INTRODUCTION

The combined topical application of riboflavin and ultra violet-A (UV-A) light to the corneal stroma, called corneal collagen cross-linking (CXL), is a newly accepted method for treating progressing keratoconus and ectatic corneal diseases. The standard treatment protocol includes mechanical debridement of the central area of the corneal epithelium, which is 9 mm in diameter, and the subsequent dripping of a 0.1% riboflavin solution every 3 min for 30 min before initiating UV-A irradiation (370 nm; 3 mW/cm²), which is used in combination with continued riboflavin dripping. The stiffening of the upper 200–300 µm of the corneal stroma is induced through a photo-oxidative induction of collagen cross-links.

The intact corneal epithelium, with its tight junctions, is considered the most significant barrier to riboflavin permeability. Therefore, in the standard protocol for CXL, the corneal epithelium is mechanically removed after surface anesthesia and prior to riboflavin application. Unfortunately, the detached epithelium causes mild to severe postoperative pain for 3–4 days and is associated with a risk of corneal infections. Therefore, some surgeons modify the standard protocol and perform the treatment without removing the epithelium by using benzalkonium chloride (BAC), tetracaine, pilocarpine containing...
BAC and EDTA or Ribomycin drops containing gentamycin, EDTA and BAC with Oxybuprocaine drops containing benzoate.

The first systematic study concerning the CXL procedure without epithelial debridement was conducted by Wollensak et al. who tested riboflavin 0.1% in 20% dextran T-500 + 0.005% BAC in rabbit eyes and found a slight biomechanical effect. Kissner et al. tested several BAC concentrations in riboflavin 0.1% in NaCl 0.44% solution on rabbit eyes and found good UV absorption and a biomechanical effect using a solution containing 0.02% BAC. In Kissner et al.’s study, the osmolarity of the riboflavin solution was not specified in the details because the focus of their investigation was on the concentration of BAC applied to intact cornea and its ability to alter the epithelium’s barrier function. Because of the positive effect of riboflavin solution containing 0.02% BAC and 0.44% NaCl in Kissner et al.’s study, we intend to investigate the influence of a lower BAC concentration (0.01%) on the transepithelial permeability of riboflavin.

Surprisingly, in further experiments with several 0.02% BAC-riboflavin solutions, we found different results in the penetration of riboflavin through the epithelium. Previous studies focused only on the BAC concentration, and the osmolarity of the solution has not been systematically investigated until now. The aim of this study was to investigate the effect of varying the osmolarity of the riboflavin solution on riboflavin’s transepithelial penetration.

**MATERIALS AND METHODS**

Thirty-six rabbit eyes were enucleated immediately after the animals were killed for other non-eye related animal studies, and several riboflavin solutions were applied to them. The osmolarity of the riboflavin solutions are labelled according to the osmolarity of the cornea, which is about 400–420 mOsm/l. An iso-osmolar solution has about the same osmolarity of the cornea, and all other solutions are termed hypo-osmolar with different degrees of NaCl. The osmolarity of the solutions was characterized by the NaCl content. Thus, we tested a riboflavin 0.1% in NaCl 0.9% solution, which is already used for thin corneas and a riboflavin 0.1% in NaCl 0.44% solution, which was already used in Kissner et al.’s study.

The eyes were divided into six groups of six eyes. In group A, which served as a control group, the corneal epithelium of the enucleated eyes was removed following the standard protocol for CXL prior to riboflavin application. In groups B–F, the corneal epithelium was not removed prior to riboflavin application. The following solutions were applied to each group:

- **A:** riboflavin 0.1% in NaCl 0.9% solution with 0.02% BAC
- **B:** riboflavin 0.1% in NaCl 0.44% solution with 0.02% BAC
- **C:** riboflavin 0.1% in NaCl 0.44% solution with 0.01% BAC
- **D:** riboflavin 0.1% in NaCl 0.44% without BAC
- **E:** riboflavin 0.1% in NaCl 0.9% solution with 0.02% BAC
- **F:** riboflavin 0.1% in NaCl 0.9% solution

A ring was used to restrict the riboflavin solution to the cornea. The solution was dropped into this ring, so the surrounding tissue was protected. The application time for the solutions was 30 min in all groups, and then the corneoscleral buttons were trephined from the enucleated rabbit eyes. Corneal thickness was measured by ultrasound pachymetry (Pach-Pen™XL, Accutome) before and 30 min after the solutions were applied (with the epithelium-on in groups B–F). After trephination, the buttons were put on a slide, and the transmitted UV intensity was measured. Then, the epithelium was carefully removed with a hockey knife, and the thickness and UV intensity were measured again to calculate the absorption coefficient of the stroma alone.

The absorption coefficient was measured according to Wollensak et al. or Iseli et al. Because there is a correlation between riboflavin concentration and the absorption coefficient, the absorption coefficient of the cornea was used as a surrogate for riboflavin concentration in the cornea to characterize the penetration of riboflavin into the stroma.

Briefly, a UV meter was used, and the UV source was the UVX (Company IROC AG, Zurich, Switzerland), a device used for CXL treatment in patients with progressive keratoconus. An aperture and a filter were used to block unwanted fluorescence light. $I_0$ is the intensity of incoming light and $I$ is the intensity of transmitted light, correcting for the absorption of UV light by the glass slide that the cornea was placed. According to the Lambert–Beer law, the absorption coefficient $\mu$ can be calculated by $\mu = (1/x) \ln(I_0/I)$, where $x$ is the thickness of the cornea.

The differences in the absorption coefficients of corneas in groups A–F were statistically tested with the non-parametric Mann–Whitney test.

**RESULTS**

For comparison of the different solutions, only the absorption coefficients of the stroma (measurements without epithelium) were statistically analysed (Figure 1).

As expected, the standard treatment (group A, $\mu = 40.53 ± 4.12 \text{ cm}^{-1}$) showed the highest absorption coefficient in the stroma due to the complete removal of the epithelial barrier. This absorption coefficient is significantly different than all other groups ($P = 0.001$).

Using riboflavin 0.1% in NaCl 0.44%, there is no statistically significant difference between group B ($\mu = 15.2 ± 4.5 \text{ cm}^{-1}$ (0.02% BAC) and group C ($\mu = 13.6 ± 2.2 \text{ cm}^{-1}$ (0.01% BAC) ($P = 0.90$). However,
a statistically significant difference exists between group C and group D (µ = 4.3 ± 0.5 cm⁻¹ (without BAC) (P = 0.025).

With riboflavin 0.1% in NaCl 0.9%, a slight increase is found between groups E and F due to the effect of 0.02% BAC (P = 0.088). The lowest absorption coefficient was observed in riboflavin 0.1% in NaCl 0.9% without BAC (group F, µ = 5.7 ± 2.1 cm⁻¹), and the absorption coefficient was slightly higher in group E (µ = 7.6 ± 2.5 cm⁻¹), in which BAC was added.

There was also a statistically significant difference (P = 0.004) between group B (containing riboflavin 0.1% in NaCl 0.44% with 0.02% BAC) and group E (containing riboflavin 0.1% in NaCl 0.9% with 0.02% BAC). The absorption coefficient of the corneas treated with riboflavin 0.1% in NaCl 0.9% with 0.02% BAC (group E) was more than twofold lower than the absorption coefficient of the corneas treated with riboflavin 0.1% in NaCl 0.44% with 0.02% BAC (group B).

**DISCUSSION**

The permeability of the cornea to drugs is clinically important because it is the major factor determining the efficacy of topically applied ophthalmic preparations. This permeability could be analysed based on an understanding of corneal anatomy. In the human eye, the epithelium contains 5–7 layers of cells each connected by tight junctions, which is expected to provide a large barrier to anything but small, lipophilic compounds. The stroma is a thick, fibrous, largely acellular tissue composed primarily of water, which should not provide a lipophilic barrier. Finally, the endothelium is a monolayer of cells with large intercellular junctions, which should present a leaky lipophilic barrier.24,25

Riboflavin (C₁₇H₂₀N₄O₆) has a molar mass of 376.36 g/mol (376.36 Da). Riboflavin is hydrophilic, which is why it is unable to penetrate the corneal epithelium.

In multicellular organisms, the internal environment is separated from the external environment by epithelial cell sheets. To maintain homeostasis between these compartments, movements of several substances through the paracellular pathway must be strictly regulated, and tight junctions, one mode of intercellular adhesion occurring in the most apical region of lateral membranes, play a major role in this regulation (barrier function). Tight junctions regulate paracellular movement by changing their molecular composition and thereby changing paracellular conductance. Paracellular conductance is dependent on several factors: the apical-to-basolateral hydrostatic pressure gradient, the osmotic gradient, the size of present extracellular macromolecules, and the direction of ion flux in relation to the apical or basolateral side. In vitro studies showed that NaCl flux from the apical to the basolateral side increases paracellular conductance, but large-molecule dextran inhibits this increase. This demonstrates the importance of the osmotic gradient to regulate tight junctions as a barrier and regulate paracellular conductance.26,27

Tokuda’s conclusions have in connection to our work limitations with regard to transepithelial riboflavin uptake: the experiments were done on Xenopus renal cells, measuring transepithelial conductance and permeability of NaCl. However, these observations can be adopted and formulated as a hypothesis also for the paracellular transport of drugs or molecules like riboflavin.

BAC increases epithelial permeability by loosening the tight junctions.28–32 This pharmacological modification of corneal epithelial permeability represents a novel method to avoid epithelial debridement in CXL.33,34

To date, the only comparative experimental study on transepithelial cross-linking has been performed...
by Wollensak et al. The study used rabbit corneas with intact epithelium treated by CXL and 0.005% BAC and demonstrated a biomechanical stiffening effect of about one-fifth compared to the standard treatment protocol. However, this method was not identical to the protocol suggested by Pinelli et al. Instead, Wollensak used proparacaine as a tensioactive substance (0.005% BAC) and a riboflavin in 20% dextran T-500 solution, whereas Pinelli applied a higher concentration of BAC (0.02%) in a riboflavin 0.1% in NaCl 0.44% solution, which was the same formula tested in one of our team’s previous pharmacological studies.

Nevertheless, the absolute results of this study can not be compared with the results of Kissner et al.’s study. In Kissner et al.’s study, riboflavin was applied for 60 min (30 min before irradiation and 30 min during irradiation) and only then was the absorption coefficient and biomechanical stress-strain measured. In our study, the riboflavin solutions were applied for only 30 min. This difference explains why Kissner et al. found no statistically significant difference between the standard method and riboflavin 0.1% in NaCl 0.44% solution with 0.02% BAC, whereas in our study, there was a difference. After a 30 min application of riboflavin 0.1% in NaCl 0.44% solution with BAC, it was not as effective regarding the absorption coefficient as the standard method. A direct comparison with Kissner et al.’s results due to different application times is not possible. We could speculate that the absorption coefficient values in our experiment could be similar or even equal to Kissner et al.’s study after the same time of riboflavin application. To test this hypothesis should be the aim of our next experiment. In the Kissner et al.’ study, we investigated several BAC concentrations. At that time, we were not aware of the importance of the osmolarity effect.

We know that 0.9% NaCl constitutes a physiological solution; a lower concentration of NaCl (0.44%) results in an osmotic gradient that permits the penetration of riboflavin through tight junctions between epithelial cells. A further reduction of NaCl content should be tested systematically to show the influence on permeability. Dextran, a molecule not present in our hypo-osmolar riboflavin solutions, is present in the iso-osmolar riboflavin solution; it inhibits paracellular transport.

This BAC supplemented hypo-osmolar riboflavin solution has two defining characteristics: the BAC (0.02%), which acts as a surfactant, and the 0.44% NaCl, which might act as a “power” creating an osmotic gradient.

In the literature, there is no detailed information about the concentration and intracorneal accumulation of BAC after epithelial application. After loosening the epithelial tight junctions, the corneal stroma should not function as a barrier to BAC.

Our previous experiments did not investigate the potential endothelial damage caused by the intensive application of BAC contained in eye drops in short, 1-hr intervals. Previous studies investigated only the effect of preservatives on endothelial cells after direct exposure.

The higher absorption coefficients of riboflavin in the corneal stroma in groups with riboflavin 0.1% in NaCl 0.44% solution with BAC compared to groups with riboflavin 0.1% in NaCl 0.44% without BAC or in NaCl 0.9% solution are significant; however, the values (15.2 ± 4.5 cm⁻¹, resp. 13.6 ± 2.2 cm⁻¹) are relatively small compared to the absorption coefficient of riboflavin in the group treated with the standard protocol (40.53 ± 4.12 cm⁻¹, with removal of epithelium). In our experiment, the effective riboflavin preparation, tested with the epithelium-on method, resulted in an absorption coefficient that was 33%, resp. 37% of the standard epithelium-off procedure. Whether this modification of the standard treatment might be sufficient to induce adequate biomechanical effects equivalent to the standard procedure has yet to be experimentally investigated.

CONCLUSION

The results of our experimental study may contribute to the mechanism of the transepithelial CXL and its influencing factors. The transepithelial riboflavin solution in the CXL procedure should not contain dextran, but it should include 0.01% BAC and 0.44% NaCl to increase the permeability of the epithelium, which will allow for the riboflavin to reach probably sufficient concentration in the corneal stroma. Further investigations regarding the biomechanical effects and toxicity are necessary to detect a real clinical effect and possible endothelial damage prior to introduction of this modified CXL treatment into the broad clinical practice.

Declaration of interest: The authors F.R. and E.S. report no conflict of interest. Author R.P. owns a patent for the new riboflavin solution formula. The authors alone are responsible for the content and writing of the paper.

REFERENCES


