Original Article

Iontophoretic Delivery of Riboflavin into the Rabbit Cornea: a Primary Study

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Abstract

Purpose: To determine the penetrability of riboflavin into the corneal stroma by iontophoresis and to compare the permeability effects of different solvents.

Methods: Twenty rabbits were randomly divided into four groups: a group that received 0.1% riboflavin-balanced salt solution (BSS) by iontophoresis, a group that received 0.1% riboflavin-saline solution by iontophoresis, a group that received 0.1% riboflavin-distilled water solution by iontophoresis, and a control group that received classical riboflavin instillation after corneal de-epithelialization. The degree of yellowing of the de-epithelialized corneal stromal button from each rabbit was compared.

Results: The yellow color scores for the corneal stromal buttons in the three iontophoresis groups were compared with those of control group. Iontophoretic delivery of a 0.1% riboflavin-distilled water solution yielded similar yellow changes in the corneal stromal button when compared with classical riboflavin instillation after de-epithelialization. However, the other two solvents did not sufficiently enhance the permeability of riboflavin.

Conclusion: Riboflavin can effectively penetrate into the corneal stroma to saturation levels by iontophoresis. Using distilled water as the solvent can promote penetrability. (***Eye Science 2014; 29:30-35***)

Keywords: riboflavin; iontophoresis; cornea

Keratoconus is a relatively common disease, with an incidence of 1 in 2000 in the general population, and often affects young patients. This condition is characterized by a progressive thinning and ectasia of the central cornea that can cause myopia and irregular astigmatism. Riboflavin/ultraviolet-A (UVA) -induced corneal collagen crosslinking (CXL), developed in the 1990s at Dresden University, Germany, is the first treatment available that may be able to stabilize the keratoconic process by affecting its pathophysiology[1-2]. In this procedure, the photosensitizer riboflavin, which is absorbed by the corneal stroma, is irradiated by and excited with UVA. The excited riboflavin can interact directly, or by generating reactive oxygen species, to form chemical covalent bonds that bridge the amino groups of collagen fibrils. This increases the biomechanical stabilization and stiffness of the cornea. Riboflavin can also absorb UVA and prevent the injury of the inner ocular tissue. Therefore, the concentration of riboflavin delivered to the corneal stoma is important[3,4].

The intact epithelium constitutes a diffusion barrier for riboflavin (molecular weight 376.36; hydrophilic); consequently, classical CXL requires removal of the epithelium before riboflavin instillation. The saturation level of riboflavin is confirmed by the presence of the substance in the anterior chamber[5-7]. However, the possible risks, such as corneal infections, ulcers, postoperative pain, and long recovery periods, make research on transepithelial CXL challenging. Since a sufficient concentration of riboflavin in the corneal stroma is vital for efficacious and safe transepithelial CXL[8-11]; many penetration-promoting methods have been evaluated. However, biomechanical efficiency tests of riboflavin in rabbits using benzalkonium chloride-containing proxymetacaine eye drops confirmed that only approximately one-fifth of the effect was observed when compared with classical

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crosslinking[12-14]. The intensity of the riboflavin observed when using ultrasound treatment only approached approximately one-third of the effect of classical crosslinking[15]. Direct introduction of riboflavin using a Femtosecond laser-created pocket or needle technique was efficacious but this process can be complex [16–18]. Iontophoretic delivery of riboflavin as a non-invasive method has been presented as a possible option, but the riboflavin concentration delivered to the corneal stroma by iontophoresis is unknown[19–22].

This study first assessed the riboflavin concentration in the corneal stroma by iontophoresis, and then compared the permeability effects of different solvents.

Materials and methods

Study subjects

Twenty right eyes of 20 New Zealand White rabbits without eye disease, weighing between 2.0–2.5 kg, were used in the experiments and were divided randomly into four groups: Group 1 received 0.1% riboflavin-balanced salt solution (BSS) by iontophoresis; group 2 received 0.1% riboflavin-saline solution by iontophoresis; group 3 received 0.1% riboflavin-distilled water solution by iontophoresis; and group 4, the control group, underwent a classical riboflavin instillation after de-epithelialization of the cornea. All animal procedures were approved by the ethics committee and conformed to the ARVO Statement for Use of Animals in Ophthalmic and Vision Research.

Iontophoresis

General anesthesia was induced with an intramuscular injection of a mixture of xylazine hydrochloride(1.5 mL) and ketamine (2 mL) at a dose of 0.3 mL/kg. The rabbits were placed on the left side and the right eye that was facing upward was held open with a blepharostat; a skin electrode was attached to the skin of the right anterior thigh after shaving and cleaning. The corneal iontophoresis applicator was positioned on the surface of the central cornea and was fixed securely using a vacuum ring. The reservoir was filled with the riboflavin solution and the iontophoresis device(I-ON CXL, SOOFT, Italia)was turned on for 10 min using 1 mA current. The device was removed after completion of the iontophoresis.

The yellow color in the corneal stroma and aqueous humor was observed using a slit lamp. A central corneal button 8.5 mm in diameter was then dissected using a trephine and microscissors. The corneal epithelium was removed using a spatula and washed quickly with 10 mL of BSS. The degree of yellow coloration of the corneal stromal buttons was then compared. The rabbits were sacrificed postoperatively.

Instillation after de-epithelium

Under general anesthesia, the rabbits were placed with the right eye upward and held open using a blepharostat and the corneal epithelium was debrided with a spatula. A 0.1% riboflavin-20% dextran solution was instilled on the cornea every three minutes for a total of 30 minutes. The yellow coloration of the corneal stroma and the aqueous humor was observed using a slit lamp and corneal buttons were dissected and quickly washed with 10 mL of BSS. The degree of yellowing of the corneal stromal buttons was then compared.

Slit-lamp examination

The degree of the yellow coloration of the corneal stroma and aqueous humor was divided into four grades: (1) no coloration, (2) mild coloration, (3) moderate coloration, or (4) severe coloration. The findings were scored 0, 1, 2, or 3, respectively.

Corneal stroma button examination

The corneal stroma buttons were scored using similar color grades: (1)no coloration, (2)mild coloration, (3)moderate coloration, or (4)severe coloration. The findings were scored 0,1,2,or 3,respectively.

A blinded method was applied in the total determination and scoring of the test.

Statistical analysis

Rank sum tests were used to compare the experimental and control group results. Results with a P value less than 0.05 were considered statistically significant. The data analyses were performed using SPSS 16.0 software.

Results

The three types of examination showed consistent trends. The values for the BSS and the saline groups were statistically different from the values for the

classical instillation group; however, the values for the distilled water group were not different from the control group.(Tables 1–3,Figure1).Iontophoretic delivery of a 0.1% riboflavin-distilled water solution can lead to similar yellowing changes in the rabbit corneal stroma and aqueous humor to those that occur after instillation using the de-epithelialization method. The riboflavin penetration with the BSS and saline solvents after iontophoresis were insufficient; both the riboflavin saturation in the corneal stroma achieved by iontophoresis and the amount of penetration were influenced by the solvent component.

Discussion

Iontophoresis is a non-invasive technique whereby a small electric current is applied to enhance pene-

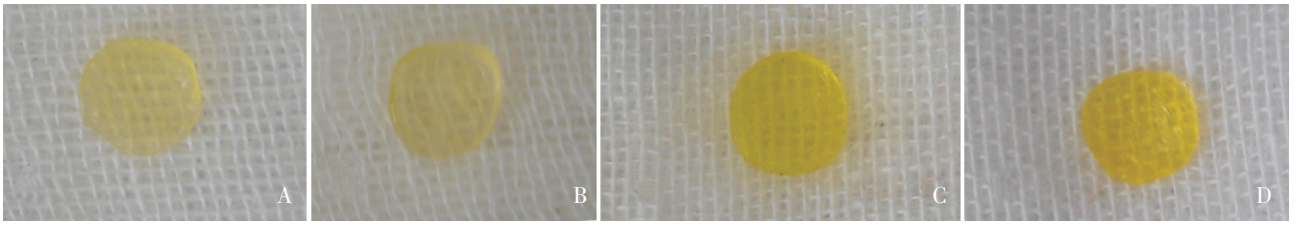

Figure 1 The different yellow colored corneal stroma buttons in four groups. A: 0.1% riboflavin-BSS by iontophoresis, B: 0.1% riboflavin-saline solution by iontophoresis,C:0.1% riboflavin-distilled water solution by iontophoresis,D:conventional riboflavin instillation after de-epithelialization as controls.

Table 1 Yellow degree of rabbit corneal stroma after iontophoretic delivery by slit lamp examination

	Eyes	BSS	Saline	Distilled water	Instillation without epithelium
Yellow degree	1	2	2	3	3
	2	2	2	3	3
	3	2	2	3	3
	4	2	2	3	3
	5	1	1	3	3
H		15.000	15.000	27.500	
P		<0.01	<0.01	>0.1	

Table 2 Yellow degree of rabbit aqueous humor after iontophoretic delivery by slit lamp examination

	Eyes	BSS	Saline	Distilled water	Instillation without epithelium
Yellow degree	1	2	1	3	3
	2	1	0	3	3
	3	1	0	3	3
	4	1	0	3	3
	5	1	0	2	3
H		15.000	15.000	25.000	
P		<0.01	<0.01	> 0.1	

Table 3 Yellow degree of rabbit corneal stroma button after iontophoretic delivery

	Eyes	BSS	Saline	Distilled water	Instillation without epithelium
Yellow degree	1	2	2	3	3
	2	2	1	3	3
	3	1	1	3	3
	4	1	1	3	3
	5	1	1	3	3
H		15.000	15.000	27.500	
P		<0.01	<0.01	>0.1	

tration of an ionized drug into tissue. The drug is applied using an electrode that carries the same charge as the drug. An electrode with the opposite charge, placed elsewhere on the body, completes the circuit. The ionized drug molecule penetrates the tissue by electric repulsion. The ease of application, the minimization of systemic side effects, and the increased drug penetration directly into the target region has resulted in an extensive clinical use of iontophoresis, especially in the transdermal field[23,24].

Ocular iontophoresis has been applied since its first application in 1908 by Wirtz. Ophthalmic drugs delivered this way have included dyes, antibacterials, antivirals, antifungals, steroids, antimetabolites, and even genes. The main approach for this delivery is to fill an eye cup with the drug solution. Ocular iontophoresis has received much attention[25,26] as a promising non-invasive drug delivery method that has the possibility of overcoming permeability barriers.

Some recent research has investigated the transepithelial iontophoresis of riboflavin into the corneal stroma[19-21], but did not report the riboflavin concentration achieved in the stroma by this method. The factors that influenced the actual drug levels in the target tissue included the current density, the iontophoretic time period, the drug concentration, and the parasitic ions. The choice of the current density and the iontophoretic time period were usually fixed when the eye cup method was applied, but parasitic ions in the complex solution may have decreased the delivery. In addition, the penetrability of riboflavin, a hydrophilic macromolecule (molecular weight 376.36), by electrically-assisted delivery and the solvent influence on the delivery effectiveness were both unknown. The present study is the first to demonstrate that riboflavin can effectively penetrate into the corneal stroma to saturation levels through iontophoresis. The use of distilled water as a solvent can promote the penetrability.

In the classical instillation method, riboflavin was dissolved in 20% dextran because the high viscidity and osmotic pressure of the dextran can prevent cornea edema after de-epithelialization. However, dextran was not used in the iontophoretic delivery of riboflavin. One reason was that the intact epithelium in the iontophoretic procedure protected the cornea from edema; another reason was that the high viscidity of dextran would perhaps interfere with the movement of the riboflavin. Therefore, BSS, saline, and distilled water were selected for investigation in this study.

In 2009, Baiocchi et al. reported high-performance liquid chromatography (HPLC) determinations of riboflavin concentrations in the corneal stroma after instillation in tissue with and without epithelium. They concluded that the instillation process required debridement of the epithelium to avoid interference of riboflavin penetration by the epithelium[8]. We used a similar method when dealing with the corneal buttons; the epithelial debridement and quick BSS wash ensured that the results reflected the riboflavin enrichment of the corneal stroma. Riboflavin has a yellow color and the corneas were transparent, so the degree of yellow coloring of the corneal stromal button was directly proportional to the concentrations of riboflavin. This provided a simple way to assess the relative degree of riboflavin in the corneal buttons.

All three types of examination yielded values that showed consistent trends, but we still found higher average yellowing scores for the corneal stroma in vivo using slit lamp examination than for the *in vitro* corneal stromal buttons. In our opinion, the *in vivo* examination results were influenced by riboflavin on the corneal surface and in the epithelium. Consequently, the coloring in the corneal stromal buttons after de-epithelialization was more accurate.

Distilled water was more effective in delivering the drug than BSS and saline. Possible reasons included the presence of fewer parasitic ions in the distilled water, leading to less interference with riboflavin penetration. Second, the hypo-osmotic pressure in the riboflavin-distilled water solution may have damaged the barrier function of the epithelium because it could cause mild edema in the epithelium, although obvious edema was not observed. This speculation needs confirmation by further pathologic evaluation.

The iontophoretic delivery used in the present study consisted of 10 min of 1 mA current. Improvements might be seen with BSS and saline by

changing the duration and current power, but that would increase the treatment time and potentially lead to patient discomfort. The effectiveness of distilled water is therefore meaningful to further research in this respect.

The initial results from this primary study were promising, but some questions remain to be answered. For example, the method used to compare the yellow coloration was not ideal; quantitative analysis of riboflavin concentration by HPLC is more accurate. On the other hand, to obtain an enough crosslinking effect, the UVA irradiation parameters may have required further adjustments because of the epithelial interference with UVA following iontophoretic delivery of sufficient riboflavin to the stroma compared to classical CXL. All these questions need further study.

In conclusion, the riboflavin was successfully and adequately delivered into the rabbit cornea by iontophoresis. The use of distilled water as the solvent can promote riboflavin penetrability.

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