Evaluation of the penetration through human cornea of riboflavin 0.1% in solution with other molecules after trans-epithelial application: diffusion study and biological effects

Authors: Ciro Caruso, MD[3]; Salvatore Troisi, MD[2] ; Antonio Del Prete, MD[1]; Luigi Pacente, MD[3]; Gaetano Barbaro, EngD[1]; Carmine Ostacolo, PharmD, Senior Researcher[5]; Salvatore Del Prete, MD[4].

1) Department Of Ophthalmology University "Federico II" Naples Italy.
2) Department Of Ophthalmology Salerno University Hospital Salerno Italy.
3) Eye Center Corneal Transplants Pellegrini Hospital Naples Italy.
4) Interdepartmental Electron Microscope Centre, University "Federico II", Naples, Italy.
5)Department of Pharmaceutical Chemistry, School of Pharmacy, University of Naples "Federico II", Naples, Italy.

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Introduction

Cross-linking combines the use of riboflavin (vitamin B2) and ultraviolet-A rays (UV-A) irradiation to induce corneal stroma strengthening by the creation of new molecular bridges (called cross-links) among the collagen fibers, so that cornea acquires greater strength, stiffness and stability over time [1,2].

The cornea is composed of three layers:

- The outermost layer - epithelium - lipophilic in nature;
- The middle layer - acellular matrix with 85% water - hydrophilic in nature;
- The innermost layer - endothelium - lipophilic, it does not give any significant resistance to the transport of molecules. The molecule has to have a balance between its lipophilic and hydrophilic character.

Other transport mechanisms such as carrier-mediated transport, endocytosis, etc. are poorly understood [3].

The corneal epithelium:

- 35–50 mm thick - five cell layers = superficial, wing, and basal cells represents less than 10% of the entire corneal thickness;
- Contributes 99% of the resistance to solute;
- The superficial epithelial cells contribute as much as 60%;
- Solutes are transported through a transcellular and a paracellular routes;
- Hydrophilic compounds mainly diffuse through the paracellular route.

It is well known that EDTA and BENZALKONIUM may be used for enhancing the penetration of drugs throughout the corneal epithelium[4],but those are cytotoxic for it [5,6].

Permeability of cornea has been measured using molecules of different molecular weight ranging from 200 to 1000 Daltons [7], and a threshold of 500 Daltons has been proposed as critical molecular weight [8].

Tests carried out in vitro to evaluate epithelium permeability gave the same results as tests carried out in vivo. Several studies illustrated tests carried out in vivo and repeated in vitro, in order to evaluate the passage of drugs throughout the corneal epithelium in these two different test conditions. The reported results have been considered substantially identical in both test conditions [9].

UV irradiation caused statistically significant metabolic changes in the rabbit corneas; a decrease in metabolites, as amino acids, was observed [10-15].

It would be therefore desirable to find a solution of riboflavin mixed with other substances, capable to cross quickly corneal epithelium, with effective concentrations, without producing cellular damage and with less cytotoxic effects. Extensive studies seeking to determine how much riboflavin penetrates alone or mixed with other products ("carriers") through human corneas without disepithelization have been carried out. The substances to be tested in combination with riboflavin have been selected in the group comprising amino acids, vitamin E and Ubiquinone Q10.

Mainly, none of the substances selected to be tested had to be cytotoxic and potentially dangerous for epithelium cells: for this reason, EDTA and benzalkonium chloride have been excluded. Amino acids are neither cytotoxic or

potentially dangerous. The corneal epithelium is relatively impermeable to water-soluble compounds such as amino acids derived from tears; the limbal blood supplies less than 20% of the corneal nutrients, with the aqueous humor being the primary source of amino acids.

As far as vitamin E and Ubiquinone Q10 are concerned, they are neither cytotoxic or potentially dangerous too. Moreover, it is know that topical vitamin E and Ubiquinone Q10 treatments may be useful for reducing the harmful effects of reactive oxygen radical after epithelial scraping and PRK, because it increases corneal glutathione peroxidase activity and prevent corneal keratocyte apoptosis in UV-exposed rabbit corneas. Therefore, vitamin E and Ubiquinone Q10 appear potentially useful at least for repairing eventual damages caused by UV-A rays [16-17].

Materials and Methods

How much riboflavin penetrates alone or mixed with other products through human corneas with epithelium? Tested ophthalmic solutions were:

- Riboflavin-dextran 0.1 mg/100ml, benzalkonium chloride 0.01%;
- Riboflavin-dextran 0.1%, vitamin E TPGS (D-alfa-tocopheryl polyethylene-glycol 1000 succinate) 500 mg % ml, coenzyme Q 100 mg % ml, L-proline 0.1 mg %, glycine 0.1 mg %, lysine hydrochloride 0.05 mg % and L-leucine 0.08 mg %;
- Riboflavin–dextran 0,1%, vitamin E TPGS (D-alfa-tocopheryl polyethylene-glycol 1000 succinate) 500 mg % ml;
- Riboflavin-dextran 0,1% + coenzyme Q 100 mg % ml;
- Riboflavin-dextran 0,1% + L-proline 0,1 mg % + glycine 0,1 mg %, lysine hydrochloride 0,05 mg %, L-leucine 0,08 mg %.

Three corneas, not suitable for a transplant, were used as sample for every test group (n=3), to minimize the variability in results due to the quality of tissue provided by the eye bank.

Only corneas with good transparency, thickness between 500 and 600 microns and with healthy endothelial mosaic were used. A device for measuring the passage of solutions containing riboflavin through human corneas is composed of a cylindrical container, made of inert polyvinylchloride (P.V.C.) filled with sodium hyaluronate + xanthan gum 0.4 ml. Corneoscleral rings were displaced on this ring, epithelial side up, and endothelium in contact with hyaluronate and xanthan gum. Another little cylindrical container with the same diameter was set on the epithelial side of corneas, allowing the application of the solutions to be tested (Fig.1).

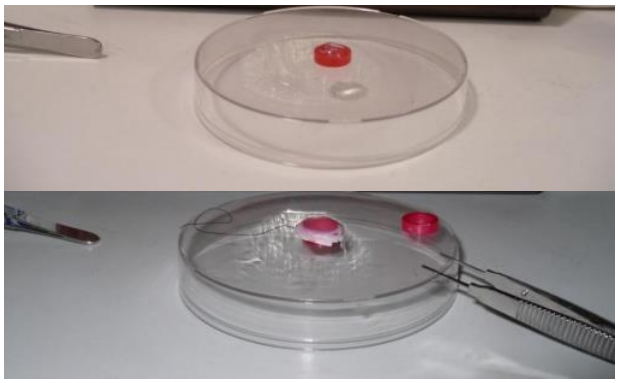


Figure 1 - Experimental device.

The presence of riboflavin in the solution of sodium hyaluronate + xanthan gum 0.4 ml, demonstrating the passages through the cornea with epithelium, was evaluated both qualitatively by adopting a visual and fluorimetric scale, as well as quantitatively, by using a color chart. The observation times were set to 15' and 30' after the application of the solutions. A reference scale has been realized preparing dilutions of riboflavin-dextran 0.1 mg/100ml with xanthan gum + sodium hyaluronate in the following proportions (unit / µl): 50/0, 40/10, 30/20, 20/30, 10/40, 0/50. Visual and fluorimetric scales with corresponding values of units / µl defined were so prepared, and a score from 10 to 0 for each dilution ratio was assigned. This color chart includes a minimum percentage of yellow at 20% in the absence of riboflavin, that corresponds to the spectrum colorimetry of the substance chosen as diluent.

In standard lighting conditions, a direct assessment of the findings obtained from the experiments has been performed with this samples pre-defined, also using digitized photographic technique. The attribution of the global score after visual and fluorimetric detection was performed by a third examiner, averaging the values obtained with both methods. Color evaluation was performed by inserting the material, present at the end of the experiment inside the container, in a transparent bag and subsequently carrying out a computer analysis with high-resolution scanning of dilutions default and the percentage of yellow using an imaging tool. It was thus possible to relate the percentage of yellow, prevalent and characteristic, detected with this method to a specific range of concentration in units / µl of riboflavin 0.1 mg/100ml (Table 1).

Results

Fluorimetric evaluation of corneas was further performed using a fluorescence microscope equipped with digital camera in the darkroom.

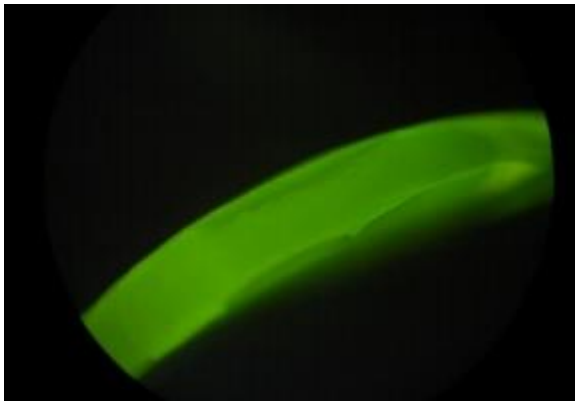


Figure 2 - Fluoroscopic picture of a section of a cornea after 15 minutes from application of the first novel solution in a trans-epithelial application.

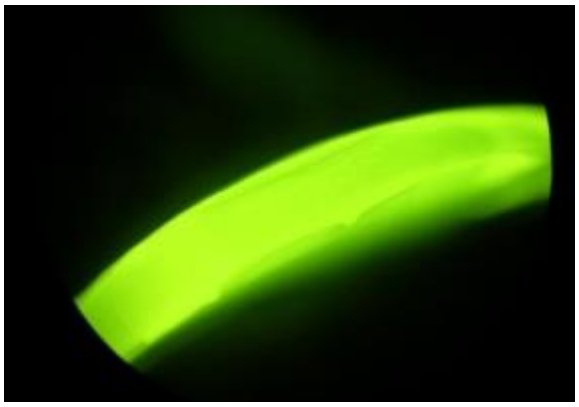


Figure 3 - Fluoroscopic picture of a section of a cornea after 30 minutes from application of the first novel solution in a trans-epithelial application.

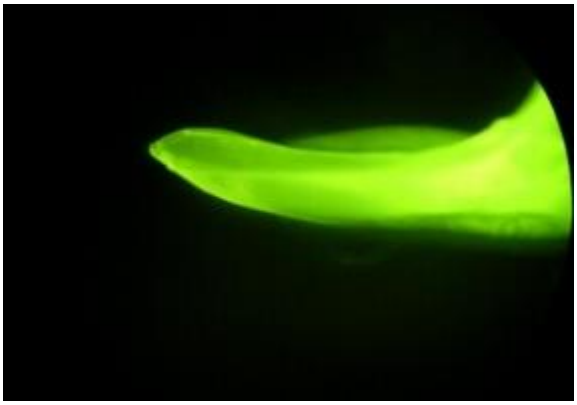
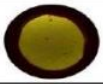
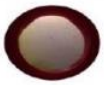


Figure 4 - Fluoroscopic picture of a section of a cornea after application of the first novel solution in a trans-epithelial application and cross-linking treatment. Riboflavin penetrated the whole corneal thickness and the tissue is more rigid after the cross-linking treatment.

The results showed that each of the novel tested solutions is suitable to promote the penetration of riboflavin through corneas with integer epithelium. The first novel solution: riboflavin-dextran 0.1%, vitamin E TPGS (D-alfa-tocopheryl polyethyleneglycol 1000 succinate) 500 mg % ml, coenzyme Q 100 mg % ml, L-proline 0.1 mg %, glycine 0.1 mg %, lysine hydrochloride 0.05 mg % and L-leucine 0.08 mg % gave the best results, both as visual and fluoroscopic dye detection as well as computer analysis of fluorescent substance inside the container after 15 and after 30 minutes of trans-epithelial application of the solution: - Score 5-6 at 15 minutes, with 88-91% percentage of yellow; - Score of 6-7 at 30 minutes, with a percentage of yellow over 90%. The highest concentration of fluorescent substance present in the container ring under the corneas is particularly evident after 15 minutes when the first novel solution is used. The standard solution riboflavin - dextran 0,1% not mixed with any enhancer is not even detectable in the material placed inside the container after 15 minutes.

Table 1 –Visual scale, fluorimetric scale and color scale.

Score	Standardized Visual Scale	Dilution riboflavin 0.1%/ (xanthan gum + sodium hyaluronate) mg/100ml	Fluorimetric Scale mg/100ml	Color Scale Yellow Percentage
10		50/0		100%
8		40/10		96%
6		30/20		92%
4		20/30		87%
2		10/40		73%
0		0/50		20%

EPI-ON or EPI-OFF?

Collagen cross-linking with riboflavin (C3-R) is mostly performed after removal of corneal epithelium. Several authors sustain that the disepithelization step would not be necessary because a certain amount of riboflavin passes through the epithelium, though requires a longer time to obtain complete stroma penetration [18]. Tests have been carried out on human and porcine corneas, but the reported results are in sharp contrast with each other [18-20]. Tests on ex vivo human corneas coherently demonstrated that removal of epithelium is necessary in order to obtain sufficient collagen cross-linking in the deep stroma [21-22].

Trans-epithelial novel solution vs standard riboflavin

Figures 5-6 show a keratoconus affected cornea before and after trans-epithelial cross-linking with our novel solution.

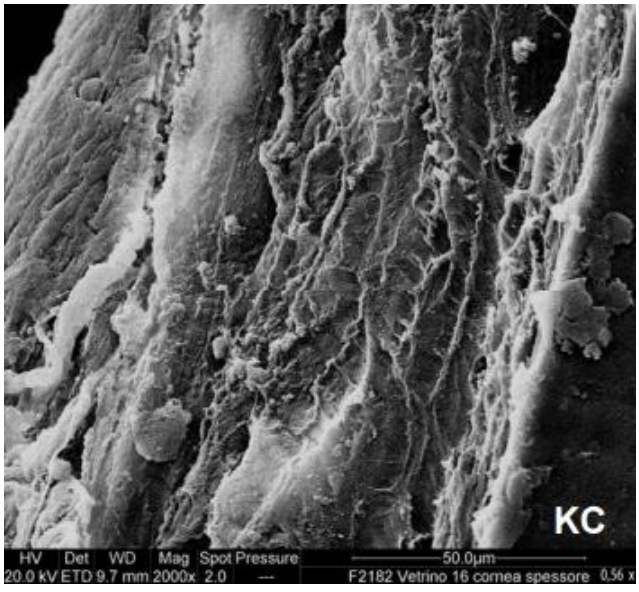


Figure 5 - Keratoconus affected cornea before treatment.

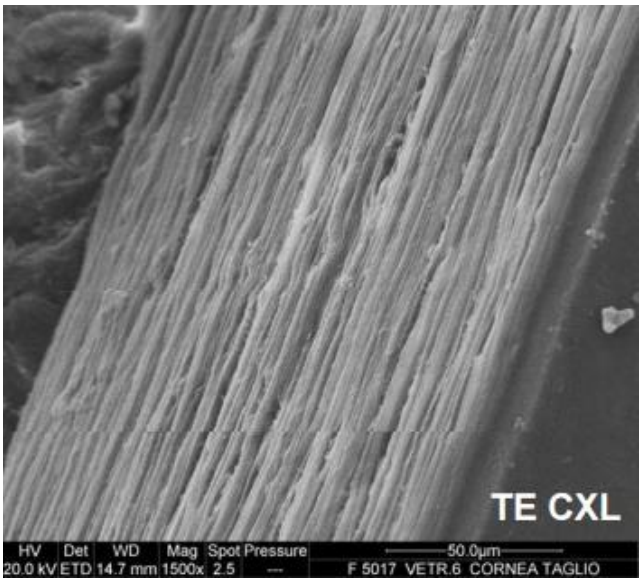


Figure 6 - Cornea after TE-CXL.

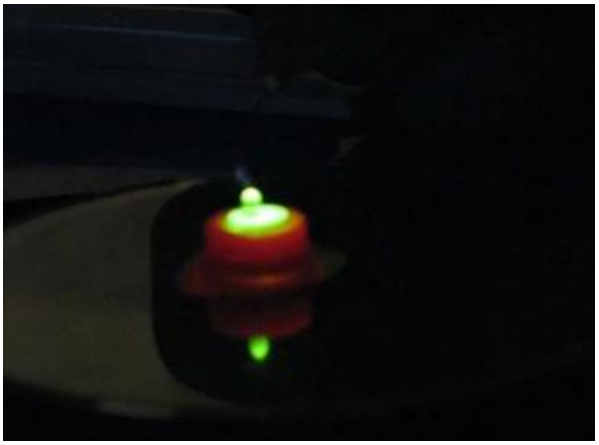


Figure 7 - TE-CXL procedure.

Before the treatment we can see weakened lamellae in the corneal limb, belonging to a patient affected by keratoconus. The keratoconic limb treated with the first novel solution with a trans-epithelial technique and a standard dose of UV-A irradiation for thirty minutes, has a dense and compact distribution of corneal lamellae, demonstrating that new biochemical cross-linkings have been generated.

The TE CXL treatment exposes the corneal epithelium to the well-known dangerous effects of UV-A [23-24]. Figures 8-9 show the complete loss of all epithelial layers and the exposition of the Bowman membrane after a TE-CXL treatment using the balanced salt solution (BSS) according to the standard protocol.

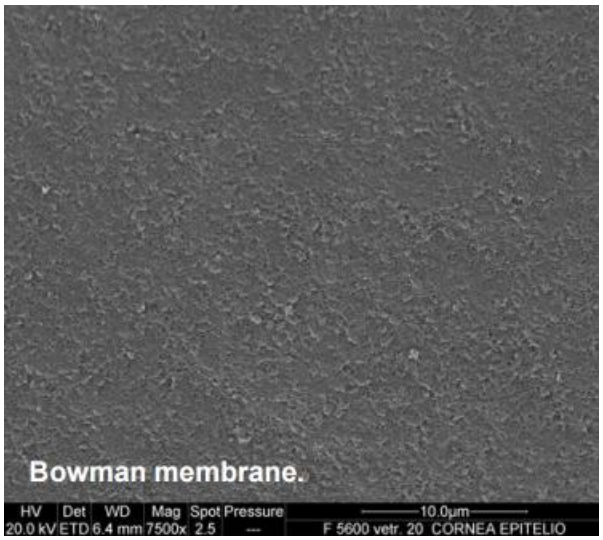


Figure 8 - Scanning Electron Microscopy showing the morphology of microvilli and of superficial layers of the epithelium in a normal cornea.

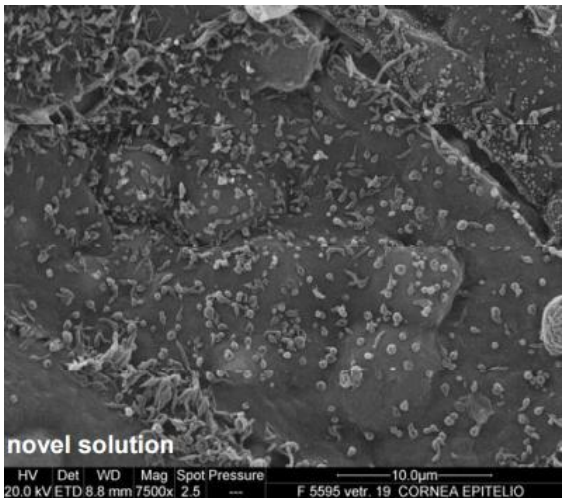


Figure 9 - Scanning Electron Microscopy of a cornea treated with UV-A after having applied the balanced salt solution (BSS) according to the standard protocol.

Figures 10-11 show the comparison between our novel solution and a standard riboflavin-dextran solution: with our solution the result show a better keeping of epithelial layers, of the cell nuclei and of inter-cellular tight junctions although the damages of UV-A radiations are evident; moreover, it is notable a significant reduction of density of distribution of microvilli, though the remaining ones appear to be morphologically integer. The standard solution, instead, produces several cellular gaps in superficial epithelial layer due to rupture of intercellular tight junction; some cells have lost their cytoplasmic nuclei and almost all microvilli are missing.

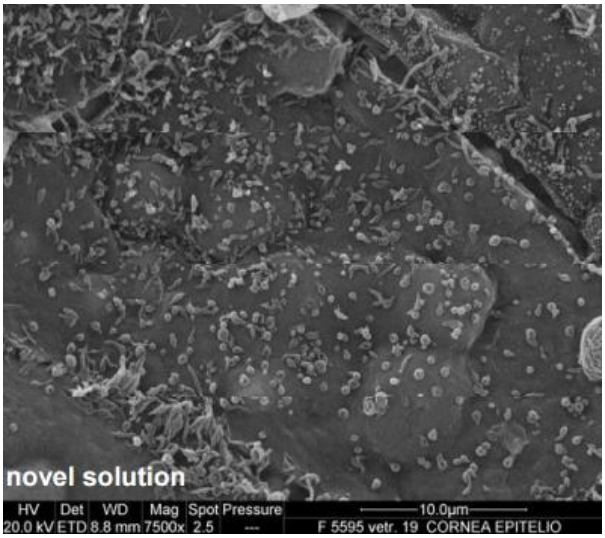


Figure 10 - Cornea treated with UV-A after having applied the first novel solution.

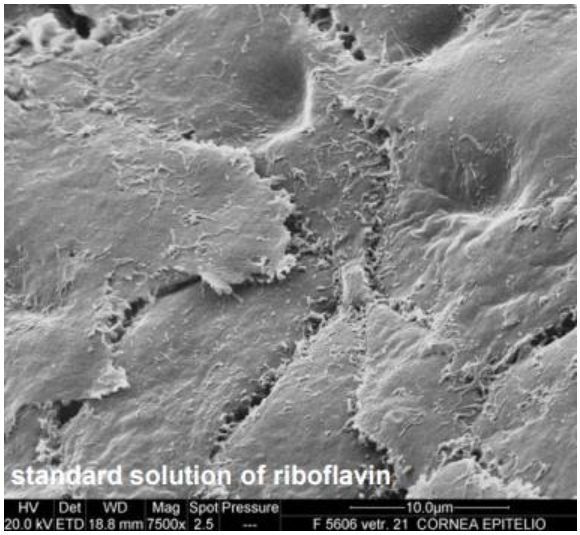


Figure 11 - Cornea treated with UV-A after having applied a standard solution of riboflavin-dextran.

Conclusions

A key issue appears to be the quantitative measurement of the riboflavin passing through the epithelium and of the diffusion in the stroma; above all, whether the mix of riboflavin-dextran mg/100ml together with other substances enhances its passage or not. This topic is the object of our tests. Our choice to evaluate in this study substances as penetration enhancers is based on two parameters:

- The capability of penetration through corneal epithelium;
- The capability of protecting the corneal structures against toxic effects of UV- rays and oxidant injuries.

At least the following substances: vitamin E;coenzyme Q; L-proline; glycine; lysine; L-leucine, alone or in combination promote and facilitate the penetration of riboflavin through the corneal epithelium by far faster than the standard solution of riboflavin-dextran and in a sufficient concentration for protecting the eye bulb from ultraviolet rays irradiated during cross-linking. The new solution therefore displays optimal characteristics of effectiveness, safety and tolerability, which make it suitable to the use through trans-epithelial route. Figures 12-13 show the trans-epithelial application of the new solution in vivo.

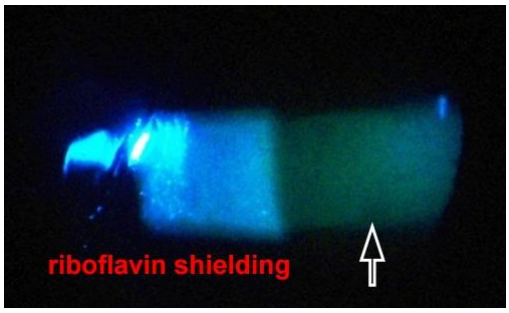


Figure 12 - Slit lamp inspection of the anterior chamber reveals a "green Tyndall" phenomenon.

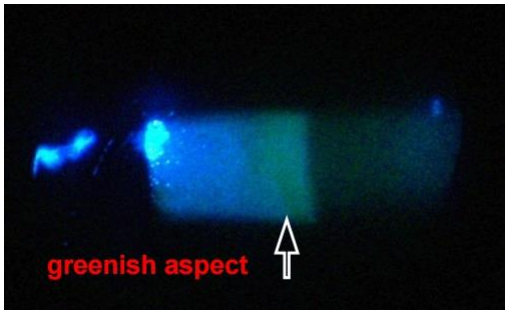


Figure 13 - complete penetration of the corneal stroma after TE application of the first new solution.

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