A histological examination of corneal epithelium after iontophoresis with different riboflavin solutions

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To the Editor: Currently, from a pathological point of view, corneal collagen crosslinking (CXL) with riboflavin is the only new therapy for controlling keratoconus progression. Riboflavin, as a photosensitizer, can be activated after the ultraviolet radiation in the cornea and prevent it from damaging intraocular organizations. Therefore, the concentration of riboflavin in the corneal stroma must be maintained at a sufficient level during the treatment.[1] Riboflavin, a type of water-soluble macromolecule, is resistant to permeating the corneal epithelial barrier. Thus, de-epithelial eye-dripping was adopted conventionally with complications such as inevitable pain, long recovery time, potential risk of infection, and so on; transepithelial corneal CXL is increasingly becoming a research hotspot. It is thus critically important to improve the transepithelial penetration of riboflavin. Although iontophoresis was reported, it had a slightly higher effect with no clear mechanism analysis.[2-4] Corneal epithelium is lipophilic and hydrophobic, and hydrophilic drugs enter corneal stroma via intercellular pathways. In this study, permeability effects of riboflavin in different solvents into the cornea via iontophoresis were compared, and the changes of corneal epithelial cell layer organizations were analyzed to examine the solvent components that are most beneficial to iontophoresis and investigate the underlying mechanism of the penetration effect.

A total of 32 New Zealand, white, male or female rabbits, weighing 2.0 to 2.5 kg, were selected. They were healthy with no eye diseases. Only the right eye of each animal was selected for the experiment. Rabbits were randomized into eight groups, each consisting of four rabbits. Three subgroups of the test group received iontophoresis of 0.1% riboflavin balanced saline solution (including NaCl, KCl, $CaCl_2$, and $MgCl_2$), 0.1% riboflavin normal saline solution, and 0.1% riboflavin distilled water solution for 5 min, respectively. Meanwhile, three eye-dripping-alone groups were given eye-dripping with 0.1% riboflavin balanced saline solution, 0.1% riboflavin normal saline solution, and 0.1% riboflavin distilled water solution for 5 min, respectively. Rabbits of the deepithelial eye-dripping group were given eye-dripping with 0.1% riboflavin dextran solution for 30 min after de-epithelization. Rabbits of the normal cornea control group did not receive any treatment. At the end of the experiment, two eyes were selected from each group for observing the corneal stroma yellowing after their central corneal epithelium was removed. Also, corneas were collected from two eyes of each group for observing the corneal epithelial cell layer structure with optical and electron microscopes. All animal procedures were approved by the ethics committee and conformed to the Association for Research in Vision and Ophthalmology (ARVO) Statement for Use of Animals in Ophthalmic and Vision Research.

In the iontophoresis group, the animals were generally anesthetized using intramuscular injection of a mixture of sumianxin and ketamine (one injection of 1.5 mL sumianxin + one injection of 2 mL ketamine; 0.3 mL/kg). They were subsequently placed in lateral position, with the test eye (right eye) facing up. Skin in the right foreleg was prepared, disinfected, adhered, and connected to the skin electrode. The eyelid was opened using an eye speculum, after which the eyecup was placed and stabilized using vacuum suction in the center of the cornea, followed by connection of corneal electrode of the iontophoresis apparatus and injection of riboflavin solution until it was slightly higher than the electrode nest. Finally, the iontophoresis apparatus was turned on after the electrodes were rechecked. The current for the iontophoresis was 1 mA and the duration was 5 min. After the iontophoresis apparatus automatically stopped, the corresponding devices were removed.

In the eye-dripping-alone group, the animals were anesthetized and placed in the same position as in the iontophoresis group. The eyelid was opened using an eye speculum, after which the eyecup was placed and

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stabilized using vacuum suction in the center of the cornea, followed by injection of riboflavin solution until it was slightly higher than the electrode nest. After 5 min, the eyecup was removed.

In the de-epithelial eye-dripping group, the animals were anesthetized and placed in the same position as in the iontophoresis group. The eyelid was opened using an eye speculum, 8.5 mm corneal epithelium was removed from the central part, and then the eye was dripped with 0.1% dextran solution, once every 3 min for 30 min.

When the operation concluded, all corneal stroma and aqueous humor yellowing, as well as corneal epithelium status, were observed under a slip-lamp. For further comparing the degree of yellowing of corneal stroma, after compressing with a corneal trephine, an 8.5 mm corneal stroma button was excised immediately from the central part, followed by quick washing using 10 mL balanced saline after the corneal epithelium was removed, as well as comparison of the degree of yellowing. For pathological examination, an 8.5 mm corneal button was excised immediately from the central part, quickly washed using 10 mL balanced saline, fixed with formalin, followed by light microscopy, or cut into 2 mm × 3 mm blocks and fixed with formaldehyde, followed by electron microscopy. At the end of the experiment, the animals were euthanized.

During slip-lamp observation, in the iontophoresis group, 5 min after iontophoresis, the corneal epithelium was smooth, and stroma was faint yellow with no edema in the balanced saline and normal saline subgroups. However, there was a mild fog-like edema in the corneal epithelium and the stroma was significantly yellow in the distilled water subgroup. In the eye-dripping-alone group, the corneal epithelium was smooth, and the stroma was slightly yellow with no edema in each subgroup. Further, the corneal stroma was significantly yellow with no edema in the de-epithelial eye-dripping group.

Comparing the yellowing of corneal stroma buttons, corneal stroma was significantly yellow in the distilled water iontophoresis and de-epithelial eye-dripping groups, whereas it was faint yellow in the balanced saline and normal saline iontophoresis groups. Further, corneal

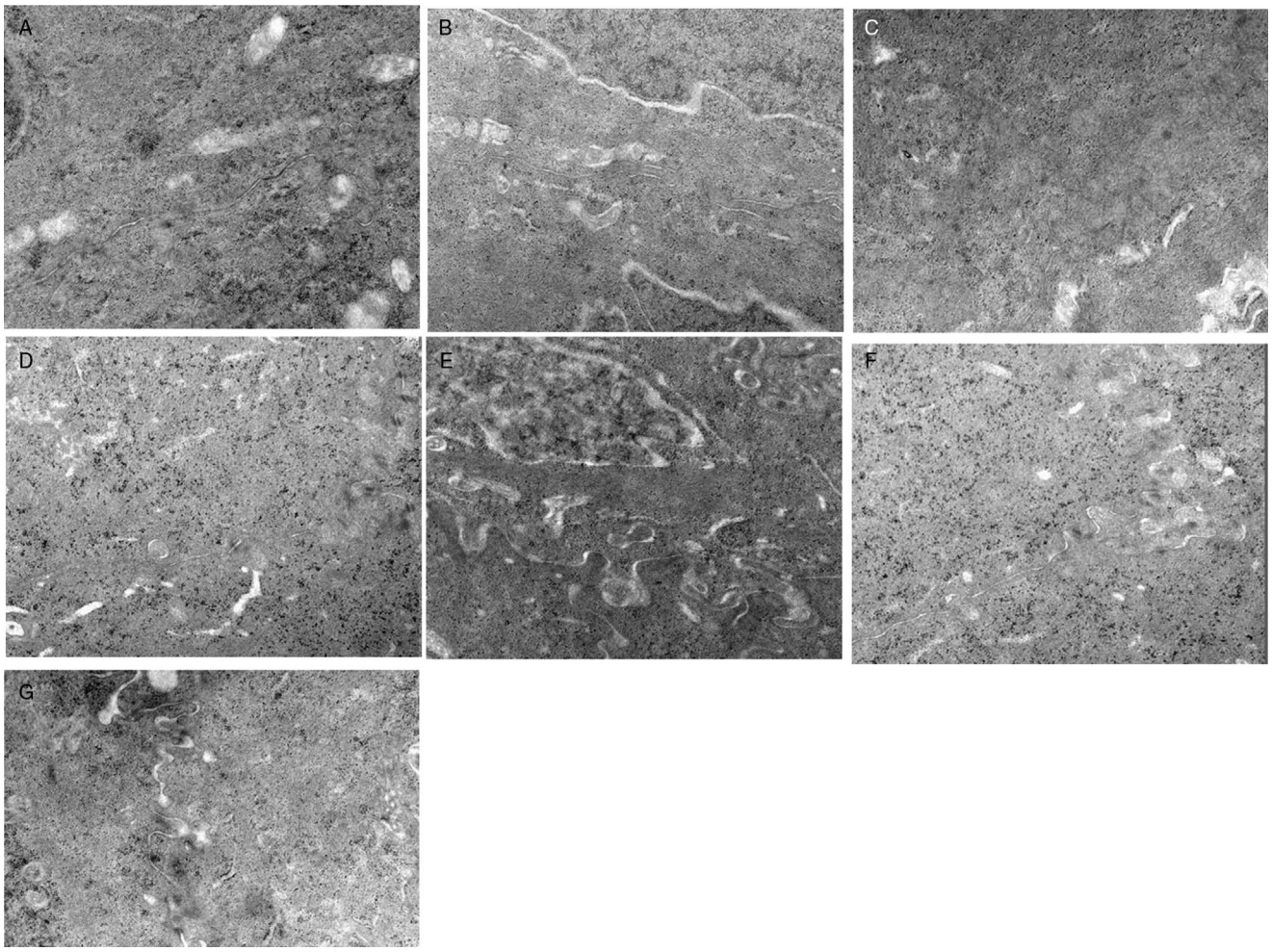

Figure 1: The transmission electromicroscopic observation of the corneal epithelial cell layer structure showed cellular edema, and the Intercellular space was significantly reduced to nearly nonexistent, with unclear tight junctions, desmosomes, and other structures only in the distilled water iontophoresis group. (A) Iontophoresis of 0.1% riboflavin balanced saline solution. (B) Iontophoresis of 0.1% riboflavin normal saline solution. (C) Iontophoresis of 0.1% riboflavin distilled water solution. (D) The eye-dripping alone with 0.1% riboflavin balanced saline solution. (E) The eye-dripping alone with 0.1% riboflavin normal saline solution. (F) The eye-dripping alone with 0.1% riboflavin distilled water solution. (G) Normal cornea.

stroma was slightly yellow in various subgroups of the eye-dripping-alone group.

Under the light microscope, no corneal epithelium was found in the de-epithelial eye-dripping group, while it showed normal structure in the other groups. Under the electron microscope, the corneal epithelial cell layer of the balanced saline and normal saline iontophoresis groups showed intact organizations; the intercellular space was slightly dilated and presented a uniform course, with reduced tight junctions, desmosomes, and other structures. In the distilled water iontophoresis group, the corneal epithelial cell layer showed cellular edema; the intercellular space was significantly reduced to nearly non-existent, with unclear tight junctions, desmosomes, and other structures. In the eye-dripping-alone group, the corneal epithelial cell layer showed intact organizations, intercellular space was not significantly changed, and desmosomes were slightly decreased [Figure 1].

Particularly, the degree of yellowing of the corneal stroma after iontophoresis of 0.1% riboflavin distilled water solution for 5 min was similar to the conventional de-epithelial eye-dripping method. However, an unfavorable permeability effect was obtained through iontophoresis using balanced saline and normal saline as well as eye-dripping alone.

Iontophoresis effect of distilled water was superior to balanced saline and normal saline, perhaps because of the following reasons. First, distilled water contains the least parasitic ions, which results in minimum resistance for migration of riboflavin ions in the electric field. Second, the solution has a low osmotic pressure and there is slight edema in the corneal epithelium, which affects the corneal epithelial barrier function.

Corneal epithelial barrier function is divided into intercellular and intracellular pathways. Since the corneal epithelium is lipophilic and hydrophobic, hydrophilic drugs can enter the corneal stroma via the intercellular pathway, while lipophilic drugs can enter via the intracellular pathway. Theoretically, since riboflavin is a water-soluble macromolecule, only a small volume can enter the corneal stroma via the intercellular pathway. Our electron microscopy results confirmed that only desmosomes were slightly decreased and the intercellular space was not significantly altered in the eye-dripping-alone group, which was indicative of insignificant increase in permeability effect. In balanced saline and normal saline iontophoresis groups, tight junctions,[5] desmosomes, and other structures were decreased; intercellular space was slightly dilated and presented uniform course, which meant increase in permeability effect. Meanwhile, in the distilled water iontophoresis group that showed the most prominent permeability effect, corneal epithelial cell edema appeared; intercellular space was significantly reduced to nearly non-existent, with unclear tight junctions, desmosomes, and other structures, indicating that the intercellular space was reduced and not conducive to penetration. We speculated that the cell edema increased the permeability of cell membrane, which enhanced the intracellular pathway and promoted the penetration of riboflavin.

Although definite results have been obtained in this study, the molecular mechanism underlying the changes of corneal epithelial membrane permeability as well as the transport mechanism of riboflavin across the cell membrane require further studies.

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Conflicts of interest

None.

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