UV-A rays absorption in human corneas before and after trans-epithelial riboflavin application: an experimental study

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Purpose: testing of a new riboflavin solution with enhancers to promote the riboflavin penetration in the cornea [1,2,3,4].

Background

Current treatments downsides:

- EPI-ON CXL: the solutions of riboflavin currently used penetrate superficially and slowly through the corneal epithelium the treatment is substantially superficial and takes a long time;
- EPI-OFF CXL: deeper treatment, but with discomfort for the patient and greater risk of infections.

A mathematical model to calculate the time-related rate of riboflavin degrading in the corneal tissue and the progressive increase of the intensity of the UV beam that crosses the cornea has been calculated. This mathematical model allowed us to design a new protocol for treatment Cross-linking [5].

It is known that:

- The amount of riboflavin absorbed by corneas in EPI-ON treatments is not sufficient to attenuate a UV-A beam from 3mW/cm^2 below the level of 0.3mW/cm^2 , with risk of biological damage [6];
- The transmittance values of a UV-A beam of 3 mW/cm^2 in human cornea, according with our studies, are in agreement with those found by Wollensak et al [6];
- The presence of a superficial layer of riboflavin on the corneal surface is mandatory in the current protocol to ensure the safety of the patient, as noted and suggested by Wollensak and Sidney [6];
- The selected dose is based on a 3mW/cm^2 energy level delivered for 30 minutes, which translates into a dose of 5.4 J/cm^2 [7];
- A histological study on in-vivo exposed rabbits showed that UVA irradiation in this wavelength and dose caused a complete

endothelial and keratocyte loss in the exposed zone [8];

- The average transmittance in terms units of energy after the 7th, 8th, 9th, 10th ,11th and 12th drop were respectively 0.915, 0.841, 0.793, 0.731, 0.687, 0.643 mW/cm^2. All these results are above the assumed endothelium safety limit of toxicity of 0.35 mW/cm^2 [9].

The treatment of standard EPI-ON Cross-linking is superficial because with the current protocol the UV-A energy is strongly attenuated by the superficial layer of riboflavin present on the corneal surface, before entering into the corneal tissue.

Moreover, the current protocol, that suggests a soaking time of 30 minutes, does not allow the penetration of a sufficient amount of riboflavin to perform a safe treatment without the protection of the surface layer of riboflavin.

We also showed that riboflavin alone slowly penetrates with EPI-ON procedure [4] but

- Not in a sufficient amount to allow a safe CXL treatment;
- Only in the upper layers;
- It takes more than 2 hours to completely saturate the corneal tissue.

Study Design

- Time-related measures of the UV-A transmittance of a riboflavin film;
- Measures of the UV-A transmittance of human corneas;
- Time-related measures of the UV-A transmittance of riboflavin-soaked corneas during a 30 minutes continuous irradiation;
- 10 Eye-bank human Corneas not suitable for surgery due to donor marker positivity (Intramural Ethics Committee approval, n°1269);
- VEGA CBM X-Linker® (CSO, Italy) as UV-A source emitter (370 nm wavelength), working at 3 mW/cm^2;
- UV-METER (Peak Tech 5085);

Figure 1 - UV-Meter.

- Solution of riboflavin 0.1% with enhancers [4];
- 15 minutes topical application of the riboflavin solution on the epithelized corneas, then successive washing with BSS (IMPORTANT!);
- 30 minutes UV-A irradiation at 3 mW/cm^2, measuring the energy exiting from the posterior surface of the corneas.

Results

1) 0.1 mL of riboflavin solution placed on a glass slide spanned over a circular surface with a diameter of about 9mm (mean thickness of about 1.57 mm).

The intensity of a UV-A circular shaft of a diameter of 9 mm impinging on the riboflavin solution was attenuated by about 285 times.

Evidence: even a thin layer of riboflavin solution is capable of a remarkable attenuation of the UV-A energy entering in the cornea.

2) Corneas not soaked with the riboflavin solution were exposed to an UV-A irradiation of 3 mW/cm^2.

The mean UV-A intensity exiting from the corneas was 1.21 mW/cm^2.

Evidence: the corneal UV-A transmittance is about 40%.

3) One drop of riboflavin solution (0.02ml) was applied every 3 minutes on corneas for 15 minutes (0.1 mL in total), the superficial layer of riboflavin was washed away with BSS, then the corneas were subjected to a UV-A shaft having an intensity of 3 mW/cm^2.

The intensity of the UV-A shaft exiting from the corneas increased with time, with average values starting from 0.623 mW/cm^2 at time 0, up to 0.903 mW/cm^2 after 30 minutes.

It is estimated that about 9 µg of riboflavin - out of 125 µg in 0.1 mL of administered riboflavin solution - were absorbed by the cornea, the remaining 116 µg of riboflavin constituted the superficial layer that was washed away.

Evidence: without the superficial layer of riboflavin solution, an intensity of 3mW/cm^2 would be dangerous for patients.

It is estimated that a layer of 0.1% riboflavin solution containing 116 µg of riboflavin uniformly disposed on a plane circular surface with a diameter of 9 mm would attenuate a UV-A shaft by about 200 times.

4) It has been experimentally measured how much riboflavin may be absorbed at most by human corneas [4].

A rubber ring filled with the proposed riboflavin solution, was applied on human corneas for 15 minutes. Then the superficial layer of riboflavin was washed away with BSS and the amount of riboflavin in the corneas was measured.

Human corneas may absorb about 15 µg of riboflavin.

Evidence: 15 µg of riboflavin are enough to perform a safe cross-linking treatment, without the superficial layer of riboflavin, using a UV-A source having an intensity of 1.5 mW/cm^2 for only 10 minutes.

Before irradiation: complete saturation of the cornea with riboflavin In vitro corneal accumulation studies [4]: results obtained were compared with that of solution A applied on intact and non-epithelized corneas (EPI-OFF) at the same time interval. As expected, epithelium strongly limited riboflavin permeability from standard solution, while epithelium debridement provided up to 10-fold increase in riboflavin corneal recovery.

HPLC Analysis:

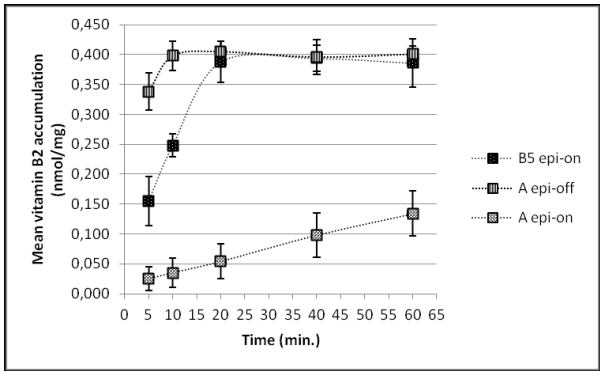


Table 1: Time course of vitamin B2 corneal accumulation from solution A and B5 on epithelized (EPI-ON) and disepitelized (EPI-OFF) corneas.

After treatment: increased corneal rigidity.

Calculated Young's modulus at 6% and 8% strain. Values are expressed in Pa

Stress in %	Group A	Group B	Group C	Group D
6	$1.13^{*}10^{5}$	$1.29^{*}10^{5}$	$3.01^{*}10^{5}$	$2.46^{*}10^{5}$
8	$2.62^{*}10^{5}$	$2.84^{*}10^{5}$	$5.71^{*}10^{5}$	$4.64^{*}10^{5}$

Chart 1: Biomechanical measurements (electronic dynamometer AG/MS1).

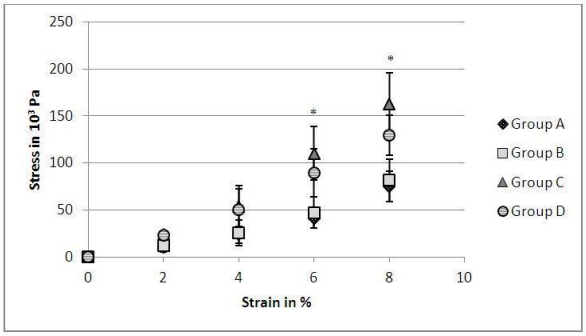


Table 2: Stress-strain behavior of corneal tissue after CXL treatment.

Discussion

New Custom Fast Corneal Cross-linking protocol:

- New enhanced solutions;
- Topical administration of riboflavin for 15 minutes;
- Removal of the surface layer of riboflavin with BSS;
- Reduction of the intensity of the UV-A source to 1.5 mW/cm^2;
- Reduction of the irradiation time to 10 minutes, divided in two step of 5 minutes each, separated by application of BSS.

We can now make an EPI-ON Cross-linking treatment with effects in depth [5].

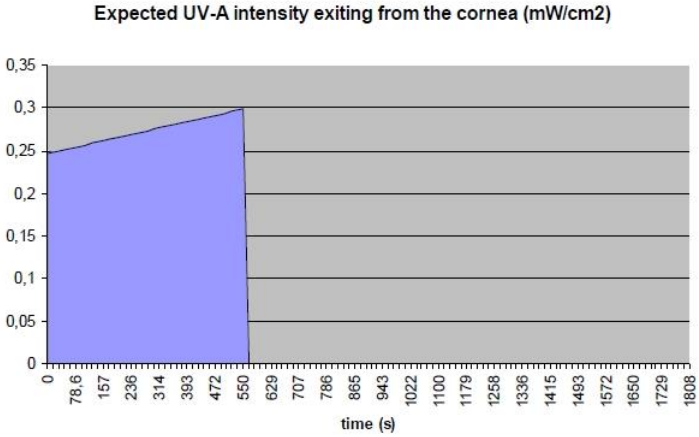


Figure 2 - Computer assisted simulation: expected UV-A intensity exiting from the cornea (mW/cm^2).

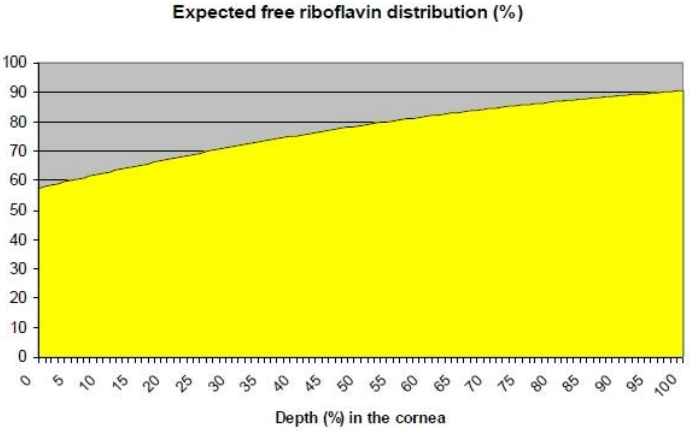


Figure 3 - Computer assisted simulation: expected free riboflavin distribution (%).

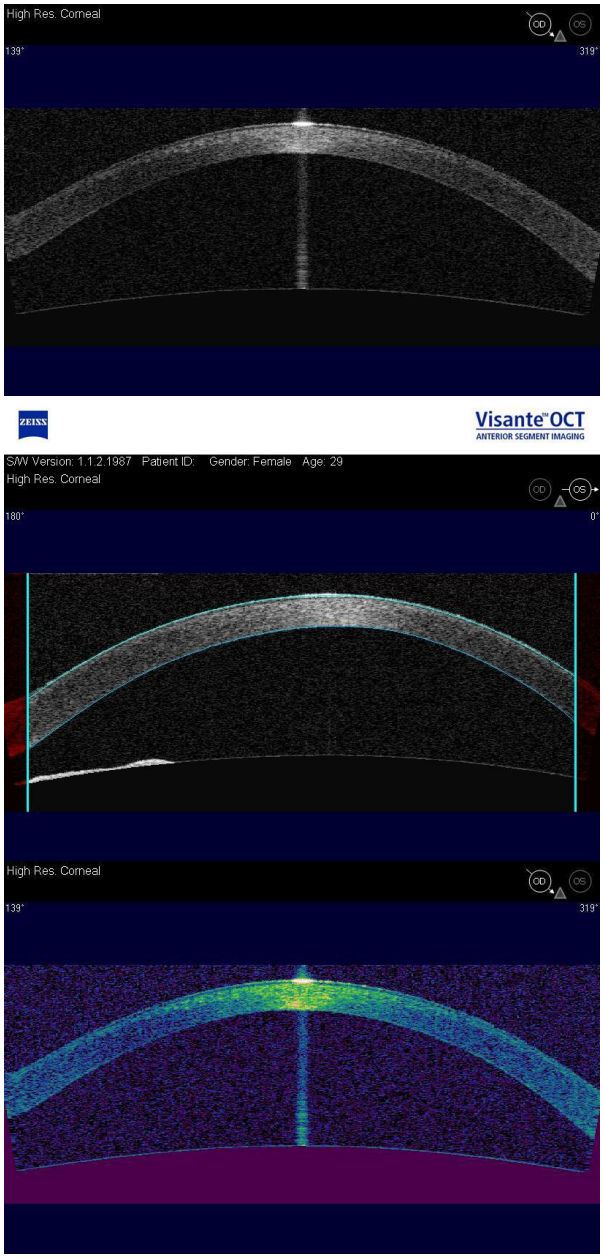


Figure 4 - Stromal area details.

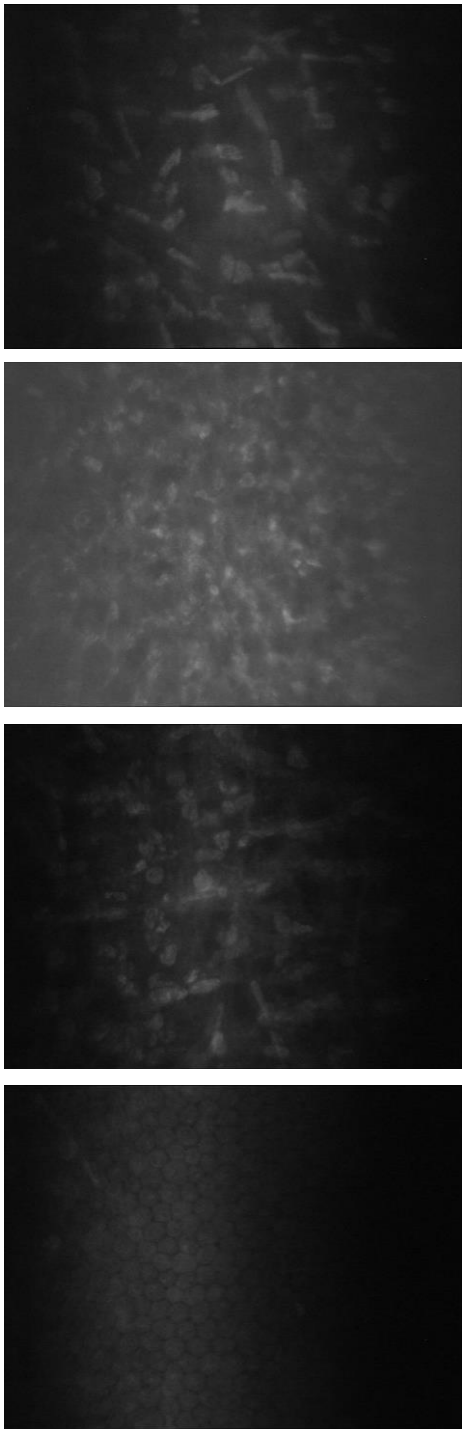


Figure 5 - Cellular details.

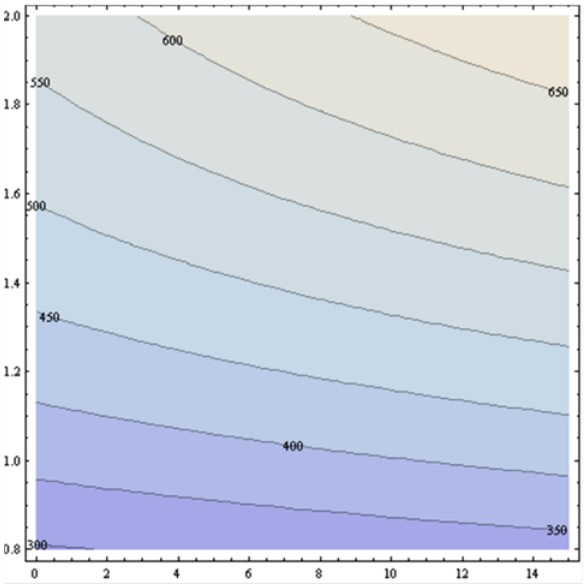
Table 3 - Treatment duration (minutes)

Intensity (mW/cm²)

Thickness (µm)	**1,2**	**1,3**	**1,4**	**1,5**	**1,6**	**1,7**	**1,8**	**1,9**	**2**	**2,1**
350	12	7	4	2	0	0	0	0	0	0
360	13	8	5	2	0	0	0	0	0	0
370	14	9	6	3	1	0	0	0	0	0
380	15	10	6	3	1	0	0	0	0	0
390	15	11	7	4	2	0	0	0	0	0
400	15	12	8	4	2	0	0	0	0	0
410	15	13	8	5	3	1	0	0	0	0
420	15	13	9	6	3	1	0	0	0	0
430	15	14	10	7	4	2	0	0	0	0
440	15	15	11	7	4	2	0	0	0	0
450	15	15	12	8	5	3	1	0	0	0
460	15	15	13	9	6	3	1	0	0	0
470	15	15	13	9	6	4	2	0	0	0
480	15	15	14	10	7	4	2	1	0	0
490	15	15	15	11	8	5	3	1	0	0
500	15	15	15	12	8	6	3	2	0	0
510	15	15	15	13	9	6	4	2	1	0
520	15	15	15	14	10	7	5	3	1	0
530	15	15	15	15	11	8	5	3	2	0
540	15	15	15	15	12	8	6	4	2	1
550	15	15	15	15	12	9	7	4	3	1

An arbitrary maximum duration of 15 minutes is pre-established.

Mathematical model graph



This graph is a product of the mathematical model. The effects of the treatment of cross-linking are : UV-A intensity – irradiation time – corneal thickness – riboflavin consumption rate dependent (intensity-dependent and not energy-dependent). To avoid, however, that patients can be subject to excessive doses of UV-A, it is necessary to fix the irradiation time and intensity of the source as a function of corneal thickness, using the graph (CUSTOM FAST CXL) and not the law of Bunsen-Roscoe, respecting, always the limit output of 0.3 mW / cm^2, beyond which the endothelium would be irremediably damaged. This graph allows to determine, depending on the thickness corneal expressed in micrometers, the source intensity UV-A and the irradiation time of a cornea soaked with riboflavin plus vitamin E TPGS and washed with BSS. It identifies the corresponding curve on the chart: the abscissa and the ordinate, the points belonging to this curve, represent respectively corneal thickness and intensity of the UV-A source.

Benefits of the new protocol:

- Shorter duration (25 minutes vs. 60 minutes according to the present protocol): more patient compliance, more rational use of the treatment room and the doctor's time;
- Lower intensity of the UV-A beam (safer condition for the patient): less risks of damaging the other ocular structures and all the corneal tissues;
- Reduction of the administered energy (about 0.9 J/cm^2, and very low damage corneal epithelium);
- Simpler post-operative management;
- No removal of the corneal epithelium. This is the less innovative aspect, but is the more appreciable for the patient and the surgeon: less post-treatment discomfort, faster clinical recovery, and a lower complication risk;
- Deeper cross-linking effect in the corneal stroma. This issue is more interesting, because the amount of cross-linked stromal tissue is strictly related to the stabilization of the ectasia over time. Our observations demonstrate a greater lamellar involvement with this protocol respect to other TE-CXL treatments

Conclusions

- This novel enhanced solution penetrates the cornea even in the presence of epithelium;
- It takes just 15 minutes of topical application on the cornea EPI-ON to saturate the cornea;
- The surface layer of riboflavin after the application is to be removed;
- The intensity of the UV-A shaft must be reduced to 1.5 mW/cm^2;
- The duration of irradiation should be reduced to 10 minutes – preferably in two step of 5 minutes, with a short interval for the application of BSS.

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