Enhancement of corneal permeation of riboflavin-5′-phosphate through vitamin E TPGS: A promising approach in corneal trans-epithelial cross linking treatment

Carmine Ostacolo [a,*], Ciro Caruso [b], Diana Tronino [a], Salvatore Troisi [c], Sonia Laneri [a], Luigi Pacente [d], Antonio Del Prete [e], Antonia Sacchi [a]

[a] Department of Pharmaceutical Chemistry, School of Pharmacy, University Federico II, Via D. Montesano 49, 80131 Naples, Italy
[b] Corneal Transplant Centre, Pellegrini Hospital, Via Portamedina alla Pignasecca 41, 80135 Naples, Italy
[c] Department of Ophthalmology, Salerno University Hospital, Largo Città di Ippocrate, 84131 Salerno, Italy
[d] Department of Ophthalmology, Pellegrini Hospital, Via Portamedina alla Pignasecca 41, 80135 Naples, Italy
[e] Department of Ophthalmology, School of Medicine, University Federico II, Via Pansini 5, 80131 Naples, Italy

ARTICLE INFO

ABSTRACT

Corneal accumulation of riboflavin-5′-phosphate (riboflavin) is an essential step in the so called corneal cross-linking (CXL), an elective therapy for the treatment of progressive keratoconus, corneal ectasia and irregular astigmatism. CXL is usually performed after surgical debridement of corneal epithelium, since it impedes the stromal penetration of riboflavin in a relatively short time.

D-Alpha-tocopheryl poly(ethylene glycol) 1000 succinate (VE-TPGS) is an effective permeation enhancer used to increase adsorption of drugs trough different biological barriers. Moreover, belonging to the group of tocopherol pro-drugs, VE-TPGS exerts a protective effect on biological membrane against free-radical damage.

The aim of this work is the evaluation of VE-TPGS effects on riboflavin corneal permeability, and the assessment of its protective effect against free-radicals generated during CXL procedures. Different solutions containing riboflavin (0.125% w/w), dextran (20.0% w/w) and increasing concentration of VE-TPGS were tested. Corneal permeation was evaluated in vitro by the use of modified Franz-cell type diffusion cells and freshly excised porcine corneas as barrier. The effect of VE-TPGS on riboflavin corneal penetration was compared with a standard commercial solution of riboflavin in dextran at different times. Accumulation experiments were conducted both on epithelized and non-epithelized corneas. Moreover, epithelized porcine corneas, treated with the tested solutions, were subjected to an in vitro CXL procedure versus non-epithelized corneas, treated with a commercial solution of riboflavin. Differences were measured by means of corneal rigidity using Young's modulus. The photo-protective effect of tested solutions on corneal epithelium was, finally, evaluated. CXL treatment was applied, in vitro, on human explanted corneas and resulting morphology of corneal epithelium was investigated by scanning electron microscopy.

1. Introduction

Keratoconus is a degenerative non-inflammatory disorder of the cornea, characterized by stromal thinning and ectasia leading to irregular astigmatism and progressive visual loss (Krachmer et al., 1984). Keratoconus-affected patients can be treated with rigid contact lenses, but surgery may be necessary in case of contact lenses intolerance, progressive ectasia or corneal scarring (Reeves et al., 2005).

Corneal cross-linking (CXL), is a new therapeutic approach for the treatment of keratoconus, initially described by Spoerl et al. (1998). This technique is based on corneal accumulation of riboflavin-5′-phosphate (riboflavin) followed by UVA irradiation at a wave length of 370 nm, in order to start a photodynamic reaction, in which riboflavin is excited into a triplet state, that generates singlet oxygen and superoxide radicals (Wollensak, 2006). Reactive oxygen species, thus produced, covalently bond to stromal collagen fibers, increasing the degree of bonding between collagen molecules and finally enhancing corneal stiffness. Moreover, riboflavin acts as an UVA absorber, preventing photo-damage for deeper ocular structures (Wollensak et al., 2003a,b). Cross-linking obtained by CXL technique is able to slow, or even stop the progression of keratoconus (Wollensak et al., 2003a; Kohlhaas et al.,

2006) and has been suggested also for the treatment of different types of ocular ectasia and diseases (Franzco, 2010). Standard CXL procedure usually comprises the removal of corneal epithelium. In fact, epithelium impedes the passage of riboflavin, limiting the concentration of the molecule in corneal stroma and, thus, the effectiveness of therapy. At the same time, reduced riboflavin stromal concentrations increases the risks of tissues photo-damage after UVA irradiation (Wollensak et al., 2003b). Removal of corneal epithelium usually causes ocular itches and burns, as well as a transient blurring. These symptoms persist until the corneal epithelium has not been restored and are treated with the use of lubricant and antibiotic eye drops, analgesic oral therapy and therapeutic contact lenses. Occasionally epithelium removal can lead to complication such as infections, keratitis, edema and scarring that may lead to further loss of vision (Koppen et al., 2009; Pollhammer and Cursiefen, 2009). Corneal thickness is also an essential parameter in CXL treatments. The UV damage of deeper structures, above all the endothelium layer, is a more probable occurrence in thinner corneas. The minimum safe corneal thickness in order to protect from endothelial damages is $400\,\mu m$ (Hafezi, 2011). Unfortunately, many patients with progressive keratoconus have corneas thinner than this threshold and, thus, are excluded from treatment. Since human corneal epithelium thickness is reported to be about $50\,\mu m$ (Reinstein et al., 2008) avoiding epithelium debridement a greater number of patients could be subjected to CXL treatments. Different approaches have been described, so far, to overcome these problems. Hyposmolar solutions of riboflavin have been used to swell the corneal stroma over $400\,\mu m$, and thereby allow the treatment of thinner corneas, but there's a lack of evidence about the real effectiveness and safety of this procedure (Hafezi, 2011). Another attempt described is the use of benzalkonium chloride (BAC) as permeation enhancer, rather than preservative, in eye drops containing riboflavin (Kissner et al., 2010). Treatment with BAC 0.02% seems to induce sufficient epithelial permeability for the passage of riboflavin and to guarantee effectiveness to cross-linking treatment (Wollensak and Iomdina, 2009), but no data are available about its safety, since it is well known that BAC may lead to chemical rather than surgical debridement of epithelium, due to its cytotoxicity (Collin, 1986). In the last period, a new technique defined as trans-epithelial corneal cross-linking (TE-CXL) has been introduced. TE-CXL is based on the same protocols of standard CXL avoiding epithelium debridement and increasing corneal concentration of riboflavin using corneal permeation enhancers (Leccisotti and Islam, 2010).

D-Alpha-tocopheryl poly(ethylene glycol) 1000 succinate (VE-TPGS) is a well-known non-ionic surfactant widely used as solubilizer, emulsifier and vehicle for lipid-based drug delivery formulations. Recently it has been recognized as oral absorption enhancer, due to its interaction with P-glycoprotein and, perhaps, with other drug transporter proteins (Collnot et al., 2006). P-glycoprotein itself has been detected in human and rabbit corneas (Katragadda et al., 2006), as well a specific riboflavin transporter has been described in rabbit corneal epithelium (Hariharan et al., 2006) and in human-derived retinoblastoma cells (Kansara et al., 2005). Moreover, non-ionic surfactants have been extensively described as ocular permeation enhancer, with a particular effectiveness for more hydrophilic molecules (Saettone et al., 1996), and, although the mechanism at the basis of drug permeation enhancement has not been completely clarified, interactions with specific transporters can be rationally supposed (Jiao, 2008). Furthermore, VE-TPGS has shown effectiveness in quenching potentially harmful oxidation-inducing substances, such as reactive oxygen species (Constantinides et al., 2006; Traber et al., 1988). The epithelial layer is, indeed, the first and more heavily corneal structure irradiated by UVA during TE-CXL, and consequently it absorbs a large amount of radiation. Irradiation-induced damage to

Table 1
% composition (w/w) in riboflavin and VE-TPGS of donor solutions.

Formulation code	Riboflavin %	VE-TPGS %
A	0.125	–
B1	0.125	0.010
B2	0.125	0.050
B3	0.125	0.100
B4	0.125	0.250
B5	0.125	0.500
B6	0.125	1.000

epithelial structures is well documented (Podskochy et al., 2000). Starting from these evidences, the aim of this work is the evaluation of D-alpha-tocopheryl poly(ethylene glycol) 1000 succinate as ocular permeation enhancer for riboflavin, and as photo-protective agent against UVA radiations in TE-CXL procedures.

2. Materials and methods

All solvents used were of HPLC grade and were furnished by Sigma–Aldrich (MI, Italy) as well chemicals and buffering agents that were of analytical grade. Dextran T500 was purchased by Pharamacosmos A/S (Holbaek, Denmark), riboflavin-5′-phosphate was furnished by ACEF (Fiorenzuola D'Arda, PC, Italy), while vitamin E TPGS was obtained from Cognis spa (Fino Mornasco, CO, Italy).

2.1. Corneal accumulation studies

The extent of riboflavin corneal accumulation was evaluated using modified Franz-type diffusion cell (Ø 9 mm, 5 mL receptor volume, SES GmbH – Analysesysteme, Bechenheim, DE). Porcine corneas were used. Pig eyes with intact epithelium were harvested in a local slaughter house, stored at $5\,°C$ and used within $24\,h$ post-mortem. When necessary, epithelium was removed using a surgical scraper after epithelial melting by 20% ethanol solution applied for $20\,s$. Then, corneas with about $2\,mm$ ring of sclera, were excised and mounted on diffusion cells, with endothelium facing the receptor compartment. This compartment was constituted by isotonic phosphate buffer at pH 7.4, and was maintained at $37 \pm 1\,°C$ and under magnetic stirring during experiments. Different aqueous iso-osmolar solutions, buffered at pH 7.2, were used as donor compartment. They all contained 20% w/w of dextran T500, and different concentrations of riboflavin and VE-TPGS, as shown in Table 1. Solution **A** was commercially available (Sooft Italia spa, Montegiorgio, AP, Italy). Before each experiment corneas were equilibrated with the use of 0.5 mL of balanced salt solution (BSS) for 10 min. BSS was then replaced by 0.5 mL of the proper soaking solution. Solution **A** was used as control and applied both on disepithelized and intact corneas. Solutions from **B1** to **B6** were applied on epitelized corneas in order to evaluate the effect of the enhancer concentration on riboflavin accumulation. At predetermined intervals, cells were dismantled, the excess of donor solution removed and the corneal area available for diffusion was excised by the use of a scalpel. Corneal tissue was weighted and homogenized in 1 mL of water using 5 mL polypropylene tubes and a Polytron PT1200E homogenizer (Kinematica AG, Lucern, Switzerland). Tubes were then centrifuged at 12,000 rpm for 5 min in a refrigerated centrifuge. The liquid phase was filtered through $0.45\,\mu m$ nylon filters (Lida, Kenosha, USA) and analyzed by HPLC. The extraction method was validated in blank experiments by spiking corneas with known amount of riboflavin. No interfering peaks were detected and percentage of recovery was in the range 98–105%. All experiments were conducted shielding diffusion cells and samples from lights, and were repeated at least six times.

2.1.1. HPLC analysis

A new analytical method was set up in order to analyze riboflavin. The LC system consisted of a Jasco LC-200 equipped with a quaternary gradient pump PU-2089 plus and with a diode array UV detector HD-2010 plus (Jasco Europe, Cremella, LC, Italy). Wavelength selected was 264 nm. Stationary phase was a Supelco C_{16} RP amide (150 mm × 4.6 mm, 5 μm. Supelco, Bellefonte, PA, USA), and a guard column was also used, as suggested by Viñas et al. (2004). Mobile phase was pumped at a flow of 1.2 mL/min and consisted of acetonitrile (A) and 0.25 mmol KH_2PO_4 in water (B). Gradient elution was used. The initial conditions were 100% A. After 1 min the mobile phase was changed linearly over 8 min to 100% B and then initial conditions were restored in the following 8 min. The analytical system suitability was assessed by checking the linearity range (0.70–72.4 μg/mL, $R^2 = 0.9891$), reproducibility, limit of detection (0.70 μg/mL) and limit of quantification (1.2 μg/mL). Retention time for riboflavin was 5.75 min. 25 μl of samples were injected in all cases.

2.2. Stress–strain measurement

A slight modification of the method reported by Wollensak et al. (2003a) was used to evaluate the biomechanical effect of tested solutions on corneas after UVA irradiation. 24 porcine corneas were divided in 4 groups of 6 samples. Group 1 consisted of intact corneas, treated with BSS and not exposed to UVA rays (baseline). Group 2 consisted of intact corneas treated with a solution of VE-TPGS (0.5%, w/w) in 20% dextran T500 before UVA irradiation (negative control). Group 3 was treated with solution **A** after epithelium removal and then irradiated (positive control). For the last group (group 4) soaking solution B5 without epithelium debridement was used. Solutions were applied for 20 min in 0.5 mL volume using Franz-type diffusion cells, as previously described. Then, from each sample a corneal strip of 5.0 ± 0.2 mm width, 890 ± 20 μm thickness and 11.0 ± 0.4 mm length, comprising 1 mm of sclera at both ends were cut. Dimensions of corneal strips were assessed by a digital caliper. Corneal strips were then subjected to cross-linking by UVA rays. The CBM-X LINKER medical device (CSO, Florence, Italy), was used for UVA irradiation at a distance of 1 cm from samples. Wavelength selected was 370 nm, with an irradiance of 2.986 mW/cm², as assessed by an UVmeter (LaserMate Q-2000, LaserMate Group, Wessling Germany). Static stress–strain measurement were performed using an electronic dynamometer AG/MS1 (Acquati Giuseppe srl, Milano, Italy). Young's modulus was calculated at 6% and 8% strain. Procedure and force parameters used were as described by Wollensak et al. (2003a).

2.3. Electron microscopy studies

Electron microscopy studies were carried out to evaluate the effect of different riboflavin solutions on the human corneal epithelium when UVA irradiation is used during trans-epithelial cross-linking. This research adhered to the tenets of the Declaration of Helsinki. Informed consent was obtained for each cornea; Institutional Review Board (IRB)/Ethics Committee approval was obtained (authorization n. 1269). The corneas used in this study were obtained from our regional eye bank and corneal transplant center and were not suitable for transplant due to donor marker positivity. They were examined before the study by light microscopy of the endothelial cell layers. Only corneas with good transparency, thickness between 500 and 600 μm and a normal endothelial mosaic were selected. Three groups of five corneas were studied, while a fourth group of three corneas remained untreated and was used as control. Initially, in this last group, the morphology of the microvilli on the surface of epithelial cells was examined with scanning electron microscopy (SEM). The three groups were
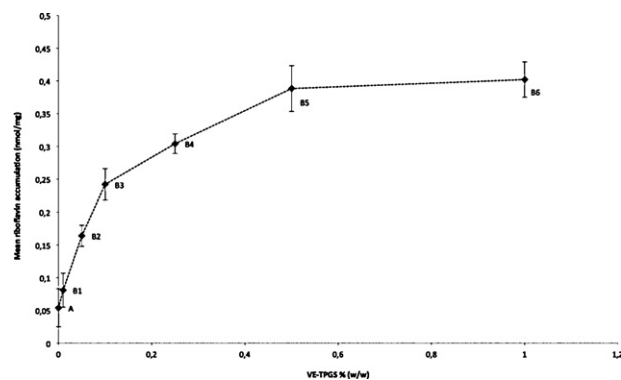

Fig. 1. Effect of different VE-TPGS concentrations on riboflavin corneal accumulation.

treated as follows: group 1 with BSS, group 2 with solution **A** and group 3 with solution **B5**. Treatment included instillation of solution on the corneal surface every 5 min (for a total of 20 min) before UVA irradiation and every 3 min for a total of 30 min during irradiation. Radiant energy (370 nm wavelength) was 2.986 mW/cm²; it was applied at 1 cm from the corneal surface using the same device previously described. Soon after treatment, the morphology of the superficial epithelial layer and of the microvilli on the epithelial cells of each cornea was examined using SEM (7500×, 20 kV).

2.4. Statistical analysis

Results are expressed as mean ± SEM. Data obtained were analyzed by Student's T test for statistical significance. A p value ≤0.05 was considered significant in this study.

3. Results and discussion

3.1. Effect of VE-TPGS concentration on riboflavin corneal accumulation

Solutions from **B1** to **B6** (Table 1) were tested on epithelized corneas to assess the correlation between enhancer concentration and riboflavin corneal accumulation. The results, shown in Fig. 1, are expressed as nmol of riboflavin per mg of corneal tissue after 20 min of exposition. Solution **A** was used as control. Amount of riboflavin extracted was statistically higher than control for all the formulated solution, except **B1**, and increased with increasing concentration of VE-TPGS until a concentration of 0.5% w/w. No significant differences were found between **B5** and **B6** (Table 2). It must be evidenced that high concentration of non-ionic surfactants are not advised for ophthalmic formulations. In fact, when the concentration of these surfactant exceeds the Critical Micelle Concentration (CMC), at a certain level ophthalmic preservatives biocidal activity drops down (DeLuca and Kostenbauder, 1960). Furthermore, a potential safety risk for disruption of the precorneal tear film, associated with inflammatory changes of ocular surfaces is addressed to higher concentration of non-ionic surfactants (Maurer et al., 1998). For these reasons, solution **B5** was used in the following permeation studies.

3.2. In vitro corneal accumulation studies

The effects of solution **B5** on riboflavin corneal permeability were assessed at 5, 10, 20, 40 and 60 min on corneas with intact epithelium (epi-on). 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

Table 2
Mean riboflavin accumulation ± SEM at different concentration of VE-TPGS on intact corneas.

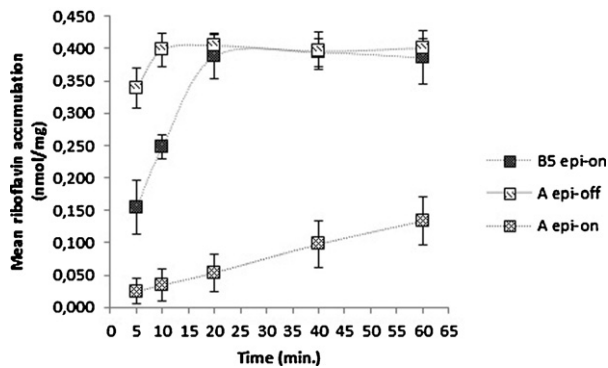| Formulation code	A	B1	B2	B3	B4	B5	B6
Vitamin B2 accumulation (nmol/mg)	0.054 ± 0.029	0.081 ± 0.026	0.164 ± 0.016	0.242 ± 0.024	0.304 ± 0.015	0.388 ± 0.035	0.402 ± 0.027

Fig. 2. Time course of riboflavin corneal accumulation from solution A and B5 on epithelized (epi-on) and disephitelized (epi-off) corneas.

Table 3
Time course of riboflavin accumulation in cornea from tested solutions without epithelial debridement (epi-on) or after (epi-on).

Time (min)	Riboflavin accumulation (nmol/mg)		
	Solution B5 epi-on	Solution A epi-off	Solution A epi-on
5	0.155 ± 0.041	0.338 ± 0.031	0.025 ± 0.020
10	0.248 ± 0.019	0.398 ± 0.025	0.035 ± 0.025
20	0.388 ± 0.035	0.405 ± 0.017	0.054 ± 0.029
40	0.394 ± 0.022	0.396 ± 0.029	0.098 ± 0.037
60	0.386 ± 0.040	0.401 ± 0.014	0.134 ± 0.038

epithelium debridement provided up to 10-fold increase in riboflavin corneal recovery (Fig. 2). These data are consistent with literature knowledge (Baiocchi et al., 2009). Moreover, a saturation of stromal compartment was displayed. Epithelium removal led to a rapid increase in riboflavin accumulation in the first 10 min that remained stable afterwards. A similar accumulation profile was recorded for solution **B5** applied in epi-on conditions, the main difference being the saturation time of stromal compartment. In fact, after 20 min no statistical differences were found between permeation enhancer-containing and control solution (Table 3), thus demonstrating the effectiveness of VE-TPGS in overcoming the epithelium resistance to riboflavin corneal permeability, despite prolonged exposure time is needed.
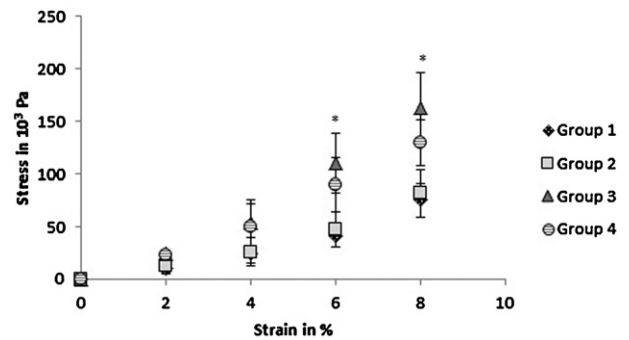

Fig. 3. Stess–strain behavior of porcine corneal tissue after in vitro CXL treatment.

Table 4
Calculated Young's modulus at 6% and 8% strain. Values are expressed in Pa.

Stress in %	Group 1	Group 2	Group 3	Group 4
6	1.13×10^6	1.29×10^6	3.01×10^6	2.46×10^6
8	2.62×10^6	2.84×10^6	5.71×10^6	4.64×10^6

3.3. Biomechanical measurements

Young's modulus was calculated at 6% and 8% strain since these are the values producing the largest difference between treated and untreated corneas, in accordance with literature (Wollensak et al., 2003a). No differences in stress–strain values were found between group 1 and group 2, while groups 3 and 4 showed an improved stress starting from 6% strain ($p = 0.045$, Fig. 3). The largest difference between treated and untreated groups was recorded at 8% strain ($p = 0.021$). No statistical differences were found between group 3 and 4 at all strain rates used ($p = 0.795$), besides Young's modulus values were higher for group 3 (Table 4). We can argue that stiffness of epithelized corneas after in vitro CXL treatment is comparable to non-epithelized corneas when VE-TPGS is used as permeation enhancer. Otherwise, it must be pointed out that differences between treated and untreated porcine corneas, in this experimental model, are not as pronounced as reported for human corneas (Wollensak et al., 2003a). This is mainly due to the greater thickness of porcine cornea, compared to human cornea, and to the reduced relative cross-linked portion. At the same time, measurements are affected by large standard deviations. For these reasons
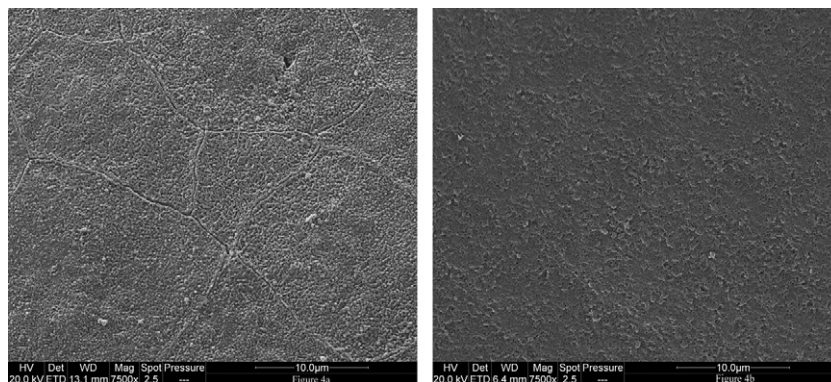

Fig. 4. Scanning electron microscopy of an untreated corneal epithelial surface **(4a)** in comparison with the epithelial surface of a cornea from group 1 **(4b)**.
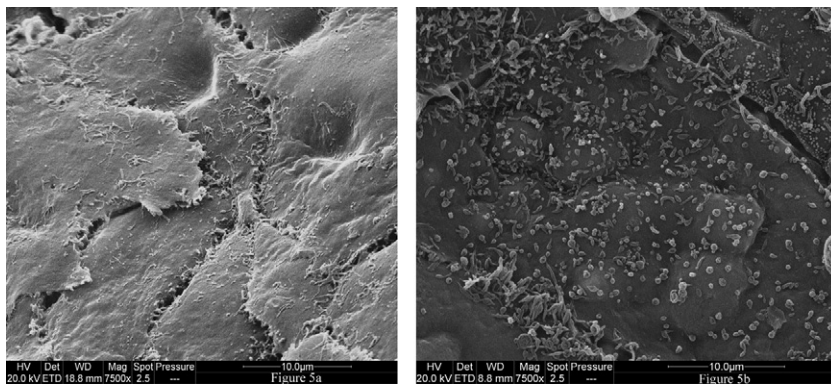
Fig. 5. Scanning electron microscopy of the epithelial surface of a cornea from group 2 (**5a**) in comparison with the epithelial surface of a cornea from group 3 (**5b**).

differences between non-epithelized and epithelized corneas, need to be further explored, maybe using more sensible experimental procedures.

3.4. Microscopic analysis of corneal epithelium after CXL procedure

Microscopic analyses of untreated corneas and of corneas treated with BSS are shown in Fig. 4a and b. In group 1, treatment resulted in the destruction of all epithelial layers thereby exposing the Bowman membrane, in comparison with the intact cellular architecture of the control group. In corneas of group 2, there were several cellular gaps in the superficial epithelial layer mostly due to the rupture of intercellular tight junctions. In addition, there was a remarkable loss of microvilli and of cytoplasmic nuclei (Fig. 5a). In corneas of group 3, the epithelial layers, the cell nuclei and the intercellular tight junctions were less damaged compared with group 2. Although there was UVA-radiation damage in group 3 corneas, the reduction in microvilli density was less evident than in group 2, and the surviving microvilli appeared morphologically intact (Fig. 5b). Ultra-structural analysis of corneal epithelium revealed that the soaking solution used in group 3 is more effective in protecting the corneal epithelium than the standard solution due to the presence of VE-TPGS.

4. Conclusions

The epithelium represents the rate-limiting step in corneal permeation of riboflavin-5′-phosphate, as well for many other hydrophilic molecules (Klyce and Crosson, 1985). It is generally agreed that the epithelium barrier function resides on the outer surface, and that permeation enhancers act by modifying the permeability of superficial cells. Inside the group of permeation promoters, non-ionic surfactants are really well known for their effectiveness, easiness of use in aqueous media and general safety, although their molecular mechanism remains mainly unknown. Among these surfactants, D-alpha-tocopheryl poly(ethylene glycol) 1000 succinate has also been described for its protective role on biological membrane against radical oxygen species. These findings make VE-TPGS an ideal candidate as permeation enhancer in CXL procedures, since a deeper stromal penetration of riboflavin could be supplemented by photo-protection against UVA and free radicals formed during photo-induced processes. The present investigation, indeed, confirmed the effectiveness of VE-TPGS, on porcine corneas, in increasing riboflavin corneal penetration, as well corneal stiffness after in vitro CXL procedures, without epithelium debridement. Also the protective role of this molecule on human corneal epithelium was proven by scanning electron microscopy. Although many other aspects have to be deeply

investigated, the evidences collected make the new proposed ophthalmic solutions a promising strategy for development of safer, non-invasive corneal trans-epithelial cross linking procedures.

References

Baiocchi, S., Mazzotta, C., Cerretani, D., Caporossi, T., Caporossi, A., 2009. Corneal crosslinking: riboflavin concentration in corneal stroma exposed with and without epithelium. J. Cataract Refract. Surg. 35, 893–899.
Collin, H.B., 1986. Ultrastructural changes to corneal stromal cells due to ophthalmic preservatives. Acta Opthalmol. 64, 72–78.
Collnot, E.M., Baldes, C., Wempe, M.F., Hyatt, J., Navarro, L., Edgar, K.J., Schaefer, U.F., Lehr, C.M., 2006. Influence of vitamin E TPGS poly(ethylene glycol) chain length on apical efflux transporters in Caco-2 cell monolayers. J. Control. Release 111, 35–40.
Constantinides, P.P., Han, J., Davis, S.S., 2006. Advances in the use of tocols as drug delivery vehicles. Pharm. Res. 23, 243–255.
DeLuca, P.P., Kostenbauder, H.B., 1960. Interaction of preservatives with macromolecules IV. Binding of quaternary ammonium compounds by nonionic agents. J. Am. Pharm. Assoc. 49, 430–437.
Franzco, G.R.S., 2010. Collagen cross-linking: a new treatment paradigm in corneal diseases–a review. Clin. Exp. Ophthalmol. 38, 141–153.
Hafezi, F., 2011. Limitation of collagen cross-linking with hypoosmolar riboflavin solution: failure in an extremely thin cornea. Cornea 30, 917–919.
Hariharan, S., Janoria, K.G., Gunda, S., Zhu, X., Pal, D., Mitra, A.K., 2006. Identification and functional expression of a carrier-mediated riboflavin transport system on rabbit corneal epithelium. Curr. Eye Res. 31, 811–824.
Jiao, J., 2008. Polyoxyethylated nonionic surfactants and their applications in topical ocular drug delivery. Adv. Drug Deliv. Rev. 60, 1663–1673.
Kansara, V., Pal, D., Jain, R., Mitra, A.K., 2005. Identification and functional characterization of riboflavin transporter in human-derived retinoblastoma cell line (Y-79): mechanisms of cellular uptake and translocation. J. Ocul. Pharmacol. Ther. 21, 275–287.
Katragadda, S., Talluri, R.S., Mitra, A.K., 2006. Modulation of P-glycoprotein-mediated efflux by prodrug derivatization: an approach involving peptide transporter-mediated influx across rabbit cornea. J. Ocul. Pharmacol. Ther. 22, 110–120.
Kissner, A., Spoerl, E., Jung, R., Spekl, K., Pillunat, L.E., Raiskup, F., 2010. Pharmacological modification of the epithelial permeability by benzalkonium chloride in UVA/riboflavin corneal collagen cross-linking. Curr. Eye Res. 35, 715–721.
Klyce, S.D., Crosson, C.E., 1985. Transport processes across the rabbit corneal epithelium: a review. Curr. Eye Res. 4, 323–331.
Kohlhaas, M., Spoerl, E., Schilde, T., Unger, G., Wittig, C., Pillunat, L.E., 2006. Biomechanical evidence of the distribution of cross-links in corneas treated with riboflavin and ultraviolet A light. J. Cataract Refract. Surg. 32, 279–283.
Koppen, C., Vryghem, J.C., Gobin, L., Tassignon, M.J., 2009. Keratitis and corneal scarring after UVA/riboflavin cross-linking for keratoconus. J. Refract. Surg. 25, 819–823.
Krachmer, J.H., Feder, R.S., Belin, M.W., 1984. Keratoconus and related noninflammatory corneal thinning disorders. Surv. Ophthalmol. 28, 293–322.
Leccisotti, A., Islam, T., 2010. Transepithelial corneal collagen cross-linking in keratoconus. J. Refract. Surg. 26, 942–948.
Maurer, J.K., Parker, R.D., Carr, G.J., 1998. Ocular irritation: microscopic changes occurring over time in the rat with surfactants of known irritancy. Toxicol. Pathol. 26, 217–225.

Podskochy, A., Gan, L., Fagerholm, P., 2000. Apoptosis in UV exposed rabbit corneas. Cornea 19, 99–103.

Pollhammer, M., Cursiefen, C., 2009. Bacterial keratitis early after corneal crosslinking with riboflavin and ultraviolet-A. J. Cataract Refract. Surg. 35, 588–589.

Reeves, S.W., Stinnett, S., Adelman, R.A., Afshari, N.A., 2005. Risk factors for progression to penetrating keratoplasty in patients with keratoconus. Am. J. Ophthalmol. 140, 607–611.

Reinstein, D.Z., Archer, T.J., Gobbe, M., Silverman, R.H., Coleman, D.J., 2008. Epithelial thickness in the normal cornea: three-dimensional display with Artemis very high-frequency digital ultrasound. J. Refract. Surg. 24, 571–581.

Saettone, M.F., Chetoni, P., Cerbai, R., Mazzanti, G., Braghiroli, L., 1996. Evaluation of ocular permeation enhancers: in vitro effects on corneal transport of four β-blockers, and in vitro/in vivo toxic activity. Int. J. Pharm. 142, 103–113.

Spoerl, E., Huhle, M., Seiler, T., 1998. Induction of cross-links in corneal tissue. Exp. Eye Res. 66, 97–103.

Traber, M.G., Thellman, C.A., Rindler, M.J., Kayden, H.J., 1988. Uptake of intact TPGS (d-alpha-tocopheryl polyethylene glycol 1000 succinate) a water-miscible form of vitamin E by human cells in vitro. Am. J. Clin. Nutr. 48, 605–611.

Viñas, P., Balsalobre, N., López-Erroz, C., Hernández-Córdoba, M., 2004. Liquid chromatographic analysis of riboflavin vitamers in foods using fluorescence detection. J. Agric. Food Chem. 52, 1789–1794.

Wollensak, G., Spoerl, E., Seiler, T., 2003a. Stress–strain measurements of human and porcine corneas after riboflavin–ultraviolet-A-induced cross-linking. J. Cataract Refract. Surg. 29, 1780–1785.

Wollensak, G., Spoerl, E., Wilsch, M., Seiler, T., 2003b. Endothelial cell damage after riboflavin-ultraviolet-A-treatment in the rabbit. J. Cataract Refract. Surg. 29, 1786–1790.

Wollensak, G., 2006. Crosslinking treatment of progressive keratoconus: new hope. Curr. Opin. Ophthalmol. 17, 356–360.

Wollensak, G., Iomdina, E., 2009. Biomechanical and histological changes after corneal crosslinking with and without epithelial debridement. J. Cataract Refract. Surg. 35, 540–546.