

# Effect of corneal epithelium on ultraviolet-A and riboflavin absorption

## Efeito do epitélio na absorção corneana de raios ultravioleta-A e riboflavina

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### ABSTRACT

**Purpose:** To determine if the corneal epithelium prevents the collagen cross-linking effect. Using immunofluorescence microscopy after CXL, we indirectly analyzed the role of the epithelium as ultraviolet-A (UVA) shield as well as a barrier to riboflavin penetration.

**Methods:** Fifteen freshly enucleated porcine eyes were divided into 3 groups. The corneal epithelium was kept intact in all groups. Five eyes served as control (Group 1). On group 2, eyes received tetracaine anesthetic drops and topical 0.1% riboflavin solution (10 mg riboflavin-5-phosphate in 10 mL 20% dextran-T-500). On Group 3, riboflavin was injected into the anterior chamber to allow penetration of the drug through the endothelium. Groups 2 and 3 were exposed to UVA (365 nm, 3 mW/cm<sup>2</sup>) for 30 minutes. Ultra-thin sections (8 µm) of the corneas were stained with anti-collagen type I and DAPI (4,6-diamidino-2-phenylindole dihydrochloride) and analyzed with fluorescence microscopy.

**Results:** Corneas treated with UVA irradiation and intracameral injection of riboflavin (Group 3) showed greater pattern of collagen organization compared to groups 1 (Control) and 2 (riboflavin and tetracaine eye drops). A yellow stromal staining, which represents the riboflavin diffusion into the stroma, was only observed in eyes injected with riboflavin into the anterior chamber.

**Conclusion:** Using immunofluorescence microscopy in porcine corneas, we demonstrated that the corneal epithelium reduces the effectiveness of CXL by preventing the penetration of the drug and not by limiting the UVA transmittance. An inadequate intrastromal concentration of riboflavin may impair CXL effect.

**Keywords:** Cross-linking reagents/therapeutic use; Riboflavin/pharmacokinetics; Epithelium, corneal/physiology; Microscopy, immunofluorescence; Photosensitizing agents; Ultraviolet rays; Swine

### RESUMO

**Objetivo:** Determinar se o epitélio corneano pode impedir ou diminuir o efeito do tratamento com "cross-linking" (CXL). Por meio de microscopia por imunofluorescência, foi indiretamente analisado o efeito do epitélio como escudo aos raios ultravioleta-A (UVA), assim como barreira à penetração da riboflavina.

**Métodos:** Quinze olhos enucleados de porcos foram divididos em 3 grupos. O epitélio corneano foi mantido intacto em todos os grupos. Cinco olhos serviram como controle (Grupo 1). No grupo 2, os olhos foram instilados com colírio anestésico de tetracaína, assim como colírio de riboflavina 0,1% (10 mg de riboflavina-5-fosfato em 10 ml de dextran 20% T-500). No grupo 3, solução de riboflavina foi injetada na câmara anterior para permitir a penetração da droga através do endotélio. Os grupos 2 e 3 foram então expostos à radiação UVA (365 nm, 3 mW/cm<sup>2</sup>) por 30 minutos. Subsequentemente, cortes ultrafinos (8 µm) das córneas foram marcados com anticógeno tipo I e DAPI (4,6-diamidino-2-fenilindole dihydrochloride) e analisados com microscópio de imunofluorescência.

**Resultados:** As córneas que receberam injeção intracameral de riboflavina e foram irradiadas com UVA (Grupo 3) mostraram um padrão maior de organização das fibras de colágeno em relação aos grupos 1 (Controle) e 2 (instiladas com colírio anestésico e de riboflavina). Macroscopicamente, a coloração amarelada do estroma, que representa a difusão da riboflavina, foi apenas observada nos olhos que receberam riboflavina intracameral.

**Conclusão:** Foi demonstrado, através de microscopia por imunofluorescência em córneas de porcos, que o epitélio corneano integro diminui a efetividade do CXL por reduzir a penetração da riboflavina, e não por impedir a penetração dos raios UVA. Uma concentração intraestromal inadequada de riboflavina limita o efeito do tratamento.

**Descritores:** Reagentes para ligações cruzadas/uso terapêutico; Riboflavina/farmacocinética; Epitélio corneano; Microscopia de imunofluorescência; Agentes fotossensibilizantes; Raios ultravioleta; Suíno

### INTRODUCTION

Corneal collagen cross-linking (CXL) has been described as a promising therapy for keratoconus and post-refractive surgery keratectasia<sup>(1,2)</sup>. Previous studies<sup>(3-7)</sup> have shown that riboflavin/ultraviolet-A (UVA) treatment leads to an increase in the biomechanical stabilization and stiffness of the cornea. It is already known that corneal tissue from keratoconus eyes are biomechanically weakened compared to normal corneas<sup>(8)</sup>. This minimally invasive procedure treats and prevents the underlying pathophysiological cause of cornea ectasia as it attempts to modify the inadequate mechanical stability of these corneas.

The CXL protocol recommended by Wollensak et al.<sup>(9)</sup> states that the central epithelium should be debrided before treatment to allow penetration of riboflavin into the corneal stroma (epi-off technique). However, removal of the epithelium can increase the risks of corneal infections, ulcers, haze, scarring and infiltrates, beyond longer recovery time, discomfort and postoperative pain experienced by patients<sup>(9-12)</sup>. Recently, slight modifications of this technique were proposed to overcome the potential disadvantages of epithelium removal: one approach advocates the use of multiple applications of topical anesthetic eye drops to loosen the tight junctions of the corneal epithelial cells, thus facilitating riboflavin penetration through an intact epithelium<sup>(13,14)</sup>. Others per-

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form a grid-like pattern de-epithelialization instead of complete epithelial removal, attempting to hasten postoperative healing<sup>(15)</sup>. Another method allows the stromal diffusion of the riboflavin through a femtosecond laser-created central corneal pocket<sup>(16,17)</sup>. This pocket enables for superficial intrastromal administration of riboflavin solution without de-epithelialization. All of these techniques, however, have not considered the potential effect of the corneal epithelium as an UVA filter.

The corneal epithelium plays an important role in the UV absorbing processes. Ultraviolet rays can induce production of reactive oxygen species and cell death in cultured corneal cells. To protect against these effects, there is a high ascorbate concentration and anti-oxidant enzymes in the corneal epithelium that absorbs portions of the irradiation, thereby protecting deeper eye structures<sup>(18,19)</sup>. While the integrity of the epithelium can protect the underlying structures from UVA penetration, it may act as a barrier, reducing the effects of CXL.

Our group has already suggested that CXL without previous epithelial debridement has a decreased effect comparing to the standard epi-off technique<sup>(20)</sup>. However, at that time, we did not clarify whether this reduced effect was due to an inadequate absorption of UVA or due to poor penetration of the riboflavin through the epithelium. To investigate this question, the present study was conducted to determine if the epithelium, acting as a UV filter, prevents the CXL effect. The occurrence of CXL in corneas with intact epithelium was evaluated using immunofluorescence microscopy of collagen type I.

## METHODS

The research was approved and conducted in compliance with the Declaration of Helsinki and the Federal University of São Paulo Ethical Committee - Investigational Review Board (CEP 01565/07).

The study was performed on 15 enucleated porcine eyes within 6 hours postmortem from the slaughterhouse. Each specimen underwent slit lamp evaluation. If there was evidence of corneal scarring, opacity or other abnormalities, the specimen was discarded. The epithelium was not removed in any groups and its anatomical integrity was assured by slit lamp examination. Eyes were divided into 3 groups, each one with five porcine eyes:

Group 1 (control - no treatment): no treatment was performed.

Group 2 (riboflavin + tetracaine eye drops): Anesthetic drops of 0.5% tetracaine (to simulate a clinical scenario) and 0.1% riboflavin eye drops (10 mg riboflavin-5-phosphate in 10 mL dextran T-500 20%) were applied to the anterior corneal surface every 5 minutes, beginning 30 minutes prior, and continuing during the UVA treatment. One minute interval between anesthetic and riboflavin drops was given.

Group 3 (riboflavin injected into the anterior chamber): 0.1% riboflavin solution was injected into the anterior chamber through a limbal port to allow the endothelial penetration of the drug. UVA exposure was carried out after 30 minutes of the injection. Our purpose was to indirectly analyze the role of the epithelium as a UVA shield in the CXL process.

## ULTRAVIOLET-A EXPOSURE

Ultraviolet-A irradiation (365 nm) was applied 45 mm from the cornea for 30 minutes using a solid-state device (X-Link; Opto Eletronica, Sao Carlos, Brazil) with a surface irradiance of 3 mW/cm<sup>2</sup>. Total radiant exposure to the cornea was 5375 J/cm<sup>2</sup>. The surface irradiance was guaranteed by the micro processed, continuous, self-controlled monitoring system of the device that utilizes an internal power meter. The UVA source consisted of a homogenized 9 mm beam that uses a capsulated, matrix light emitting diode as its source.

## IMMUNOFLUORESCENCE MICROSCOPY

After dissection with a 9 mm trephine, corneal buttons were embedded in tissue freezing media (Leica Microsystems Inc., Bannockburn, IL, USA) and immediately frozen at -70°C. Ultra-thin sections (8 mm) were performed on a cryostat at -21°C.

After washing with phosphate-buffered saline (PBS), the slides were incubated for 1 hour with the primary antibody anti-type I collagen 1:500 (Calbiochem, Darmstadt, Germany) in PBS containing 1% bovine serum albumin (BSA) and 0.1% saponin. Afterwards, the slides were washed in PBS and incubated with anti-mouse IgG secondary antibody conjugated with AlexaFluor 488 (Molecular Probes, Carlsbad, CA, USA) (1:300, 30 min). The slides were washed and the nuclei were stained using DAPI (4,6-diamidino-2-phenylindole, dihydrochloride - Molecular Probes) 1:1000 in PBS containing 0.1% saponin for 30 minutes. Sections were observed under a Nikon E800 fluorescence microscope using a B-2E/C filter, 494 nm wavelength for excitation and 518 nm for emission (Nikon, Melville, NY, USA).

Images were digitized using a CoolSNAP-Pro charge-coupled device (CCD) digital camera and Image-Pro Express Software (Media Cybernetics, Silver Spring, Md., USA). Slides untreated with primary antibodies were used as negative controls.

## RESULTS

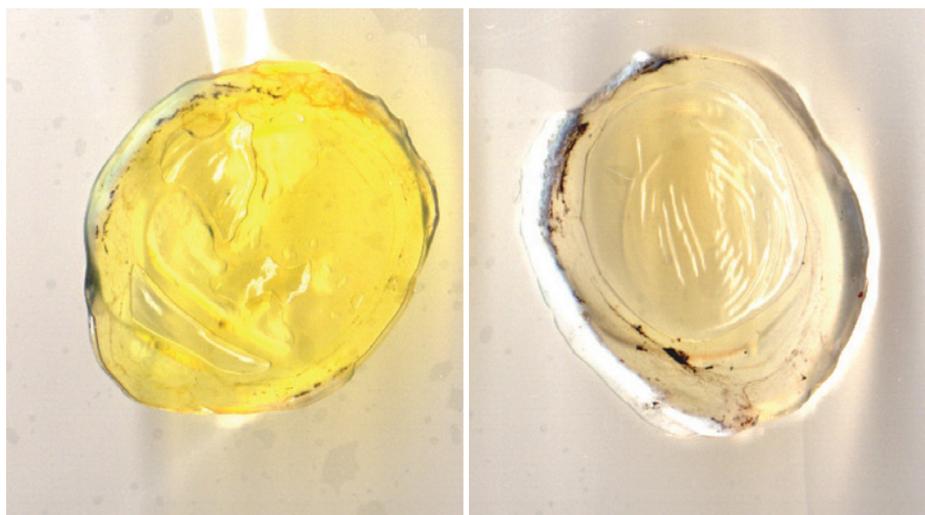
Macroscopically, only corneas from group 3 (riboflavin injected into the anterior chamber) showed a yellow stromal staining, which represents the riboflavin diffusion into the stroma (Figure 1). Microscopically, porcine corneas from group 3 showed a greater pattern of collagen organization compared to groups 1 (Control) and 2 (riboflavin + tetracaine eye drops). Interfiber spaces were similarly displaced on groups 1 and 2, whereas in group 3, the collagen fibers were more compacted on the anterior portion (Figure 2).

Immunofluorescence analysis using anti-collagen type-I and DAPI for nuclei was performed to assess the organization of collagen fibers and epithelium integrity respectively (Figure 2). DAPI was used to assess the keratocytes and epithelial cells distribution rather than to analyze apoptosis. The apoptosis reaches its peak after 24 hours. In our study, our samples were immediately submitted to immunofluorescence microscopy after the experimental procedure. Therefore, we assume that images suggesting apoptosis would not be found in the present study. Collagen fibers appeared in green and were higher organized on group 3 compared to the other groups. The epithelial cells and keratocytes nuclei could be identified as blue bodies.

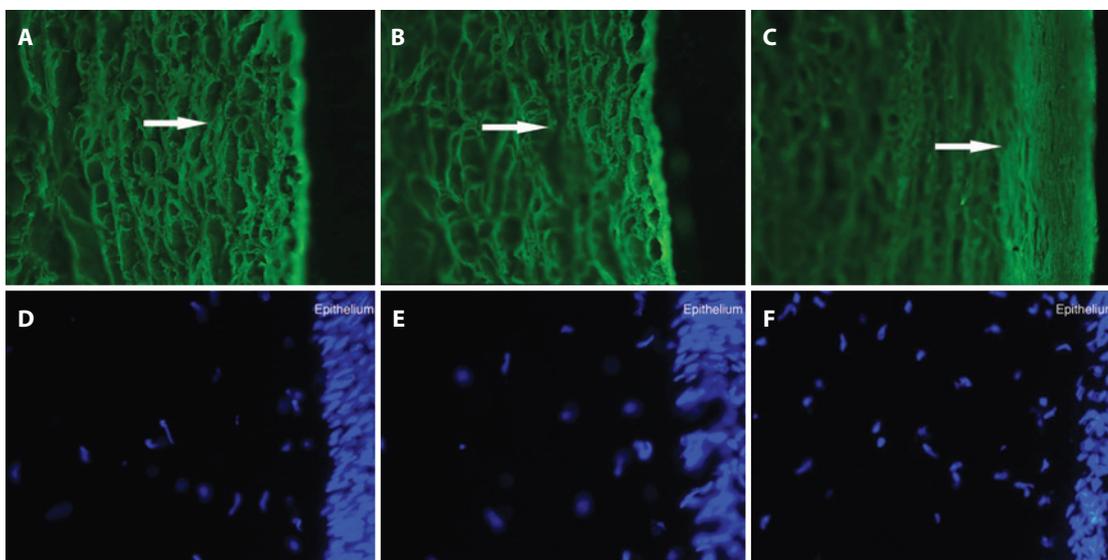
## DISCUSSION

The complete removal of the epithelium has been recommended as an initial step of the CXL procedure since its lipophilic nature reduces the diffusion of riboflavin into the corneal stroma<sup>(20-26)</sup>. Moreover, the epithelium may block UV rays<sup>(27-29)</sup>. Despite this recommendation, some ophthalmologists have adopted the "epi-on" technique, with intact epithelium<sup>(13,14)</sup>. This technique, also called transepithelial CXL, attempts to minimize possible complications due to epithelial debridement such as corneal ulcer, infections, haze as well as photophobia, prolonged recovery time and pain.

In the CXL process, the synergism of UVA rays and riboflavin is crucial<sup>(20)</sup>. It is known that the corneal epithelium strongly absorbs ultraviolet (UV) radiation due to high amounts of tryptophan residues and high ascorbate content<sup>(28)</sup>. It protects deeper corneal structures against UV damage by absorbing a substantial amount of the irradiation applied to the eye. However, Kolozsvári et al.<sup>(29)</sup> reported that the corneal epithelium has a significantly higher absorption coefficient for UV rays with wavelengths shorter than 300 nm, thus higher UV waveband, such as 365 nm used in CXL treatment, is



**Figure 1.** Photographs of corneal buttons, both with intact epithelium. Left cornea: riboflavin was injected into the anterior chamber. Right cornea: Topical tetracaine and riboflavin drops were instilled on the anterior surface of the cornea.



**Figure 2.** Immunofluorescence microscopy using anti collagen-I (A,B,C) and corresponding images of fluorescence microscopy for nuclei (DAPI) (D, E, F). Original magnification X40. A) Control cornea, no treatment was performed. Arrows show the interfiber spaces. B) Group 2: Topical riboflavin and tetracaine drops were applied. Arrows show the interfiber spaces. C) Group 3: Riboflavin solution was injected into the anterior chamber. Note the reduced interfiber spaces compared to the other groups.

mainly absorbed by the corneal stroma and lens rather than by the epithelium. To evaluate the role of the epithelium in the CXL process as a potential UV filter, we injected riboflavin into the anterior chamber, bypassing the epithelium. In spite of the lipophilic single cell layer architecture of the endothelium, the stroma was stained by the riboflavin (yellow), demonstrating that riboflavin crossed the endothelium. It is known that the endothelium is permeable to riboflavin<sup>(2,22)</sup>. This phenomenon is observed during the classic epi-off treatment by the presence of a yellow flare in the aqueous humor after instillation of riboflavin. We clearly demonstrated that corneas injected with riboflavin into the anterior chamber showed higher organization of the collagen fibers compared to corneas with intact epithelial basal membrane instilled with riboflavin and tetracaine drops. Therefore, although the epithelium can partially block the UV rays, the amount of UVA that reached the stroma was sufficient for the occurrence of CXL.

Due to the pharmacological properties (hydrophilia) and its high molecular weight (376.37 g/mol), riboflavin has a poor penetration through the epithelium. In 2008, Bottós et al.,<sup>(20)</sup> demonstrated that CXL did not occur in porcine eyes with intact epithelium. In their study, however, riboflavin was instilled alone. Nevertheless, in a clinical setting riboflavin is instilled with topical anesthetic, which may increase epithelium permeability by disrupting tight junctions. In the current study, however, even with the addition of tetracaine we were still not able to detect CXL. Our results are in accordance with previous reports. In 2008, Hayes et al.<sup>(21)</sup> used spectrophotometry to assess the ability of the riboflavin to penetrate the epithelium. They concluded that the presence of an intact epithelial basal membrane acts as a barrier to riboflavin absorption, which is not disrupted by trauma nor tetracaine eye drops. Samaras et al.,<sup>(15)</sup> reported that the absorption of riboflavin was still not adequate despite

the aggressive attempt to loosen the epithelial tight junctions with 20% alcohol or the grid-like pattern of de-epithelialization. In a clinical study, Leccisotti et al.<sup>(14)</sup> found a limited but favorable effect of transepithelial CXL in keratoconus eyes. They reported that this effect appeared to be less pronounced than described in the literature after CXL with de-epithelialization. Their approach involved the preoperative use of other drugs such as: Ribomicin eye drops, 0.3% gentamicin, 0.01% EDTA (ethylenediamine tetraacetic acid), 0.01% benzalkonium chloride and 0.4% oxybuprocaine eye drops. Since our goal was to follow the standard technique proposed by Wollensak et al.<sup>(3)</sup> rather than test new modalities of transepithelial CXL, these medications were not tested; therefore, our results may not be applicable to these transepithelial techniques.

Safety should be carefully analyzed when using riboflavin drops on an intact epithelium: even though the epithelium may protect the underlying ocular structures by absorbing some amount of UV rays (without impeding CXL effect), riboflavin penetration will be impaired. It is well known that riboflavin is essential to CXL process, not only by acting as a photosensitizer, but also by preventing the harmful effects of irradiation<sup>(20,22,23)</sup>. This drug absorbs UVA preventing injuries to deeper ocular structures such as the endothelium, crystalline lens and retina. Wollensak et al.<sup>(7)</sup> determined the damage threshold of the combination of UVA and riboflavin to be 0.35 mW/cm<sup>2</sup>, which is 10 times lower than UV alone (4 mW/cm<sup>2</sup>). However, because of the riboflavin shielding effect, the clinically applied UV irradiance at the endothelium level is smaller than the damage threshold. An adequate amount of riboflavin is of paramount importance because it has an amplification effect on UVA absorption, thus reducing the irradiance transmission through the cornea.

Immunofluorescence microscopy was proposed by Bottós et al.<sup>(20)</sup> in 2008 as new approach to indirectly visualize the effect of collagen cross-linking, which was later validated by other authors<sup>(30)</sup>. Bottós et al.<sup>(20)</sup> addressed the isolated effect of riboflavin on the collagen organization and corneal dehydration by applying 0,1% riboflavin solution to de-epithelialized corneas without UVA exposure. It is expected that 20% dextran may cause some stromal dehydration. Therefore, the analysis of riboflavin solution alone is important to clarify whether the stromal compaction was due to dehydration or due to the CXL effect. It was not possible to distinguish corneas receiving only riboflavin drops from those of the control group (without any treatment), meaning that riboflavin solution alone is not sufficient to cause the same level of collagen organization than the CXL treatment with riboflavin/UVA in debrided corneas. For this reason, in the present study, we assume that the high collagen organization observed when riboflavin was injected into the anterior chamber was a result from the CXL affect.

Limitations of this study include the small number of eyes tested and the use of enucleated porcine corneas instead of human corneas. Further studies addressing this issue should be performed, using a larger sample of human eye-bank eyes.

The present study may have significant implications for the development of future CXL techniques. As new transepithelial approaches have been proposed, it is important to define which are the critical steps for CXL effectiveness. Using immunofluorescence microscopy, we demonstrated that the intact epithelial basal membrane reduces the effect of CXL by preventing the penetration of the drug and not by limiting the UVA transmittance. Therefore, our study provides additional evidence that the CXL should be performed either without the epithelium or by using an efficient method to deliver riboflavin into corneal stroma.

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