with CXL, which clearly indicates that there is no need for irradiances higher than 45 mW/cm^2, while maintaining fluences under 20 J/cm^2, to be applied for crosslinking the corneal stromal collagen. In reality, even lower values are currently used in most clinics around the world. Moreover, all UVA devices for CXL currently on the market (a 1.2-billion-dollar market) are limited to deliver a maximum irradiance of 45 mW/cm^2. For purely safe practicality, we believe that such values are also suitable for the therapeutic irradiation of the tarsal conjunctiva, and there is no reason to apply irradiances near 60 mW/cm^2 as suggested by the experimental photochemomechanical response in our study.

5 Conclusion

By applying a graphical method based on the relation between irradiance and certain photoresponses, the validity interval of Bunsen-Roscoe law can be established and employed to select suitable conditions for exposing the tarso-conjunctival tissue to UVA radiation for therapeutic purposes. When the local radiation-induced temperature increase at the conjunctival surface was chosen as a photothermal response, the method indicated failure of the law occurring at an irradiance of approximately 30 mW/cm^2 for a radiant exposure (fluence) between 6 and 18 J/cm^2. When changes in the mechanical properties of the tissue (stiffness, strength) were investigated as photochemomechanical responses, the law broke down at around 60 mW/cm^2. However, following the safest application route principle, a biologically safe performance of the procedure should be prioritized over other considerations. Therefore, based on the law's validity limit disclosed by the analysis of photothermal response, an irradiance less than 30 mW/cm^2, associated with exposure times able to assure a radiant exposure within an estimative range of 5 to 20 J/cm^2, are recommended for crosslinking the tarsal collagen.

Symbols and Abbreviations

ΔT – temperature increase;
E_e – irradiance;
H_e – radiant exposure;
m – slope ($\tan\theta$);
M_Y – Young's modulus;
p – Schwarzschild's coefficient;
$\mathcal{P}$ – photoresponse;
R^2 – coefficient of determination (for linear regression);
σ – stress;
t – time;
θ – inclination angle;
BRL – Bunsen-Roscoe law;
CXL – corneal collagen crosslinking;
PBS – phosphate-buffered solution;
RF-5P – riboflavin 5'-phosphate monosodium salt;
s.e.m. – standard error of the mean;
TXL – tarsal collagen crosslinking;
UV – ultraviolet radiation;
UVA – region A of ultraviolet radiation (wavelength from 315 to 400 nm).

Author Contributions

T.V.C.: Conceptualization, Methodology, Investigation, Writing – original draft, Writing – review & editing, Supervision, Project administration; S.S.: Methodology, Investigation, Resources, Data curation, Writing – review & editing; N.E.P.: Methodology, Investigation, Resources; C.C.: Methodology, Investigation; A.I.M.: Methodology, Investigation, Writing – review & editing. All authors contributed to the experiments, checked the final draft, and approved its submission. The authors assume full responsibility for the results reported in this article.

Ethics Approvals, Consent for Publication

None applicable.

Data Sharing and Availability

The data supporting the findings of this study are provided within the article. Additional information is available upon reasonable request addressed to the corresponding author.

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